Structure-function studies on human granulocyte-macrophage colony-stimulating factor

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Contents

Summary

The aim of the work described in this thesis was to study the structure-function properties of the human cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF) in order to generate molecules with novel biological properties. The approach used was to generate mutated forms of GM-CSF cDNA, express mutant proteins and examine their biological activity and receptor binding properties.

At the time the work for this thesis commenced the structure of GM-CSF had not yet been determined. However, the predicted structure of GM-CSF was a bundle of four alpha helices containing hydrophilic regions. Initial studies using COS and CHO cell expression systems focussed on residues within the first predicted helix of GM-CSF that contribute to a prominent hydrophilic peak. Deletion analysis and substitution mutagenesis of residues 20 and 21 indicated that Glu^{21} was a functionally significant residue as charge reversal mutations at this position reduced GM-CSF activity 300-fold. A variety of amino acid substitutions at position 21 indicated a hierarchy of tolerance with Glu,Asp > Asn > Ala > Ser > Gln > Lys,Arg. The results indicated that Glu^{21} was essential for multiple GM-CSF activities including cell proliferation and mature cell activation.

The GM-CSF receptor comprises a GM-CSF specific, low affinity receptor α chain (GMRα) and a β chain (β_c) that does not by itself detectably bind GM-CSF but confers high affinity binding when co-expressed with the α chain and is required for signal transduction. The β_c chain is shared with the IL-3 and IL-5 receptors. Receptor binding studies with residue 21 analogues indicated that Glu^{21} is essential for binding to the high affinity (GMR$\alpha\beta_c$) but not the low affinity (GMRα) receptor. The results identified the presence of two functional domains of GM-CSF required for either GMRα

or GMR$\alpha\beta_c$ interaction and demonstrated that GM-CSF stimulation of both proliferation and mature cell activation are mediated through high affinity receptors.

The functional role of other residues in the first alpha helix was examined in light of the critical role determined for Glu^{21}. Oligonucleotide cassette mutagenesis (OCM) was developed to generate large numbers of mutants for expression in COS cells. Residues 14 and 17 to 28 of the first alpha helix of human GM-CSF were subjected to extensive substitution mutagenesis. Mutation of most amino acids buried in the hydrophobic core did not significantly impair biological activity or receptor binding apart from a modest decrease in biological activity observed with mutation of residues Ala^{22} and Leu^{26}. Mutation of Ile^{19} produced a marked decrease in biological activity and receptor binding, probably as a result of structural perturbations. Mutation of amino acids located on the surface of the first helix did not significantly impair biological activity or receptor binding, with the notable exception of Glu^{21} (discussed above). The conclusion was that residue 21 is the only significant β_c chain contact on the surface of the first helix.

The carboxy terminus of GM-CSF contains a prominent hydrophilic peak centred over the fourth alpha helix with a number of charged amino acids conserved across several species. The importance of these residues was examined by charge reversal mutagenesis and indicated a role in GM-CSF biological activity for Asp^{112}. Binding studies showed a reduction in high- and low-affinity binding for the D112R analogue. In this respect residue 112 appears to be functionally distinct from residue 21 and is apparently involved in binding to the GMRα chain.

An *E. coli* expression system was used to produce larger quantities of GM-CSF residue 21 analogues for detailed receptor binding studies and to enable structural characterisation. The analogues which included hydrophobic (Ala, Phe), hydrophilic (Gln) and basic (His, Lys, Arg) substitutions at position 21, proved refractive to expression but techniques were devised that enabled their expression and purification.

The *E. coli*-derived GM-CSF residue 21 analogues displayed a range of biological activities, yet they all exhibited low affinity binding characteristics on both GMRα and GMRαβ_c, similar to CHO cell-derived analogues. The E21R and E21K charge reversal analogues, were surprisingly devoid of activity. The lack of activity from the *E. coli*-derived E21R analogue was in marked contrast to the activity observed for the CHO cell-derived E21R analogue. These results demonstrated that although in some cases low affinity binding can lead to biological signalling, in other cases such as with the E21R and E21K analogues, low affinity binding can be dissociated from receptor activation and biological signalling.

The *E. coli*-derived E21R and E21K analogues, which bind GMRα chain without eliciting a functional response, were tested for antagonistic activity. Both analogues were effective antagonists of the GM-CSF-mediated proliferation of leukaemic cells and the GM-CSF-mediated release of superoxide anions from neutrophils. The antagonism was effective against glycosylated and non-glycosylated forms of GM-CSF and was specific for GM-CSF in that no antagonism of IL-3-mediated leukaemic cell proliferation or TNF-α-mediated neutrophil superoxide production was observed.

On the basis of the observations presented in this thesis, a model for the interaction of GM-CSF with the receptor complex is proposed that should also be applicable to related cytokines, IL-3 and IL-5 in particular. The critical feature of this model is that GM-CSF contains two functionally distinct receptor binding sites, one for the GMRα chain and another for the β_c chain. The GMRα chain binding site includes residues from the fourth helix and in particular the Asp^{112} residue. The β_c chain binding site includes only Glu^{21} of the first helix. A model to account for the antagonism of GM-CSF activity is presented which proposes that the antagonist sequesters the available GMRα chain into complexes unable to associate with the β_c chain and therefore prevents wild type GM-CSF interaction with the GM-CSF receptor complex. Possible clinical implications for GM-CSF antagonists are discussed.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

Signed **Date:** 30/8/94

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Chapter 1

Introduction

1.1 Haemopoiesis and the colony stimulating factors

Haemopoiesis is the process of blood cell production that under normal conditions is required to maintain the steady-state levels of mature blood cells. A typical feature of blood cells is their short life-span. In the human adult erythrocytes survive for only a few weeks, and every hour approximately 1 x 10^{10} erythrocytes die and must be replaced. The process of haemopoiesis is also flexible and responds to situations of haemopoietic stress such as blood loss or infection. All of the mature blood cells generated by the process of haemopoiesis originate from a population of self-renewing, pluripotential stem cells found predominantly in the bone marrow of adults. During haemopoiesis these stem cells proliferate and differentiate via a number of committed intermediate progenitors and eventually undergo terminal differentiation into the multiple components of the haemopoietic system. These include cells of the lymphoid, myeloid, megakaryocyte and erythroid lineages (reviewed in Metcalf, 1985; Whetton and Dexter, 1986).

Over many years, *in vitro* cell culture techniques were developed to study the process of haemopoiesis and established that the clonal proliferation and differentiation of haemopoietic progenitor cells was dependent upon the presence of soluble peptide growth factors. These growth factors were termed the haemopoietic colony-stimulating factors and are members of the larger family of regulatory molecules called cytokines. Members of the haemopoietic growth factor family, which now number more than 20, exhibit considerable functional overlap as well as functional pleiotropy by promoting cell proliferation, survival, differentiation commitment and the functional activation of mature cell responses.

Initially four haemopoietic colony stimulating factors were identified in both human and murine systems that were able to stimulate the formation of colonies of the granulocyte-macrophage lineage (reviewed in Metcalf, 1986; Clark and Kamen, 1987; Sieff, 1987; Morstyn and Burgess, 1988). Two of these factors, G-CSF and M-CSF, are relatively lineage specific giving rise to colonies of granulocytes and macrophages respectively. The other two factors are less lineage specific with GM-CSF able to generate colonies of both granulocytes and macrophages and IL-3, also known as multi-CSF, able to generate colonies of many different lineages. The development of other lineages requires the presence of appropriate growth factors such as erythropoietin for erythrocyte development and IL-2 which is a lymphocyte growth factor (Clark and Kamen, 1987).

1.2 Granulocyte-macrophage colony-stimulating factor

The initial characterisation of the biological activities of haemopoietic growth factors such as GM-CSF used material purified from natural sources. The advent of recombinant material enabled detailed characterisation of the *in vitro* and *in vivo* biological activities of GM-CSF. Experiments *in vitro* using purified native or recombinant GM-CSF have demonstrated that GM-CSF is a pleiotropic cytokine able to stimulate both the production of different haemopoietic lineages and the effector function of mature myeloid cells (reviewed in Clark and Kamen, 1987; Gasson, 1991). Human GM-CSF stimulates the formation of colonies of the granulocyte-macrophage lineage (Sieff *et al.*, 1985; Tomonaga *et al.*, 1986; Metcalf *et al.*, 1986) as well as the proliferation of leukaemic cell lines (Hoang *et al.*, 1986; Begley *et al.*, 1987b) and differentiation of the HL-60 cell line (Tomonaga *et al.*, 1986; Begley *et al.*, 1987a). Human GM-CSF is also able to stimulate the functional activation of mature cells of the granulocyte-macrophage lineage. Thus GM-CSF is able to enhance macrophage

activation (Grabstein *et al.*, 1986), adherence (Gamble *et al.*, 1989; Elliott *et al.*, 1990), HIV-1 production (Koyanagi *et al.*, 1988) and cytokine production (Bender *et al.*, 1993). Neutrophils (Gasson *et al.*, 1984; Weisbart *et al.*, 1985), eosinophils (Vadas *et al.*, 1983; Lopez *et al.*, 1986) and basophils (Haak-Frendscho *et al.*, 1988) are all functionally activated by GM-CSF incubation. The surface expression of adhesion molecules on neutrophils and monocytes (Griffin *et al.*, 1990) and the adhesion of neutrophils to endothelial cells (Gamble *et al.*, 1990) are also enhanced by GM-CSF.

The biological activities attributed to GM-CSF *in vitro* have in general been observable *in vivo* following recombinant GM-CSF administration in animal models or human patients. Recombinant human GM-CSF has been used *in vivo* to stimulate haemopoiesis in primates (Donahue *et al.*, 1986b) or humans following chemotherapy (Antman *et al.*, 1988; Brandt *et al.*, 1988; Socinski *et al.*, 1988) or bone marrow transplantation (Nemunaitis *et al.*, 1991) and in situations of myelodysplasia (Vadhan-Raj *et al.*, 1987; Ganser *et al.*, 1989). In patients with AIDS, administration of recombinant GM-CSF enhances neutrophil production and function (Baldwin *et al.*, 1988).

In contrast to these therapeutic applications for recombinant GM-CSF, a number of studies have implicated *in vivo* GM-CSF activity in disease conditions that include chronic inflammation and leukaemia. The presence of elevated levels of GM-CSF (Williamson *et al.*, 1988; Xu *et al.*, 1989; Alvaro-Gracia *et al.*, 1989) and activated neutrophils (Emery *et al.*, 1988) in the synovial fluid of patients with rheumatoid arthritis suggests that GM-CSF plays a pathological role in rheumatoid arthritis. The expression of GM-CSF by activated eosinophils (Moqbel *et al.*, 1991) and the presence of GM-CSF in bronchoalveolar lavage fluids from allergen-challenged atopic subjects (Kato *et al.*, 1992) and asthmatic subjects (Broide *et al.*, 1992), suggested an important role for GM-CSF in allergic inflammation. The over-expression of GM-CSF in transgenic mice carrying additional copies of the murine GM-CSF gene produces an accumulation of macrophages in the eyes and striated muscles leading to blindness, muscle damage and

premature death (Lang *et al.*, 1987). Myeloid leukaemia's such as AML (Hoang *et al.*, 1986; Begley *et al.*, 1987b), ALL (Freedman *et al.*, 1993) and CMMoL (Everson *et al.*, 1989) have demonstrated a proliferative response to paracrine GM-CSF as have other non-haemopoietic tumours such as certain small cell lung carcinomas (Baldwin *et al.*, 1989), osteogenic sarcoma's (Dedhar *et al.*, 1988) and certain colon adenocarcinoma's (Berdel *et al.*, 1989). Certain GM-CSF responsive AML populations are also able to express GM-CSF suggesting the possibility of an autocrine response to GM-CSF (Young and Griffin, 1986; Young *et al.*, 1987, 1988). The observation that factor-independent proliferation of an ALL cell line able to secrete and respond to GM-CSF was abolished by a GM-CSF neutralising MoAb is consistent with an autocrine response to GM-CSF (Freedman *et al.*, 1993).

GM-CSF is expressed from many different cell types including T-cells, macrophages, fibroblasts and endothelial cells (reviewed in Gasson, 1991). GM-CSF expression from T-cells is induced by factors such as lectin (Wong *et al.*, 1985a), anti-CD28 MoAb (Lindstein *et al.*, 1989), HTLV (Chan *et al.*, 1986) and IL-1 (Herrmann *et al.*, 1988) while expression from fibroblasts and endothelial cells is induced by the inflammatory stimuli TNF-α (Munker *et al.*, 1986; Broudy *et al.*, 1986) and IL-1 (Bagby *et al.*, 1986; Broudy *et al.*, 1987; Kaushansky *et al.*, 1988a). Human bone marrow stromal fibroblasts are able to produce GM-CSF (Charboard *et al.*, 1991) in response to factors in human serum as well as the inflammatory stimuli TNF-α and IL-1 (Guba *et al.*, 1992). Bacterial LPS increases the expression of GM-CSF from macrophages (Sieff *et al.*, 1988), osteoblasts (Horowitz *et al.*, 1989) and endothelial cells (Seelentag *et al.*, 1987). Stimulation of GM-CSF expression in the lungs and the presence of GM-CSF colony stimulating activity in the serum, has also been observed in mice following treatment with TNF-α or TNF-β (Kaushansky *et al.*, 1988b).

Under normal conditions GM-CSF is undetectable in the circulation and constitutive GM-CSF expression by populations of normal cells has not been observed (Chan *et al.*, 1986). These observations suggest that *in vivo*, GM-CSF is likely to

function in response to stress conditions such as blood loss or infection rather than as a regulator of steady-state processes. This idea is supported by the observation that GM-CSF-deficient mice exhibit no major perturbation of haemopoiesis up to 12 weeks of age but do develop abnormal lungs, frequently infected with opportunistic bacterial and fungal organisms and displaying a pathology similar to some forms of the human disorder, alveolar proteinosis (Stanley *et al.*, 1994; Lieschke *et al.*, 1994). This highlights the functional redundancy that exists within the haemopoietic system but also indicates that GM-CSF activity is clearly required *in vivo* for optimum functioning of the immune system.

Clones encoding the cDNA for human GM-CSF have been isolated from libraries made with human T-cell line mRNA as well as human peripheral blood T-lymphocyte mRNA (Wong *et al.*, 1985a; Lee *et al.*, 1985; Cantrell *et al.*, 1985). The cDNA for human GM-CSF encodes a 144 amino acid precursor protein and includes a 17 residue signal peptide that is cleaved off to yield a mature protein of 127 amino acids (Fig. 1.2.1). The amino acid sequence of GM-CSF from a number of different species has been deduced from the cloned GM-CSF cDNA. The other species for which GM-CSF sequences have been obtained include gibbon, bovine, canine, ovine, rat and murine GM-CSF. Comparison of the seven GM-CSF sequences shows that residues at 44 positions (35%) are absolutely conserved across all species while residues at 68 positions (54%) are conserved across at least six of the seven species (Fig. 1.2.2). The conserved residues are spread throughout the entire sequence with only one region, from residues 28 to 36, devoid of highly conserved residues. Despite the conservation of sequence, murine and human GM-CSF exhibit absolute species specificity (Metcalf *et al.*, 1986; Maliszewski *et al.*, 1988) while others such as human, bovine and canine GM-CSF exhibit partial cross reactivity (Maliszewski *et al.*, 1988; Nash *et al.*, 1991). The reasons for the differences in species-specificity exhibited by GM-CSF are unknown but are almost certainly a consequence of differences in the amino acid sequence. All of the GM-CSF sequences contain at least one potential N-linked glycosylation site (Asn-X-Thr/Ser) and four absolutely conserved Cys residues (Fig. 1.2.2). In human GM-CSF

Figure 1.2.1 Sequence of the human GM-CSF cDNA.

The human GM-CSF cDNA sequence (Wong *et al.*, 1985a), is flanked by EcoRI and HindIII restriction enzyme sites. The cDNA encodes a single open reading frame of 144 amino acids that includes a 17 amino acid signal peptide and 127 amino acid mature protein sequence. The sequence also contains a short, 8 nucleotide, 5' untranslated sequence and a significantly longer, 227 nucleotide, 3' untranslated sequence.

EcoRI(1) PstI(22) PstI(54)

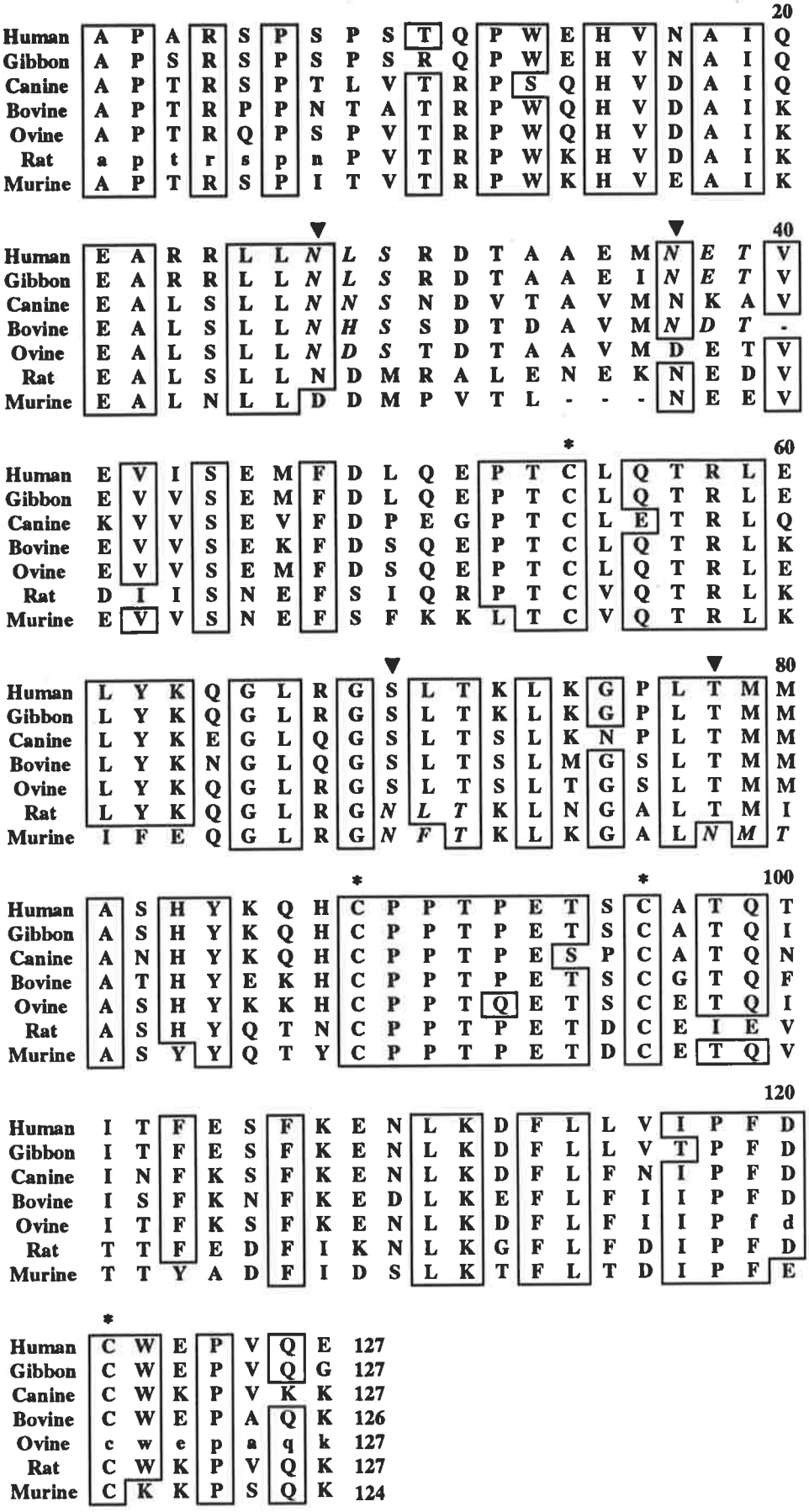
```
        10         20         30         40         50         60
GAATTCCGCTGGAGGATGTGGCTGCAGAGCCTGCTGCTCTTGGGCACTGTGGCCTGCAGC
               METTrpLeuGlnSerLeuLeuLeuLeuGlyThrValAlaCysSer

        70         80         90        100        110        120
ATCTCTGCACCCGCCCGCTCGCCCAGCCCCAGCACGCAGCCCTGGGAGCATGTGAATGCC
IleSerAlaProAlaArgSerProSerProSerThrGlnProTrpGluHisValAsnAla
       1                                  10

       130        140        150        160        170        180
ATCCAGGAGGCCCGGCGTCTCCTGAACCTGAGTAGAGACACTGCTGCTGAGATGAATGAA
IleGlnGluAlaArgArgLeuLeuAsnLeuSerArgAspThrAlaAlaGluMetAsnGlu
    20                                 30

       190        200        210        220        230        240
ACAGTAGAAGTCATCTCAGAAATGTTTGACCTCCAGGAGCCGACCTGCCTACAGACCCGC
ThrValGluValIleSerGluMetPheAspLeuGlnGluProThrCysLeuGlnThrArg
    40                                 50

       250        260        270        280        290        300
CTGGAGCTGTACAAGCAGGGCCTGCGGGGCAGCCTCACCAAGCTCAAGGGCCCCTTGACC
LeuGluLeuTyrLysGlnGlyLeuArgGlySerLeuThrLysLeuLysGlyProLeuThr
    60                                 70

       310        320        330        340        350        360
ATGATGGCCAGCCACTACAAGCAGCACTGCCCTCCAACCCCGGAAACTTCCTGTGCAACC
MetMetAlaSerHisTyrLysGlnHisCysProProThrProGluThrSerCysAlaThr
    80                                 90

       370        380        390        400        410        420
CAGACTATCACCTTTGAAAGTTTCAAAGAGAACCTGAAGGACTTTCTGCTTGTCATCCCC
GlnThrIleThrPheGluSerPheLysGluAsnLeuLysAspPheLeuLeuValIlePro
   100                                110

       430        440        450        460        470        480
TTTGACTGCTGGGAGCCAGTCCAGGAGTGAGACCGGCCAGATGAGGCTGGCCAAGCCGGG
PheAspCysTrpGluProValGlnGluEnd
   120

       490        500        510        520        530        540
GAGCTGCTCTCTCATGAAACAAGAGCTAGAAACTCAGGATGGTCATCTTGGAGGGACCAA

       550        560        570        580        590        600
GGGGTGGGCCACAGCCATGGTGGGAGTGGCCTGGACCTGCCCTGGGCCACACTGACCCTG

       610        620        630        640        650        660
ATACAGGCATGGCAGAAGAATGGGAATATTTTATACTGACAGAAATCAGTAATATTTATA

                          HindIII(682)
       670        680
TATTTATATTTTTACGTCGGGAAGCTT
```

Figure 1.2.2 Alignment of the GM-CSF protein sequence from seven different species.

The sequence of GM-CSF cDNA from seven different species has been determined and the amino acid sequence deduced. The largest GM-CSF comprises 127 amino acid with only bovine GM-CSF (126 amino acids) and murine GM-CSF (124 amino acids) differing in size. Sequences were aligned from the amino terminus of the mature protein and residues conserved in at least 6 of the seven sequences boxed. The sequence of GM-CSF exhibits conservation in all 7 species at 44 positions (35%) and conservation in at least 6 species at 68 positions (54%). The location of potential N-linked glycosylation sites are marked with a "▼" and the sites (N X T/S) italicised while the position of the conserved Cys residues are indicated with an asterisk.

The cDNA for rat and ovine GM-CSF was cloned by PCR using primers derived from murine and bovine GM-CSF respectively. Rat GM-CSF cDNA was amplified using primers designed from residues 1 to 7 of mature murine GM-CSF, and the 3' untranslated region of murine GM-CSF (Smith *et al.*, 1994). Ovine GM-CSF cDNA was amplified using primers designed from the amino terminus of the bovine GM-CSF signal peptide and residues 118 to 126 of bovine GM-CSF (O'Brien *et al.*, 1991). As a result, the actual sequence of rat GM-CSF residues 1 to 7 and ovine GM-CSF residues 119 to 127 have yet to be determined so the published sequence in this region is included in lower case only.

GM-CSF sequences; human (Wong *et al.*, 1985a), gibbon (Wong *et al.*, 1985b), canine (Nash *et al.*,1991), bovine (Maliszewski *et al.*,1988), ovine (O'Brien *et al.*, 1991), rat (Smith *et al.*,1994), murine (Gough *et al.*,1984, 1985).


20
Human A P A R S P S P S T Q P W E H V N A I Q
Gibbon A P S R S P S P S R Q P W E H V N A I Q
Canine A P T R S P T L V T R P S Q H V D A I Q
Bovine A P T R P P N T A T R P W Q H V D A I K
Ovine A P T R Q P S P V T R P W Q H V D A I K
Rat a p t r s p n P V T R P W K H V D A I K
Murine A P T R S P I T V T R P W K H V E A I K
40
Human E A R R L L N L S R D T A A E M N E T V
Gibbon E A R R L L N L S R D T A A E I N E T V
Canine E A L S L L N N S N D V T A V M N K A V
Bovine E A L S L L N H S S D T D A V M N D T -
Ovine E A L S L L N D S T D T A A V M D E T V
Rat E A L S L L N D M R A L E N E K N E D V
Murine E A L N L L D D M P V T L - - - N E E V
60
Human E V I S E M F D L Q E P T C L Q T R L E
Gibbon E V V S E M F D L Q E P T C L Q T R L E
Canine K V V S E V F D P E G P T C L E T R L Q
Bovine E V V S E K F D S Q E P T C L Q T R L K
Ovine E V V S E M F D S Q E P T C L Q T R L E
Rat D I I S N E F S I Q R P T C V Q T R L K
Murine E V V S N E F S F K K L T C V Q T R L K
80
Human L Y K Q G L R G S L T K L K G P L T M M
Gibbon L Y K Q G L R G S L T K L K G P L T M M
Canine L Y K E G L Q G S L T S L K N P L T M M
Bovine L Y K N G L Q G S L T S L M G S L T M M
Ovine L Y K Q G L R G S L T S L T G S L T M M
Rat L Y K Q G L R G N L T K L N G A L T M I
Murine I F E Q G L R G N F T K L K G A L N M T
100
Human A S H Y K Q H C P P T P E T S C A T Q T
Gibbon A S H Y K Q H C P P T P E T S C A T Q I
Canine A N H Y K Q H C P P T P E S P C A T Q N
Bovine A T H Y E K H C P P T P E T S C G T Q F
Ovine A S H Y K K H C P P T Q E T S C E T Q I
Rat A S H Y Q T N C P P T P E T D C E I E V
Murine A S Y Y Q T Y C P P T P E T D C E T Q V
120
Human I T F E S F K E N L K D F L L V I P F D
Gibbon I T F E S F K E N L K D F L L V T P F D
Canine I N F K S F K E N L K D F L F N I P F D
Bovine I S F K N F K E D L K E F L F I I P F D
Ovine I T F K S F K E N L K D F L F I I P f d
Rat T T F E D F I K N L K G F L F D I P F D
Murine T T Y A D F I D S L K T F L T D I P F E
127
Human C W E P V Q E 127
Gibbon C W E P V Q G 127
Canine C W K P V K K 127
Bovine C W E P A Q K 126
Ovine c w e p a q k 127
Rat C W K P V Q K 127
Murine C K K P S Q K 124

the four Cys residues are involved in two disulphide bonds between Cys^{54}/Cys^{96} and between Cys^{88}/Cys^{121} (Schrimsher *et al.*, 1987).

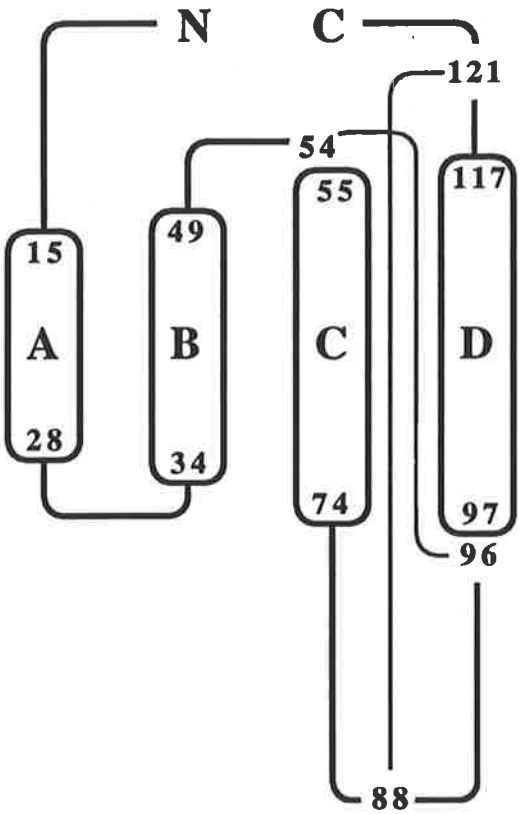
Naturally occurring human GM-CSF is extensively modified by the addition of both N- and O-linked carbohydrate (Lusis *et al.*, 1981; Donahue *et al.*, 1986a). The carbohydrate is not required for biological activity (Kaushansky *et al.*, 1987) and actually reduces the specific activity of GM-CSF (Moonen *et al.*, 1987; Cebon *et al.*, 1990), probably as a result of a reduced affinity for the GM-CSF receptor (Cebon *et al.*, 1990). The role of the carbohydrate moieties in GM-CSF activity remains unclear although *in vivo* studies have shown that the effective half-life of GM-CSF in the serum of rats (Donahue *et al.*, 1986a) or humans (Denzlinger *et al.*, 1993) is greatly enhanced by the presence of carbohydrate on GM-CSF.

At the beginning of this study the structure of human GM-CSF and many of the other cytokines had been predicted from molecular modelling studies to be based around a bundle of four α-helices (Parry *et al.*, 1988, 1991) (Fig. 1.2.3). The predicted structure of GM-CSF was consistent with circular dichroism measurements on purified human GM-CSF which indicated a high content of α-helical structure (Wingfield *et al.*, 1988). The three-dimensional structure for one of the cytokines, human IL-2, had been determined by x-ray crystallography (Brandhuber *et al.*, 1987) and was in agreement with the models proposed by Parry *et al.*, (1988, 1991). Although the original structure misinterpreted the location and connectivity of the helical regions within the IL-2 molecule, the essentially helical nature of IL-2 was correctly identified. Alignment of the amino acid sequences of GM-CSF and IL-2 demonstrated a low but significant level of sequence identity, indicating that these cytokines may be structurally homologous (Schrader *et al.*, 1986). During the course of this study, the three-dimensional structure of human GM-CSF was published (Diederichs *et al.*, 1991; Walter *et al.*, 1992a) and revealed a bundle of four α-helices with considerable structural homology to IL-2 (Rozwarski *et al.*, 1994). The empirically determined structure of human GM-CSF displayed some significant differences when compared with the predicted structure

Figure 1.2.3 The predicted structure of human GM-CSF.

A model for the structure of human GM-CSF was proposed by Parry *et al*, (1988) based on an analysis of the GM-CSF amino acid sequence with a number of algorithms predictive for protein secondary structure. The key feature of the GM-CSF fold illustrated in this topology diagram, is the presence of four α-helices (A-D) in an up-down-up-down arrangement.

 α helices A,B,C & D.


N
C
121
54
55
117
49
15
A
B
C
D
28
34
74
97
96
88

although as described in Chapter 3, these differences did not effect the work presented here.

Human GM-CSF exerts its biological effects by binding to receptors located on the surface of responsive cell types. Receptors capable of specifically binding ^{125}I-labelled human GM-CSF with high (K_d=10-50pM) or low (K_d=1-10nM) affinity have been detected on a variety of haemopoietic cells. These include bone marrow cells, primary myeloid leukaemia's and leukaemic cell lines (Gearing *et al.*, 1989; Chiba *et al.*, 1990) as well as neutrophils, eosinophils, monocytes (Gasson *et al.*, 1986; Park *et al.*, 1986; DiPersio *et al.*, 1988; Baldwin *et al.*, 1989; Elliott *et al.*, 1989) and basophils (Lopez *et al.*, 1990a, b, 1991). Similar high (K_d=20-60pM) and low (K_d=0.8-1.2nM) affinity receptors have also been detected for murine GM-CSF binding to bone marrow cells, myeloid cell lines and neutrophils (Walker and Burgess, 1985). Certain non-haemopoietic cells such as small cell lung carcinoma lines (Baldwin *et al.*, 1989) and endothelial cells (Bussolino *et al.*, 1989) express high affinity GM-CSF receptors while purified human placental cell membranes (Gearing *et al.*, 1989), melanoma cell lines (Baldwin *et al.*, 1991) and the simian COS cell line (Baldwin *et al.*, 1989), express low affinity GM-CSF receptors. The receptor binding studies suggested that cell types which are functionally responsive to GM-CSF are able to bind GM-CSF with high-affinity (Gasson, 1991).

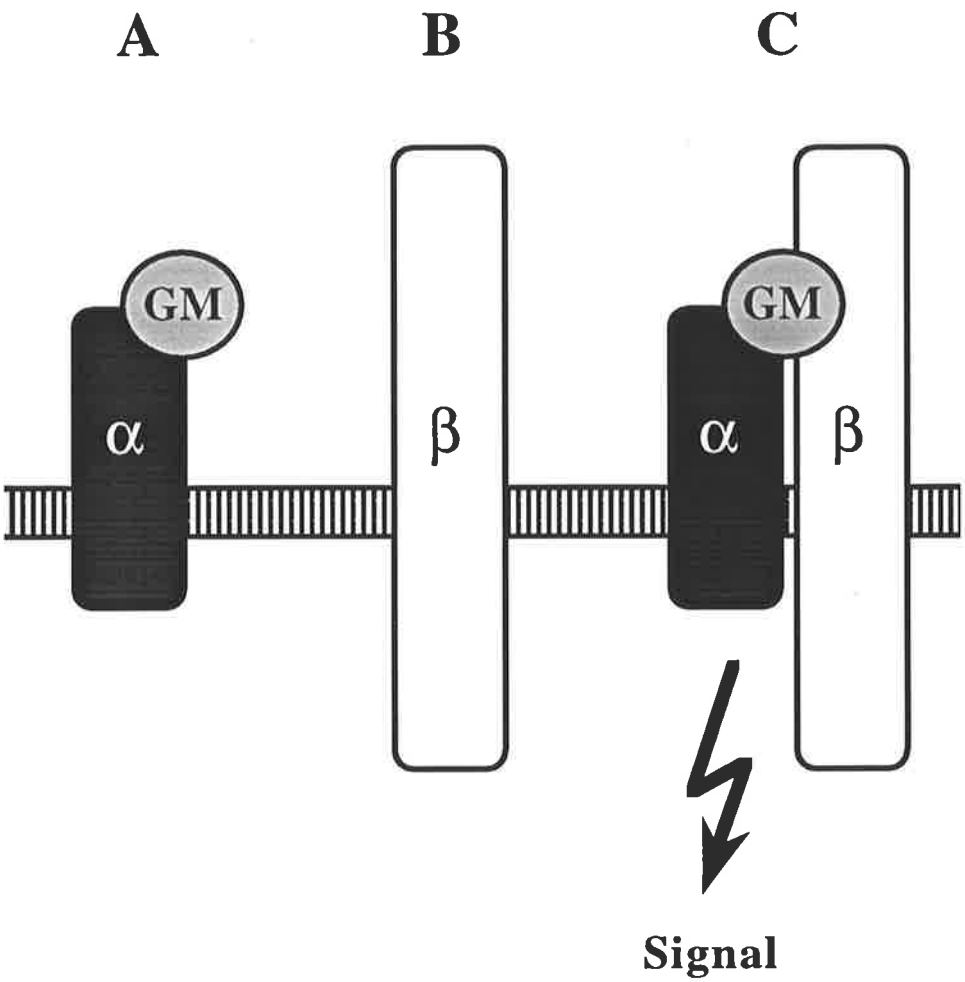
The specific binding of GM-CSF to certain cell types can be at least partially competed for by the heterologous ligands IL-3 and IL-5. This cross-competition of specific binding has been observed for GM-CSF and IL-3 on KG-1 cells (Park *et al.*, 1989a; Gesner *et al.*, 1989), acute nonlymphocytic leukaemia (Park *et al.*, 1989b), eosinophils (Lopez *et al.*, 1989) and monocytes (Park *et al.*, 1989a; Elliott *et al.*, 1989) and for GM-CSF, IL-5 and IL-3 on basophils (Lopez *et al.*, 1990a) and eosinophils (Lopez *et al.*, 1989, 1991). These results indicated that the receptors for GM-CSF, IL-3 and IL-5 are closely associated on the surface and suggested the existence of a common

receptor or at least a common receptor subunit that is shared by the receptors for these cytokines (Gearing *et al.*, 1989; reviewed in Lopez *et al.*, 1992a).

Receptor crosslinking experiments identified a number of potential receptor components, suggesting that the GM-CSF receptor is a multichain complex. A GM-CSF-specific receptor component with a molecular mass of 84-kDa was observed on neutrophils, myeloid cell lines (DiPersio *et al.*, 1988), CML cells, small cell lung carcinoma lines and COS cells (Baldwin *et al.*, 1989) and additional receptor components with molecular masses of 135-kDa and 100-kDa have been detected on neutrophils and the myeloid cell lines U937 and TF-1 (Chiba *et al.*, 1990). A cDNA clone encoding a GM-CSF binding protein was isolated from a placental cDNA library which, when transfected into COS cells, enables ^{125}I-GM-CSF to bind with low affinity (K_d=2-8nM) (Gearing *et al.*, 1989). Cross-linking studies estimated the molecular mass of the cloned low affinity receptor to be 85-kDa (Gearing *et al.*, 1989) in good agreement with the cross-linking studies on a number of different cell types. A second GM-CSF receptor chain was isolated which does not detectably bind GM-CSF by itself but when co-expressed with the cloned low affinity GM-CSF receptor forms a high-affinity GM-CSF receptor (Hayashida *et al.*, 1990). Cross-linking studies estimated the molecular mass of the second GM-CSF receptor chain to be 120-kDa and also indicated the presence of a complex containing the 80-kDa and 120-kDa receptor components in the presence of GM-CSF (Hayashida *et al.*, 1990). The low-affinity GM-CSF binding protein has been termed the α subunit (GMRα) of the GM-CSF receptor while the second, affinity converting protein has been termed the β subunit. Thus the low-affinity GM-CSF receptor comprises the GMRα chain alone while the biologically active, high-affinity receptor comprises a GMRαβ complex (Fig. 1.2.4). The discovery that the GM-CSF receptor β chain is shared by the receptors for IL-3 (Kitamura *et al.*, 1991b) and IL-5 (Tavernier *et al.*, 1991) led to this protein being termed the common β subunit (β_c). The existence of a receptor subunit that is shared by GM-CSF, IL-3 and IL-5 may account for some of the overlapping biological activities displayed by these cytokines and for the cross-competition of receptor binding.

Figure 1.2.4 The human GM-CSF receptor

A model illustrating the components of the high and low affinity GM-CSF receptors. Human GM-CSF is able to bind with low affinity to the GMRα chain by itself (A) but does not detectably bind to the β chain by itself (B). The high affinity GM-CSF receptor comprises a complex of the GMRα and β chains and is the biologically functional complex (C).


A
B
C
GM
α
β
GM
α
β
Signal
Low affinity
Not detected
High affinity
GM-CSF binding

Although the biological actions of GM-CSF were well described at the beginning of this study, very little detailed information was available concerning the structure-function properties of this cytokine and as a result, the mechanism of GM-CSF action was very poorly understood. Structure-function information can be obtained using many different approaches although many of the most powerful techniques utilise recombinant DNA technology. This enables the generation of protein analogues with precisely defined alterations. Analysis of the structural and functional properties of the protein analogues identifies regions and specific residues that are sensitive to mutation and that are therefore either structurally or functionally important in the wild type protein.

1.3 Project aims

The aim of this project was to study the structure-function properties of human GM-CSF and specifically to identify regions and residues that are important for the functional interaction of human GM-CSF with the high- and low-affinity GM-CSF receptor. Functionally important regions on the surface of GM-CSF are identified by comparing the biological activity and receptor binding properties of mutated GM-CSF analogues with wild type GM-CSF. The generation of human GM-CSF analogues with altered biological and receptor binding activities might also give rise to analogues with clinically useful properties such as the ability to antagonise GM-CSF function or selectively interact with restricted cell populations.

Chapter 2

Materials & Methods

2.1 Abbreviations

Standard abbreviations were as described for The Journal of Biological Chemistry, 1993, volume 268, pages 750-753. Non-standard abbreviations are listed below.

ALL	acute lymphoblastic leukaemia
AML	acute myeloid or myeloblastic leukaemia
AU	absorbance units
β_c	common beta chain of the GM-CSF, IL-3 and IL-5 receptors
BCIG	5-bromo-4-chloro-3-indolyl-β-D-galactoside
bp	base pair
BSA	bovine serum albumin
CAPS	3-cyclohexylamino-1-propanesulfonic acid
CML	chronic myeloid leukaemia
CMMoL	chronic myelomonocytic leukaemia
CNTF	ciliary neurotrophic factor
DAB	3,3' diaminobenzidine
DMEM	Dulbecco's Modified Eagle's medium
DTT	dithiothreitol
ED_{50}	effective dose, 50%
EPO	erythropoietin
FCS	foetal calf serum
FMLP	N-formyl methionyl leucyl phenylalanine (f-Met-Leu-Phe)
G-CSF	granulocyte colony-stimulating factor
GH	growth hormone
GHbp	growth hormone binding protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMRα	GM-CSF receptor alpha chain
HBSS	Hank's balanced salt solution
HFBA	heptafluorobutyric acid
HIV	human immunodeficiency virus
HP-SEC	high performance size exclusion chromatography
HTLV	human T-cell leukaemia virus
IC_{50}	inhibitory concentration, 50%
IL	interleukin
IL-3Rα	IL-3 receptor alpha chain

IPTG	isopropyl β-D-thiogalactoside
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
K_d	dissociation constant
kDa	kilodalton
mA	milliamps
M-CSF	macrophage colony-stimulating factor
MoAb	monoclonal antibody
Mr	relative molecular mass
NEM	N-ethyl morpholine
NP-40	Nonidet P-40
OCM	oligonucleotide cassette mutagenesis
OSM	oncostatin M
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol 8,000
PVP	polyvinyl pyrrolidone
RP-HPLC	reversed phase high performance liquid chromatography
RIA	radioimmunoassay
RNase A	ribonuclease A
RSV-LTR	rous sarcoma virus long terminal repeat
S.A.	specific activity
SCF	stem cell factor
SEM	standard error of the mean
SV40	simian virus 40
TEMED	N,N,N',N'-tetramethyl-ethene-diamine
TFA	trifluoroacetic acid
TNF	tumour necrosis factor
TMACl	tetramethylammonium chloride
Tween 20	polyoxyethylene (20)-sorbitan monolaurate
USP	universal sequencing primer

2.2 Chemicals, reagents and consumables

Standard chemicals were obtained from Ajax Chemicals (Auburn, NSW), BDH Chemicals (Poole, UK) and Sigma Chemical Company (St. Louis, MO)

Ethanolamine : Ajax Chemicals, Auburn, NSW.

TMACl : Aldrich Chemical Company Inc., Milwaukee, WI.

Centricon-10, Centriprep-10, Diaflo YM10 membrane : Amicon, Danvers, MA.

PEG, TFA Spectrosol, thioglycolic acid, Tween 20, urea : BDH Chemicals, Poole, UK.

Acrylamide, bisacrylamide, broad range biotinylated SDS/PAGE calibration standards, Coomassie brilliant blue R-250, Econo columns, anti-mouse immunobeads, anti-rabbit immunobeads, anti-sheep immunobeads, Econo-Pac Q columns, Macro-Prep Q anion exchange support, TEMED : Bio-Rad, Richmond, CA.

Chondroitin sulphate : Calbiochem, La Jolla, CA.

BSA, penicillin : Commonwealth Serum Laboratories, Melbourne, Victoria.

Gentamicin, heparin : Delta West, Bentley, Western Australia.

E. coli-derived TNF-α : Genentech, South San Francisco, CA.

CHO cell-derived human GM-CSF, *E. coli*-derived human IL-3, sheep anti-GM-CSF polyclonal antisera : gift from Dr. S. Clark, Genetics Institute, Cambridge, MA.

Agar, caesium chloride, DMEM, foetal bovine serum, G-418 (Geneticin), Ham's F12 nutrient mixture, peptone, RPMI, yeast extract : Gibco Laboratories, Glen Waverly, Victoria.

Yeast-derived human GM-CSF : gift from Dr. Linda Park, Immunex Corporation, Seattle, WA.

E. coli-derived human GM-CSF : gift from Dr. George Morstyn, Melbourne Tumour Biology branch, Ludwig Institute for Cancer Research, Melbourne, Vic.

Isopropanol ChromAR HPLC : Mallinckrodt Specialty Chemicals Co., Paris, KY.

Acetonitrile LiChrosolv, amido black : Merck, Dormstadt, FRG.

Imobilon PVDF membrane, PLGC 10,000 Mr regenerated cellulose membrane : Millipore Corporation, Bedford, MA.

Lymphoprep : Nycomed, Oslo, Norway.

Tryptone : Oxoid, Basingstoke, UK.

CM-Sepharose CL-6B columns, CNBr-activated Sepharose 4B, Dextran T-500, dideoxyribonucleotide triphosphates, deoxyribonucleotide triphosphates, NP-40, ribonucleotide triphosphates, low molecular weight protein standards for SDS/PAGE, Sephadex G-100, Sephadex G-25 PD-10 columns, Sephacryl S-200 : Pharmacia, Uppsala, Sweden.

BCIG, IPTG : Promega Corporation, Madison, WI.

Nitrocellulose : Schleicher and Schuell, Dassel, FRG.

Agarose, ampicillin, BSA, chloramphenicol, cytochrome-*c*, DTT, EDTA, ethidium bromide, ficoll, FMLP, HFBA, NEM, PVP, salmon sperm DNA, SDS, tetracycline, Tris base, tunicamycin : Sigma Chemical Company, St. Louis, MO.

Avidin DH, biotinylated rabbit anti-sheep IgG immunoglobulin : Vector Laboratories, Burlingame, CA.

No. 6 filter paper, 3MM : Whatman International Ltd. Maidstone, U.K.

2.3 Radiochemicals and radiolabelled reagents

Reagents were obtained from the following sources:

[α-^{32}P]dATP (3,000 Ci/mmol), [α-^{35}S]dATP (1,500 Ci/mmol), [γ-^{32}P]ATP (4,000 Ci/mmol) : BRESATEC, Adelaide, S.A.

$Na_2{}^{51}CrO_4$ (200 Ci/mmol), $Na^{125}I$ (2,500 Ci/mmol), ^{125}I-GM-CSF CHO cell-derived (98μCi/μg) : DuPont Australia, Melbourne, Vic.

[^{3}H]thymidine (6.7 Ci/mmol) : ICN, Costa Mesa, CA.

2.4 Enzymes

All restriction enzymes were purchased from New England Biolabs (Beverly, MA.) or Pharmacia (Uppsala, Sweden).

Other enzymes were obtained from the sources listed;

Calf intestinal phosphatase : Boehringer Mannheim, Mannheim, FRG.

T4 DNA ligase, T4 polynucleotide kinase : New England Biolabs, Beverly, MA.

Thermus aquaticus DNA polymerase : The Perkin Elmer Corporation, Norwalk, CT.

E. coli DNA polymerase I Klenow fragment, T7 DNA polymerase, T4 DNA ligase, T4 polynucleotide kinase : Pharmacia, Uppsala, Sweden.

Lysozyme, ribonuclease A : Sigma Chemical Company, St. Louis, MO.

Sequenase sequencing kit : United States Biochemical Corporation, Cleveland, OH.

Biotinylated horseradish peroxidase H : Vector Laboratories, Burlingame, CA.

2.5 Bacterial strains and genotypes

AB1899	thr-1 leuB6 thi-1 argE3 his G4 proA2 lon-1 lacY1 galK2 mtl-1 xyl-5 ara-14 strA31 tsx-33 λ supE44 Gift from Dr. Robert Kastelein, DNAX Research Institute, Palo Alto, CA. (Greenberg *et al.*, 1988)
B178htrA63	degP Gift from Dr. Renato Moreno, Department of Microbiology and Immunology, University of Adelaide, S.A.
BB4	F'[proAB$^+$ lacI^q lacZΔM15 Tn10 Tet^r], supF58 supE44 hsdR514 (r_k^- m_k^-) galK2 galT22 trpR55 metB1 tonA λ$^-$ Δ(arg-lac)U169 (Stratagene Inc., La Jolla, CA.)
BL21	F$^-$ ompT hsdS$_B$ ($r_B^-m_B^-$) *E. coli* B strain so is probably deficient in lon protease Gift from Dr. Renato Moreno, Department of Microbiology and Immunology, University of Adelaide, S.A. (Studier and Moffatt, 1986; Studier *et al.*, 1990)
E299	F$^-$ lacΔ lonΔ100 Tn10 Tet^r strA thi araD139 Gift from Dr. Justin Dibbens, Department of Biochemistry, University of Adelaide, S.A.
MC1061	araD139 Δ (araABC-leu)$_{7679}$ galE15 galK16 Δ (lac) X74 rpsL (Str^r) galU thi hsdR2 (r_k^- m_k^+) mcrA mcrB1 (New England Biolabs, Beverly, MA.)
JM101	F'traD36 lacI^q Δ (lacZ) M15 proA$^+$B$^+$ / supE thi Δ (lac-proAB) (New England Biolabs, Beverly, MA.)

TOPP 1,2,4,5,6 F'[proAB$^+$ lacI^q lacZ Δ M15 Tn10 Tet^r] rif^r

TOPP 3 F'[proAB$^+$ lacI^q lacZ Δ M15 Tn10 Tet^r] rif^r kan^r

(Stratagene Inc., La Jolla, CA.)

2.6 Cloning and expression vectors

M13mp19 was purchased from New England Biolabs, Beverly, MA.

pGEX-2T was purchased from Pharmacia, Uppsala, Sweden.

pIN-III-OmpH3 (Lundell *et al.*, 1990) was a gift from Dr. Robert Kastelein, DNAX Research Institute, Palo Alto, CA.

pJL4 (Gough *et al.*, 1985) was a gift from Dr. Nicholas Gough, Melbourne Tumour Biology branch, Ludwig Institute for Cancer Research, Melbourne, Vic.

pRc/CMV was purchased from Invitrogen Corporation, San Diego, CA.

pRSVN.07 was a gift from Dr. Allan Robbins, Department of Biochemistry, University of Adelaide, S.A.

2.7 Cloned DNA sequences

Human GM-CSF cDNA (Wong *et al.*, 1985a) was a gift from Dr. Steve Clark, Genetics Institute, Cambridge, MA.

Human GM-CSF synthetic cDNA (Shanafelt and Kastelein, 1991a) in pIN-III-OmpH3 was a gift from Dr. Robert Kastelein, DNAX Research Institute, Palo Alto, CA.

Human GM-CSF receptor alpha chain cDNA (Gearing *et al.*, 1989) in πH3 was a gift from Dr. Nicos Nicola, The Walter and Eliza Hall Institute, Melbourne, Vic.

2.8 Standard solutions and bacterial media

2.8.1 Standard solutions

Coupling buffer	0.5M NaCl, 0.1M Na_2CO_3, 0.1M $NaHCO_3$ pH8.3.
20mg/ml BCIG	Dissolved in dimethyl formamide and stored at -20°C.
Binding medium	RPMI 1640, 10mM HEPES, 0.5% (w/v) BSA and 0.1% (w/v) sodium azide
Blotto solution	1xPBS, 0.05% (v/v) Tween 20, 2% (w/v) BSA and 5% (w/v) Skim milk powder.
Denhardt's solution (1x)	0.02% (w/v) Ficoll, 0.02% (w/v) BSA, 0.02% (w/v) PVP.
Dextran	5% (w/v) Dextran T-500 in 1 x PBS.
1xDSS	900mM NaCl, 90mM sodium citrate pH7.4, 0.5% (w/v) SDS, 0.02% (w/v) Ficoll, 0.02% (w/v) BSA, 0.02% (w/v) PVP.
1xHBSS	136mM NaCl, 5.4mM KCl, 4.2mM $NaHCO_3$, 0.4mM KH_2PO_4, 0.3mM Na_2HPO_4.
HEPES buffered saline	20mM HEPES, 137mM NaCl, 5mM KCl, pH7.0
24mg/ml IPTG	Dissolved in sterile MilliQ and stored at -20°C.
6xNET	900mM NaCl, 90mM Tris-HCl pH7.6, 9mM EDTA, 0.5% (v/v) NP-40, 0.02% (w/v) Ficoll, 0.02% (w/v) BSA, 0.02% (w/v) PVP.
PAT buffer	1xPBS, 0.1% (w/v) sodium azide, 0.01% (v/v) Tween 20.
PBS (1x)	136mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4.
PTB buffer	1xPBS, 0.05% (v/v) Tween 20, 0.5% (w/v) BSA.
10mg/ml RNase A (DNase free)	10mg/ml bovine pancreatic RNase A in 10mM Tris-HCl pH7.5, 15mM NaCl. Incubated for 15' at 100°C, cooled slowly to 25°C and aliquots stored at -20°C.
3M Sodium acetate	3M sodium acetate adjusted to pH4.6-5.5 with glacial acetic acid
SSC (1x)	150mM NaCl, 15mM sodium citrate pH7.4.
TAE buffer (1x)	40mM Tris, 20mM acetic acid, 0.9mM EDTA
TBE buffer (1x)	50mM Tris, 42mM boric acid, 1mM EDTA.
TE	10mM Tris-HCl pH7.5, 0.1mM EDTA.
TEG	25mM Tris-HCl pH8.0, 10mM EDTA, 50mM glucose. Autoclaved.
TES	25mM Tris-HCl pH8.0, 10mM EDTA, 15% (w/v) sucrose. Autoclaved.
TMACl solution	3M TMACl, 50mM Tris-HCl pH8.0, 0.1% (w/v) SDS 2mM EDTA.

TS	10mM Tris-HCl pH8.0, 20% (w/v) sucrose. Autoclaved.

2.8.2 Bacterial media

0.7% agar	170mM NaCl, 1.0% (w/v) tryptone, 0.7% (w/v) agar. Autoclaved.
L-agar	L-broth, 1.5% (w/v) agar. Autoclaved.
Luria broth (L-broth)	170mM NaCl, 0.5% (w/v) yeast extract, 1.0% (w/v) tryptone or peptone. Autoclaved.
Minimal medium	60mM K_2HPO_4, 33mM KH_2PO_4, 7.6mM $(NH_4)_2SO_4$, 1.7mM sodium citrate. After autoclaving, final concentrations of 1.7mM $MgSO_4$, 14.8µM Thiamine and 0.2% (w/v) glucose were added.
Minimal agar	1.5% (w/v) agar. After autoclaving, all components of minimal medium were added.
SOC medium	10mM NaCl, 2.3mM KCl, 0.5% (w/v) yeast extract, 2.0% (w/v) tryptone, or peptone. After autoclaving, final concentrations of 10mM $MgCl_2$, 10mM $MgSO_4$ and 0.36% (w/v) glucose were added.
Terrific broth	2.4% (w/v) yeast extract, 1.2% (w/v) tryptone, 0.4% (v/v) glycerol. After autoclaving, a final concentration of 100mM KPO_4 pH7.0 was added.
2xYT	85mM NaCl, 1.0% (w/v) yeast extract, 1.6% (w/v) tryptone or peptone. Autoclaved.

2.9 Molecular weight standards

EcoRI digested bacteriophage SPP-1 DNA molecular weight markers were obtained from BRESATEC (Adelaide, SA). Approximate fragment sizes in Kbp are: 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, 0.36.

HpaII digested pUC19 DNA molecular weight markers were obtained from BRESATEC (Adelaide, SA). Fragment sizes in base pairs are: 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 34, 26.

Low molecular weight protein standards for SDS/PAGE from Pharmacia (Uppsala, Sweden).

Phosphorylase b (rabbit muscle)	94kDa
Albumin (bovine serum)	67kDa
Ovalbumin (hen egg white)	43kDa

Carbonic anhydrase (bovine erythrocyte)	30kDa
Trypsin inhibitor (soybean)	20.1kDa
α-Lactalbumin (bovine milk)	14.4kDa

Broad range, biotinylated SDS/PAGE standards from Bio-Rad (Richmond, CA).

Biotinylated myosin (rabbit muscle)	200kDa
Biotinylated β-galactosidase (*E. coli*)	116.5kDa
Biotinylated phosphorylase B (rabbit muscle)	97.4kDa
Biotinylated albumin (bovine serum)	66.2kDa
Biotinylated ovalbumin (hen egg white)	45kDa
Biotinylated carbonic anhydrase B (bovine erythrocyte)	31kDa
Biotinylated trypsin inhibitor (soybean)	21.5kDa
Biotinylated lysozyme (hen egg white)	14.4kDa
Biotinylated aprotinin (bovine pancreas)	6.5kDa

2.10 Methods

2.10.1 Processing oligonucleotides

2.10.1A Synthesis, cleavage and deprotection

Oligonucleotides were synthesised on an Applied Biosystems model 381A DNA synthesiser (Applied Biosystems, Foster City, CA) by Heath Suskin and Julie Phillips. Following trityl-off synthesis, oligonucleotides were cleaved from the support by incubating in 700µl of ammonium hydroxide for 15' at room temperature. The cleavage reaction was repeated twice and the pooled fractions of crude oligonucleotide deprotected by incubation at 56°C overnight. Oligonucleotides prepared using FOD (fast oligonucleotide deprotection) chemistry were deprotected by incubation at 56°C for 2 hours or at room temperature overnight.

The mixture containing crude oligonucleotide was thoroughly mixed with 30mls of water-saturated butanol and centrifuged at 15,000 rpm, 4°C for 10' in a Beckman JA-20 rotor (Beckman Instruments, Palo Alto, CA). The pelleted oligonucleotide was dried and

resuspended in 500µl of MilliQ. As an alternative, oligonucleotide was recovered by lyophilisation and resuspended in 500µl of MilliQ. Oligonucleotides were quantified by measuring the OD_{260nm} and assuming 1AU = 33µg/ml.

2.10.1B PAGE purification

Full length oligonucleotides were purified from the crude synthesis mixture by polyacrylamide gel electrophoresis. Typically gels contained 20% (w/v) acrylamide with an acrylamide:bisacrylamide ratio of 30:1, 8M urea, 1xTBE buffer and were polymerised by adding final concentrations of 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulphate. For oligonucleotides larger than 40 nucleotides in length, the concentration of acrylamide was reduced to 10%. Gels were pre-electrophoresed at 350 volts for at least 30' in 1xTBE buffer. Samples were prepared in a load buffer containing final concentrations of 40% (v/v) deionised formamide, 1mM EDTA, 0.002% (w/v) bromophenol blue, 0.002% (w/v) xylene cyanole and incubated at 100°C for 5' before loading onto the gel. Gels were electrophoresed at 400 to 500 volts and then stained for 15' in a solution of 10µg/ml ethidium bromide. Gels were visualised using ultra violet light, and the full length oligonucleotides excised and eluted in 5mls of TE at 37°C overnight. Eluates were then filtered through a 0.45µM cellulose acetate filter and DNA precipitated by adding 500µl of 3M sodium acetate pH5.5, 12.5mls of 100% ethanol and incubating at -20°C overnight. Purified oligonucleotides were pelleted by centrifugation at 15,000 rpm, 4°C for 30' in a JA-20 rotor, washed with 5mls 70% (v/v) ethanol and centrifuged at 15,000 rpm, 4°C for 15' in a JA-20 rotor. The oligonucleotide pellets were dried, resuspended in TE and quantified.

Oligonucleotides were also separated from un-incorporated γ-^{32}P-ATP following phosphorylation (2.10.1C) by polyacrylamide gel electrophoresis. Following electrophoresis, as described above, gels were carefully removed from the glass plates, wrapped in cling film and autoradiographed. The ^{32}P-labelled, full length oligonucleotides, identified from the autoradiograph, were excised and eluted at 37°C overnight in 1ml of TE.

2.10.1C 5' phosphorylation of oligonucleotides

Oligonucleotides were 5' phosphorylated with γ-^{32}P-ATP for use as probes. A 20µl reaction containing 100 to 500ng of purified oligonucleotide, 50mM Tris-HCl pH 7.4, 1mg/ml BSA, 10mM $MgCl_2$, 5mM DTT, 20µCi γ-^{32}P-ATP and 20 units of T4 polynucleotide kinase was incubated at 37°C for 45'. Un-incorporated γ-^{32}P-ATP was separated from 5' phosphorylated oligonucleotides by electrophoresis on an 8M urea/20% acrylamide gel (2.10.1B).

Oligonucleotides were 5' phosphorylated with ATP for use in the M13 mutagenesis procedure (2.10.4). A 20µl reaction containing 100ng of purified oligonucleotide, 50mM Tris-HCl pH 7.4, 1mg/ml BSA, 10mM $MgCl_2$, 5mM DTT, 1mM ATP and 20 units of T4 polynucleotide kinase was incubated at 37°C for 45'. Enzyme was inactivated by incubation at 65°C for 5'.

2.10.1D Preparation of oligonucleotide fragments for OCM

Complimentary oligonucleotides were 5' phosphorylated with ATP and annealed to form double stranded fragments used in the OCM procedure. A 40µl reaction containing 500ng of each of the purified, complementary oligonucleotides, 50mM Tris-HCl pH 7.4, 1mg/ml BSA, 10mM $MgCl_2$, 5mM DTT, 1mM ATP and 20 units of T4 polynucleotide kinase was incubated at 37°C for 60'. The mixture was then incubated at 100°C for 5' and allowed 15' to cool to room temperature, allowing the complementary oligonucleotides to anneal. Volume was adjusted to 100µl with MilliQ and reaction extracted once with 100µl of buffer-saturated phenol:chloroform:iso-amyl alcohol (25:24:1), vortexed vigorously and centrifuged for 5' in a micro-centrifuge. DNA was precipitated by adding 10µl of 3M sodium acetate pH5.5 and 250µl of 100% ethanol to the aqueous phase and incubating at -70°C for 30'. Centrifugation for 15' in a micro-centrifuge at 4°C pelleted the DNA which was washed with 500µl of 70% (v/v) ethanol, dried and resuspended in 40µl of TE buffer. The procedure for fragments composed of more than two oligonucleotides was essentially the same with a 2x reaction volume for four oligonucleotides and a 3x reaction volume for six oligonucleotides.

2.10.2 The polymerase chain reaction

A standard PCR protocol was used to amplify defined DNA fragments with terminal restriction enzyme sites. The typical 50µl reaction contained 5µM of each of the purified oligonucleotide primers, 10mM Tris-HCl pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.01% (v/v) glycerol, 200µM dATP, 200µM dGTP, 200µM dCTP, 200µM dTTP, 1 unit of *Taq* DNA polymerase and 1ng of template DNA. The reaction mix was overlayed with 100µl of paraffin oil and subjected to 25 to 30 cycles of PCR. The standard PCR profile consisted of a 1' incubation at 94°C to denature the DNA, a 2' incubation at 52°C to anneal the primers and template and a 1' incubation at 72°C for primer extension.

A modified PCR protocol was used to screen bacterial colonies for the presence of plasmids carrying inserts in the correct orientation (Sandhu *et al.*, 1989). This same protocol was also used to screen M13 plaques for correctly orientated inserts. The typical 50µl reaction contained all the components of the standard reaction without polymerase and with DNA template obtained from a colony or plaque. The reaction mix was overlayed with 100µl of paraffin oil, incubated at 100°C for 10' and cooled slowly to 50°C. One µl of *Taq* DNA polymerase, diluted to 0.5 units/µl in 20mM Tris-HCl pH 8.4, 100mM KCl, 200mg/ml gelatin, was added and the reaction mix subjected to 25 cycles of the standard PCR profile.

2.10.3 Maintenance and transformation of *E. coli*

2.10.3A Maintenance of strains

The genotypes of the *E. coli* strains used during the course of the work described in this thesis are listed in Section 2.5. Glycerol stocks of all strains were prepared by mixing 500µl of fresh overnight culture with 500µl of 80% (v/v) glycerol and storing at -70°C.

AB1899, BL21 and MC1061 were maintained on L-agar plates and incubated at 37°C.

B178htrA63 was maintained on L-agar plates and incubated at room temperature.

BB4, E299 and TOPP strains were maintained on L-agar plates containing 10µg/ml tetracycline and incubated at 37°C.

JM101 was maintained on minimal agar and incubated at 37°C.

2.10.3B Transformation of *E. coli* made competent with calcium chloride

1. Transformation of MC1061

A fresh single colony of MC1061 was inoculated into 10mls L-broth and incubated at 37°C overnight. The following day, 500µl of the overnight culture was added to 50mls of L-broth and incubated at 37°C with shaking, to an OD_{600nm} of 0.4 to 0.6. The culture was chilled on ice for 30' and the cells pelleted by centrifugation at 5,000 rpm, 4°C for 5' in a JA-20 rotor. The cells were gently resuspended in 25mls of ice-cold, sterile 100mM $MgCl_2$ and then pelleted again by centrifugation at 5,000 rpm, 4°C for 5' in a JA-20 rotor. The cells were gently resuspended in 2mls of ice-cold, sterile 100mM $CaCl_2$ and incubated on ice for 1 hour.

Competent MC1061 were gently resuspended and 200µl aliquots mixed with 1 to 20µl of DNA and incubated on ice for 30'. Mixture was then heat shocked at 42°C for 2' and left at room temperature for 5'. One ml of L-broth was added and the mixture incubated at 37°C for 30'. Cells were pelleted by centrifugation for 1' in a micro-centrifuge at room temperature and 1ml of the supernatant discarded. The bacterial pellet was resuspended in the remaining supernatant, spread onto L-agar plates containing 100µg/ml ampicillin and incubated at 37°C overnight.

The procedure described for MC1061 was also effective at transforming the AB1899, BL21, BB4 and E299 strains. The B178htrA63 strain was also transformed using this procedure but the bacteria were cultured at room temperature and the heat shock was performed at 37°C.

2. Transformation of JM101

A fresh single colony of JM101 was inoculated into 10mls minimal media and incubated at 37°C overnight. The following day, 500µl of the overnight culture was added to 50mls of 2xYT broth and incubated at 37°C, with shaking, to an OD_{600nm} of 0.4 to 0.6. The culture was chilled on ice for 30' and the cells pelleted by centrifugation at 5,000 rpm, 4°C for 5' in a JA-20 rotor. The cells were gently resuspended in 25mls of ice-cold, sterile 100mM $CaCl_2$ and then pelleted again by centrifugation at 5,000 rpm, 4°C for 5' in a JA-20 rotor. The cells were gently resuspended in 2mls of ice-cold, sterile 100mM $CaCl_2$ and incubated on ice for 1 hour.

Competent JM101 were gently resuspended and 200µl aliquots mixed with 1 to 20µl of DNA and incubated on ice for 30'. Cells were heat shocked at 42°C for 2' and added to 3mls of 0.7% agar seeded 1:100 with a log-phase culture of JM101. The mixture was plated on minimal media plates and incubated at 37°C overnight. For blue-white colour selection, 20µl of 24mg/ml IPTG and 20µl of 20mg/ml BCIG was added to the plating mixture.

2.10.3C Transformation of *E. coli* by electroporation

This procedure was specifically used to transform all six of the TOPP *E. coli* strains which exhibit a very low transformation efficiency as a result of their non-K12 background but can be used for all strains of *E. coli*.

A fresh single colony of TOPP *E. coli* was inoculated into 20mls of L-broth with 10µg/ml tetracycline and incubated at 37°C overnight. The following day, 10mls of the overnight culture was added to 1L of L-broth with 10µg/ml tetracycline and incubated at 37°C, with shaking, to an OD_{600nm} of 0.6. The culture was chilled on ice for 30' and the cells pelleted by centrifugation at 5,000 rpm, 4°C for 10' in a Beckman JA-10 rotor (Beckman Instruments, Palo Alto, CA). Pelleted bacteria were then washed by gentle resuspension in 250mls of ice-cold 1mM HEPES, 10% (v/v) glycerol followed by a second wash with 125mls. Bacteria were pelleted by centrifugation at 5,000 rpm, 4°C for

10' in a JA-10 rotor. Pelleted bacteria were then washed by gentle resuspension in 50mls of ice-cold 10% (v/v) glycerol followed by a second wash with 25mls. Bacteria were pelleted by centrifugation at 5,000 rpm, 4°C for 10' in a JA-20 rotor. Finally, bacteria were resuspended in 1ml 10% (v/v) glycerol and 40µl aliquots stored at -70°C.

An aliquot of electrocompetent cells was thawed and mixed with 1µl of DNA in low salt buffer. The mixture of cells plus DNA was then placed in a chilled 2mm cuvette and pulsed at 25µF and 2,500 volts with a resistance of 200Ω using a Bio-Rad Gene Pulser (Richmond, CA). Typical time constants were between 4.1 and 4.9. Immediately after the pulse, 1ml of SOC medium at room temperature was added to the cuvette and the cells incubated at 37°C for 30'. Cells were pelleted by centrifugation for 1' in a micro-centrifuge at room temperature and 1ml of the supernatant discarded. The bacterial pellet was resuspended in the remaining supernatant, spread onto L-agar plates containing 10µg/ml tetracycline, 100µg/ml ampicillin and incubated at 37°C overnight.

2.10.4 M13 mutagenesis protocol

The standard protocol for the mutagenesis of genes carried by the M13 bacteriophage was based on the procedure described by Zoller and Smith (1984).

2.10.4A The mutagenesis reaction

A standard reaction comprised 100ng of single stranded M13 DNA containing the GM-CSF cDNA, 5ng of each 5'-phosphorylated oligonucleotide (2.10.1C), USP and the mutagenic primer, 60mM Tris-HCl pH7.5, 9mM $MgCl_2$ and 67mM NaCl in a volume of 15µl. The mixture was incubated at 65°C for 5' and cooled at room temperature for 5'. The reaction mixture was increased to a final volume of 50µl containing 50µM of dATP, dGTP, dCTP and dTTP, 1mM ATP, 1mM DTT, 2 units of *E. coli* DNA I polymerase Klenow fragment, 1 unit of T4 DNA ligase and incubated at room temperature overnight. Competent JM101 were then transformed (2.10.3B) with 0.5, 1 and 2µl aliqouts of the mutagenesis reaction, plated on minimal media and incubated at 37°C overnight.

2.10.4B Screening the mutagenesis

1. Plaque lifts.

The following day the plates were chilled at 4°C for at last 30' and duplicate lifts, 90" for the first and 5' for the second, taken from one of the transformation plates containing approximately 200 plaques. Nitrocellulose or nylon membranes were used although nylon membranes were reserved for mutagenic oligonucleotides with Tm°C >65°C. Filters were air dried and nitrocellulose filters baked in a vacuum oven at 80°C for 2 hours while nylon filters were cross-linked with ultra violet light for 5'.

2. Prehybridisation.

Nitrocellulose filters were prehybridised in 6xNET while nylon filters were prehybridised in 1xDSS with approximately 50mls of prehybridisation solution for each pair of filters. Sonicated salmon sperm DNA at 10mg/ml, was incubated at 100°C for 5', snap-chilled on ice and added to the prehybridisation solution at a final concentration of 10µg/ml just before use. Prehybridisation and hybridisation was done at 37°C when using oligonucleotides < 25-mers and at 42°C when using oligonucleotides > 24-mers.

3. Hybridisation.

Hybridisation was performed in the same buffer and at the same temperature as the prehybridisation. Hybridisation mix was prepared in n+1mls where n is the number of filters being probed. The eluted, 5' phosphorylated, oligonucleotide probe (2.10.1C) was adjusted to 500µg/ml with sonicated salmon sperm DNA, incubated at 100°C for 5', snap-chilled on ice and added to the hybridisation solution. Filters were sandwiched between pieces of Whatman 1MM filter paper, soaked in hybridisation mix and incubated overnight.

4. Washing filters.

Filters were washed at room temperature for 5', twice in 6xSSC, 0.1% (w/v) SDS and then once in TMACl solution. Filters were then washed twice at the appropriate Tm°C-Δ for 30' in TMACl solution. The Tm°C of an oligonucleotide is a function of

length in the TMACl solution (Wood *et al.*, 1985). Deductions of 2°C per base substitution and 5-10°C for an insertion/deletion, enabled the temperature at which a mutagenic oligonucleotide melts from the wild type sequence (Tm°C-Δ) to be calculated. Filters were blotted dry and autoradiographed. Plaques that aligned to duplicating positive signals were picked and single stranded DNA prepared (2.10.5A) for sequence analysis (2.10.6).

2.10.5 Preparation of M13 and plasmid DNA

2.10.5A Small scale isolation of phage M13, single stranded DNA

This procedure was used to isolate the single stranded DNA from M13 clones. An M13 plaque was inoculated into 2mls 2xYT, seeded 1:100 with an overnight culture of JM101, and incubated at 37°C for 6 hours. Bacteria were pelleted by centrifugation of 1.5mls of culture for 10' in a micro-centrifuge, 1ml of the supernatant transferred to a fresh tube and mixed with 270µl of a solution containing 2.5M NaCl and 20% (w/v) PEG. After a 15' incubation at room temperature, centrifugation for 10' in a micro-centrifuge was used to pellet M13 phage. The phage pellet was resuspended in 100µl of TE and extracted twice with 100µl of buffer-saturated phenol:chloroform:iso-amyl alcohol (25:24:1), vortexed vigorously and centrifuged for 5' in a micro-centrifuge. DNA was precipitated by adding 10µl of 3M sodium acetate pH5.5 and 250µl of 100% ethanol to the final aqueous phase and incubating at -20°C overnight. The DNA was pelleted by centrifugation for 15' in a micro-centrifuge at 4°C, washed with 500µl of 70% (v/v) ethanol, dried, resuspended in 20µl of TE buffer and stored at -20°C.

2.10.5B Small scale isolation of plasmid DNA and phage M13 replicative form DNA

The procedure for isolating plasmid DNA from bacteria was based on the alkaline lysis procedure described by Birnboim and Doly (1979). Using suitable antibiotic selection, usually 100µg/ml ampicillin, a single, fresh colony of *E. coli* harbouring the appropriate plasmid was inoculated into 2mls L-broth + ampicillin and incubated at 37°C overnight. The same procedure was also used to isolate the double stranded, replicative

form DNA of phage M13. An M13 plaque was inoculated into 2mls 2xYT, seeded 1:100 with an overnight culture of JM101, and incubated at 37°C for 6 hours.

Bacteria were pelleted by centrifugation of 1.5mls of culture for 10' in a micro-centrifuge and the pellet resuspended in 100µls TES containing 2mg/ml lysozyme. After a 30' incubation on ice, 200µls of a solution containing 0.2M NaOH and 1% SDS was added, mixed in gently and incubated on ice. After 5', 150µls of ice-cold 3M sodium acetate pH4.6 was added, mixed in gently and incubated on ice for 40'. Cellular and chromosomal debris was pelleted by centrifuging twice for 15' in a micro-centrifuge at 4°C. The supernatant was then incubated with 5µl of 10mg/ml RNase A (DNase free) at 37°C for 30' and extracted twice with 400µl of buffer-saturated phenol:chloroform:iso-amyl alcohol (25:24:1). Mixture was vigorously vortexed, centrifuged for 5' in a microcentrifuge and 1ml of 100% ethanol added to the final aqueous phase. DNA was precipitated at -70°C for 30' or at -20°C overnight then pelleted by centrifugation for 15' in a micro-centrifuge at 4°C, washed with 800µl of 70% (v/v) ethanol, dried, resuspended in 20µl of TE buffer and stored at -20°C.

2.10.5C Large scale isolation of plasmid DNA

Large scale preparation of plasmid DNA used alkaline lysis followed by banding of covalently closed circular DNA in a caesium chloride gradient. Using suitable antibiotic selection, usually 100µg/ml ampicillin, a single, fresh colony of *E. coli* harbouring the appropriate plasmid was inoculated into 10mls L-broth + ampicillin and incubated at 37°C overnight. The following day 5ml of overnight culture was added to 500mls of L-broth containing 100µg/ml ampicillin in a 2L conical flask. The culture was incubated at 37°C with shaking to an OD_{600nm} of 0.8 to 1.0 before being adjusted to a final concentration of 10µg/ml chloramphenicol, 2mM $MgCl_2$ (Frenkel and Bremer, 1986) and the incubation at 37°C continued overnight.

Bacteria were pelleted by centrifugation at 5,000 rpm, 4°C for 10' in a JA-10 rotor, vigorously resuspended in 5mls of TEG containing 2mg/ml lysozyme. After a 30'

incubation on ice, 10mls of fresh 0.2M NaOH,1% SDS was added, mixed in gently and incubated on ice. After 10', 6.25mls of ice-cold 3M sodium acetate pH4.6 was added, mixed in gently and incubated on ice for 40'. Cellular and chromosomal debris was pelleted by centrifuging twice at 16,000 rpm, 4°C for 30' in a JA-20 rotor. The final supernatant was extracted twice with 20mls of buffer-saturated phenol:chloroform:iso-amyl alcohol (25:24:1), vortexed and centrifuged at 10,000 rpm, 20°C for 10' in a JA-20 rotor. The DNA was precipitated by adding 50mls of 100% ethanol and incubating at -20°C for 2 to 16 hours. DNA was pelleted by centrifugation at 15,000 rpm, 4°C for 30' in a JA-20 rotor and resuspended in 5mls of TE. Protein and RNA were precipitated by adding 5mls of 5M ammonium acetate, incubating on ice for 30' and pelleting debris by centrifugation at 15,000 rpm, 4°C for 10' in a JA-20 rotor. DNA in the supernatant was precipitated by adding 12.5mls of 100% ethanol and incubating at -20°C for 2 to 16 hours. DNA was pelleted by centrifugation at 15,000 rpm, 4°C for 30' in a JA-20 rotor, washed with 5mls 70% ethanol and centrifuged at 15,000 rpm, 4°C for 10' in a JA-20 rotor.

The DNA was resuspended in 2.3mls of TE, 2.45g of optical grade caesium chloride dissolved in and 60µl of 10mg/ml ethidium bromide added. The solution of DNA was loaded into a 13x32mm Quick-seal tube, sealed and centrifuged overnight at 65,000 rpm, 20°C in a Beckman TLA 100.3 rotor (Beckman Instruments, Palo Alto, CA). The lower band, containing covalently closed circular plasmid DNA, was collected using a 25 gauge needle and the ethidium bromide removed by extracting three times with 1ml of MilliQ-saturated butanol. The DNA was precipitated by adding 6mls of MilliQ, 700µl of 3M sodium acetate pH5.5, 18mls of 100% ethanol and incubating at -20°C overnight. DNA was pelleted by centrifugation at 15,000 rpm, 4°C for 30' in a JA-20 rotor, washed with 5mls 70% ethanol and centrifuged at 15,000 rpm, 4°C for 10' in a JA-20 rotor. The DNA was resuspended in 400µl TE and re-precipitated by adding 400µl of 3M sodium acetate pH5.5, 1ml of 100% ethanol and incubating at -20°C for 30'. DNA was pelleted by centrifugation for 15' in a micro-centrifuge at 4°C, washed

with 800µl of 70% (v/v) ethanol, dried and resuspended in 300µl of TE buffer. The DNA was quantified by measuring the OD_{260nm} and assuming 1AU = 50µg/ml.

2.10.6 DNA sequencing

2.10.6A Denaturation of double stranded DNA for sequencing

This method was based on a protocol described by Chen and Seeburg (1985).

To 2-5µg of double stranded DNA in a volume of less than 5µl, was added 40µl of denaturation buffer containing 200mM NaOH and 2mM EDTA. After incubation at 37°C for 30', 4µl 2M ammonium acetate pH4.5 and 125µl 100% ethanol were added and DNA precipitated at -70°C for 25'. DNA was pelleted by centrifugation for 15' in a micro-centrifuge at 4°C, washed with 1ml of 70% (v/v) ethanol, dried and resuspended in 10µl of annealing mix (2.10.6B).

2.10.6B Sequencing with Sequenase

DNA was sequenced by the chain termination method originally described by Sanger *et al.* (1977), using a Sequenase kit (United States Biochemical Corporation, Cleveland, OH). The method was used to sequence single- and double-stranded DNA using either [α-^{32}P]dATP or [α-^{35}S]dATP.

The annealing reaction contained 40mM Tris-HCl pH7.5, 20mM $MgCl_2$, 50mM NaCl, 10ng of purified or 100ng of crude primer and 3-5µg of single-stranded DNA template in a final volume of 10µl. DNA was denatured at 65°C for 5' and cooled slowly to room temperature. For double-stranded DNA the denatured DNA (2.10.6A) was resuspended in 10µl of annealing mix which contained 40mM Tris-HCl pH7.5, 20mM $MgCl_2$, 50mM NaCl and 10ng of purified or 100ng of crude primer. Template and primer were annealed at 45°C for 20'.

The labelling reaction contained 6.3mM DTT, 190nM dGTP, 190nM dCTP, 190nM dTTP, 5 to 10µCi [α-^{32}P]dATP or [α-^{35}S]dATP and 1 unit of Sequenase

enzyme added to the annealing reaction with a final volume of 16µl. The labelling reactions was incubated at 15°C for 5'.

Reactions were terminated by mixing 3.5µl from the labelling reaction with 2.5µl of each of the A,G,C,T termination mixes in tubes pre-warmed to 45°C. The termination mixes contained 80µM of dATP, dGTP, dCTP and dTTP as well as 8µM of the appropriate dideoxynucleotide triphosphate. Termination reactions were incubated at 45°C for 5'. Reactions were stopped by adding 4µl of stop solution containing 95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanole and placing at -20°C until electrophoresed.

Modifications of the standard protocol that enabled the sequence close to the primer to be determined included using 1:10 or 1:20 dilutions of the labelling mix, reduced incubation times (3') for the labelling reaction and the use of Mn buffer. The Mn buffer contains 150mM sodium isocitrate, 100mM $MnCl_2$ and 1µl was included in the standard labelling reaction for short sequencing runs.

2.10.6C Electrophoresis of the sequencing reactions

The sequencing reactions from 2.10.6B were analysed on 400mm x 400mm x 1mm sequencing gels. Gels contained 6% acrylamide with an acrylamide:bisacrylamide ratio of 19:1, 8M urea, 1xTBE buffer and were polymerised by adding final concentrations of 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulphate. Gels were pre-electrophoresed at 35mA for at least 30' in 1xTBE buffer prior to loading. Samples, denatured at 100°C for 5', were loaded at 1-3µl per well, depending on the comb used and electrophoresed at 35mA. Standard conditions included the addition of a final concentration of 333mM sodium acetate to the buffer in the lower tank during electrophoresis to compress the 5' sequence and running the bromophenol blue to the end of the gel. The gel was then fixed in 3L of 10% (v/v) methanol, 10% (v/v) acetic acid for 30', transferred to Whatman 3MM, covered in cling film and dried under vacuum for 1hour at 80°C. Once dry the gel was autoradiographed.

2.10.7 Transfection of COS and CHO cells

COS and CHO cells were maintained by Bronwyn Cambareri who also routinely performed the transfection of plasmid constructs into COS cells.

2.10.7A Transient transfection of COS cells

COS cells were maintained in DMEM containing 10mM HEPES, 0.2% (w/v) sodium bicarbonate, 1.7mM glutamine, 10.5µg/ml penicillin, 14µg/ml gentamicin and supplemented with 10% (v/v) FCS. Cells were usually grown to 50-70% confluence in 150cm^2 canted neck flasks containing 35mls of medium, before being harvested for transfection. Medium was aspirated off and cells rinsed briefly in 0.8% (w/v) NaCl, 0.02% (w/v) EDTA before adding 3mls of 0.8% (w/v) trypsin in 0.8% (w/v) NaCl, 0.02% (w/v) EDTA and incubating at 37°C for 1'. Cells were detached by "knocking", 15mls of fresh DMEM added immediately and cells collected by centrifugation at 1,600 rpm for 5'. Harvested cells were counted, pelleted by centrifugation at 1,600 rpm for 5' and resuspended in 1xHBSS supplemented with 6mM glucose to 1 x 10^7 cells/ml.

Plasmid constructs were introduced into COS cells by electroporation (Chu *et al.*, 1987) using a Bio-Rad Gene Pulser (Richmond, CA). For each transfection, 20µg of plasmid DNA, 25µg of sonicated salmon sperm DNA and 50µl of FCS were mixed in 4mm electroporation cuvettes chilled on ice before adding 500µl of resuspended COS cells. For mock transfections, 20µl of HEPES buffered saline, 6mM glucose was substituted for the plasmid DNA. The mixture was electroporated at 300V and 250µF, left at room temperature for 10' and plated out in petri dishes with 10mls of DMEM. After a 24 hour incubation at 37°C, the medium was replaced with FCS-free DMEM and the incubation continued for a further 72 hours. Finally the condition medium was harvested and assayed for GM-CSF.

2.10.7B Transfection of CHO cells and cloning permanently transfected cell lines

CHO cells were maintained in Ham's F12 nutrient mixture containing 10.5µg/ml penicillin, 14µg/ml gentamicin and supplemented with 10% (v/v) FCS. CHO cells were

grown to 80% confluence in 150cm^2 canted neck flasks containing 35mls of medium and harvested using essentially the procedure described for COS cells. The harvested CHO cells were washed twice in cold 1 x PBS before being resuspended in 1xPBS to 6.3 x 10^5 cells/ml.

For electroporation, 10µg of plasmid DNA was mixed with 800µl of CHO cells in chilled 4mm electroporation cuvettes and incubated on ice for 10'. Mock transfections contained 10µl of 1xPBS substituted for the plasmid DNA. The mixture was electroporated at 1300V and 25µF using a Bio-Rad Gene Pulser (Richmond, CA) and left at room temperature for 10' before mixing with 30mls of Ham's F12 nutrient mixture. Transfected CHO cells were plated out in 6 x 60mm gridded petri dishes and after a 24 hour incubation at 37°C, the medium was replaced with selective Ham's F12 containing 350µg/ml G-418.

The selective medium was replaced every 72 hours until all cells in the mock transfection were dead. Individual colonies of G-418 resistant CHO cells were cloned by placing cloning rings over the colonies and harvesting the transfected CHO cells with a 1' incubation at 37°C in 200µls of 0.8% (w/v) trypsin in 0.8% (w/v) NaCl, 0.02% (w/v) EDTA. Cloned CHO cells were grown in selective Ham's F12 containing 350µg/ml G-418. A number of colonies for each transfection were cloned and the conditioned medium for each cell line assayed for GM-CSF using an RIA (2.10.11A). Transfected CHO cell lines expressing the highest level of GM-CSF mutant analogue were selected and used to produce analogues for purification.

2.10.8 Osmotic shock protocol

The procedure for isolating periplasmic proteins was based on a protocol described by Koshland and Botstein (1980). Using suitable antibiotic selection, usually 100µg/ml ampicillin, a single, fresh colony of *E. coli* harbouring the appropriate GM-CSF expression construct was inoculated into 10mls L-broth + ampicillin and incubated at 37°C overnight. The following day the overnight culture was pelleted at 3,000 rpm for

10' and the cell pellet washed twice in fresh L-broth to remove accumulated β-lactamase. The entire, washed overnight culture was then added to 1L of L-broth containing 100μg/ml ampicillin in a 2L conical flask. The culture was incubated at 37°C with shaking to an OD_{600nm} of 0.8 to 1.0. IPTG was then added to a final concentration of 0.1mM and the culture incubated at room temperature for a further 3 hours or left overnight.

Cells were then pelleted by centrifugation at 5,000 rpm, 4°C for 20' in a JA-10 rotor. The pellet was then resuspended in 5mls TS buffer with aggressive pipetting and transferred to Oakridge tubes before adding 250μl 0.25M ice-cold EDTA and incubating the mixture on ice for 30'. Tubes were filled with ice-cold MilliQ, mixed rapidly and incubated on ice for a further 30'. Cellular debris was pelleted by centrifugation at 12,000 rpm and 4°C for 15' in a JA-20 rotor. The supernatant was transferred to a clean tube and centrifuged at 19,500 rpm and 4°C for 15'. The final supernatant containing periplasmic contents, was filtered through Whatman No. 6 filter paper and adjusted to 0.1% (w/v) sodium azide. Preparations of periplasmic proteins were stored at 4°C for up to a few weeks but normally GM-CSF was purified from this crude preparation immediately.

2.10.9 Preparation and use of immunoaffinity columns

2.10.9A Preparation of MoAb from ascites fluid

Ascites fluid containing crude anti-GM-CSF monoclonal antibodies, was generated from BALB/c mice. Solids were removed and MoAb precipitated by slowly adding 660μl of saturated ammonium sulphate solution per ml of ascites fluid. The mixture was stirred for 30' at room temperature, pelleted by centrifugation for 10' in a micro-centrifuge and the pellet washed twice with a 50% saturated ammonium sulphate solution. The precipitated monoclonal antibody was resuspended in MilliQ to twice the volume of the original ascites fluid, dialysed 1:10,000 (final) against fresh coupling buffer and concentrated in Centricon-10 mini concentrators to 5-10mg/ml. Coomassie

stained SDS/PAGE indicated that the preparations typically contained 80-90% immunoglobulin.

2.10.9B Coupling MoAb to CNBr-activated Sepharose

Partially purified monoclonal antibodies were coupled to CNBr-activated Sepharose 4B according to the manufacturer's protocol. Freeze-dried CNBr-activated Sepharose 4B was swollen in 1mM HCl for 15' and washed in several changes of 1mM HCl. One gram of freeze-dried Sepharose swelled to a gel volume of 3.5mls. Swollen gel was then washed briefly with coupling buffer and mixed with monoclonal antibody for 2 hours at room temperature. The gel was drained of coupling mixture and the liquid assayed for uncoupled protein which was usually <5% of the starting material for a successful coupling. The gel was then mixed with 10 volumes of blocking agent for 2 hours at room temperature. Typical blocking agents were 200mM glycine, 1.0M Tris-HCl or 1.0M ethanolamine all at pH8.0.

The gel was washed with 5 cycles of alternating high pH and low pH buffers to remove uncoupled protein using 5 volumes of buffer for each wash. Coupling buffer was used for the high pH washes and 100mM sodium acetate, 500mM NaCl pH4.0 used for the low pH washes. Gel was then washed 5 times with 10 volumes of coupling buffer. Finally gel was washed 5 times with 10 volumes of PAT buffer containing 1 x PBS, 0.1% (w/v) sodium azide, 0.01% (v/v) Tween 20. The immunoaffinity matrix was stored in PAT buffer at 4°C where it was stable for at least 12 months.

2.10.9C Immunoaffinity chromatography

Immunoaffinity matrix was used to purify GM-CSF mutant analogues expressed from COS or CHO cells or *E. coli*. Aliquots of immunoaffinity matrix, usually 0.5-1.0mls, were mixed with crude GM-CSF overnight and the following day the matrix was collected in an Econo column. Alternatively the immunoaffinity matrix was packed into an Econo column and crude GM-CSF passed over the column 2-3 times. For ease of handling, the volume of crude GM-CSF either in COS or CHO cell conditioned medium

or *E. coli* osmotic shock preparations, was always less than 50mls. Crude samples were concentrated from as much as 1L using a stirred-cell apparatus (Amicon, Danvers, MA) with a 10,000 Mr cutoff membrane.

The immunoaffinity matrix with bound GM-CSF was then washed 5 times with 10 volumes of PAT buffer. Typically, the specifically bound GM-CSF was eluted using 4 x 500µl aliquots of 100mM glycine, 100mM NaCl, 0.02% (w/v) sodium azide pH3.0. For some immunoaffinity matrices milder conditions were sufficient to elute bound GM-CSF so 100mM sodium acetate, 100mM NaCl, 0.02% (w/v) sodium azide pH5.0 was used instead. The eluted GM-CSF was immediately neutralised by adding 1.0M Tris-HCl pH10.0. The immunoaffinity matrix was then washed 5 times with 10 volumes of PAT buffer and stored in PAT buffer at 4°C.

2.10.10 Radioiodination of recombinant cytokines

The radioiodination of GM-CSF was performed by Mara Dottore or Dr. J. Woodcock. Recombinant GM-CSF produced in CHO cells, yeast or *E. coli*, was radioiodinated by the iodine monochloride method as previously described (Contreras *et al.*, 1983). Iodinated GM-CSF was separated from $Na^{125}I$ by chromatography with a Sephadex G-25 PD-10 column equilibrated in 1 x PBS, 0.02% (v/v) Tween 20 and stored at 4°C for up to 1 month. For use in receptor binding experiments, non-protein-associated radioactivity and Tween 20 were removed by cation exchange chromatography on a 300µl CM-Sepharose CL-6B column and the iodinated GM-CSF stored at 4°C for up to 5 days. Radioiodinated GM-CSF retained at least 90% of wild type biological activity compared with unlabelled GM-CSF.

2.10.11 Quantification of GM-CSF

2.10.11A Radioimmunoassay

A modification of the radioimmunoassay described in Lopez *et al* (1992c) was used to quantify crude GM-CSF analogues in COS (2.10.7A) or CHO (2.10.7B) cell conditioned medium and osmotic shock supernatants (2.10.8). Briefly, 10µl ^{125}I-GM-

CSF (0.1ng) was mixed with 50µl crude GM-CSF sample and 50µl of suitably diluted polyclonal anti-GM-CSF serum and incubated at room temperature for 2 to 4 hours. The serum was either sheep anti-GM-CSF diluted 1:40,000 (final) or rabbit anti-GM-CSF diluted 1:4,000 (final). An equal volume of reconstituted anti-sheep or anti-rabbit Immunobeads was then added and the incubation continued for a further 2 to 4 hours. The beads were then pelleted, washed twice in 1 x PBS containing 0.1% (w/v) BSA and counted in a gamma counter (Cobra Auto Gamma, Packard Instrument Company, Meriden, CT). The amount of GM-CSF in the samples was calculated from a standard curve constructed with known amounts of wild type GM-CSF.

2.10.11B High performance size exclusion chromatography

Wild type GM-CSF and mutant analogues purified from *E. coli* were directly quantified by high performance size exclusion chromatography (HP-SEC). All HPLC applications used a Bio-Rad 1350 dual pump HPLC with a series 600 gradient controller and either a Bio-Rad model 1306 UV monitor (Bio-Rad, Richmond, CA) or a Waters 490E programmable multiwavelength detector using Maxima 820 software (Waters, Milford, MA). For HP-SEC samples were chromatographed on a 7.5 x 300mm Ultraspherogel SEC 3000 column (Beckman Instruments, San Ramon, CA) using a mobile phase containing 0.1M sodium phosphate pH7.0, 0.1M sodium sulphate and 0.05% (w/v) sodium azide run at 0.5mls/min. The GM-CSF absorbance peak was integrated and the amount of protein determined using a calculated extinction coefficient of 0.95 $AU.ml^{-1}.mg^{-1}$ or 1.1 $AU.ml^{-1}.mg^{-1}$ for the P6YY analogue.

2.10.12 Electrophoresis of proteins

2.10.12A SDS/PAGE

The procedure for fractionating proteins by SDS/PAGE was based on the system described by Laemmli (1970). A 30% (w/v) acrylamide stock solution was used for all protein gels and contains an acrylamide:bisacrylamide ratio of 36:1. Separating gels were composed of 12.5 or 15% (w/v) acrylamide, 0.1% (w/v) SDS, 375mM Tris-HCl pH8.8 and were polymerised by adding final concentrations of 0.125% (v/v) TEMED and

0.025% (w/v) ammonium persulphate. Stacking gels were composed of 4.8% (w/v) acrylamide, 0.1% (w/v) SDS, 125mM Tris-HCl pH6.8 and were polymerised by adding final concentrations of 0.2% (v/v) TEMED and 0.06% (w/v) ammonium persulphate. Samples to be analysed by SDS/PAGE under reducing conditions were prepared in a load buffer containing final concentrations of 31mM Tris-HCl pH6.8, 0.6% (w/v) SDS, 6.1% (v/v) glycerol, 0.01% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol. Prior to loading on the gel samples were incubated at 100°C for 5'. For analysis of samples under non-reducing conditions, the β-mercaptoethanol was omitted from the sample. Gels were electrophoresed at 15 to 30mA for standard 160x160mm gels in a buffer composed of 83mM Tris, 192mM glycine and 0.1% (w/v) SDS.

2.10.12B High pH native PAGE

The procedure described by Goldenberg (1990) was used for native PAGE of GM-CSF. The separating gel was composed of 10% (w/v) acrylamide, 375mM Tris-HCl pH8.9 and 0.06% (v/v) TEMED and was polymerised by adding ammonium persulphate to a final concentration of 0.05% (w/v). The stacking gel was composed of 4.8% (w/v) acrylamide, 60mM Tris-PO_4 pH 6.9 and 0.06% (v/v) TEMED and was polymerised by adding ammonium persulphate to a final concentration of 0.012% (w/v). Samples were prepared in a load buffer containing final concentrations of 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue. Gels were electrophoresed at 10 to 20mA for standard 160x160mm gels in a buffer composed of 5mM Tris base and 38mM glycine.

2.10.12C Transverse urea gradient PAGE

Transverse urea gradient PAGE was performed using a protocol described by Creighton (1979). The gradient, which was formed perpendicular to the direction of electrophoresis, contained a gradient of urea from 0M to 8M with a compensatory gradient of acrylamide from 10% to 8%. Two acrylamide solutions were prepared containing either 0M or 8M urea. The 0M solution contained 10% (w/v) acrylamide, 375mM Tris-HCl pH8.9, 0.06% (v/v) TEMED and 0.04% (w/v) ammonium persulphate while the 8M solution contained 8% (w/v) acrylamide, 8M urea, 375mM Tris-HCl

pH8.9, 0.06% (v/v) TEMED and 0.04% (w/v) ammonium persulphate. Using 11mls of each solution a linear gradient from 0M to 8M urea was formed using a gradient former and pumped into the bottom of a gel casting apparatus containing two 80 x 80 x 1mm gel cassettes at 5ml/min. A solution of 50% (v/v) glycerol, 0.1% (w/v) methyl green pumped at 3ml/min was used to ensure that all of the acrylamide solution enters the gel cassette. Once set, the gel was rotated by 90° for electrophoresis. Samples were prepared in a load buffer containing final concentrations of 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue. Gels were electrophoresed for 400 volt/hours at 50 volts and at 4°C in a buffer composed of 5mM Tris base and 38mM glycine.

2.10.12D Western transfer, Towbin protocol

The electrophoretic transfer of proteins fractionated by SDS/PAGE to nitrocellulose was performed using one of two methods. The original method was based on the protocol of Towbin *et al.* (1979). Following electrophoresis, the gel was placed on three sheets of Whatman 3MM soaked in transfer buffer containing 20mM Tris base, 150mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol. A sheet of nitrocellulose soaked in transfer buffer was placed over the gel and all air bubbles carefully squeezed out. Three more sheets of Whatman 3MM soaked in transfer buffer were then placed on top. Two Scotchbrite pads soaked in transfer buffer were placed on either side of the sandwich and the entire assembly supported in a plastic grid which was then placed in the transfer tank. The assembly was completely immersed in transfer buffer and transfer performed at 25V o/n or 60V for 4 hours if a cooling coil was used. Protein migration was towards the anode so the nitrocellulose sheet faced the anode while the gel faced the cathode.

2.10.12E Western transfer, semi-dry protocol

The second method used a different, semi-dry apparatus that enabled faster transfer. Immediately prior to transfer, the graphite anode was washed with MilliQ. A stack of nine sheets of Whatman 3MM, trimmed to match the gel and soaked in transfer buffer containing 50mM Tris base, 40mM glycine, 0.0375% (w/v) SDS and 20% (v/v) methanol, was then assembled. Alternatively the Towbin transfer buffer used in the

original transfer method can be used. A sheet of nitrocellulose, trimmed to match the gel and soaked in transfer buffer was then placed on the Whatman 3MM and following electrophoresis, the gel was placed on the nitrocellulose and all air bubbles carefully squeezed out. Another stack of nine sheets of Whatman 3MM, trimmed to match the gel and soaked in transfer buffer was then placed on the gel. The graphite cathode was then rinsed with MilliQ and placed on top. Transfer was performed for 60 to 90' at 0.8mA x surface area, which was typically 160mA.

2.10.13 Visualisation of proteins after electrophoresis

2.10.13A Coomassie blue staining

Following SDS/PAGE or native PAGE electrophoresis, the gel was stained for 30' in a solution of 0.1% (w/v) Coomassie Brilliant blue R-250, 30% (v/v) methanol, 10% (v/v) acetic acid. Staining solution was filtered through Whatman 3MM prior to use and used at most three times. Gel was destained in 30% (v/v) methanol, 10% (v/v) acetic acid although after initial de-staining, native PAGE gels and urea gradient gels were destained in 5% (v/v) methanol, 5% (v/v) acetic acid.

2.10.13B Silver staining

The procedure for silver staining SDS/PAGE gels was based on a protocol described by Morrissey (1981) but did not include fixing in 10% glutaraldehyde. Because silver staining is approximately 40x more sensitive than Coomassie blue staining, the apparatus used for SDS/PAGE was cleaned thoroughly to remove contaminating proteins. Protein molecular weight standards were diluted 40-fold because of the increased sensitivity of silver staining. The following protocol was based on a standard 160x160mm gel and volumes were doubled for the 320x160mm gels.

Following electrophoresis the gel was fixed using 500mls of 50% (v/v) methanol, 10% (v/v) acetic acid for 30' followed by 500mls of 5% (v/v) methanol, 7% (v/v) acetic acid for 30'. Throughout the procedure the gel was gently agitated. Gel was then soaked

overnight in MilliQ with a 30' soak the following day in fresh MilliQ. Alternatively the gel was soaked in several changes of MilliQ for 1 to 2 hours.

Washed gel was then soaked in 200mls of 5µg/ml DTT for 30' followed immediately by 200mls of 0.1% (w/v) silver nitrate for 30'. Gel was then briefly rinsed with MilliQ and then twice with a small volume (50mls) of developer solution, containing 3% (w/v) sodium carbonate and 0.02% (v/v) formaldehyde, before soaking gel in developer solution until appropriately stained. The developer solution was discarded and the reaction stopped with 5% (v/v) acetic acid. The gel could be stored in 5% acetic acid for months if sealed in a plastic bag.

2.10.13C Amido Black staining

Protein molecular weight standards transferred to nitrocellulose by Western blot transfer, were stained with amido black for 1' in a solution of 0.1% (w/v) Amido black, 45% (v/v) methanol, 10% (v/v) acetic acid. The nitrocellulose was destained in 45% (v/v) methanol, 10% (v/v) acetic acid.

2.10.13D Western blot staining

Following transfer of proteins to nitrocellulose (2.10.12D,E) the track containing non-biotinylated, protein molecular weight standards was cut off and stained with amido black (2.10.13C). Non-specific protein binding sites on the remainder of the nitrocellulose filter were blocked by incubation at 4°C overnight in Blotto solution. The filter was then probed with sheep anti-GM-CSF polyclonal antibody diluted 1:2,000 (final) in PTB buffer and incubated in a sealed plastic bag at 4°C for 3 hours. The filter was then washed in four changes of PTB, 5' each wash with agitation. The filter was then probed with biotinylated, rabbit anti-sheep immunoglobulin diluted 1:100 in PTB and incubated in a sealed plastic bag at 4°C for 1 hour. Filter was then washed and probed with a conjugate of avidin and biotinylated horseradish peroxidase diluted 1:1,000 in PTB and incubated in a sealed plastic bag at 4°C for 1 hour. Filter was then washed and placed in substrate solution and stain allowed to develop. Substrate solution

was prepared by dissolving 50mg DAB in 1ml 1M HCl and adding to 100mls of MilliQ. A small amount of solid Tris base was then added to adjust to pH7.0 and immediately prior to use H_2O_2 was added to 0.018% (v/v) final. When the filter was appropriately stained, development was halted by placing in a solution of 0.5% (w/v) sodium azide. The filter was photographed immediately as discolouration occurred rapidly, <1 hour.

2.10.14 Protein sequencing

This is a method from Richard Simpson's laboratory (Ludwig Institute for Cancer Research, Melbourne, Vic). The separating gel was composed of 15% (w/v) acrylamide, 750mM Tris-HCl pH8.8, 0.1% (w/v) SDS and was polymerised by adding final concentrations of 0.06% (v/v) TEMED and 0.09% (w/v) ammonium persulphate. The separating gel was pre-electrophoresed at 20mA overnight in a buffer composed of 375mM Tris-HCl pH8.8, 0.1% (w/v) SDS and 0.07% (v/v) thioglycolic acid. Stacking gel was composed of 4.7% (w/v) acrylamide, 123mM Tris-HCl pH6.8, 0.1% (w/v) SDS and was polymerised by adding final concentrations of 0.1% (v/v) TEMED and 0.03% (w/v) ammonium persulphate. Samples were prepared in a load buffer containing final concentrations of 31mM Tris-HCl pH6.8, 0.6% (w/v) SDS, 6.1% (v/v) glycerol, 0.01% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol. Prior to loading on the gel samples were incubated at 100°C for 5'. The gel was electrophoresed at 10 to 35mA in a buffer composed of 50mM Tris, 192mM glycine, 0.1% (w/v) SDS. The cathode buffer also contained 0.007% (v/v) thioglycolic acid.

Following electrophoresis the gel was soaked for 20' in transfer buffer containing 10mM CAPS pH11, 10% (v/v) methanol and placed on three sheets of Whatman 3MM soaked in transfer buffer. Two sheets of Imobilon PVDF membrane, wet in methanol for 5' and soaked in transfer buffer for 15', were placed over the gel and all air bubbles carefully squeezed out. Three more sheets of Whatman 3MM soaked in transfer buffer were then placed on top. Two Scotchbrite pads soaked in transfer buffer were then placed on either side of the sandwich and the entire assembly supported in a plastic grid

which was then placed in the transfer tank. The assembly was completely immersed in transfer buffer and transfer performed at 90V for 45'.

Following transfer, the membrane was washed in MilliQ for 5' and then stained in a solution of 0.1% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol for 5'. The membrane was then destained in 50% (v/v) methanol, 10% (v/v) acetic acid for 15', washed in MilliQ for 5' and blotted dry. The GM-CSF band was then excised ready for sequence determination.

2.10.15 Purification of neutrophils and monocytes

Neutrophils were typically purified by Bronwyn Cambareri while monocytes were purified by Julie Halsall, Michelle Parsons or Betty Zacharakis.

2.10.15A Purification of human neutrophils

Neutrophils were prepared from the peripheral blood of normal donors as previously described (Vadas *et al.*, 1979). Fresh blood was collected in 50ml tubes containing 1,000 units of heparin, mixed with a 1/3 volume of Dextran solution and left at room temperature for 30'. The buffy coat was collected and washed with 50mls of supplemented RPMI 1640 medium containing 10mM HEPES and 0.1% (w/v) BSA. Cells were pelleted by centrifugation at 1,500 rpm, 25°C for 5' in a benchtop centrifuge. Supernatant was discarded and the cell pellet resuspended in 10mls of supplemented RPMI 1640 medium, carefully underlaid with 10mls of Lymphoprep and centrifuged at 1,800 rpm, 25°C for 30' in a Beckman JS-4.2 rotor (Beckman Instruments, Palo Alto, CA) with minimal deceleration. Supernatant was discarded and cell pellet resuspended in 5mls of 0.2% (w/v) NaCl and mixed gently for 45" to lyse erythrocytes. Cell suspension was made isotonic by adding 5mls of 1.6% (w/v) NaCl before mixing with 40mls of supplemented RPMI 1640 medium. Cells were pelleted by centrifugation at 1,500 rpm, 25°C for 5', the supernatant discarded and cells resuspended in supplemented RPMI 1640 medium to 1 x 10^7 cells/ml. The neutrophil purity was typically >95%.

2.10.15B Purification of human monocytes

Monocytes were purified from the peripheral blood of normal donors, obtained from the Adelaide Red Cross Transfusion Service, as previously described (Elliott *et al.*, 1990). Whole blood was diluted 1:1 in 1xHBSS and carefully underlaid with 0.5 x volumes of Lymphoprep and centrifuged at 1,800 rpm, 25°C for 25' in a Beckman JS-4.2 rotor (Beckman Instruments, Palo Alto, CA) with minimal deceleration. Mononuclear cells were collected, washed twice in HBSS, centrifuged at 1,000 rpm for 5' to remove platelets and resuspended in elutriation buffer consisting of HBSS, 0.1% (w/v) glucose, 0.01% (w/v) EDTA and 0.1% (v/v) FCS. Monocytes were purified in a Beckman J-6M/E elutriator (Beckman Instruments, Palo Alto, CA) using the Sanderson chamber, a flow rate of 12 ml/min and a constant rotor speed of 2,050 rpm. Cells remaining in the chamber after 30' were collected, washed twice in HBSS and used immediately. The monocyte purity was typically >95%.

2.10.16 GM-CSF biological activity assays

2.10.16A Cellular proliferation assays

Cellular proliferation assays were routinely performed by Bronwyn Cambareri. Primary cells from patients with CML or AML were selected for their ability to incorporate [^{3}H]thymidine in response to GM-CSF and were maintained in RPMI 1640 containing 10% (v/v) FCS, 10.5µg/ml penicillin, 14µg/ml gentamicin, 1.7mM glutamine and 0.23% (w/v) $NaHCO_3$. The erythroleukaemic cell line TF-1, incorporates [^{3}H]thymidine in response to GM-CSF or IL-3 (Kitamura *et al.*, 1989) and was maintained in RPMI 1640 containing 10% (v/v) non-heat inactivated FCS, 10.5µg/ml penicillin, 14µg/ml gentamicin, 1.7mM glutamine, 0.23% (w/v) $NaHCO_3$ and 2ng/ml GM-CSF.

Haemopoietic cell proliferation assays were performed essentially as described (Kitamura *et al.*, 1989). Briefly, 100µl of cells at 1 x 10^5 cells/ml in supplemented RPMI 1640, was incubated with 100µl of growth factor in 96-well plates for 48 hours (TF-1) or 72 hours (CML, AML) at 37°C. Cells were then pulsed with 1µCi/well [^{3}H]thymidine

for 5 hours at 37°C, harvested with a Skatron automated cell harvester (Lier, Norway) and cell associated radioactivity counted in a Packard TriCarb liquid scintillation counter (Meriden, CT.).

The percent relative activity for GM-CSF analogues was calculated as described (Hercus *et al.*, 1994b). The equation for an asymmetric sigmoid curve that fitted the dose-response for each GM-CSF analogue was obtained using Fig.P (Biosoft, Cambridge, UK). From this equation, the concentration of GM-CSF analogue required to elicit a biological response equivalent to the ED_{50} response of wild type GM-CSF was calculated. The percent relative activity of wild type GM-CSF was defined as 100% and that of the GM-CSF analogues calculated using the formula,

$$\frac{\text{wild type GM-CSF } ED_{50}\text{, (ng/ml) x 100}}{\text{analogue dose equivalent to wild type } ED_{50} \text{ response, (ng/ml)}}$$

Using the $\log_{10}$ value of the percentage relative activity, the means and standard deviations were calculated for each analogue and then expressed as percent relative activity.

2.10.16B Neutrophil superoxide release assay

The production of superoxide anions by neutrophils was measured as previously described (Lopez *et al.*, 1986) and the assays were performed by Bronwyn Cambareri. Purified neutrophils at 1 x 10^7 cells/ml in supplemented RPMI 1640 medium, were mixed with 0.5 volumes of medium or cytokine stimulus, pre-incubated at 37°C for 45' and then placed on ice. A reaction mixture was prepared on ice containing 650µl of supplemented RPMI 1640 medium, 100µl of FMLP at 1µM in medium, 100µl of fresh cytochrome-*c* at 12.4 mg/ml in medium and finally 150µl of pre-incubated neutrophils at 6.7 x 10^6 cells/ml. Samples were vortexed and incubated at 37°C for 15', chilled to 4°C and centrifuged at 2,500 rpm at 4°C for 5'. The supernatant was transferred to plastic disposable cuvettes and the absorbance at 550nm measured using a Beckman DU-50

spectrophotometer (Beckman Instruments, San Ramon, CA). A blank sample was always included for calibration of the spectrophotometer where the neutrophils were pre-incubated in medium alone and no FMLP was included in the reaction mixture. Superoxide production was measured by the reduction of cytochrome-*c* using an extinction coefficient of $21.1mM^{-1}$.

2.10.16C Monocyte adherence assay

Monocyte adherence was measured as previously described (Elliott *et al.*, 1990) and the assays were performed by Julie Halsall or Betty Zacharakis. Purified monocytes (1×10^7) were resuspended in 1ml of elutriation buffer and incubated at 37°C for 40' with 500μCi of $Na_2{}^{51}CrO_4$. Cells were then washed three times in RPMI and resuspended to 2×10^6 cells/ml in culture medium consisting of RPMI, 10% (v/v) FCS, 10.5μg/ml penicillin, 14μg/ml gentamicin and 0.2% (w/v) sodium bicarbonate. Monocytes were aliquoted into 96-well plates at 2×10^5 cells/well, mixed with culture medium or GM-CSF in a total volume of 100μl and incubated at room temperature for 21 hours. At harvest, samples of culture supernatant were collected to assess spontaneous ^{51}Cr release and wells carefully washed three times with RPMI at 37°C. Residual adherent cells were lysed in 10mM Tris, 150mM NaCl and 1% (v/v) NP-40 at 37°C for 30' and lysates transferred to tubes and counted in a gamma counter (Cobra Auto Gamma, Packard Instrument Company, Meriden, CT). The percent adhesion was calculated using the formula,

$$\frac{\text{residual adherence (cpm)} \times 100}{\text{total cpm added - spontaneously released cpm}}$$

2.10.17 GM-CSF receptor binding assays

Receptor binding experiments were routinely performed by Mara Dottore.

2.10.17A Receptor binding on neutrophils

Competition for binding to the GM-CSF high affinity receptor used freshly purified human neutrophils which only express this type of receptor (Gasson *et al.*,

1986). Neutrophils were resuspended in binding medium consisting of RPMI 1640 supplemented with 10mM HEPES, 0.5% (w/v) BSA and 0.1% (w/v) sodium azide. Typically, equal volumes (50µl) of 2-4 x 10^6 neutrophils, 100-200pM ^{125}I-GM-CSF and different concentrations of wild type GM-CSF or GM-CSF analogues were mixed in siliconised glass tubes for 2-3 hours at room temperature.

The cell suspensions were then overlaid on a 200µl cushion of cold FCS, centrifuged at room temperature and the tip of each tube containing the visible cell pellet cut off and the radioactivity counted in a gamma counter (Cobra Auto Gamma, Packard Instrument Company, Meriden, CT). Typically, the maximum specifically bound counts was between 5,000 and 10,000 cpm. Data from receptor binding experiments were analysed using the EBDA/LIGAND programs (Munson and Rodbard, 1980) obtained from Biosoft (Cambridge, UK).

2.10.17B Receptor binding on monocytes

Competition for binding to high and low affinity GM-CSF receptors used freshly purified human monocytes which express both types of receptor (Elliott *et al.*, 1989). Equal volumes (50µl) of 2 x 10^6 monocytes, ^{125}I-GM-CSF at different concentrations and a 100-fold excess of wild type GM-CSF to establish non-specific binding or a 35-fold excess of the E21R analogue, were mixed in siliconised glass tubes for 16 hours at 4°C. The cell suspensions were then overlaid on a 200µl cushion of cold FCS, centrifuged at room temperature and the tip of each tube containing the visible cell pellet cut off and the radioactivity counted in a gamma counter. Specific counts were determined by subtracting the counts obtained in the presence of a 100-fold excess of wild type GM-CSF. Typically, the maximum specifically bound counts was between 25,000 and 30,000 cpm. Results are expressed in the form of equilibrium binding data and Scatchard transformation of these data as described (Scatchard, 1949).

2.10.17C Receptor binding on A9/C7 cells

Competition for binding to the cloned low affinity GM-CSF receptor also used the A9/C7 CHO cell line which express 2 to 5 x 10^5 low affinity GM-CSF binding sites per cell (Hercus *et al.*, 1994b). A9/C7 CHO cells were maintained in 150cm^2 canted neck flasks containing 35mls of medium consisting of Ham's F12 nutrient mixture, 10.5µg/ml penicillin, 14µg/ml gentamicin, 10% (v/v) FCS and 350µg/ml G-418. A9/C7 cells grown to 80% confluence, were detached from flasks by incubating in 1xPBS containing 40mM EDTA and 100µg/ml chondroitin sulfate for 5-10', washed and resuspended in binding medium. Typically, equal volumes (50µl) of 6 x 10^5 A9/C7 CHO cells, 500pM ^{125}I-GM-CSF and different concentrations of wild type GM-CSF or GM-CSF analogues were mixed in siliconised glass tubes for 2-3 hours at room temperature. Cell suspensions were spun through an FCS cushion, bound ^{125}I-GM-CSF counted and data analysed as described (2.10.17A). Typically, the maximum specifically bound counts was between 5,000 and 10,000 cpm.

Chapter 3

Structure-function studies on human GM-CSF: Identification of two potential receptor binding sites

3.1 Introduction

At the commencement of this project to study human GM-CSF, little information was available regarding the structure-function relationships of GM-CSF or other members of the cytokine family. Two preliminary structure-function studies had identified residues within GM-CSF that appeared to be important for activity. The generation of a series of deletion and clustered point mutations of murine GM-CSF, revealed four regions comprising residues 11-15, 24-37, 47-49 and 81-89, that were required for a functional murine GM-CSF molecule (Gough *et al.*, 1987). Chemical synthesis of truncated human GM-CSF analogues demonstrated that the structural integrity of residues 14-25, coincident with the predicted first α-helix and residues in the C-terminus, were critical for biological activity (Clark-Lewis *et al.*, 1988). These studies demonstrated that residues in the N- and C-termini of both human and murine GM-CSF were important for GM-CSF activity.

Structure-function studies of other related cytokines suggested that residues in the first and last α-helices are required for biological activity. Deletion and substitution mutagenesis studies on murine IL-2 (Zurawski *et al.*, 1986; Zurawski and Zurawski, 1988) and human IL-2 (Ju *et al.*, 1987) revealed the presence of residues in the N- and C-terminus critical for biological activity. These biologically important regions coincide with regions that were predicted to form the first and last α-helices of IL-2 (Zurawski and Zurawski, 1988). Homologue-scanning and alanine-scanning mutagenesis studies on human growth hormone identified three regions that modulate the binding of growth hormone to the growth hormone receptor from human liver (Cunningham *et al.*, 1989; Cunningham and Wells, 1989). These studies, in general, point to the existence of functionally important regions in the N- and C- termini of these cytokines.

3.1.1 Hydropathy analysis of human GM-CSF

The primary structure of human GM-CSF was analysed for regions likely to be available for interaction with the GM-CSF receptor based on physical properties rather than structure-function studies. To this end the hydropathy (hydro = water, pathy = feeling for) profile of human GM-CSF was analysed using three different hydropathy scales (Fig. 3.1.1). Hydrophilic regions which exhibit a relatively high affinity for water are likely to occur on the surface of a protein where they are closely associated with antigenic determinants (Hopp and Woods, 1981). The presence of these regions on the surface of a protein also indicates that they are likely to be available for interaction with receptor molecules. Analysis of the hydropathy plots of human GM-CSF, revealed the presence of three hydrophilic peaks (Fig. 3.1.1). Two of the hydrophilic peaks are associated with the predicted α-helices between residues 15 to 28 and residues 97 to 117 (Fig. 1.2.3).

Helical wheel plots of the first and fourth predicted alpha helices (Fig. 3.1.2) indicated that these helices are amphipathic with a hydrophobic face that exhibits a very high degree of sequence conservation from species to species (Fig. 1.2.2). The conserved and hydrophobic nature of this region suggests that is likely to contribute to the hydrophobic core of GM-CSF. Residues on the hydrophilic surfaces of these helices, which tend to exhibit less overall sequence conservation, are likely to be located on the surface of GM-CSF and are candidates for interaction with receptor molecules. Mutational analysis of these residues should provide information on which residues are required for the interaction of GM-CSF and its receptor.

On the basis of the hydropathy analysis and the available structure-function data on the cytokines, hydrophilic regions of the first and fourth predicted α-helices of GM-CSF were targeted for mutagenesis.

Figure 3.1.1 Hydropathy analysis of human GM-CSF.

The human GM-CSF sequence of Wong et al. (1985a) was analysed for hydrophilic and hydrophobic regions. The hydropathy scales of Kyte and Doolittle (1982) (Panel A), Hopp and Woods (1981) (Panel B) and Eisenberg *et al.* (1984) (Panel C) were used with an averaging window of 5. In each panel, points above the line are hydrophilic while points below the line are hydrophobic. The arrows highlight 2 prominent hydrophilic peaks identified with all 3 hydropathy scales. The solid arrow indicates a peak centred around residues 20 and 21 of the predicted α-helix spanning residues 15 to 28 (Fig. 1.2.3). The empty arrow indicates a peak centred around residues 107 to 112 of the predicted α-helix spanning residues 97 to 117 (Fig. 1.2.3).

The GM-CSF sequence is represented by a numeric scale from residue 1 to 127 inclusive.

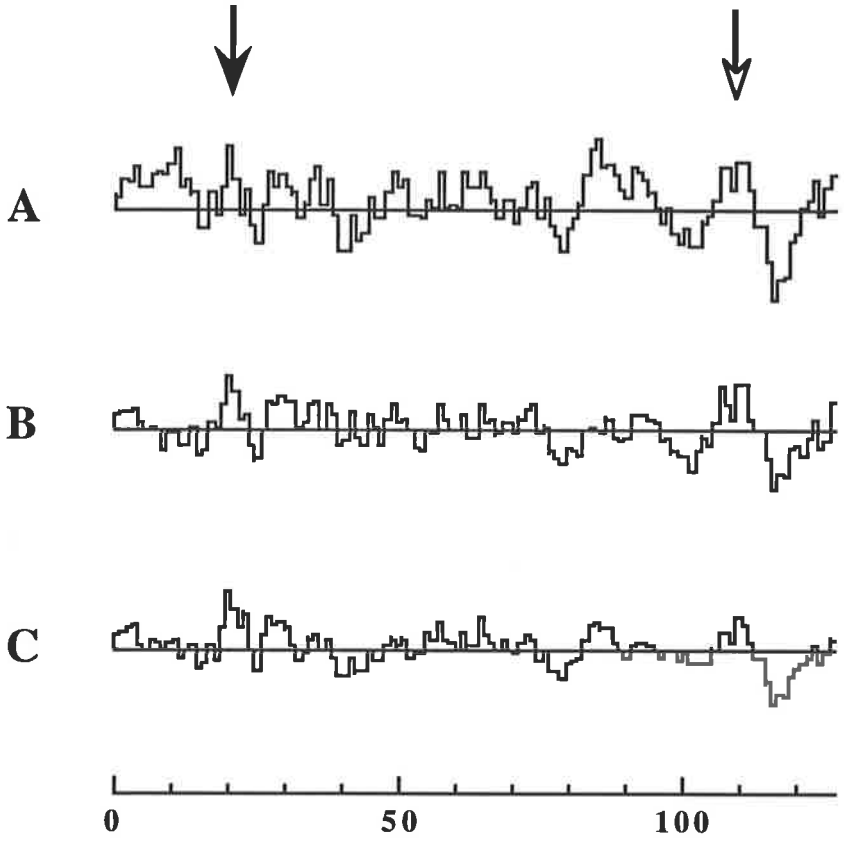

A
B
C
0
50
100
GM-CSF sequence

Figure 3.1.2 The amphipathic nature of the first and fourth α-helices of human GM-CSF.

Helical wheel plots (Schiffer and Edmundson, 1967) of residues within the first and fourth predicted α-helices, illustrate the amphipathic nature of these helices. The first residue of the proposed helix is marked with an asterisk and subsequent residues are arranged with 100° of clockwise rotation around the helical axis between each residue. The helix is viewed from the initiating residue and is projected into the page. Residues are identified using the single letter code while the hydrophilic residues are also identified with their position number.

Panel A) The first predicted α-helix from residues 15 to 28 inclusive. The residues in the hydrophobic face, His^{15}, Ala^{18}, Ile^{19}, Ala^{22}, Leu^{25} and Leu^{26} are completely conserved between the GM-CSF from seven different species (Fig. 1.2.2).

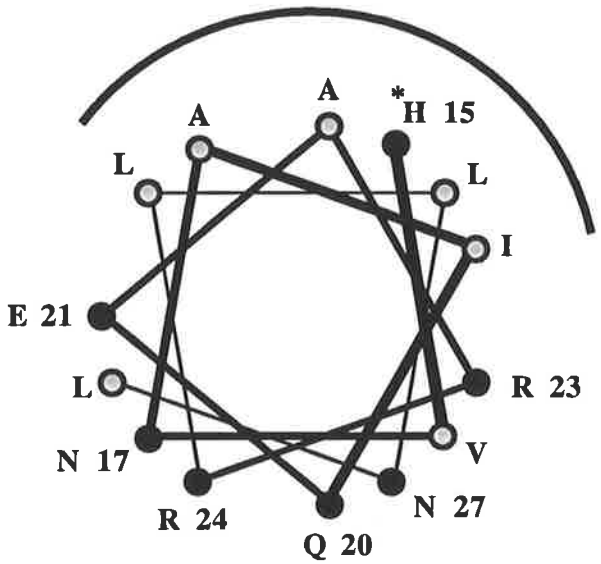
Panel B) The fourth predicted α-helix from residues 97 to 117 inclusive. The residues in the hydrophobic face, Gln^{99}, Phe^{103}, Phe^{106}, Leu^{110}, Phe^{113}, Leu^{114} and Leu^{117} are highly conserved between the GM-CSF from seven different species (Fig. 1.2.2).

● = Hydrophilic residues

 = Hydrophobic residues

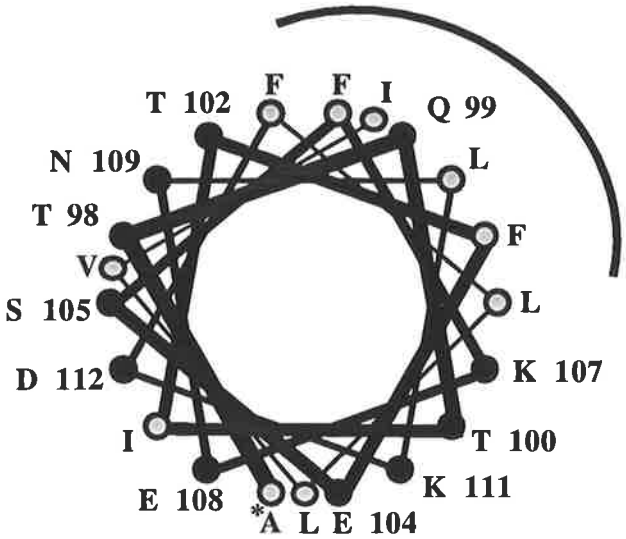
A

Hydrophobic face



B

Hydrophobic face



3.2 Methods

3.2.1 Protocol for the identification of functionally important regions of GM-CSF

A protocol was devised for the generation of mutated GM-CSF cDNA's and expression and analysis of the mutant GM-CSF protein (Fig. 3.2.1). The protocol contained three fundamental procedures;

A. Mutagenesis

The oligonucleotide-directed mutagenesis procedure of Zoller and Smith (1984) provided a simple and efficient method for generating a range of insertion, deletion or substitution mutations throughout the entire GM-CSF cDNA (Fig. 1.2.1). Deletion analogues are described with a Δ symbol followed by the position of deleted residues, thus Δ14-18 represents a GM-CSF analogue with a deletion of residues 14 to 18, inclusive. Substitution analogues are described using the single letter amino acid code where the wild type residue and residue number are listed, followed by the mutant residue, thus E21A represents a GM-CSF analogue where Glu^{21} is replaced by Ala^{21}.

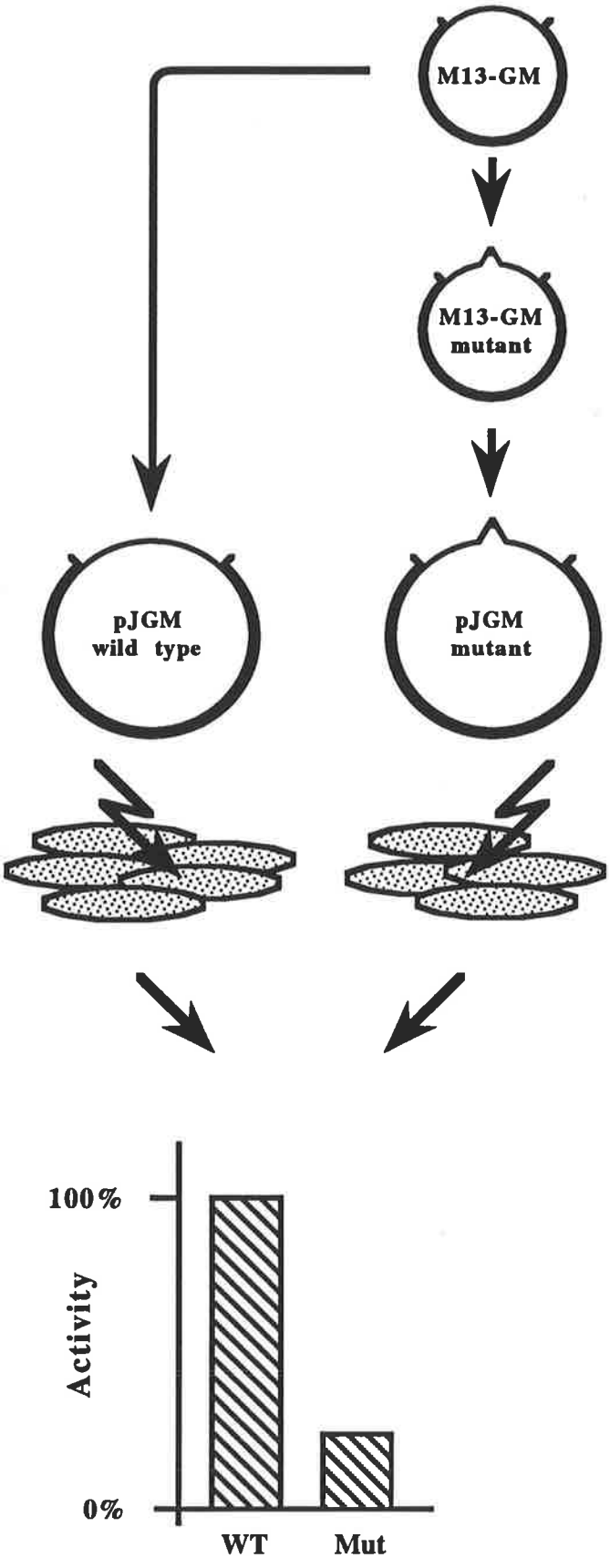
B. Expression of GM-CSF mutant analogues

Expression of mutated GM-CSF cDNA's using a suitable plasmid expression vector was achieved by transfection of COS cells. COS cell-derived human GM-CSF is functionally indistinguishable from native human GM-CSF isolated from the HTLV-transformed T-lymphocyte cell line, Mo (Wong *et al.*, 1985a) or the U5637 bladder cancer cell line (Metcalf *et al.*, 1986). Transient expression in COS cells also enabled rapid analysis of biological activity. GM-CSF analogues in COS cell conditioned medium were quantified by means of a radioimmunoassay (RIA) using a rabbit polyclonal anti-GM-CSF antibody. The level of expression of the GM-CSF analogues

Figure 3.2.1 Protocol for the generation and analysis of human GM-CSF analogues.

Human GM-CSF cDNA (Fig. 1.2.1) cloned into the M13mp19 vector was mutated by the method of Zoller and Smith (1984) and plaques screened for specific binding to the mutagenic oligonucleotide using TMACl (Wood *et al.*, 1985). The presence of the correct mutation was confirmed by chain termination sequencing (Sanger *et al.*, 1977). Double stranded (replicative form) DNA was prepared from M13 clones and the GM-CSF cDNA fragment excised with EcoRI and HindIII. The GM-CSF fragment was incubated with Klenow and deoxy nucleotides and the resulting blunt end fragment cloned into SmaI digested pJL4 (Appendix A.3.2.1). The orientation of the GM-CSF fragment in the resulting pJGM series of plasmids was determined by PstI digestion, utilising the asymmetric PstI sites located at the 5' end of the GM-CSF cDNA (Fig. 1.2.1). The presence of the correct mutation in the pJGM plasmids was also confirmed by chain termination sequencing of the mutated region.

GM-CSF was expressed from COS cells after electroporation with the pJGM plasmids (Chu *et al.*, 1987). Serum-free conditioned medium was collected after incubation for 72 hours and the GM-CSF concentration determined by radioimmunoassay. The biological activity of GM-CSF mutant analogues was typically assessed in a GM-CSF dependent cell proliferation assay. The ability of GM-CSF mutant analogues to bind the GM-CSF receptor on human neutrophils was assessed in competitive binding experiments using ^{125}I-GM-CSF.



Human GM-CSF cDNA in M13mp19

Mutagenesis of GM-CSF

Subclone mutant GM-CSF cDNA into pJL4 expression vector

Transfect COS cells with pJGM plasmid

Harvest conditioned media

Quantify GM-CSF with RIA

Assay GM-CSF mutant for biological activity and receptor binding

was also confirmed by Western blot analysis using a sheep polyclonal anti-GM-CSF antibody.

C. Biological activity and receptor binding of GM-CSF analogues

The biological activity of wild type GM-CSF and mutant analogues were typically compared in a GM-CSF-dependent, cell proliferation assay. Primary leukaemic cells from a patient with CML that incorporate [^{3}H]thymidine in response to GM-CSF (Metcalf, 1984) were used for proliferation assays. The ability of wild type GM-CSF and mutant analogues to stimulate mature cell function was also compared by analysing the GM-CSF mediated release of superoxide anions from neutrophils (Weisbart *et al.*, 1985) or the GM-CSF mediated adherence of monocytes to cultured endothelial cells or plastic surfaces (Gamble *et al.*, 1989: Elliott *et al.*, 1990). The binding of wild type GM-CSF and analogues to the high affinity (GMR$\alpha\beta_c$) receptor of neutrophils (Gasson *et al.*, 1986; Park *et al.*, 1986) was compared in competition assays using ^{125}I-GM-CSF.

Crude wild type GM-CSF produced by transfected COS cells and quantified by RIA, displayed essentially identical activity compared with purified, CHO cell-derived GM-CSF in CML cell proliferation and neutrophil superoxide anion release assays (data not shown). Conditioned media from mock transfected COS cells failed to stimulate CML proliferation or neutrophil superoxide anion release (data not shown).

3.3 Results

3.3.1 Analysis of residues in the predicted first α-helix of GM-CSF

The initial mutants (Table 3.3.1) were designed to delete regions of the amino terminus of GM-CSF, specifically the predicted first α-helix and the residues responsible for the prominent hydrophilic peak in this region (Fig. 3.1.1).

Table 3.3.1 Plasmid constructs for the expression of GM-CSF first helix mutants in COS cells.

#	Plasmid[a]	Mutation/s	Oligonucleotide[b]
1.	pJGM3	wild type	
2.	pJGMΔ9.2	Deletion 1-24	OL-9
3.	pJGMΔ10.8	Deletion 7-24	OL-10
4.	pJGMΔ11.3	Deletion 14-18	OL-11
5.	pJGMΔ12.1	Deletion 14-24	OL-12
6.	pJGMΔ45.5	Deletion 20-21	OL-45
7.	pJGM42.12	Q20A	OL-42
8.	pJGM43.2	E21A	OL-43
9.	pJGM31.16	E21R	OL-31
10.	pJGM44.10	Q20A/E21A	OL-44
11.	pJGM32.12	Q20A/E21R	OL-32

[a] The pJGM series of plasmids was generated by cloning the mutated GM-CSF cDNA into the pJL4 expression vector (Appendix A.3.2.1). An EcoRI/HindIII fragment containing GM-CSF cDNA was excised from the M13mp19 vector, Klenow blunt-ended and cloned into the SmaI site of pJL4.

[b] The sequence of the mutagenic oligonucleotides is listed in Appendix A.3.3.1.

A. Analysis of residues at the N-terminus of GM-CSF and their role in biological activity

The mutants Δ1-24 and Δ7-24 expressed very low levels of GM-CSF protein in the conditioned medium of transfected COS cells (Fig. 3.3.1A) and were not tested for biological activity. This probably reflected degradation of unfolded or poorly secreted GM-CSF analogues within COS cells. The Δ14-24 mutant expressed moderate levels of GM-CSF while the Δ14-18 and Δ20-21 mutants expressed levels of GM-CSF protein similar to that observed for wild type GM-CSF. The wild type GM-CSF and deletion analogues that were expressed in sufficient quantities, were titrated for their ability to stimulate the dose-dependent proliferation of CML cells (Fig. 3.3.1B). Wild type GM-CSF was able to stimulate [^{3}H]thymidine incorporation by CML cells with an ED_{50} value of 0.2ng/ml but the deletion mutants were devoid of measurable activity except for the Δ14-18 analogue which, with an ED_{50} value of 1.0ng/ml, exhibited a modest decrease (5-fold) in activity. The deletion of residues 20 and 21 also abolished the ability of GM-CSF to stimulate bone marrow colony formation, neutrophil superoxide anion release and neutrophil- and eosinophil-mediated antibody-dependent cytotoxicity (Lopez *et al.*, 1992c). The observation that within the first predicted α-helix, deletion of residues 14 to 18 produced a modest decrease in activity but that deletion of residues 20 and 21 abolished activity, suggested a more important functional role for the latter two residues.

B. Analysis of the role of residues 20 and 21 in GM-CSF activity

The loss of activity observed with the Δ20-21 analogue may be attributable to disruption of the amphipathic first helix and associated structural perturbations of the analogue. Thus the role of residues 20 and 21 in GM-CSF activity was examined further by alanine substitution mutagenesis since this may be expected to cause less structural disruptions than deletion mutagenesis (Table 3.3.1). Alanine substitutions were used to examine the contribution of residues 20 and 21 to GM-CSF biological activity and reduce the hydrophilic nature of the first predicted α-helix. The activity of the Q20A analogue (ED_{50} 0.4ng/ml) was comparable with wild type GM-CSF (ED_{50} 0.2ng/ml) in stimulating the proliferation of CML cells while the E21A analogue (ED_{50} 1.3ng/ml) exhibited a 6-fold reduction in activity (Fig. 3.3.2A). In neutrophil superoxide anion

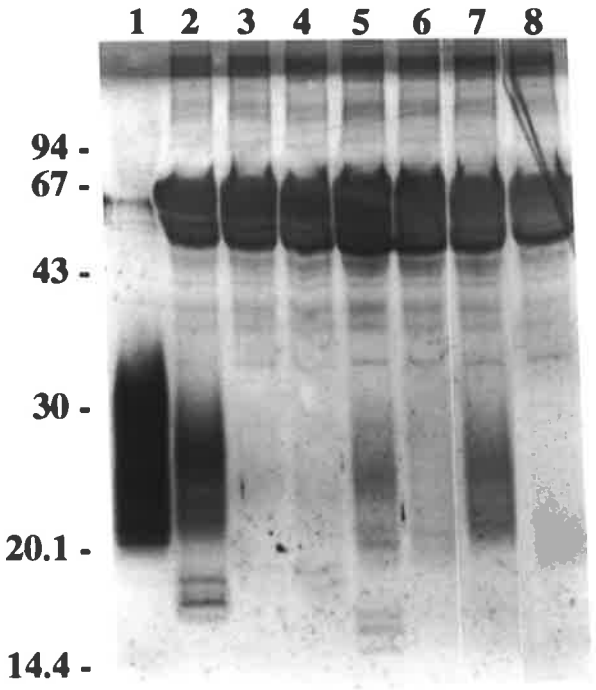
Figure 3.3.1 Expression and activity of GM-CSF deletion analogues.

Panel A) Plasmid constructs encoding wild type GM-CSF and the deletion analogues Δ1-24, Δ7-24, Δ14-18, Δ14-24 and Δ20-21 were transfected into COS cells. A mock transfection of COS cells in the absence of plasmid DNA was also performed. Samples of conditioned medium were concentrated 20-fold using Centricon-10 micro concentrators, fractionated by 12.5% SDS/PAGE, transferred to nitrocellulose and probed with sheep anti-GM-CSF polyclonal antibody as described 2.10.12-13. Molecular weight standards are indicated.

1. 200ng CHO cell-derived wild type GM-CSF
2. 25µl Wild type GM-CSF
3. 25µl Δ1-24 analogue
4. 25µl Δ7-24 analogue
5. 25µl Δ14-18 analogue
6. 25µl Δ14-24 analogue
7. 25µl Δ20-21 analogue
8. 25µl Mock transfection.

Panel B) Wild type GM-CSF (●) and the analogues Δ14-18 (■), Δ14-24 (□) and Δ20-21 (○) were quantified by RIA and titrated for their ability to stimulate [^{3}H]thymidine incorporation into CML cells as described 2.10.16. Each value represents the mean of triplicate determinations and error bars represent the SEM.

A


1 2 3 4 5 6 7 8
94 -
67 -
43 -
30 -
20.1 -
14.4 -

B

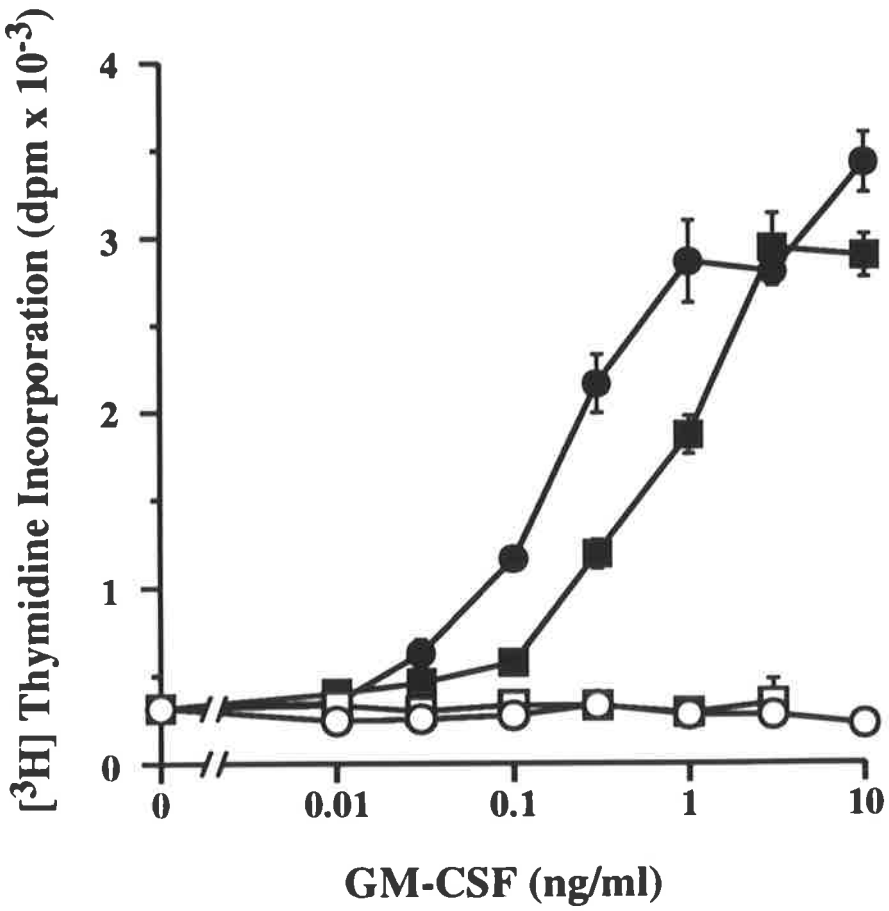

[3H] Thymidine Incorporation (dpm x 10-3)
4
3
2
1
0
0
0.01
0.1
1
10
GM-CSF (ng/ml)

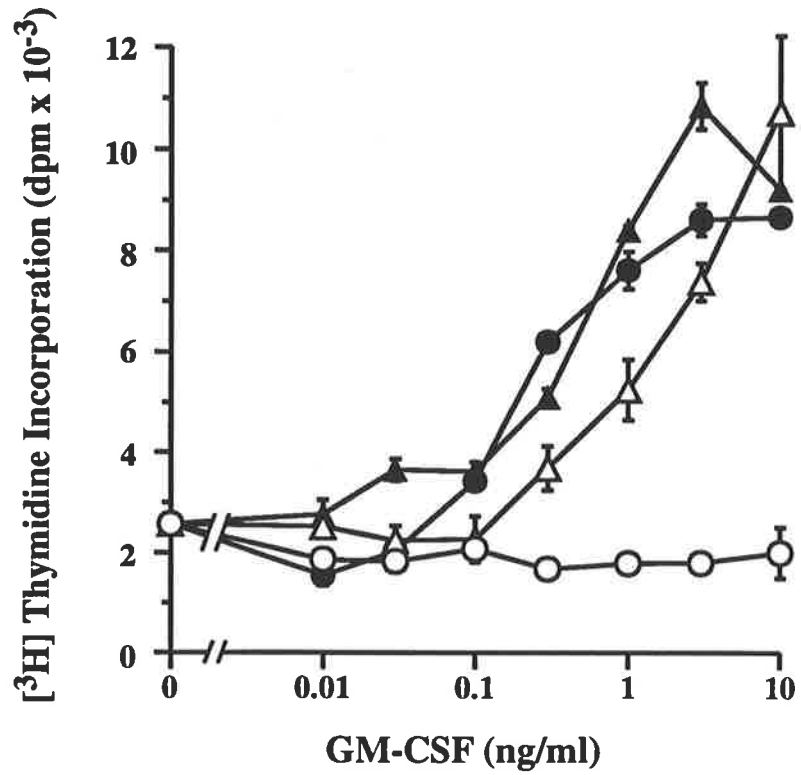
Figure 3.3.2 Activity of GM-CSF analogues with substitutions at residues 20 and 21.

Wild type GM-CSF and the analogues Δ20-21, Q20A, E21A, E21R, Q20A/E21A and Q20A/E21R were expressed in COS cells, quantified by RIA and titrated for their ability to stimulate [^{3}H]thymidine incorporation into CML cells. Each value represents the mean of triplicate determinations and error bars represent the SEM.

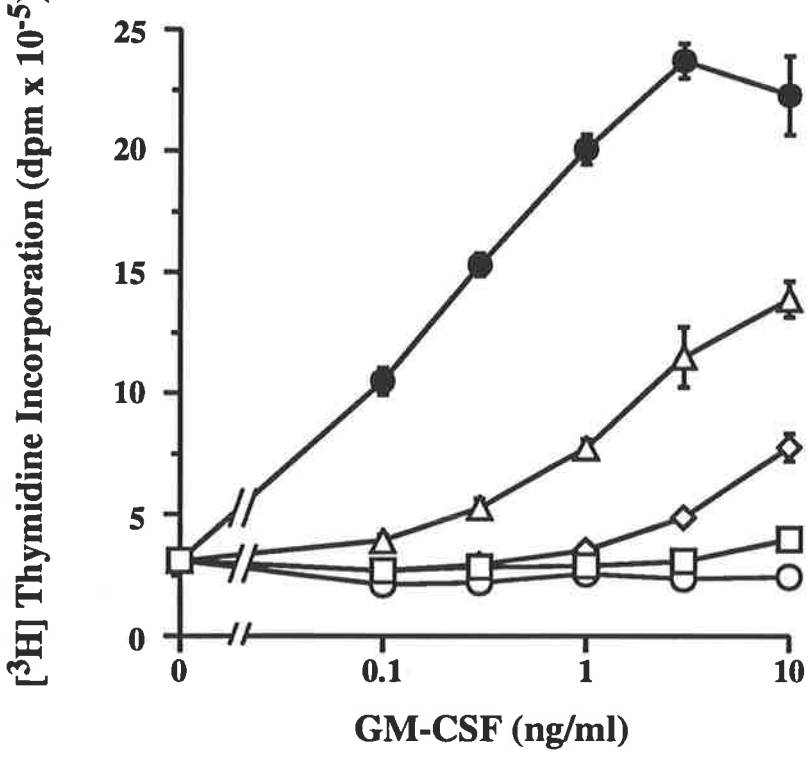
Panel A). Wild type GM-CSF (●) and the analogues Δ20-21 (○), Q20A (▲) and E21A (△).

Panel B). Wild type GM-CSF (●) and the analogues E21A (△), E21R (○), Q20A/E21A (◇) and Q20A/E21R (□).

A


[3H] Thymidine Incorporation (dpm x 10-3)
12
10
8
6
4
2
0
0
0.01
0.1
1
10
GM-CSF (ng/ml)

B


[3H] Thymidine Incorporation (dpm x 10-5)
25
20
15
10
5
0
0
0.1
1
10
GM-CSF (ng/ml)

release and neutrophil- and eosinophil-mediated antibody-dependent cytotoxicity, the Q20A analogue was indistinguishable from wild type GM-CSF while the E21A analogue exhibited a 4-fold reduction in activity (Lopez *et al.*, 1992c). The reduction in biological activity of the E21A analogue compared with wild type GM-CSF suggested that Glu^{21} was important for GM-CSF activity.

The significance of the highly conserved acidic residue at position 21 (Fig. 1.2.2) was examined more closely by generating the E21R analogue (Table 3.3.1) which, unlike the E21A analogue, retained the hydrophilic nature of this region but reversed the charge (data not shown). Dose-dependent proliferation of CML cells was observed with wild type GM-CSF (ED_{50} 0.17ng/ml) and the E21A analogue (ED_{50} 5.7ng/ml) but the E21R analogue was unable to stimulate proliferation of CML cells up to a concentration of 10ng/ml (Fig. 3.3.2B). This observation confirmed the critical role of Glu^{21} in GM-CSF activity and demonstrated that the acidic nature of this highly conserved residue is important.

The Q20A mutation, which appeared to have no affect on GM-CSF activity (Fig. 3.3.2A) was coupled with the mildly deleterious E21A mutation and the highly deleterious E21R mutation (Table 3.3.1). Interestingly the double substitution Q20A/E21A analogue displayed a marked decrease in biological activity compared to the single substitution E21A (Fig. 3.3.2B) and failed to stimulate a proliferative response equivalent to the ED_{50} response of wild type GM-CSF up to a concentration of 10ng/ml. However, comparison of the ED_{25} values suggested a 270-fold decrease in activity for the Q20A/E21A analogue compared with wild type GM-CSF. In the same assay, the E21A analogue exhibited a 30-fold decrease in activity compared with wild type GM-CSF (Fig. 3.3.2B). The difference in activity of the E21A and Q20A/E21A analogues suggested that the Q20A mutation has an effect on GM-CSF activity but only when Glu^{21} was also mutated. The Q20A/E21R analogue was unable to stimulate proliferation of CML cells up to a concentration of 10ng/ml (Fig. 3.3.2B).

C. Expression of GM-CSF residue 20 and 21 analogues from permanently transfected CHO cell lines

The GM-CSF residue 20, 21 analogues were expressed and purified from permanently transfected CHO cell lines to enable a more detailed analysis of the effects of the mutations on the multiple GM-CSF activities and receptor binding. Mutated GM-CSF cDNA's were subcloned from the pJGM series of plasmids into the pRSVN.07 expression vector (Appendix A.3.3.2) and the resulting constructs transfected into CHO cells. Following selection with geneticin and cloning, cell lines were obtained that expressed each of the GM-CSF analogues (Table 3.3.2).

In collaboration with Dr J.Cebon and Louis Fabri (Melbourne Tumour Biology Branch, Ludwig Institute for Cancer Research, Melbourne, Vic), the GM-CSF analogues expressed from CHO cell lines were immunoaffinity purified using the anti-GM-CSF monoclonal antibody, LMM 111 (Cebon *et al.*, 1988). Affinity purified GM-CSF analogues were subjected to reversed phase HPLC which separated three distinct species of GM-CSF (Fig. 3.3.3). This pattern was similar to that observed with the reversed phase fractionation of GM-CSF purified from activated human T-lymphocytes (Cebon *et al.*, 1990). The species fractionated by reversed phase HPLC represent three size classes of glycosylated GM-CSF with high Mr species eluting first, followed by intermediate and low Mr species (Cebon *et al.*, 1990). Donahue *et al* (1986a) have reported that the three size classes of CHO cell-derived GM-CSF are attributable to different degrees of occupancy of the two sites for asparagine-linked (N-linked) carbohydrate at Asn^{27} and Asn^{37}. High Mr species contain GM-CSF in which both N-linked sites are occupied, intermediate Mr species contain GM-CSF in which either site is occupied while the low Mr GM-CSF is devoid of N-linked carbohydrate (Donahue *et al.*, 1986a).

Fractions containing the first two glycosylation species of GM-CSF analogue to elute from the reversed phase column were combined and in collaboration with Dr R.Simpson (Joint Protein Structure Laboratory, Ludwig Institute for Cancer

Table 3.3.2 Plasmid constructs for the expression of GM-CSF analogues from permanently transfected CHO cells.

#	Plasmid[a]	Insert	CHO[b] cell line	Mutation/s	GM-CSF[c] (ng/ml)
1.	pRSVNGM42.5	GM42.12	42.5	Q20A	460
2.	pRSVNGM43.4	GM43.2	43.4	E21A	410
3.	pRSVNGM31.7	GM31.16	31.7	E21R	170
4.	pRSVNGM44.9	GM44.10	44.9	Q20A/E21A	270
5.	pRSVNGM32.11	GM32.12	32.11	Q20A/E21R	490

[a] The pRSVNGM series of plasmids was generated by cloning the EcoRI/BamHI fragments from the pJGM series of plasmids (Table 3.3.1), containing the mutated GM-CSF cDNA, into EcoRI/BamHI digested pRSVN.07 expression vector (A.3.3.2).

[b] Several clones of each CHO cell line were isolated and the expression of GM-CSF analogue by each cell line measured by radioimmunoassay. The cell line displaying the highest level of GM-CSF expression was selected for each GM-CSF analogue.

[c] Concentration of GM-CSF analogue secreted into the medium by the isolated CHO cell line. Each CHO cell line was cultured to approximately 80% confluence in Ham's F12 containing 400µg/ml geneticin and 10% (v/v) FCS. Medium was replaced with FCS-free Ham's F12 containing 400µg/ml geneticin and after a 72 hour incubation, conditioned medium was collected and the concentration of GM-CSF measured by radioimmunoassay.

Figure 3.3.3 Reversed phase HPLC fractionation of CHO cell-derived GM-CSF analogues.

Conditioned medium from the CHO cell lines expressing GM-CSF analogues was mixed at 4°C overnight with sufficient LMM 111 affinity matrix to bind approximately 50µg of GM-CSF. Matrix was collected, washed with PAT buffer and then 100mM NaCl, 100mM glycine HCl pH4.0. GM-CSF was eluted from the affinity matrix with 2mls of 100mM NaCl, 100mM glycine HCl pH2.0 and neutralised with Tris-HCl pH8.8. Affinity purified GM-CSF analogues were loaded onto a Brownlee Aquapore RP-300 microbore reversed phase column (2.1x30mm). Bound protein was eluted at 0.1ml/min using a 0-60% gradient of acetonitrile containing a 0.15-0.125% (v/v) gradient of TFA. The traces display the absorbance recorded at 215nm. The peaks containing the eluted GM-CSF are marked with an asterisk.

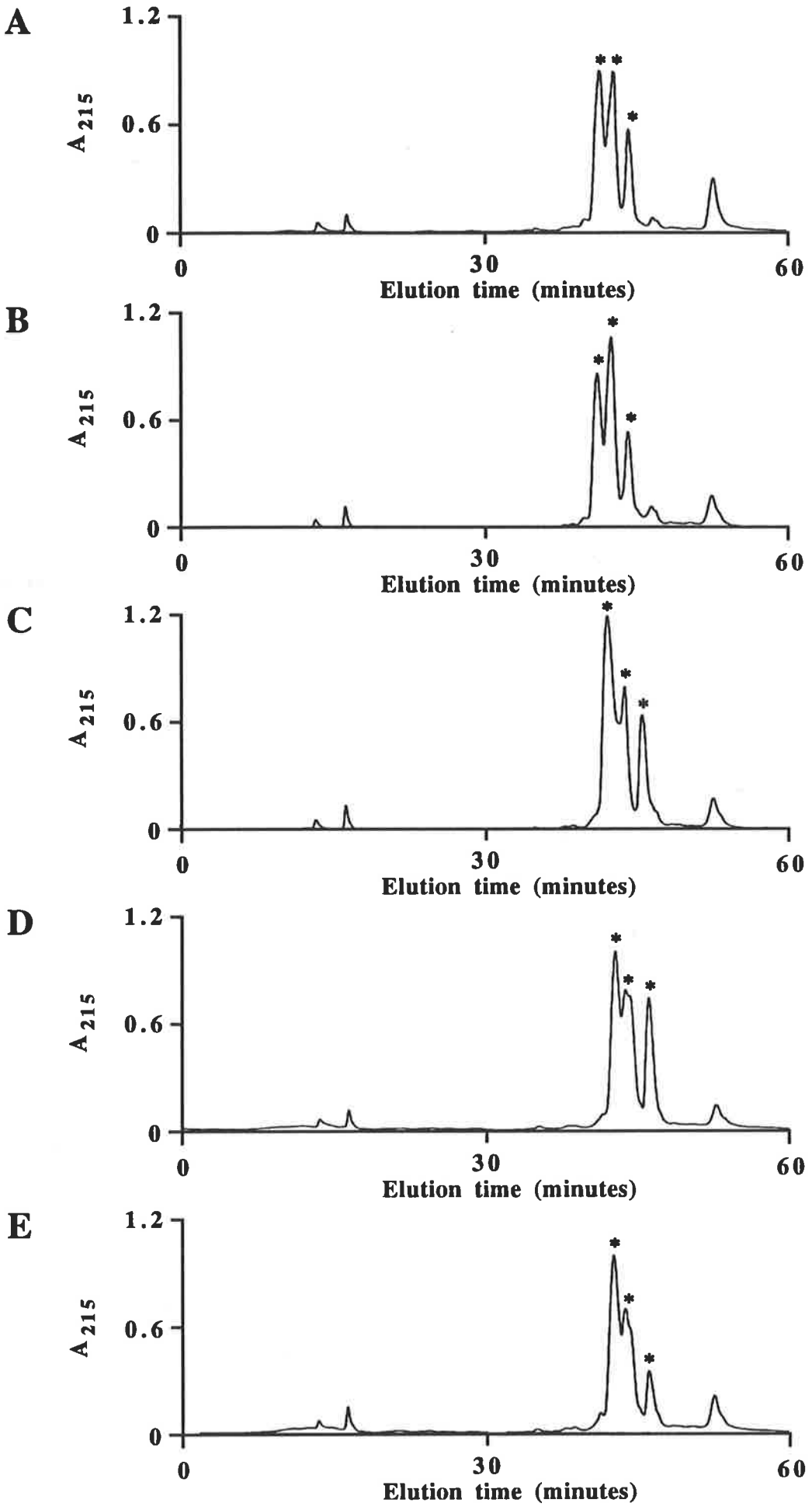
Chromatograms for, A) E21R analogue

B) Q20A/E21R analogue

C) Q20A analogue

D) E21A analogue

E) Q20A/E21A analogue


A
B
C
D
E
1.2
0.6
0
A_{215}
0
30
60
Elution time (minutes)

Research/Walter & Eliza Hall Institute for Medical Research, Melbourne, Vic), quantified by amino acid analysis. The use of amino acid analysis avoided the potential problems associated with immunological quantification where mutant analogues do not necessarily bind with wild type affinity to the antibody leading to an inaccurate estimate of mutant analogue concentration. However, there was no significant difference in the concentration of the GM-CSF analogues determined by amino acid analysis or by using the standard RIA procedure (data not shown). Comparison of the purified GM-CSF analogues by silver stained SDS/PAGE (Fig. 3.3.4A) and western blot analysis (Fig. 3.3.4B) showed very similar molecular weight heterogeneity for the analogues compared with the CHO cell-derived wild type GM-CSF obtained from Genetics Institute. The level of glycosylation was deliberately balanced as much as possible because of the known influence of carbohydrate on the specific activity of GM-CSF (Moonen *et al.*, 1987; Kaushansky *et al.*, 1987; Cebon *et al.*, 1990). Table 3.3.3 summarises the yield for each of the purified GM-CSF analogues.

D. Activity of CHO cell-derived GM-CSF residue 20 and 21 analogues

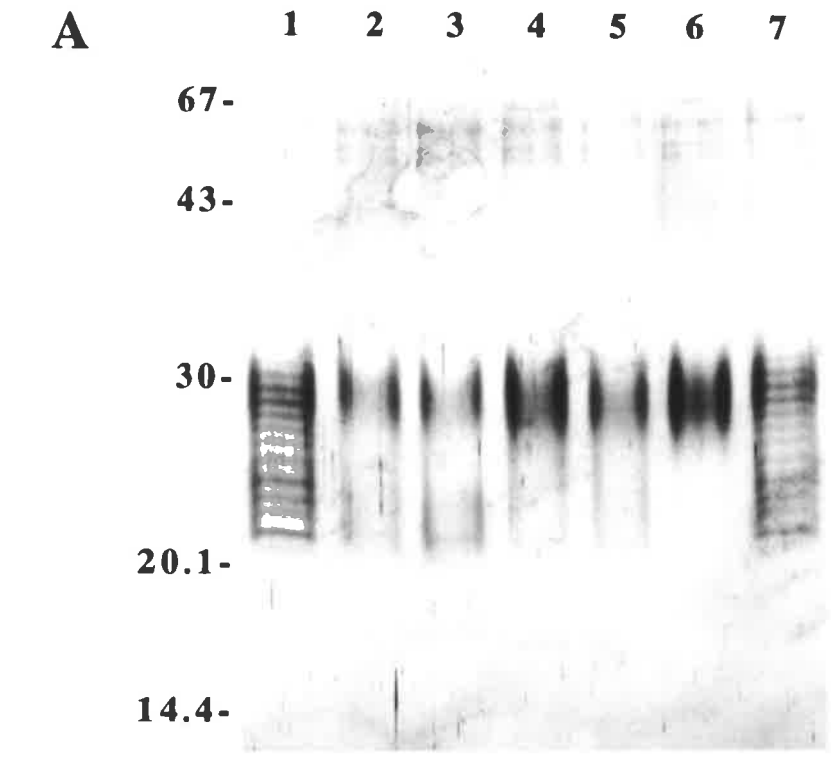
The purified, CHO cell-derived GM-CSF analogues were compared for their ability to stimulate proliferation of CML cells (Fig. 3.3.5). Wild type GM-CSF and the purified analogues stimulated dose-dependent proliferation of CML cells with ED_{50} values ranging from 0.15 ng/ml for wild type up to 40 ng/ml for the Q20A/E21R analogue (Table 3.3.4). The hierarchy of biological activity for the purified analogues was similar to that observed with crude, COS cell-derived analogues (Fig. 3.3.2). The purified E21R analogues did exhibit biological activity but only at significantly higher concentrations than previously tested with COS cell-derived material (Fig. 3.3.2) and are 200- to 270-fold less active than wild type GM-CSF.

The purified GM-CSF analogues were also compared for their ability to stimulate mature cell function (Fig. 3.3.6). Wild type GM-CSF and the purified analogues stimulated the dose-dependent production of superoxide anions by neutrophils (Fig. 3.3.6A) with ED_{50} values ranging from 0.33 ng/ml for the Q20A analogue up to 330

Figure 3.3.4 Purified GM-CSF residue 20,21 analogues.

GM-CSF analogues were expressed from permanently transfected CHO cell lines, immunoaffinity purified and fractionated by reversed phase HPLC (Fig. 3.3.3). Fractions containing high molecular weight GM-CSF, similar in mobility when compared with CHO cell-derived wild type GM-CSF, were pooled and quantified by amino acid analysis. Samples of wild type GM-CSF and analogues were fractionated by 12.5% SDS/PAGE and stained with silver (Panel A) or transferred to nitrocellulose and probed with sheep anti-GM-CSF polyclonal antibody (Panel B). Molecular weight standards are indicated.

1. 200ng Wild type GM-CSF
2. 200ng E21R analogue
3. 200ng Q20A/E21R analogue
4. 200ng Q20A analogue
5. 200ng E21A analogue
6. 200ng Q20A/E21A analogue
7. 200ng Wild type GM-CSF


A
1 2 3 4 5 6 7
67-
43-
30-
20.1-
14.4-

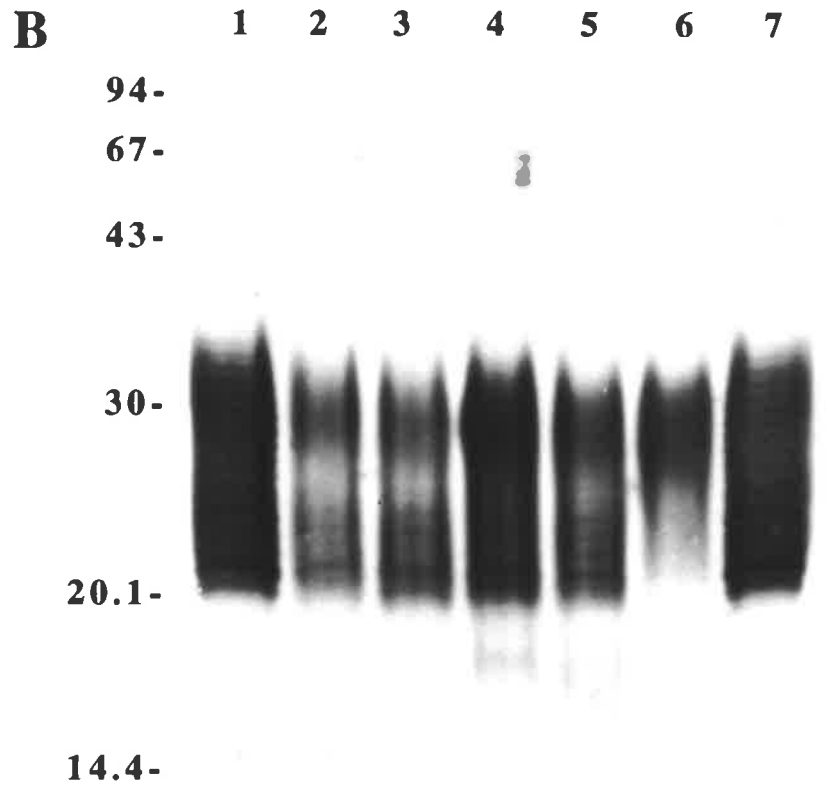

B
1 2 3 4 5 6 7
94-
67-
43-
30-
20.1-
14.4-

Table 3.3.3 Yield of GM-CSF analogues purified from CHO cell lines.

CHO cell	Analogue line[a]	Crude GM-CSF[b] (mls)	Yield[c] (μg)
42.5	Q20A	250	34.2
43.4	E21A	200	27.3
31.7	E21R	280	27.6
44.9	Q20A/E21A	250	13.6
32.11	Q20A/E21R	280	26.1

[a] Permanently transfected CHO cell lines expressing GM-CSF residue 20,21 analogues (Table 3.3.2).
[b] Volume of conditioned cell line medium containing crude GM-CSF analogue.
[c] Yield of purified GM-CSF analogue quantified by amino acid analysis. Value adjusted to account for the fact that the four Cys and two Trp residues of GM-CSF were destroyed during hydrolysis.

Figure 3.3.5 Comparison of the proliferative activity of CHO cell-derived GM-CSF analogues.

Wild type GM-CSF (●) and the analogues Q20A (▲), E21A (Δ), E21R (O), Q20A/E21A (◇) and Q20A/E21R (□), purified from CHO cell lines and quantified by amino acid analysis, were titrated for their ability to stimulate [^{3}H]thymidine incorporation into CML cells. Each value represents the mean of triplicate determinations and error bars represent the SEM.

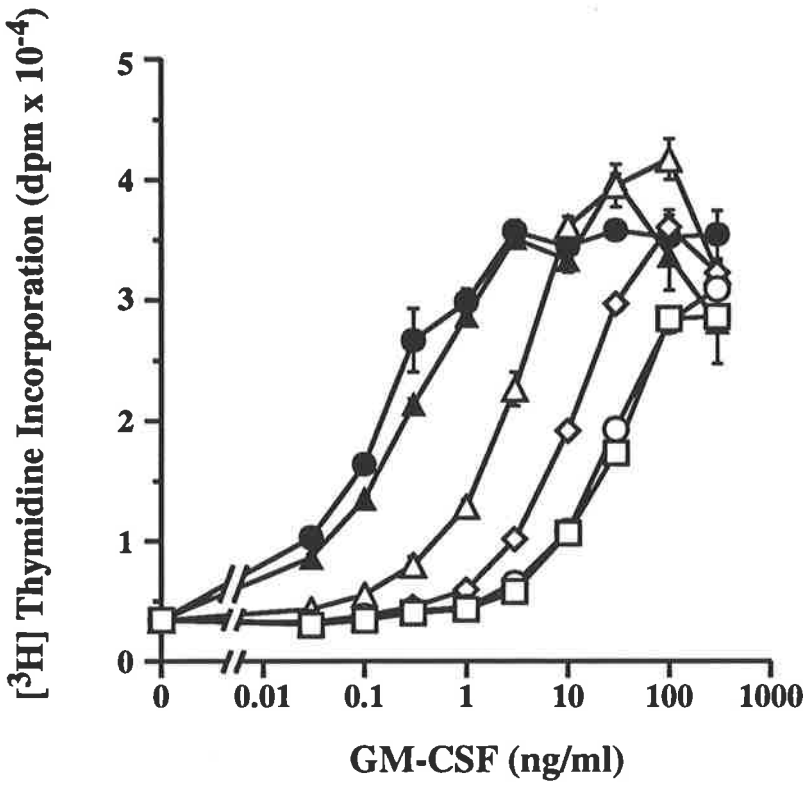
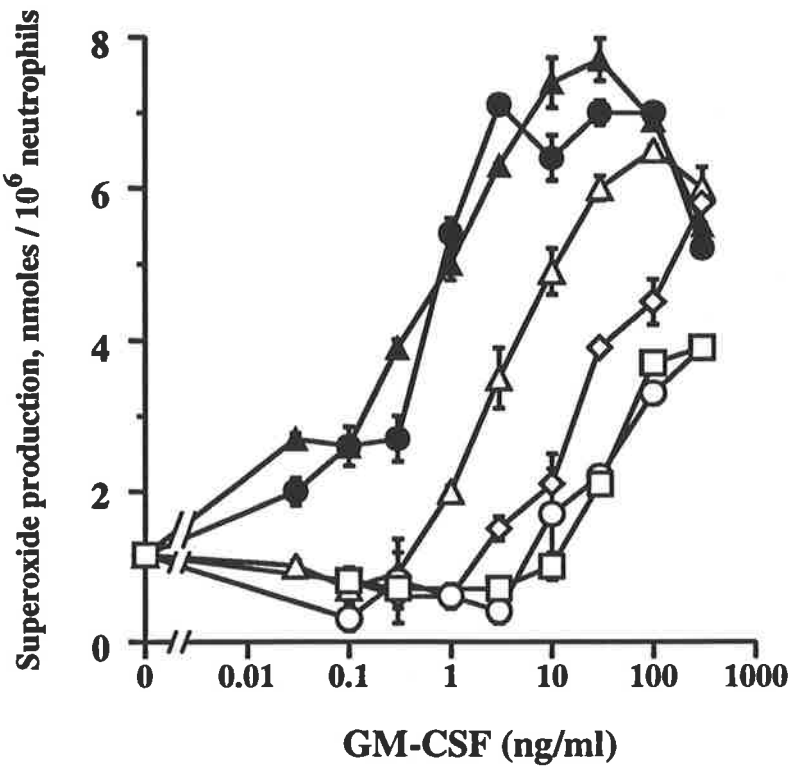

[3H] Thymidine Incorporation (dpm x 10-4)
5
4
3
2
1
0
0
0.01
0.1
1
10
100
1000
GM-CSF (ng/ml)

Figure 3.3.6 Comparison of the CHO cell-derived GM-CSF analogues ability to stimulate mature cell function.

Wild type GM-CSF (●) and the analogues Q20A (▲), E21A (△), E21R (○), Q20A/E21A (◇) and Q20A/E21R (□), were titrated for their ability to stimulate the production of superoxide anions from human neutrophils (A) or to stimulate the adherence of human monocytes as described 2.10.16 (B). Each value represents the mean of triplicate determinations and error bars represent the SEM.

A


Superoxide production, nmoles / 10^6 neutrophils
8
6
4
2
0
0
0.01
0.1
1
10
100
1000
GM-CSF (ng/ml)

B

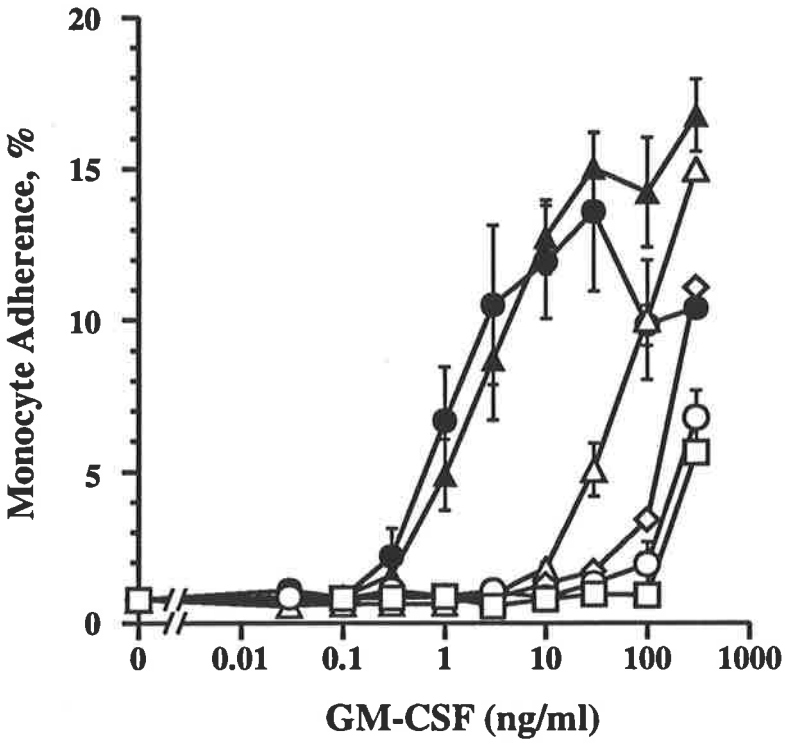

Monocyte Adherence, %
20
15
10
5
0
0
0.01
0.1
1
10
100
1000
GM-CSF (ng/ml)

Table 3.3.4 Relative biological activity of GM-CSF analogues purified from CHO cells.

Assay[a]		GM-CSF analogue					
		WT	Q20A	E21A	Q20A/ E21A	E21R	Q20A/ E21R
Proliferation	ED_{50}[b]	0.15	0.23	2.1	10	30	40
	Activity[d]	**100**	**65**	**7**	**1.5**	**0.5**	**0.4**
Superoxide	ED_{50}[b]	0.55	0.33	5.1	40	330[c]	330[c]
	Activity[d]	**100**	**170**	**10**	**1**	**0.5[e]**	**0.5[e]**
Adherence	ED_{50}[b]	1.2	2.0	55	170	330[c]	500[c]
	Activity[d]	**100**	**60**	**2**	**0.7**	**0.3[e]**	**0.2[e]**
Average activity		**100**	**90**	**5**	**1**	**0.4**	**0.3**

[a] Data obtained from the CML proliferation assay (Fig. 3.3.5), neutrophil superoxide anion release assay (Fig. 3.3.6A) and monocyte adherence assay (Fig. 3.3.6B).

[b] ED_{50} values are in ng/ml GM-CSF.

[c] Some analogues failed to reach the ED_{50} value of wild type GM-CSF at maximal stimulation so the ED_{50} values were extrapolated from the dose-response curves.

[d] The activity for each analogue, expressed as a percentage of wild type GM-CSF activity, was calculated from the ED_{50} values as described 2.10.16A.

[e] Decrease in activity compared to wild type GM-CSF was calculated from the ED_{40} values (Superoxide) or ED_{25} values (Adherence).

ng/ml for the E21R and Q20A/E21R analogues (Table 3.3.4). Wild type GM-CSF and the purified analogues also stimulated the dose-dependent adherence of monocytes to plastic (Fig. 3.3.6B) with ED_{50} values ranging from 1.2 ng/ml for wild type up to 500 ng/ml for the Q20A/E21R analogue (Table 3.3.4). The E21R and Q20A/E21R analogues were not able to maximally stimulate neutrophil or monocyte activation at the concentrations tested.

The hierarchy of biological activity for the GM-CSF analogues was very similar in both proliferation and functional activation assays. The average decrease in GM-CSF activity for each of the single amino acid substitutions was, Q20A, 1.3-fold, E21A, 24-fold and E21R, 250-fold (Table 3.3.4).

E. Receptor binding characteristics of CHO cell-derived GM-CSF residue 20 and 21 analogues

The purified, CHO cell-derived GM-CSF analogues were compared for their ability to compete for ^{125}I-GM-CSF binding to the high affinity receptor (GMR$\alpha\beta_c$) of human neutrophils (Fig. 3.3.7). Compared with wild type GM-CSF, the Q20A analogue was 3-fold less effective and the E21A analogue 40-fold less effective at competing for ^{125}I-GM-CSF binding. The Q20A/E21A, E21R and Q20A/E21R analogues were all at least 100-fold less effective at competing for the binding of ^{125}I-GM-CSF than wild type GM-CSF. Thus, the binding of the GM-CSF residue 20/21 analogues to the GM-CSF high affinity receptor (GMR$\alpha\beta_c$) paralleled the biological activity of these analogues on neutrophils, monocytes and leukaemic cells. However, these results did not indicate if binding to the low affinity receptor (GMRα) was also affected.

The question of low affinity receptor binding was addressed with the E21R analogue which exhibited the lowest level of biological activity and weakest interaction with the high affinity receptor. The purified E21R analogue was assessed for the ability to compete for ^{125}I-GM-CSF binding to the low and high affinity receptor of human monocytes (Elliott *et al.*, 1989). A binding curve for ^{125}I-GM-CSF was performed in the presence of a 35-fold excess of the E21R analogue (Fig. 3.3.8A). The E21R analogue

Figure 3.3.7 Comparison of the binding of the CHO cell-derived GM-CSF analogues to the GM-CSF receptor on human neutrophils.

The binding of wild type GM-CSF (●) and the analogues Q20A (▲), E21A (△), E21R (○), Q20A/E21A (◇) and Q20A/E21R (□) to cells expressing the high ($GMR\alpha\beta_c$) affinity GM-CSF receptors was compared. Binding experiments were performed as described 2.10.17. Unlabelled wild type GM-CSF or analogues were titrated against 70pM ^{125}I-GM-CSF and 4 x 10^6 neutrophils per tube. The values are expressed as a percentage of the ^{125}I-GM-CSF bound in the absence of competitor and are the means of duplicate determinations.

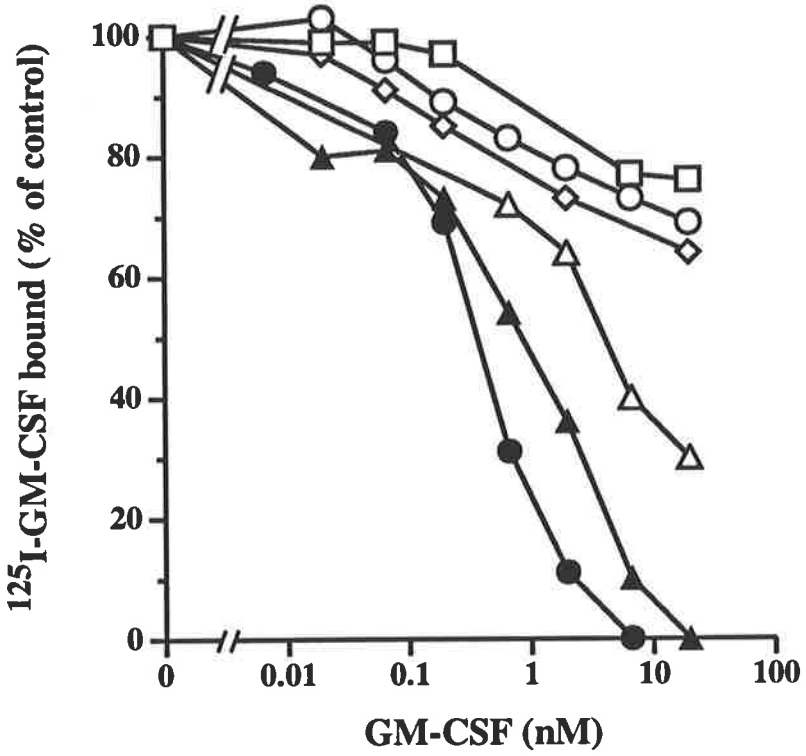
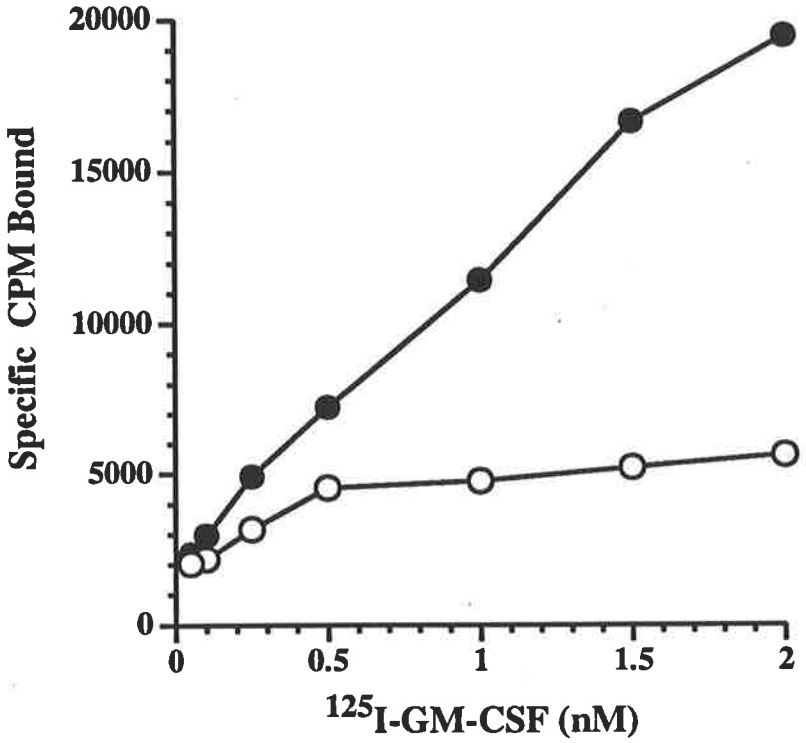

^{125}I-GM-CSF bound (% of control)
100
80
60
40
20
0
0
0.01
0.1
1
10
100
GM-CSF (nM)

Figure 3.3.8 Binding of the CHO cell-derived E21R analogue to the low affinity and high affinity GM-CSF receptors of human monocytes.

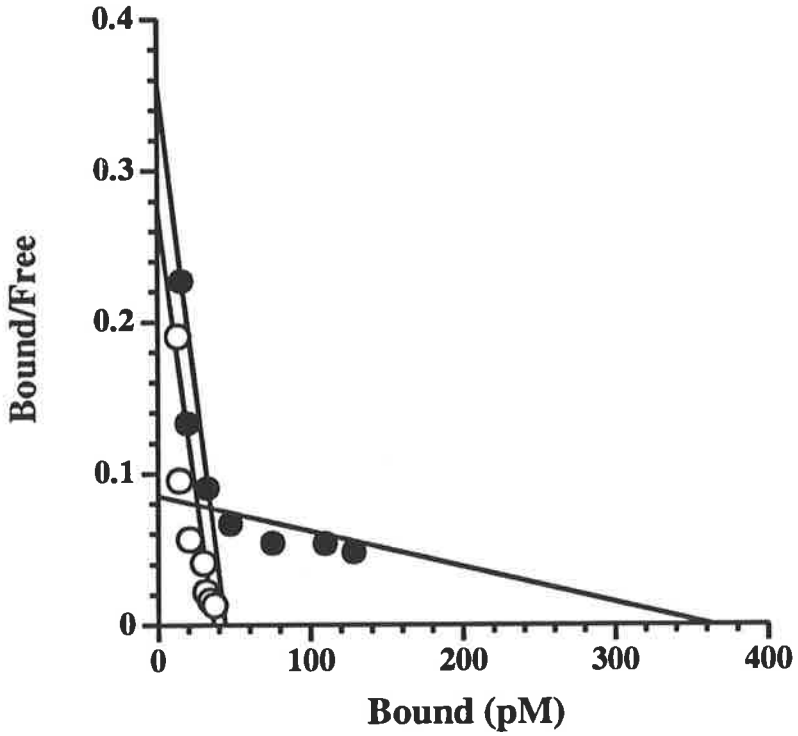
Binding curve of ^{125}I-GM-CSF to purified monocytes in the presence or absence of the E21R analogue (Panel A). Wild type ^{125}I-GM-CSF in the absence (●) or presence (O) of a 35-fold excess of the E21R analogue was mixed with 2 x 10^6 monocytes per tube. The values are expressed as specific counts bound and are the means of duplicate determinations. Specific counts were determined by subtracting the counts obtained in the presence of a 100-fold excess of wild type GM-CSF at each concentration of ^{125}I-GM-CSF.

Scatchard analysis of binding data from Panel A, showing the competition for ^{125}I-GM-CSF binding in the presence (O) or absence (●) of the E21R analogue (Panel B).

A


20000
15000
10000
5000
0
Specific CPM Bound
0
0.5
1
1.5
2
125I-GM-CSF (nM)

B


0.4
0.3
0.2
0.1
0
Bound/Free
0
100
200
300
400
Bound (pM)

slightly inhibited the binding of low concentrations of ^{125}I-GM-CSF but strongly inhibited the binding of high concentrations of ^{125}I-GM-CSF. Scatchard transformation of the data showed that the E21R analogue was able to effectively compete for the low affinity binding of ^{125}I-GM-CSF but only slightly reduced the high affinity binding (Fig. 3.3.8B). This result indicated that the E21R analogue was able to preferentially interact with the low affinity but not the high affinity receptor.

To further characterise the interaction of the E21R analogue with the low affinity receptor, competitive binding experiments were performed using COS cells expressing the cloned low affinity receptor α-chain (Gearing *et al.*, 1989). In collaboration with Dr. N.Nicola and Meredith Layton (The Walter and Eliza Hall Institute, Parkville, Vic) the ability of wild type GM-CSF and the E21R analogue to compete for the binding of ^{125}I-GM-CSF to COS cells expressing the cloned human GM-CSF receptor α-chain was compared (Fig. 3.3.9A). The results indicated that the ability of wild type GM-CSF and the E21R analogue to the compete for ^{125}I-GM-CSF binding to the low affinity receptor was indistinguishable. In parallel experiments the E21R analogue was 300-fold less potent than wild type GM-CSF at competing for ^{125}I-GM-CSF binding to the high affinity receptor of neutrophils (Fig. 3.3.9B). These results show that the reduced biological activity of the residue 21 analogues was associated with reduced binding to the high affinity receptor (GMRαβ_c) but not the low affinity receptor (GMRα).

3.3.2 Analysis of residues in the predicted fourth α-helix of GM-CSF

Using the experience gained with mutagenesis of residues in the first helix, charged residues on the hydrophilic surface of the predicted fourth α-helix were targeted for mutagenesis. The residues Lys^{107}, Glu^{108}, Lys^{111} and Asp^{112}, which exhibit a high degree of conservation amongst the seven known GM-CSF sequences (Fig. 1.2.2) and may therefore be important for GM-CSF function, were subjected to charge reversal mutagenesis (Table 3.3.5). The mutation encoding the E104A analogue arose as the result of an error during PCR amplification of the GM-CSF cDNA and was retained to

Figure 3.3.9 Comparison of the binding of the wild type GM-CSF and the E21R analogue to COS cells transfected with the human GMRα chain and neutrophils.

The binding of wild type GM-CSF (●) and the E21R (O) analogue to cells expressing the low (GMRα, Panel A) or high (GMRαβ_c, Panel B) affinity GM-CSF receptors was compared. Wild type GM-CSF or E21R analogue were titrated against 6nM of *E. coli*-derived ^{125}I-GM-CSF and 1 x 10^6 pGMR138 (Gearing *et al.*, 1989) transfected COS cells per tube (A) or 2nM of *E. coli*-derived ^{125}I-GM-CSF and 1.5 x 10^6 neutrophils per tube (B). The values are expressed as a percentage of the ^{125}I-GM-CSF bound in the absence of competitor and are the means of duplicate determinations.

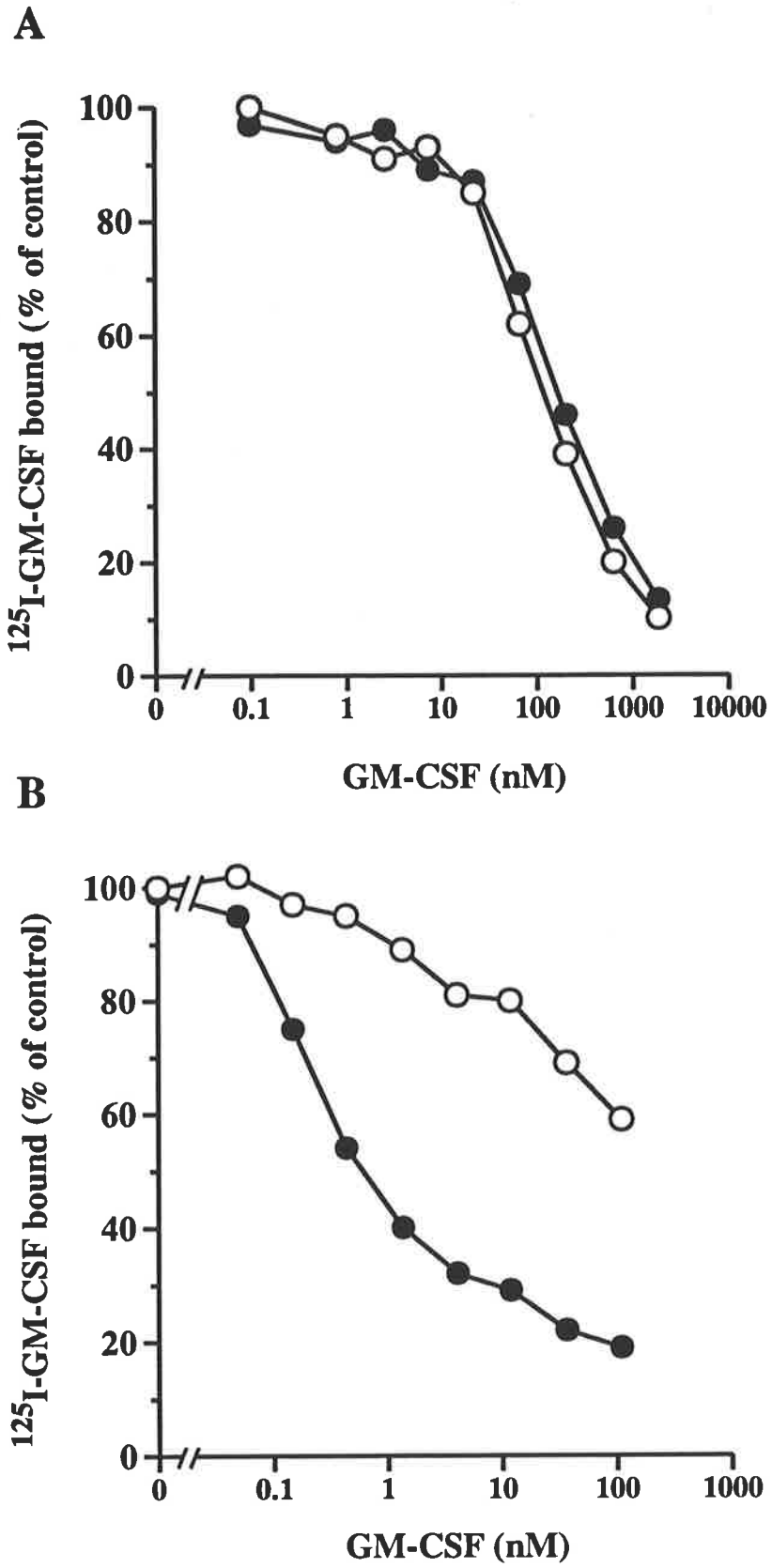

A
100
80
60
40
20
0
^{125}I-GM-CSF bound (% of control)
0
0.1
1
10
100
1000
10000
GM-CSF (nM)
B
100
80
60
40
20
0
^{125}I-GM-CSF bound (% of control)
0
0.1
1
10
100
1000
GM-CSF (nM)

Table 3.3.5 Plasmid constructs for the expression of GM-CSF fourth helix mutants in COS cells.

#	Plasmid[a]	Mutation	Oligonucleotide[b]
1.	pJSGM24	E104A	--[c]
2.	pJGM37.5	K107D	OL-37
3.	pJGM38.73	E108R	OL-38
4.	pJGM39.2	K111D	OL-39
5.	pJGM40.153	D112R	OL-40

[a] The pJGM series of plasmids was generated by cloning the mutated GM-CSF cDNA into the pJL4 expression vector (Appendix A.3.2.1). An EcoRI/HindIII fragment containing GM-CSF cDNA was excised from the M13mp19 vector, Klenow blunt-ended and cloned into the SmaI site of pJL4.

[b] The sequence of the mutagenic oligonucleotides is listed in Appendix A.3.3.1.

[c] The translated portion of wild type GM-CSF cDNA was PCR amplified from pJGM3 (Table 3.3.1) using the primers PCR#1 and PCR#2 (see below) and 25 cycles of 94°C/1', 52°C/2', 72°C/1'. The resulting 459bp product was digested with SalI and XbaI and the fragment cloned into the SalI/XbaI digested pJSV40 expression vector (Appendix A.3.3.3). One clone carried a single A→C substitution which produced the Glu^{104}(GAA) to Ala^{104}(GCA) mutation. Sequence analysis of the entire PCR product showed that the mutation at residue 104 was unique.

PCR#1 5' TAA TAT GTC GAC ATG TGG CTG CAG AGC CTG CT 3'
SalI Met

PCR#2 5' ATA CTA TCT AGA TCA CTC CTG GAC TGG CTC CC 3'
XbaI Stop

enable the role of the fifth charged residue in the predicted fourth helix to be assessed (Table 3.3.5).

A. Analysis of the role in biological activity of charged residues in the predicted fourth helix of GM-CSF

The wild type GM-CSF and fourth α-helix analogues were titrated for the ability to stimulate the proliferation of CML cells (Fig. 3.3.10). The fourth helix analogues were all able to stimulate dose-dependent proliferation of CML cells with ED_{50} values ranging from 0.42ng/ml for the K111D analogue to 6.4ng/ml for the D112R analogue compared with 0.56ng/ml for wild type GM-CSF. The effect of substitutions affecting the charged residues of the fourth predicted helix on GM-CSF biological activity appeared less dramatic than observed for Glu^{21} in the first predicted helix (Fig. 3.3.2B). However, the E108R and D112R analogues exhibited a 4- and 11-fold decrease in biological activity, respectively, which suggested that Glu^{108} and Asp^{112} are important for GM-CSF activity.

B. Receptor binding characteristics of the E108R and D112R analogues

The E108R and D112R analogues were compared for the ability to compete for ^{125}I-GM-CSF binding to the high affinity receptor (GMRαβ_c) of human neutrophils. Compared with wild type GM-CSF the E108R analogue was two-fold less effective and the D112R analogue six-fold less effective at competing for ^{125}I-GM-CSF binding to neutrophils (Fig. 3.3.11A). Thus the residue 108 and 112 analogues exhibit a modest reduction in binding to the high affinity receptor (GMRαβ_c) that is similar to the reduction in biological activity of these analogues.

The ability of the E108R and D112R analogues to compete for ^{125}I-GM-CSF binding to the low affinity receptor (GMRα) of the A9/C7 CHO cell line (Hercus *et al.*, 1994b) was compared. The A9/C7 cell line is a G-418-resistant clone of CHO cells, stably transfected with the human GMRα chain cDNA (Gearing *et al.*, 1989) and isolated by Dr J.Woodcock and Mara Dottore in our laboratory. Scatchard analysis

Figure 3.3.10 Activity of GM-CSF analogues with substitutions of the charged residues in the predicted fourth α-helix.

Wild type GM-CSF (●) and the analogues E104A (▲), K107D (■), E108R (Δ), K111D (◆) and D112R (O) were expressed in COS cells, quantified by RIA and titrated for the ability to stimulate [^{3}H]thymidine incorporation into CML cells. Each value represents the mean of triplicate determinations and error bars represent the SEM.

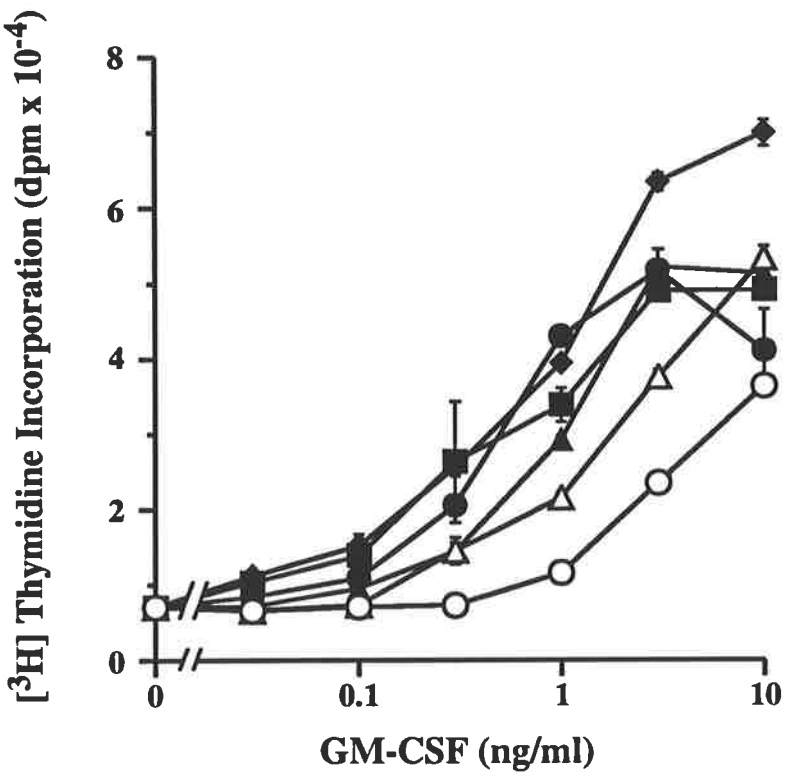
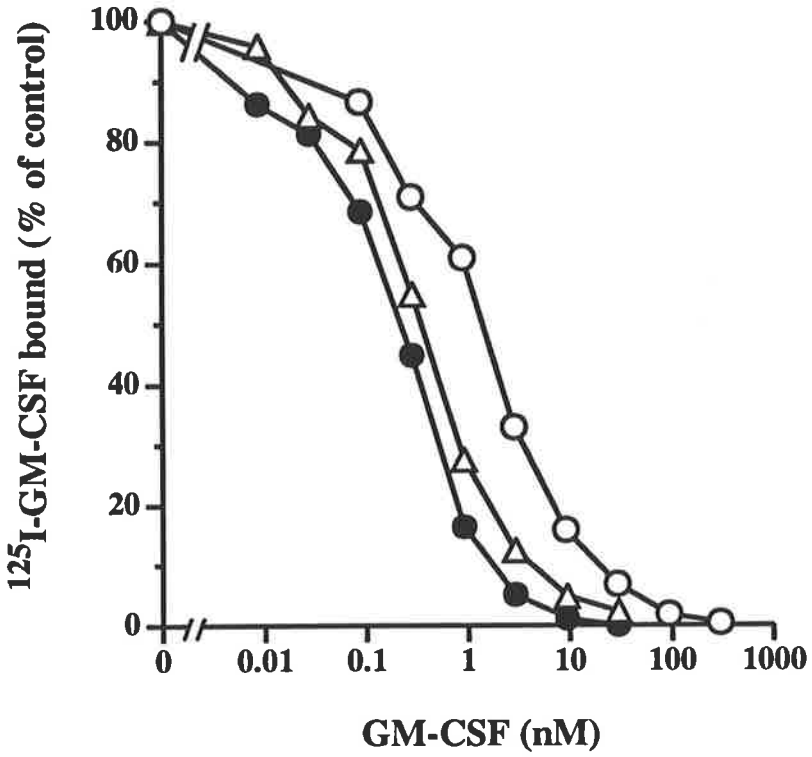

[3H] Thymidine Incorporation (dpm x 10-4)
8
6
4
2
0
0
0.1
1
10
GM-CSF (ng/ml)

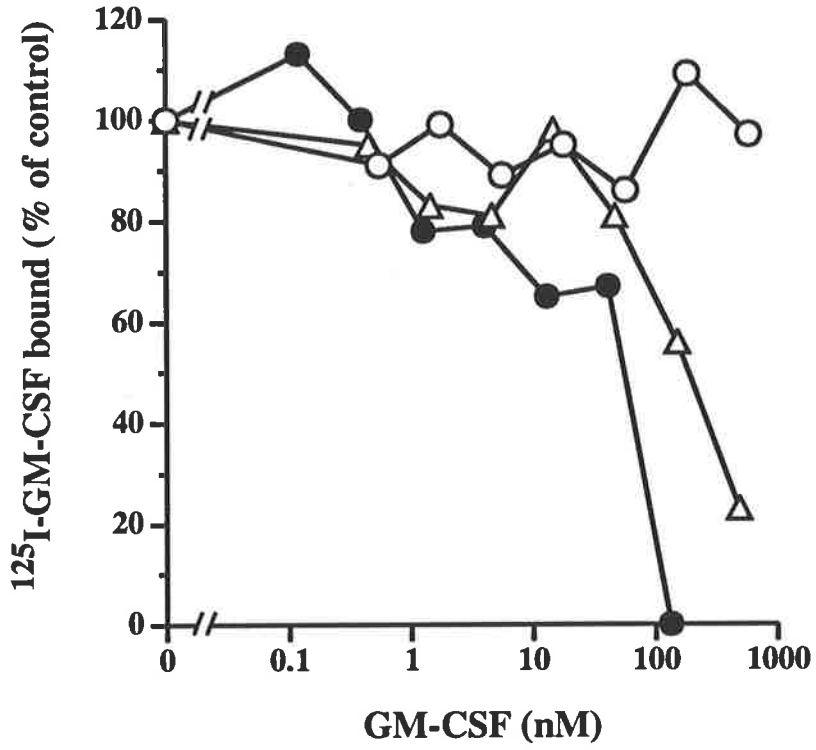
Figure 3.3.11 Comparison of the binding of wild type GM-CSF and the analogues, E108R and D112R, to neutrophils and CHO cells transfected with the human GMRα chain.

The binding of wild type GM-CSF (●) and the E108R (Δ) and D112R (O) analogues to cells expressing the high (GMR$\alpha\beta_c$, Panel A) or low (GMRα, Panel B) affinity GM-CSF receptors was compared. Binding experiments on the permanently transfected CHO cell line, A9/C7, expressing the human GMRα chain (Hercus *et al.*, 1994b), were performed as described 2.10.17. Unlabelled wild type GM-CSF or analogues were titrated against 150pM of CHO cell-derived ^{125}I-GM-CSF and 1 x 10^6 neutrophils per tube (A) or 500pM of CHO cell-derived ^{125}I-GM-CSF and 5 x 10^5 A9/C7 cells per tube (B). The values are expressed as a percentage of the ^{125}I-GM-CSF bound in the absence of competitor and are the means of duplicate determinations.

A


100
80
60
40
20
0
0
0.01
0.1
1
10
100
1000
^{125}I-GM-CSF bound (% of control)
GM-CSF (nM)

B


120
100
80
60
40
20
0
0
0.1
1
10
100
1000
^{125}I-GM-CSF bound (% of control)
GM-CSF (nM)

demonstrated from 2 to 5 x 10^5 binding sites for ^{125}I-GM-CSF per cell. The E108R analogue was four-fold less effective at competing for ^{125}I-GM-CSF binding to the A9/C7 cell line and the D112R analogue, which did not compete for ^{125}I-GM-CSF binding, was at least 20-fold less effective (Fig. 3.3.11B).

These results demonstrated a role for Glu^{108} and particularly for Asp^{112} in GM-CSF biological activity and in the interaction of GM-CSF with its receptor complex. The lack of detectable interaction between the D112R analogue and the low affinity receptor suggests that the Asp^{112} residue plays a significant role in the interaction of GM-CSF with the GMRα chain.

3.3.3 Mutagenesis of the disulphide-bonded cysteine residues of GM-CSF

Human GM-CSF, which is sensitive to sulphydryl reducing agents (Clark-Lewis *et al.*, 1988), contains four cysteine residues that are known to be connected by disulphide bonds between Cys^{54}-Cys^{96} and Cys^{88}-Cys^{121} (Schrimsher *et al.*, 1987). Alignment of the amino acid sequences of human GM-CSF and the structurally related cytokine, human IL-2, showed that Cys^{54}, Cys^{96} and Cys^{121} of GM-CSF align with the three Cys residues of IL-2 (Schrader *et al.*, 1986). Importantly, only two of the Cys residues of human IL-2 are critical for activity (Ju *et al.*, 1987). These results suggested that one of the disulphide bonds within human GM-CSF may be redundant and not required for biological activity. Mutants were designed in which pairs of disulphide-bonded Cys residues were substituted with Ala to prevent the formation of either one or both of the disulphide bonds (Table 3.3.6). In the case of the second disulphide bond between Cys^{88}-Cys^{121}, the Cys residues were also substituted with Ser which may represent a more conservative substitution.

A. Expression of GM-CSF Cys mutants

The Cys mutants of GM-CSF failed to express any detectable protein as measured by the standard RIA (data not shown). However, western blot analysis of the transfected

Table 3.3.6 Plasmid constructs for the expression of GM-CSF Cys mutants in COS cells.

#	Plasmid[a]	Mutations	Oligonucleotides[b]
1.	pJSGM51.53.3	C54A C96A	OL-51 OL-53
2.	pJGM52.54.9	C88A C121A	OL-52 OL-54
3.	pJGM55.56	C88S C121S	GM-55 GM-56A
4.	pJGMCys.8	C54A C88A C96A C121A	OL-51 OL-52 OL-53 OL-54

[a] The pJGM series of plasmids was generated by cloning the mutated GM-CSF cDNA into the pJL4 expression vector (A.3.2.1). An EcoRI/HindIII fragment containing GM-CSF cDNA was excised from the M13mp19 vector, Klenow blunt-ended and cloned into the SmaI site of pJL4.

[b] The sequence of the mutagenic oligonucleotides is listed in Appendix A.3.3.1.

COS cell conditioned media, indicated that the Cys analogues expressed at a level comparable with wild type GM-CSF expression (Fig. 3.3.12A). Thus it appeared that the polyclonal antibody used in the RIA for GM-CSF was unable to recognise GM-CSF lacking one or both of the disulphide bonds. This was later confirmed using chemically reduced GM-CSF (Fig. 5.2.16).

B. Activity of GM-CSF Cys mutants

The GM-CSF Cys mutants were quantified by RIA using the sheep anti-GM-CSF polyclonal antibody which is able to recognise chemically reduced GM-CSF in Western blots (data not shown). The concentrations of the GM-CSF Cys mutants determined by this modified RIA were consistent with the amounts of GM-CSF protein detected by Western blot (Fig. 3.3.12A). Wild type GM-CSF and the Cys mutants were titrated for the ability to stimulate the proliferation of CML cells (Fig. 3.3.12B). Dose-dependent proliferation of CML cells was observed with wild type GM-CSF, ED_{50} 0.1ng/ml, but the GM-CSF Cys mutants were unable to stimulate the proliferation of CML cells.

The increase in the apparent molecular weight of the Cys mutants compared to wild type GM-CSF by SDS/PAGE (Fig. 3.3.12A), indicated that these analogues were hyperglycosylated. As glycosylation is known to influence the specific activity of GM-CSF (Moonen *et al.*, 1987; Kaushansky *et al.*, 1987) it was possible that the loss of activity observed for the Cys mutants was attributable to hyperglycosylation. In an attempt to address this possibility, COS cells transfected with the Cys mutant constructs (Table 3.3.6), were incubated with the drug tunicamycin which inhibits the addition of N-linked carbohydrate (Donahue *et al.*, 1986a). However, the level of GM-CSF Cys mutant expression from tunicamycin treated COS cells was too low for analysis of the analogues biological activity (data not shown). The expression of non-glycosylated GM-CSF Cys mutants from *E. coli* may be a more efficient alternative to the tunicamycin treatment of transfected COS cells.

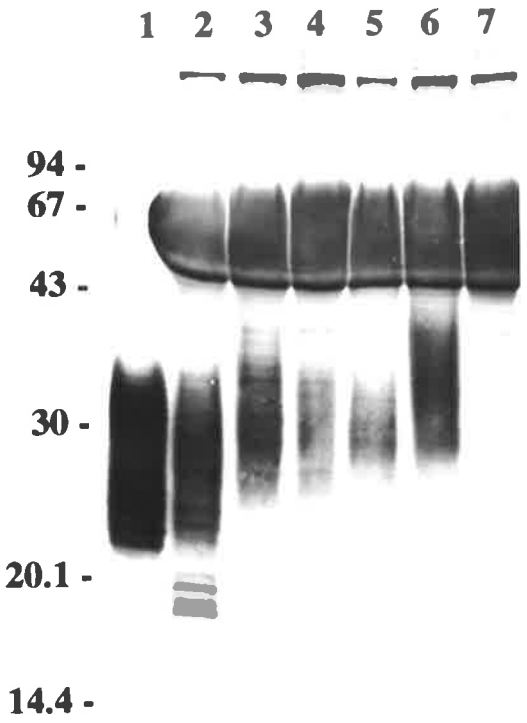
Figure 3.3.12 Expression and activity of GM-CSF Cys mutants.

Plasmid constructs encoding wild type GM-CSF and the Cys mutants C54,96A, C88,121A, C88,121S and C54,88,96,121A were transfected into COS cells. A mock transfection of COS cells in the absence of plasmid DNA was also performed. Samples of conditioned medium were concentrated 20-fold using Centricon-10 micro concentrators, fractionated by 12.5% SDS/PAGE, transferred to nitrocellulose and probed with sheep anti-GM-CSF polyclonal antibody (Panel A). Molecular weight standards are indicated.

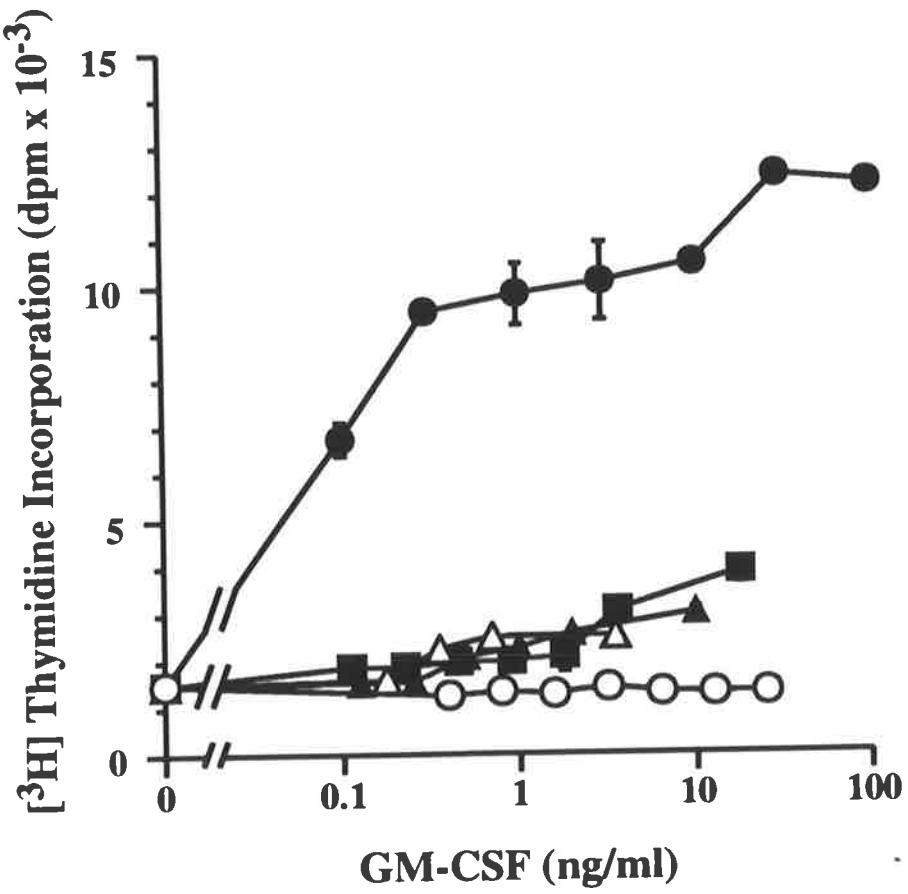
1. 200ng CHO cell-derived wild type GM-CSF
2. 15µl Wild type GM-CSF
3. 15µl C54,96A analogue
4. 15µl C88,121A analogue
5. 15µl C88,121S analogue
6. 15µl C54,88,96,121A analogue
7. 15µl Mock

Wild type GM-CSF (●) and the analogues C54,96A (■), C88,121A (▲), C88,121S (Δ) and C54,88,96,121A (O) were quantified by RIA using the sheep anti-GM-CSF polyclonal antibody and titrated for their ability to stimulate [^{3}H]thymidine incorporation into CML cells (Panel B). Each value represents the mean of triplicate determinations and error bars represent the SEM.

A


1 2 3 4 5 6 7
94 -
67 -
43 -
30 -
20.1 -
14.4 -

B


[3H] Thymidine Incorporation (dpm x 10-3)
15
10
5
0
0
0.1
1
10
100
GM-CSF (ng/ml)

3.4 Discussion

The work presented in this chapter describes the analysis of human GM-CSF analogues mutated in the predicted first and fourth α-helices. Mutation of the Glu^{21} and Asp^{112} residues demonstrated an important role for these residues in the interaction of GM-CSF with its receptor and for the biological activities of GM-CSF. These residues differed in that mutation of Asp^{112} (D112R) influenced binding to the GMRα chain but mutation of Glu^{21} (E21R) did not. On the basis of the results obtained with a number of GM-CSF mutant analogues and a consideration of the GM-CSF structure, a model for the interaction of GM-CSF with its receptor is proposed.

3.4.1 Identification of functionally important residues in human GM-CSF

A. The role of residue 21

Deletion analysis and single amino acid substitutions of residues in the N-terminus demonstrated that Glu^{21}, predicted to be exposed on the surface of the first α-helix (Fig. 3.1.2A), was critical for GM-CSF function. Mutagenesis of Glu^{21} significantly reduced the ability of GM-CSF to stimulate both proliferation (Fig. 3.3.5) and mature cell activation (Fig. 3.3.6) and also decreased the ability of GM-CSF to bind the high affinity ($GMR\alpha\beta_c$) receptor of neutrophils (Fig. 3.3.7). Significantly, while the E21R analogue displayed greatly reduced biological activity and binding to the high affinity receptor, its binding to the low affinity (GMRα) receptor was essentially the same as wild type (Fig. 3.3.9). These results linked the binding of GM-CSF to the high affinity ($GMR\alpha\beta_c$) receptor with multiple GM-CSF activities and also implicated Glu^{21} in the interaction of GM-CSF with the β_c chain.

The ability of the E21R analogue to trigger a biological response at high but not low concentrations, indicated that the low affinity receptor (GMRα) may be able to transmit a proliferative signal. This possibility was supported by the observation that high concentrations (in the low affinity binding range) of human GM-CSF were required to stimulate [^{3}H]thymidine incorporation in monocytes (Elliott *et al.*, 1989) and the proliferation of murine FDC-P1 cells transfected with the human GMRα (Metcalf *et al.*, 1990). In contrast, the murine IL-2-dependent T-cell line, CTLL2, does not proliferate in response to human GM-CSF when transfected with the human GMRα alone (Kitamura *et al.*, 1991a). This indicated that the GMRα alone is insufficient to allow proliferation. The proliferation of FDC-P1 cells transfected with the human GMRα may be the result of a functional interaction between the human GMRα and the murine β_c, as CTLL2 cell lines co-transfected with the human GMRα and human β_c or murine β_c do proliferate in response to human GM-CSF (Kitamura *et al.*, 1991a). These results demonstrated that both GMRα and β_c are required for a functional response to GM-CSF and therefore the activity of the E21R analogue must presumably be mediated through residual interaction with the β_c chain.

The Gln^{20} residue did not appear to be critical for GM-CSF activity (Fig. 3.3.5 & 3.3.6). However, the slightly impaired ability of the Q20A analogue to compete for the high affinity binding of ^{125}I-GM-CSF (Fig. 3.3.7) and the fact that the double mutant Q20A/E21A was less active than E21A alone (Fig. 3.3.5 & 3.3.6), suggested that Gln^{20} may also contribute to GM-CSF activity. It is possible that the proximity of Gln^{20} and Glu^{21} allows these residues to interact with a similar region of the GM-CSF receptor complex. Alternatively these residues may interact with slightly different regions of the receptor complex but the relatively minor influence of Gln^{20} is normally masked by the more pronounced influence of Glu^{21} on receptor interaction.

B. The role of residue 112

Analysis of the charged residues predicted to be exposed on the surface of the fourth α-helix of human GM-CSF, showed that the conserved acidic residues Glu^{108}

and Asp^{112} were important for maximal GM-CSF activity (Fig.3.3.10). The other charged residues in the predicted fourth helix, Glu^{104}, Lys^{107} and Lys^{111}, did not appear to be critical for GM-CSF activity (Fig. 3.3.10). Binding studies demonstrated that mutation of Asp^{112} significantly reduced the binding of GM-CSF to the high affinity receptor ($GMR\alpha\beta_c$) and low affinity receptor ($GMR\alpha$) GM-CSF. In fact no binding to the $GMR\alpha$ chain was observed with the D112R analogue (Fig. 3.3.11B) although, must presumably occur at higher concentrations than was used as this analogue did bind to the high affinity receptor of neutrophils (Fig. 3.3.11A). As the $GMR\alpha$ chain is common to both high and low affinity receptors, the receptor binding properties of the D112R analogue suggested that the Asp^{112} residue may interact with the $GMR\alpha$ chain.

C. The first and fourth α-helices of GM-CSF are implicated as functionally important by a number of different studies

The results described in this chapter indicated that certain residues in the first and fourth α-helices have important roles in the biological and receptor binding activity of human GM-CSF. A number of different experimental approaches have also attributed functional significance to these regions in both human and murine GM-CSF.

Fine deletion analysis revealed functionally important regions spanning residues 18-22 and 94-115 of murine GM-CSF (Shanafelt and Kastelein 1989) and residues 20-21 and 89-120 of human GM-CSF (Shanafelt *et al.*, 1991a). Interspecies chimeras of human and murine GM-CSF, which exploited the lack of cross-reactivity between these two cytokines, identified residues 17-31 as being important for GM-CSF activity (Kaushansky *et al.*, 1989; Shanafelt *et al.*, 1991b). The mapping of GM-CSF neutralising monoclonal antibodies suggested that Arg^{23} in the first predicted α-helix (Brown *et al.*, 1990) and residues 110-127 at the C-terminus (Nice *et al.*, 1990; Kanakura *et el.*, 1991) participate in the interaction of GM-CSF and its receptor. Finally, the substitution of individual amino acids demonstrated a functional role for residues 14, 15, 20 and 21 of murine GM-CSF (Shanafelt *et al.*, 1991b; Meropol *et al.*, 1992;

Shanafelt and Kastelein 1992) and residues 20 and 23 of human GM-CSF (Kaushansky *et al.*, 1989, Shanafelt *et al.*, 1991b).

The data presented in this chapter is therefore consistent with a number of structure-function studies that identified functionally important regions in the first and fourth α-helices and most significantly, identified two residues (Glu^{21}, Asp^{112}) that are critical for GM-CSF activity and receptor binding. It is worth noting that the functionally important Glu^{21} residue, which appears to be conserved amongst many of the cytokines (Shanafelt *et al.*, 1991b) as well as in all species of GM-CSF for which sequence is available (Fig. 1.2.2), is not specifically identified as important by studies that utilised interspecies chimeras.

D. The role of the two disulphide bonds in GM-CSF activity

The absence of detectable activity for any of the GM-CSF Cys mutants showed that both of the disulphide bonds were required for GM-CSF activity. In this respect human and murine GM-CSF differ as the second disulphide bond of murine GM-CSF can be eliminated by mutagenesis of Cys^{85} and Cys^{118} with no apparent influence on GM-CSF activity (Shanafelt and Kastelein, 1989). This is despite the absolute conservation of the four Cys residues in GM-CSF from the seven species of GM-CSF examined (Fig. 1.2.2). One possible explanation for this difference is that murine GM-CSF requires only the one disulphide linkage to form a stable structure while human GM-CSF requires both disulphides. GM-CSF, IL-2 and IL-4 exhibit highly superimposable three dimensional structures, particularly within the helical regions, yet display a remarkable diversity and number of disulphide bonds (Wlodawer *et al.*, 1993).

3.4.2 The structure of human GM-CSF

The main feature of the predicted structure of human GM-CSF, described in Chapter 1, was the presence of a bundle of four α-helices (Parry *et al.*, 1988; Fig. 1.2.3). Initially the topology for these helices was proposed as classical antiparallel, up-down-up-down but a later model proposed a three-down-one-up topology (Parry *et*

al.,1991). During the course of this work the three dimensional structure of recombinant human GM-CSF was solved by means of x-ray crystallography (Diederichs *et al.*,1991; Walter *et al.*, 1992a). The key features of the GM-CSF fold were the presence of four α-helices (Fig. 3.4.1A) with an unusual two-up-two-down arrangement previously observed for porcine growth hormone. Another feature of the GM-CSF fold was the presence of two short β strands, one in the loop connecting helix A and B and the other in the loop connecting helix C and D (Fig. 3.4.1A). At least five cytokines have been demonstrated to adopt a GM-CSF-like fold including M-CSF, IL-2, IL-4 and IL-5 which all exhibit a conserved structural core despite the low level of amino acid sequence identity amongst these cytokines (reviewed in Wlodawer *et al.*, 1993; Rozwarski *et al.*, 1994). Other cytokines such as IL-3, IL-7 and SCF are predicted to adopt a GM-CSF-like fold (Rozwarski *et al.*, 1994). Two other cytokines, G-CSF and GH, possess a similar topology but with longer helices (Kaushansky and Karplus, 1993).

Comparison of the GM-CSF structure determined by x-ray crystallography with the structure proposed by molecular modelling, showed only limited alignment of secondary structure components (Fig. 3.4.1B). Overall, the predicted structure accurately identified helices A & D but misplaced helices B & C and failed to correctly anticipate the unusual topology of the GM-CSF fold. Significantly, the functionally important residues identified in this chapter, Glu^{21} and Asp^{112}, are located on distinct and separate regions of the GM-CSF surface (Fig. 3.4.2). When viewed along the axis of the helical bundle, the side chains of residues Glu^{21} and Asp^{112} project at approximately 180° from each other while the functional groups are separated by at least 20Å and a number of intervening residues.

The results presented in this chapter assumed that the amino acid substitutions for the non-Cys mutants, identified specific contact points for ligand:receptor interaction. An alternative possibility is that the substitutions introduced structural perturbations that were responsible for the reduced receptor interaction and biological activity. The location of Glu^{21} and Asp^{112} on the surface of GM-CSF suggests that their substitution by other

Figure 3.4.1 Topology of the three-dimensional structure of GM-CSF determined by x-ray crystallography and a comparison with that predicted by molecular modelling.

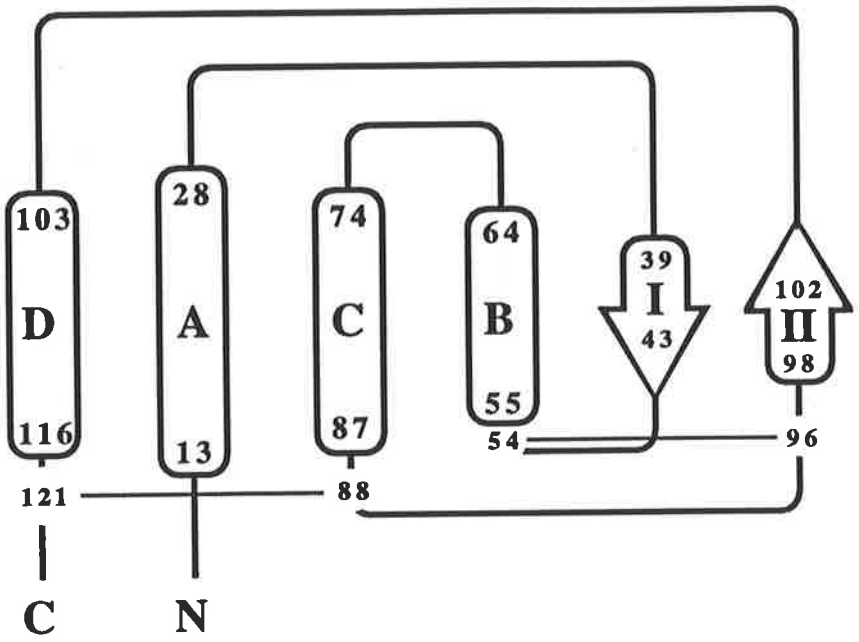
The topology of the GM-CSF fold shown in panel A was derived from Diederichs *et al.* (1991). The key feature of the GM-CSF fold is the presence of four α-helices (A-D) in a two-up-two-down arrangement. In addition to the α-helices, the long overhand loops, which connect helices A&B and helices C&D, contain short regions of β-strand (I & II). The disulphide bonded Cys residues are indicated.

The predicted and x-ray determined secondary structure elements of human GM-CSF are compared in panel B. The key features of the predicted structure (Parry *et al.*, 1988) are shown in the upper figure (i) and those of the x-ray structure (Diederichs *et al.*, 1991) in the lower figure (ii). Regions shown to be important for biological activity and receptor binding are indicated with arrows.

α helices A,B,C & D.

β strands I & II.

A


103
D
116
28
A
13
74
C
87
64
B
55
39
I
43
102
II
98
54
96
121
88
C
N

B

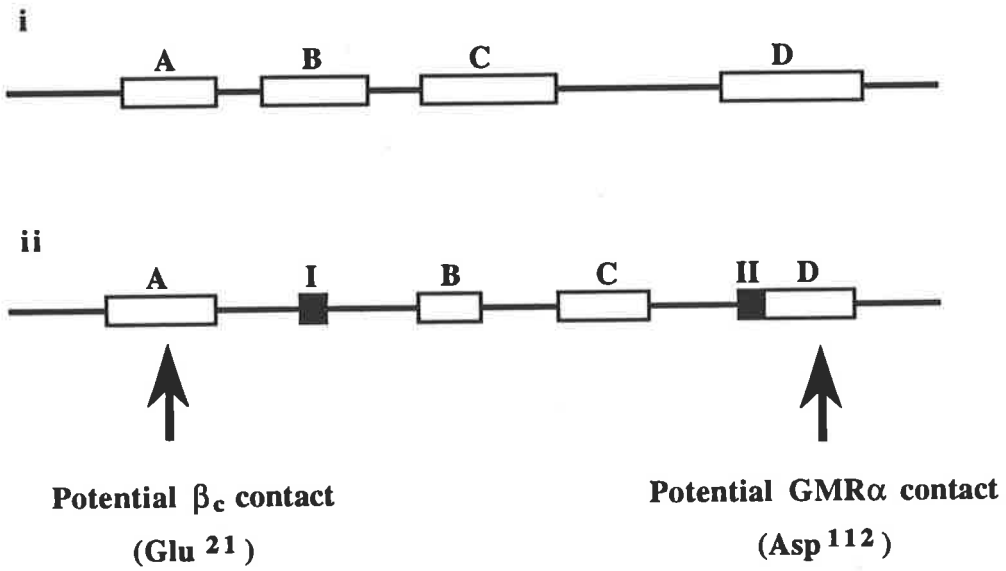
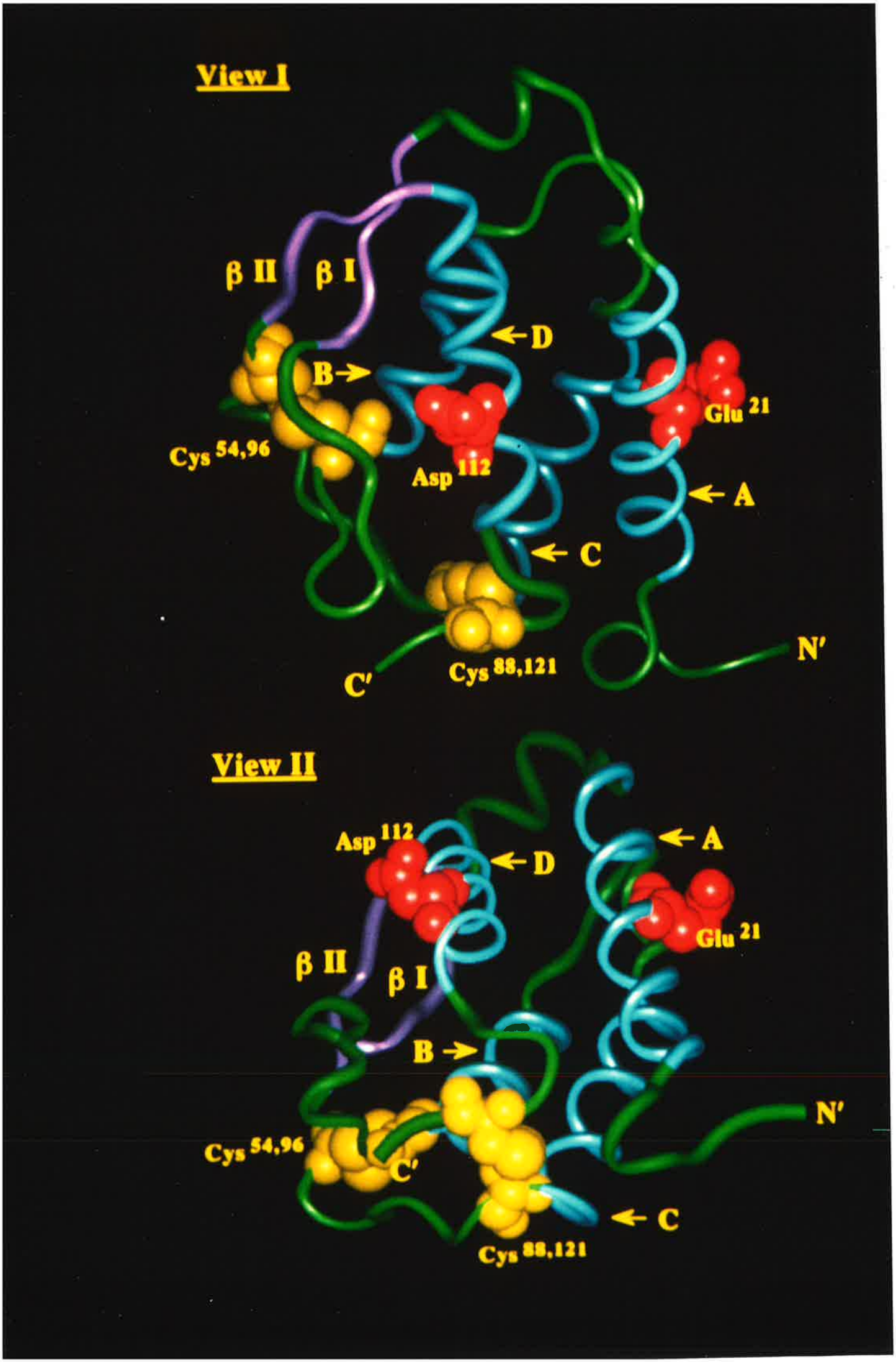

i
A
B
C
D
ii
A
I
B
C
II
D
Potential β_c contact
(Glu ^{21})
Potential GMRα contact
(Asp ^{112})

Figure 3.4.2 Structure of recombinant human GM-CSF and the location of functionally important residues.

The three-dimensional structure of recombinant human GM-CSF has been determined by x-ray crystallography (Diederichs *et al.*, 1991; Walter *et al.*, 1992a). The figure shows two views of a tube diagram of GM-CSF from residues 5 (N') to 123 (C') inclusive highlighting the different secondary structure components. Regions shown in cyan are the α-helices (A,B,C,D), regions in magenta are the β-strands (I,II) and green represents the loops. The functionally important Glu^{21} and Asp^{112} residues are shown as red spheres while the disulphide bonded Cys residues, 54-96 and 88-121, are shown as yellow spheres.


View I
β II
β I
D
B
Glu 21
Cys 54,96
Asp 112
A
C
C′
Cys 88,121
N′
View II
Asp 112
D
A
Glu 21
β II
β I
B
N′
Cys 54,96
C′
C
Cys 88,121

amino acids compatible with an α-helical structure, is not expected to perturb the tertiary structure. The wild type binding of the E21R analogue to the GMRα chain provides evidence that the binding site for this receptor has not been adversely influenced by the mutation of residue 21. Indirect evidence that the mutations have not affected the gross tertiary structure of GM-CSF, comes from two observations. The first is that western blot analysis of GM-CSF analogues revealed no evidence of hyperglycosylation that has previously been associated with mutations affecting GM-CSF folding (LaBranche *et al.*, 1990; Altmann *et al.*, 1991). The second is that the polyclonal rabbit anti-GM-CSF serum used for RIA quantification of the GM-CSF mutants described in this thesis is conformation-sensitive and unable to recognise unfolded GM-CSF (Fig. 5.2.16). Ultimately the question of mutation-induced structural changes may need to be resolved, particularly for those analogues with reduced receptor interaction, using techniques such as native gel electrophoresis, x-ray crystallography or nuclear magnetic resonance spectroscopy.

3.4.3 A model for the interaction of GM-CSF with the two chains of the GM-CSF receptor complex

The consequences of charge-reversal mutations at Glu^{21} and Asp^{112} together with the position occupied by these residues on the surface of GM-CSF, suggests that these residues interact with two different components of the GM-CSF receptor complex. The fact that the E21R analogue retained wild type binding to the low affinity receptor (GMRα) but not the high affinity receptor (GMRαβ_c), indicated that Glu^{21} probably participates in the interaction of GM-CSF with the β_c chain. In contrast, the D112R analogue exhibited reduced interaction with both the low and high affinity receptor and as the Asp^{112} residue is spatially removed from Glu^{21} in the three-dimensional structure, suggested that it may participate in the interaction of GM-CSF with the GMRα chain. On the basis of the data described in this chapter, a model for the interaction of GM-CSF and its receptor complex is proposed (Fig. 3.4.3). The model proposes two distinct receptor interaction sites on the surface of GM-CSF. The first site interacts with the GMRα chain

Figure 3.4.3 A model for the interaction of GM-CSF with the GM-CSF receptor.

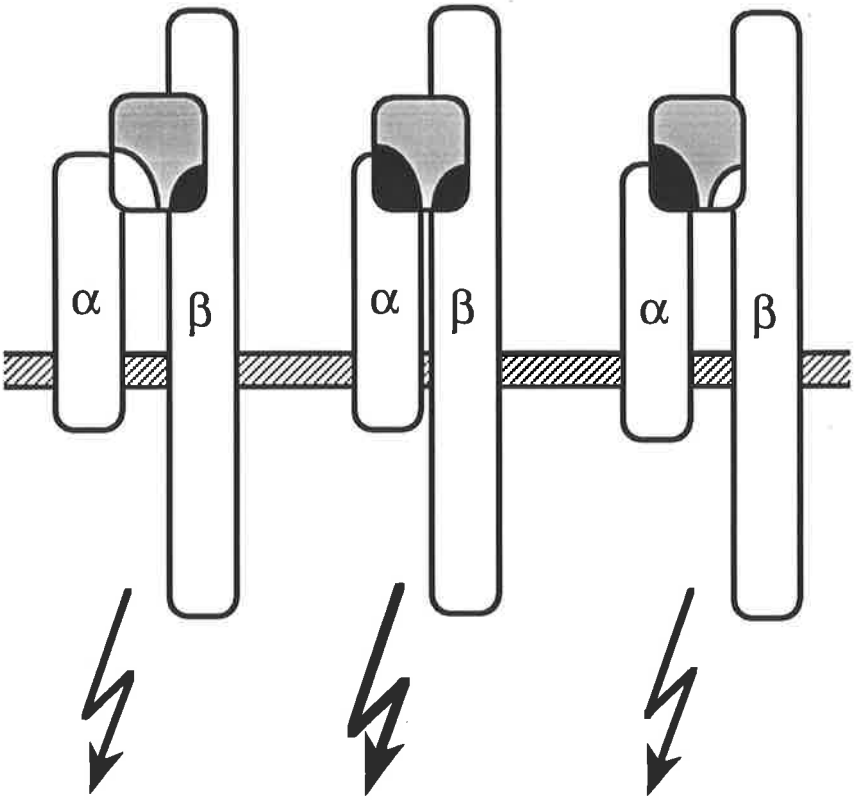
The interaction of wild type GM-CSF with the GMR$\alpha\beta_c$ complex is characterised by high affinity binding and biological activity. Substitution of the glutamic acid at residue 21 reduced biological activity and binding to the high affinity (GMR$\alpha\beta_c$) but not the low affinity (GMRα) receptor. Substitution of the aspartic acid at residue 112 reduced biological activity and high (GMR$\alpha\beta_c$) affinity receptor binding while binding to the low (GMRα) affinity receptor was not detected (N.D.).

On the basis of the location of Glu^{21} and Asp^{112} on the surface of GM-CSF (Fig. 3.4.2) and the mutagenesis results described in this chapter, the following model for the interaction of GM-CSF and its receptor is proposed. This model proposes that GM-CSF contains two receptor binding sites, one that binds the GMRα chain and one that binds the β_c chain. The GMRα binding site is predicted to include Asp^{112} while the β_c binding site includes Glu^{21}.

The GM-CSF ligand

GMRα binding site → ← βc binding site.

	Residue 112 mutants	Wild Type	Residue 21 mutants
	ie D112R		ie E21R



Biological activity	**10%**	**100%**	**0.3%**
Binding			
GMRαβ_c	**10%**	**100%**	**0.3%**
GMRα	**N.D.**	**100%**	**100%**

and may include Glu^{108} and Asp^{112} while the second site interacts with the β_c chain and includes Glu^{21}.

The E21R analogue which identified two functionally distinct receptor binding sites also suggested a mechanism whereby GM-CSF antagonists might be generated. Since binding to GMRα and β_c is required for high affinity binding and biological activity (Kitamura *et al.*, 1991a), analogues totally devoid of interaction with β_c but retaining the interaction with GMRα may be able to antagonise the activity of wild type GM-CSF. The model shown in Figure 3.4.3 illustrates the essential features of the interaction of GM-CSF and its receptor complex and also identifies regions to be mutated in order to elucidate which residues on the surface of GM-CSF make functionally relevant contacts with the two chains of the GM-CSF receptor complex.

Chapter 4

Saturation mutagenesis of residues in the first α-helix of GM-CSF

4.1 Introduction

The results presented in Chapter 3 identified residues 21 and 112 as being important for GM-CSF biological activity. Residue 112, on the surface of the fourth α-helix, potentially interacts with the GMRα chain while residue 21, on the surface of the first α-helix, was identified as playing an important role in the interaction of GM-CSF with the β_c chain of the GMR$\alpha\beta_c$ receptor complex. The fact that mutation of these residues reduced but did not abolish biological activity suggested that additional residues were likely to be involved in the interaction of GM-CSF with the GMRα and β_c chains of the GM-CSF receptor complex.

Mutation of residue 21 significantly reduced the biological activity of GM-CSF (Figures 3.3.5 & 3.3.6) as well as the ability of GM-CSF to bind the high affinity (GMR$\alpha\beta_c$) receptor but not the low affinity (GMRα) receptor (Figures 3.3.7 & 3.3.9). The identification of all functionally important β_c chain contact residues on GM-CSF may enable the generation of a GM-CSF analogue devoid of β_c chain interaction but still capable of binding the GMRα chain. A GM-CSF analogue with these receptor binding properties may act as a GM-CSF antagonist and block wild type GM-CSF function. In an attempt to identify other functionally important residues which may interact with the β_c chain, residues in the vicinity of Glu^{21} and predicted to be located on the solvent exposed surface of the first α-helix were specifically targeted for mutagenesis. As the solvent exposed residues could only be predicted prior to the determination of the three-dimensional structure of GM-CSF (Fig. 3.1.2), a number of buried residues were also targeted for mutagenesis. The first α-helix of human GM-CSF spans amino acids 13 to 28 with Trp^{13}, Glu^{14}, Asn^{17}, Gln^{20}, Glu^{21}, Arg^{23}, Arg^{24}, Asn^{27} and Leu^{28} showing a high degree of solvent accessibility (Diederichs *et al.*,1991). These residues were targeted for mutagenesis with multiple substitutions apart from Trp^{13} which has been

shown to be dispensable for GM-CSF activity (Clark-Lewis *et al.*, 1988). The Ala^{18}, Ile^{19}, Ala^{22}, Leu^{25} and Leu^{26} residues which are located in the vicinity of Glu^{21}, were also targeted for mutagenesis although they show low solvent accessibility (Diederichs *et al.*,1991).

The mutagenesis procedure described in Chapter 3 was not considered suitable for the saturation mutagenesis required to thoroughly investigate the functional role of residues in the first α-helix of GM-CSF, so an alternative procedure was pursued. The results presented in this chapter describe the development of an efficient protocol for generating large numbers of substitution mutants within the first α-helix of GM-CSF and analysis of the functional consequence of these mutations.

4.2 Results

4.2.1 A potential problem for site-directed mutagenesis in the first α-helix of GM-CSF

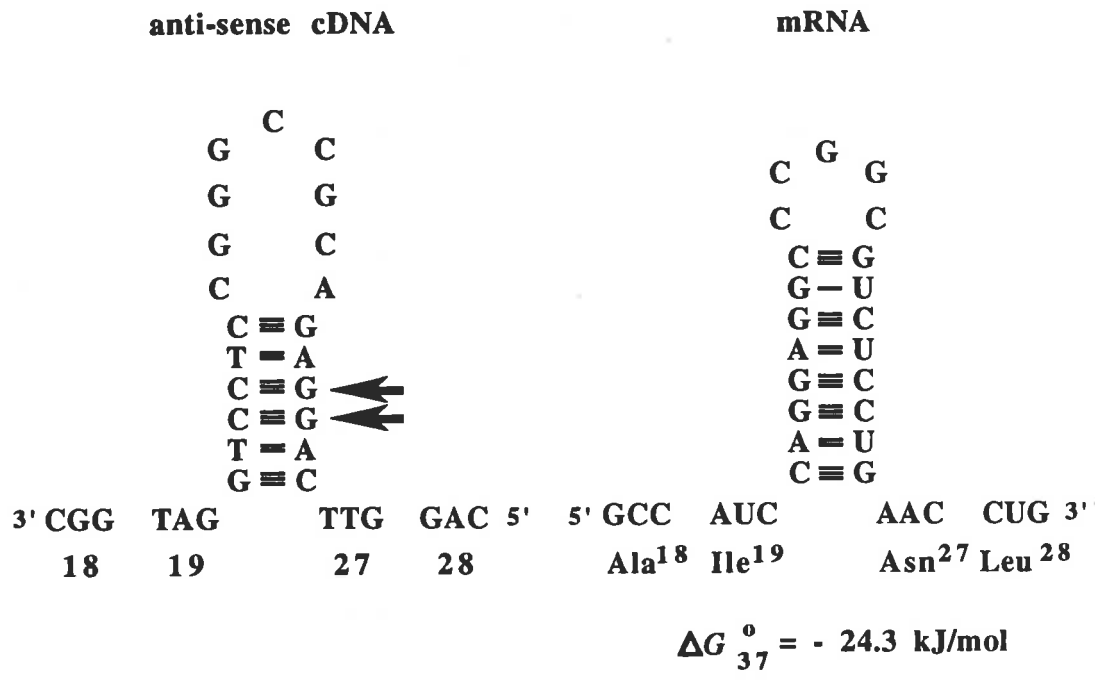
During the mutagenesis of residues in the first helix, it became apparent that mutagenesis at some positions was particularly inefficient with only 1-5% of plaques hybridising to the mutagenic oligonucleotide (data not shown). Close examination of the GM-CSF cDNA sequence showed that a region spanning residues 20 to 26 had the potential to form a stable hairpin loop structure in mRNA (Fig.4.2.1A). Using the values of Freier *et al.* (1986), the hairpin loop has a calculated stability of ΔG° = -24.3 kJ/mol at 37°C. A similar structure might also exist in the single stranded, anti-sense strand of the cDNA used for the mutagenesis procedure although the stability of such a structure is not known. From these observations it seemed possible that the presence of secondary structural features might be responsible for the low mutagenesis efficiency of this region, perhaps by preventing the mutagenic oligonucleotides from annealing to the template. Two silent mutations were introduced to disrupt the potential hairpin loop structure

Figure 4.2.1 A potential hairpin loop in the GM-CSF cDNA may influence mutagenesis efficiency.

The sequence of the GM-CSF cDNA anti-sense strand used for mutagenesis, was examined for potential secondary structure in the region encoding the first α-helix. A hairpin loop involving the codons for amino acids 20 to 26 (A) was found which may influence the annealing of mutagenic oligonucleotides to this region. The same region of the GM-CSF mRNA, equivalent to the sense strand of the cDNA, also appears able to form a hairpin loop with a calculated stability of ΔG° = -24.3 kJ/mol at 37°C (Freier *et al.*, 1986).

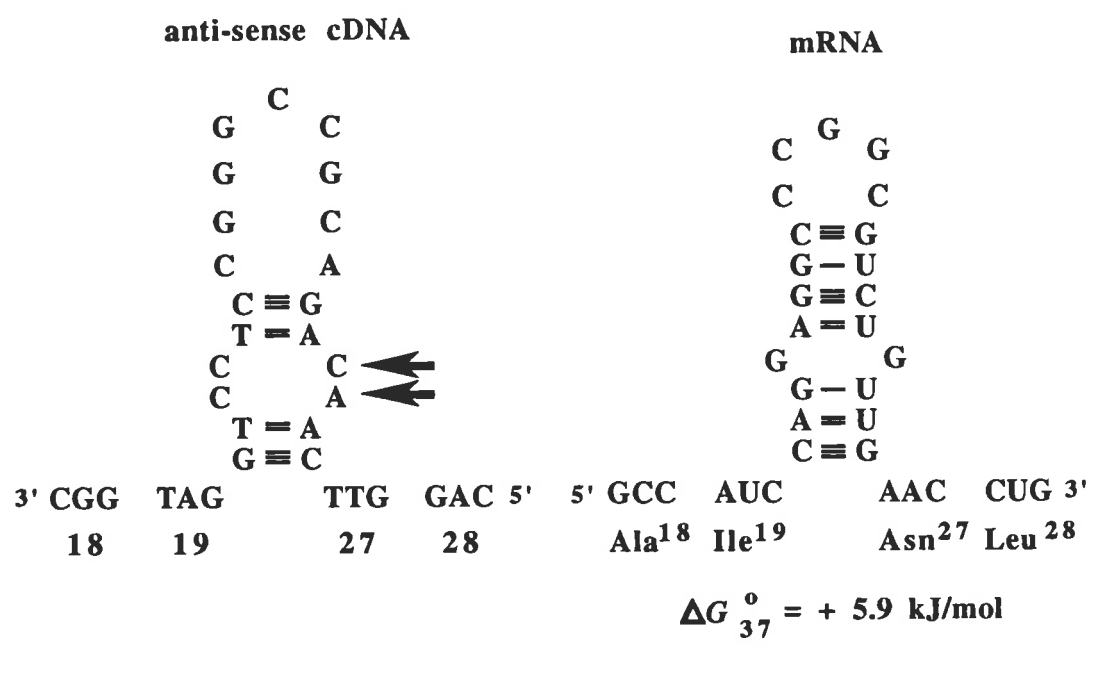
The CpC doublet at positions 141 and 142 of the GM-CSF cDNA sense strand (Fig. 1.2.1), which corresponds to a GpG in the anti-sense strand, was substituted for GpT (ApC in the anti-sense strand) using the OL-256 oligonucleotide (Appendix A.4.2.1). This mutation markedly reduced the calculated stability of the potential hairpin loop in the mRNA strand (ΔG° = +5.9 kJ/mol at 37°C) and suggested that a hairpin loop structure was unlikely to form in this region of the DNA anti-sense strand (B).

A Wild type GM-CSF



ΔG^{o}_{37} = - 24.3 kJ/mol

B "Hairpin" mutant GM-CSF



ΔG^{o}_{37} = + 5.9 kJ/mol

without altering the amino acid sequence (Figure 4.2.1B). These mutations markedly reduced the calculated stability of the potential hairpin loop in the mRNA strand (ΔG° = +5.9 kJ/mol at 37°C) and suggested that a hairpin loop structure was unlikely to form in this region of the DNA anti-sense strand.

Mutagenesis experiments were performed with the "hairpin minus" and wild type DNA templates and mutagenic oligonucleotides binding to the region of the hairpin loop but not including the residues mutated to disrupt the potential hairpin loop structure. These experiments showed a 2- to 5- fold increase in mutagenesis efficiency for the "hairpin minus" DNA template when compared to wild type DNA template (data not shown). The plasmid containing the "hairpin minus" GM-CSF cDNA, pJGM256.3, expressed essentially the same level of GM-CSF as the pJGM3 plasmid encoding wild type GM-CSF cDNA when transfected into COS cells (data not shown).

Despite the modest improvements in mutagenesis efficiency, the entire procedure was considered to be too slow and complex for the generation of large numbers of mutants, so alternatives were examined.

4.2.2 A more efficient mutagenesis protocol, OCM

Alternative site-directed mutagenesis protocols, based on the method of Zoller and Smith (1984), have been developed that by selecting against the template or wild type strand, produce mutagenesis efficiencies of 50 to 90%. The use of DNA templates grown in *dut ung* strains of *E. coli* and which as a result contain a low level of deoxyuridine in place of thymine, enables selection against the template strand in *ung*$^+$ strains of *E. coli* (Kunkel, 1985). Another approach is to use the second primer to introduce an observable phenotypic change into the mutagenesis template such as alteration of β-galactosidase activity (Setzer *et al.*, 1990) or antibiotic resistance (Lewis and Thompson, 1990). The latter approach has the advantage that the antibiotic resistance provides a convenient selection for mutated plasmids. The asymmetric polymerase chain

reaction has also been used to introduce site specific mutations (Perrin and Gilliland, 1990).

These methods, whilst presumably more efficient than method of Zoller and Smith (1984), were all considered to be too complex. However, a simpler alternative that appeared to be ideally suited to the task of saturation mutagenesis was oligonucleotide cassette mutagenesis (OCM). In essence OCM is a simple cloning strategy where the region to be mutated is deleted using suitable unique restriction enzyme sites and replaced with a fragment formed with appropriately mutated oligonucleotides (Lo *et al.*, 1984). As mutagenesis can be performed with the target gene cloned into the appropriate expression vector, DNA manipulations are minimised. In addition, the use of redundant or randomised oligonucleotides, enables the OCM procedure to conveniently generate libraries of mutations (Matteucci and Heyneker, 1983; Oliphant *et al.*, 1986; Oliphant and Struhl, 1989).

4.2.3 Preparation of plasmid constructs for OCM of the first α-helix of GM-CSF

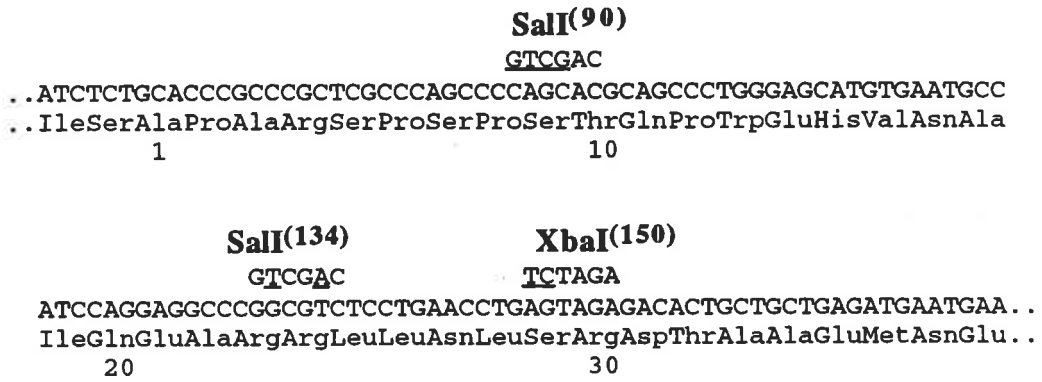
The GM-CSF cDNA sequence shown in Figure 1.2.1, contained no unique restriction enzyme sites that conveniently spanned the first α-helix. Reverse translation of the encoded amino acid sequence indicated that two SalI restriction sites and a unique XbaI restriction site could be introduced into the GM-CSF cDNA without altering the amino acid sequence and spanning residues 11 to 28 (Fig. 4.2.2A). The pJL4 expression vector, used to express the wild type and mutated GM-CSF cDNA's from transfected COS cells (Appendix A.3.2.1), contains SalI and XbaI sites in the polylinker. These sites were first destroyed by digestion with BamHI and SalI, incubation with Klenow and religation of the blunt-ended fragment to produce the BamHI$^-$, XbaI$^-$, SalI$^-$, pJL5 expression vector (Appendix A.4.2.2). Conventional site-directed mutagenesis was then used to introduce the SalI and XbaI restriction sites into the GM-CSF cDNA in pairs. The mutated cDNA's were then sub-cloned into the pJL5 expression vector to generate the pJGMV series of plasmids (Fig. 4.2.2B). The pJGMV series of plasmids and the

Figure 4.2.2 The generation of vectors for OCM of the first α-helix of GM-CSF.

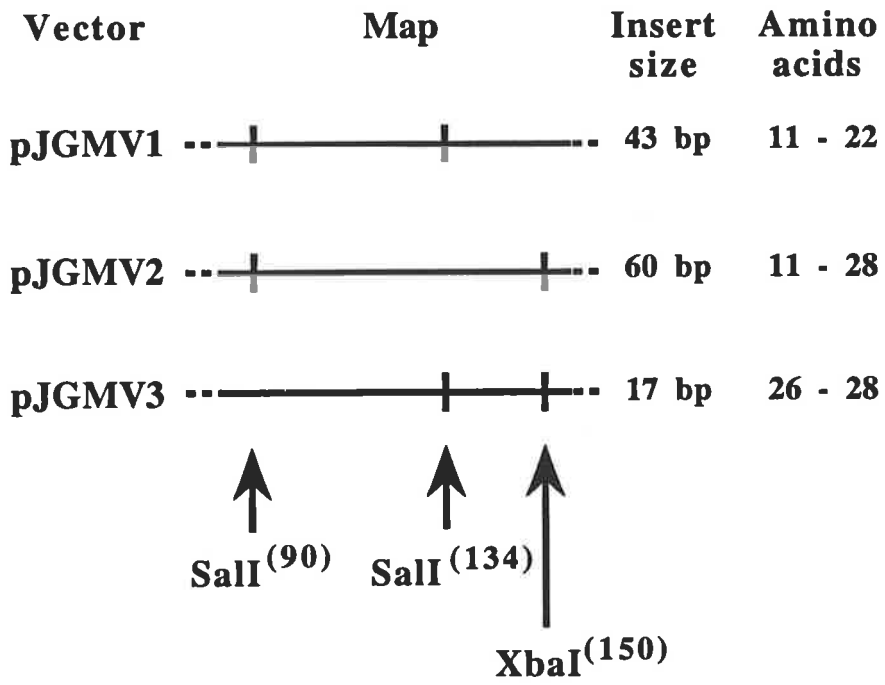
Nucleotides 61 to 180 of the human GM-CSF cDNA sequence are illustrated in Panel A (from Figure 1.2.1) along with the three potential restriction enzyme sites spanning amino acids 11 to 28. The mutations required to generate the restriction site are underlined and do not alter the amino acid sequence.

The three potential restriction enzyme sites were introduced into the GM-CSF cDNA in pairs by site-directed mutagenesis with the oligonucleotides MI-2,-3,-5 (Appendix A.4.2.1) and the mutated cDNA fragments blunt cloned into the SmaI site of the pJL5 expression vector (Appendix A.4.2.2). The pJGMV series of plasmids encoded wild type GM-CSF and carried pairs of restriction enzyme sites which flanked part or all of the GM-CSF cDNA sequence that encoded amino acids 11 to 28 (Panel B).

A


SalI(90)
GTCGAC
..ATCTCTGCACCCGCCCGCTCGCCCAGCCCCAGCACGCAGCCCTGGGAGCATGTGAATGCC
..IleSerAlaProAlaArgSerProSerProSerThrGlnProTrpGluHisValAsnAla
1
10
SalI(134)
XbaI(150)
GTCGAC
TCTAGA
ATCCAGGAGGCCCGGCGTCTCCTGAACCTGAGTAGAGACACTGCTGCTGAGATGAATGAA..
IleGlnGluAlaArgArgLeuLeuAsnLeuSerArgAspThrAlaAlaGluMetAsnGlu..
20
30

B


Vector
Map
Insert size
Amino acids
pJGMV1
43 bp
11 - 22
pJGMV2
60 bp
11 - 28
pJGMV3
17 bp
26 - 28
SalI(90)
SalI(134)
XbaI(150)

pJGM3 plasmid encoding wild type GM-CSF cDNA, expressed similar levels of GM-CSF protein when transfected into COS cells (data not shown).

4.2.4 OCM of the first α-helix of GM-CSF

Mutagenesis of the first α-helix of GM-CSF was achieved by replacing the SalI and/or XbaI defined cDNA fragments with double-stranded oligonucleotide cassettes encoding mutated cDNA fragments. Fragments cloned into the pJGMV1 ($SalI^{90}/SalI^{134}$) vector were randomly orientated so a PCR protocol was devised to enable colonies to be screened for plasmids with the correctly orientated SalI fragment. In contrast, fragments cloned into the pJGMV2 ($SalI^{90}/XbaI^{150}$) and pJGMV3 ($SalI^{134}/XbaI^{150}$) vectors were always correctly orientated because of the non-compatible SalI/XbaI sites used.

The oligonucleotide cassettes were generated in a number of different ways which emphasised the utility of OCM for generating a range of mutations (Fig. 4.2.3). Initially the cassettes used were fragments composed of two complementary, 5' phosphorylated oligonucleotides encoding single amino acid changes. Larger fragments were usually formed as two separate fragments with overlapping 6bp single-stranded regions to enable annealing and ligation. A feature of the multiple fragment oligonucleotide cassettes was that usually only one fragment contained the mutated region and so the other, wild type fragment, could be coupled with different mutated fragments. Mutations were also introduced at multiple sites which enabled the generation of double-mutant analogues. This could also be used to introduce entirely different sequences from other cytokines such as murine GM-CSF or human IL-3. The use of redundant oligonucleotides enabled libraries of recombinant plasmids to be generated with each plasmid containing only one of the potential sequences.

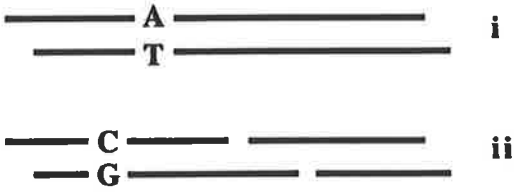
The oligonucleotide cassette mutagenesis procedure was used to generate 62 substitution mutants at 13 of the 16 residues within the first α-helix of GM-CSF (Table 4.2.1). In conjunction with substitutions generated by conventional site-directed

Figure 4.2.3 Permutations of the OCM procedure.

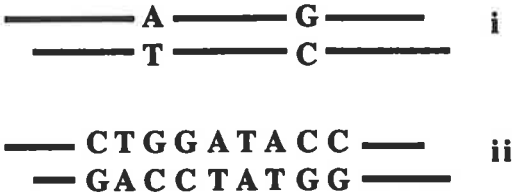
Oligonucleotide cassette mutagenesis of human GM-CSF made use of the pJGMV series of plasmids (Fig. 4.2.2) and used a number of variations to generate mutant sequences. The wild type fragment (A) was removed and replaced with one or two (B) fragments encoding a single amino acid substitution although more fragments could be used. Fragments encoding double amino acid substitutions were also used and could be extended to change multiple amino acids by inserting sequences of related cytokines such as murine GM-CSF or human IL-3 (C). Cloning of OCM fragments by the approaches described (B,C), typically generated a single, recombinant plasmid with one or more mutations. A procedure for generating multiple recombinant plasmids with different substitutions at a single amino acid position was to generate redundant oligonucleotides encoding many different amino acids. It was also possible to generate non-complementary redundancies in the upper and lower strands of an OCM fragment, thereby reducing the complexity of each oligonucleotide while retaining the same level of redundancy in the OCM fragment (D). Finally, a combination of the approaches shown in C and D could generate multiple substitutions at multiple sites (E) although this approach was not utilised during the course of this work.

OCM vector with fragment excised

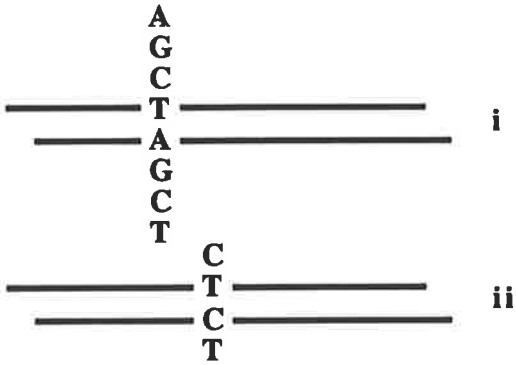
A Wild type



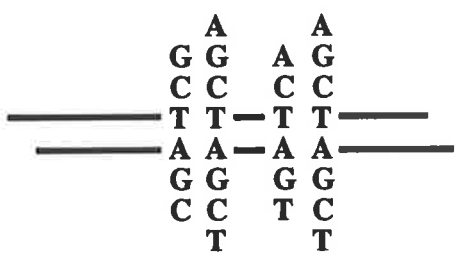
B Single amino acid substitution with one (i) or two (ii) fragments



C Double (i) or multiple (ii) amino acid substitutions



D Multiple complementary (i) or non-complementary (ii) substitutions of one amino acid residue



E Multiple substitutions of multiple amino acid residues

Table 4.2.1 Mutants of the first α-helix of GM-CSF.

Residue	Mutation[a]
14	E14Q, E14K, E14A, E14T
17	N17E, N17D, N17A, N17K, N17S, N17T
18	A18N, A18D, A18K, A18E
19	I19Q, I19E, I19R, I19G, I19K
20	Q20A[b], Q20K, Q20E
21	E21A[b], E21R[b], E21K, E21Q, E21S, E21D, E21N, E21T
22	A22N, A22D, A22K
23	R23L, R23S, R23E, R23K, R23G, R23D, R23N
24	R24D[c], R24S, R24N, R24E
25	L25Q, L25E, L25K
26	L26Q, L26E, L26K
27	N27D, N27K, N27S, N27R, N27A, N27T
28	L28K, L28M, L28G, L28V, L28Q, L28W, L28D, L28H, L28N

[a] All plasmids encoding GM-CSF mutant analogues, were generated by oligonucleotide cassette mutagenesis (OCM) apart from the exceptions described below. Details of these plasmids are described in Appendix A.4.2.3 and the sequence of the mutagenic oligonucleotides used to generate the OCM fragments is listed in Appendix A.4.2.4.

[b] Constructs encoding these mutant analogues were generated by classical site-directed mutagenesis and are described in Table 3.3.1

[c] The R24D mutant analogue was generated by classical site-directed mutagenesis using the mutagenic oligonucleotide OL-36 (Appendix A.4.2.1). The plasmid pJGM36.4 encoding the R24D mutant analogue was generated by cloning the mutated GM-CSF cDNA into the pJL4 expression vector (Appendix A.3.2.1). An EcoRI/HindIII fragment containing GM-CSF cDNA was excised from the M13mp19 vector, Klenow blunt-ended and cloned into the SmaI site of pJL4.

mutagenesis, a total of 65 single amino acid substitution mutants were generated in the first α-helix, with 3 to 9 substitutions at each position mutated.

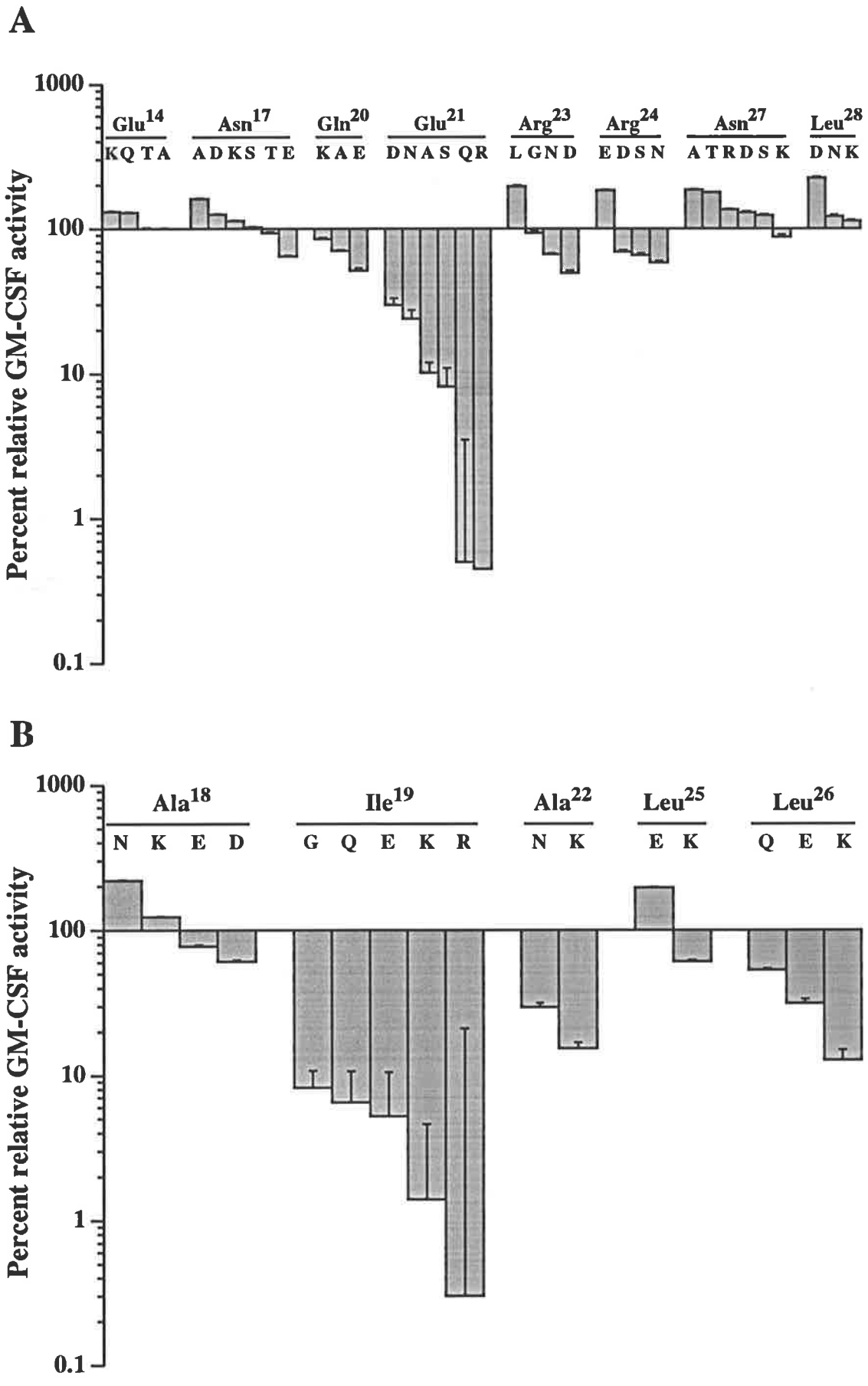
4.2.5 The biological activity of GM-CSF mutants

GM-CSF analogues were expressed from COS cells after transfection with the appropriate plasmid and the concentration of GM-CSF in the conditioned medium determined by RIA. In general the GM-CSF analogues were all expressed at levels similar to that obtained with wild type GM-CSF. However, analogues mutated at residue 22 expressed quite poorly, yielding approximately 10% of the levels observed for wild type GM-CSF and suggesting that the mutations may have had a deleterious effect on GM-CSF folding or secretion. Western blot analysis showed a similar degree of molecular weight heterogeneity for the majority of the analogues compared with wild type GM-CSF (data not shown). Analogues mutated at residue 27 showed a reduced level of glycosylation, consistent with the loss of the Asn^{27}, N-linked glycosylation site (Donahue *et al.*, 1986a). The GM-CSF analogues were then titrated for the ability to stimulate [^{3}H]thymidine incorporation into CML cells.

The data from CML proliferation assays was analysed using the Fig.P program to obtain the equation for an asymmetric sigmoid curve that fit the data. Using this equation, the concentration of GM-CSF analogue required to elicit a biological response equivalent to the half maximal response (ED_{50}) of wild type GM-CSF was calculated. The relative biological activity of each GM-CSF analogue was then expressed as a percentage of wild type activity where wild type was defined as 100%. At least two determinations for the activity of each analogue were made from independent COS cell transfections. The relative biological activity of GM-CSF analogues mutated at solvent exposed residues (Fig. 4.2.4A) and buried residues (Fig. 4.2.4B) are shown. Biological activity data for a number of the GM-CSF analogues included in Table 4.2.1 was not available or for a number of reasons was not included. The A22D analogue was very poorly expressed such that analysis of the biological activity of this analogue was not possible. The E21R, E21K and E21T analogues were devoid of measurable biological

Figure 4.2.4 Relative biological activity of GM-CSF first α-helix analogues.

Plasmid constructs encoding GM-CSF analogues were transfected into COS cells, quantified by RIA and titrated for the ability to stimulate [^{3}H]thymidine incorporation into CML cells. Data for the E21R analogue was derived from Figure 3.3.5 using purified, CHO cell-derived E21R. The relative biological activity of GM-CSF analogues is shown for solvent exposed residues (Panel A) and buried residues (Panel B). Columns represent the variation in potency from wild type GM-CSF (100%) for each of the GM-CSF analogues, using the mean from at least two experiments in which full titrations were performed. Potency was defined as the concentration of GM-CSF analogue required to elicit a response equivalent to the 50% maximal response (ED_{50}) induced by wild type GM-CSF and is expressed as a percentage of the wild type GM-CSF concentration. For certain analogues with low activity, relative potency was determined at a level equivalent to 25% of the maximal response (ED_{25}) for wild type GM-CSF. Bars indicate the single-sided standard deviation from all assays. GM-CSF analogues are identified in one letter code under the wild type residue in three letter code with the residue number.


A
1000
100
10
1
0.1
Percent relative GM-CSF activity
Glu14
KQ TA
Asn17
ADKS TE
Gln20
KAE
Glu21
DNAS QR
Arg23
LGND
Arg24
EDSN
Asn27
ATRDSK
Leu28
DNK
B
1000
100
10
1
0.1
Percent relative GM-CSF activity
Ala18
N K E D
Ile19
G Q E K R
Ala22
N K
Leu25
E K
Leu26
Q E K

activity although with the E21R analogue this is the result of insufficient GM-CSF protein in the COS cell conditioned medium. The concentrated and purified E21R analogue expressed from CHO cells displays biological activity, albeit some 300-fold lower than wild type GM-CSF (Fig. 3.3.5). The GM-CSF analogues mutated at residue 28 all exhibited essentially wild type biological activity although L28Q, L28H, L28W, L28G, L28M and L28V were not included in Figure 4.2.4A for the sake of simplicity.

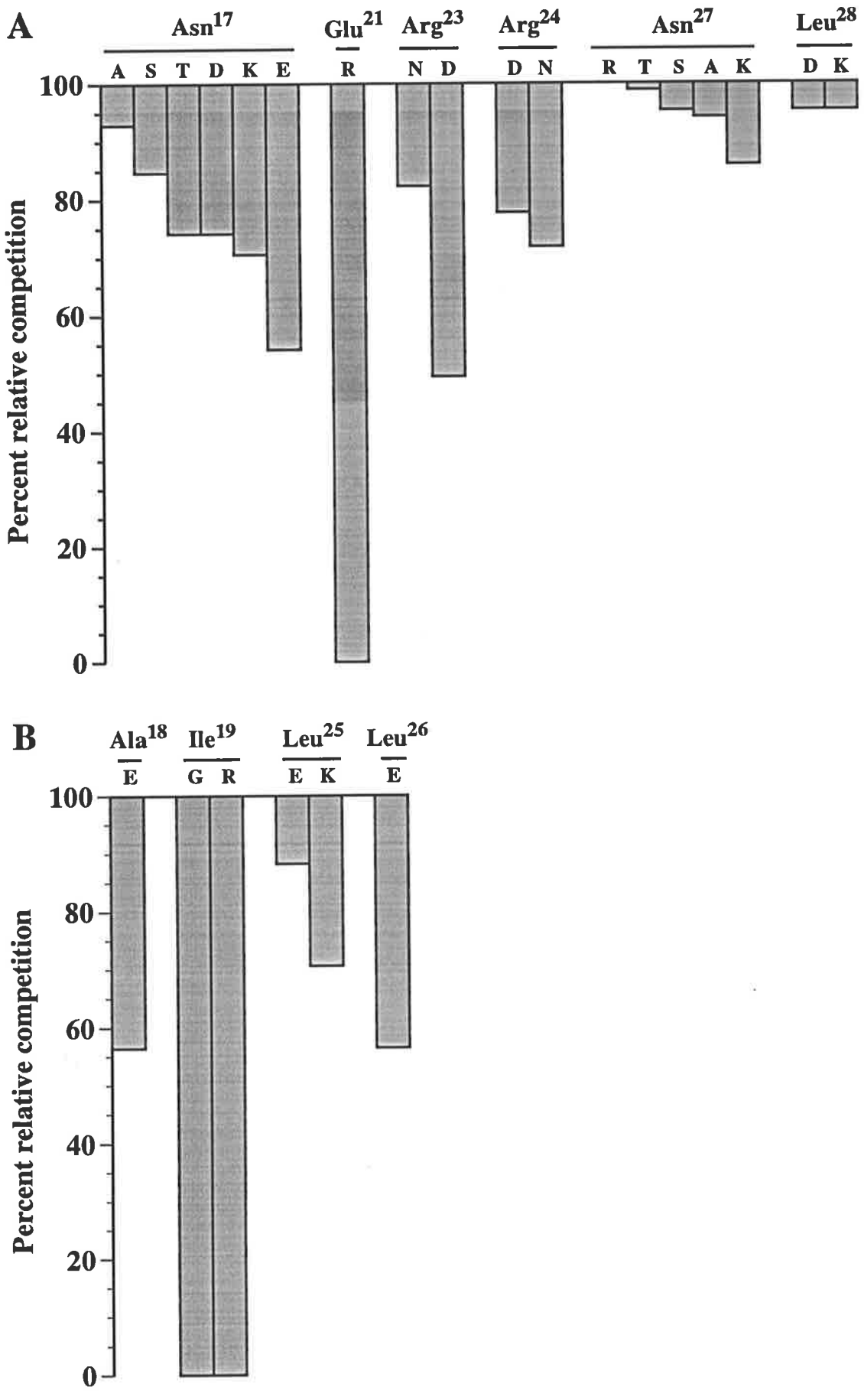
The analysis of GM-CSF analogues mutated at solvent exposed residues of the first α-helix, indicated that Glu^{21} was the only residue that contributed significantly to the biological activity of GM-CSF. Residues Asn^{17}, Gln^{20}, Arg^{23} and Arg^{24} made only minor contributions as assessed by the GM-CSF-dependent proliferation of primary human CML cells. The hierarchy of residue sensitivity to mutation and the fold decrease in activity (in parentheses) for the most deleterious mutation at each residue was, $Glu^{21}(220) >> Arg^{23}(2) = Gln^{20}(1.9) > Arg^{24}(1.7) > Asn^{17}(1.5) > Asn^{27}(1.1) > Glu^{14}(1)$ (Fig. 4.2.4A). The analysis of GM-CSF analogues mutated at buried residues of the first α-helix, indicated that these residues are in general, slightly less tolerant to substitution. The hierarchy of residue sensitivity to mutation and the fold decrease in activity (in parentheses) for the most deleterious mutation at each residue was, $Ile^{19}(330) > Leu^{26}(7.7) > Ala^{22}(6.7) > Leu^{25}(1.6) = Ala^{18}(1.6) > Leu^{28}(0.9)$ (Fig. 4.2.4B). Interestingly, certain analogues such as E14K, E14Q, N17A, A18N, R23L, R24E, L25E, most residue 27 mutants and L28D, consistently generated higher biological activity than wild type GM-CSF.

4.2.6 The binding of GM-CSF mutants to the low (α) and high ($\alpha\beta_c$) affinity GM-CSF receptors

Representative first α-helix GM-CSF mutants were compared for their ability to recognise the high affinity ($GMR\alpha\beta_c$) receptor of human neutrophils (Fig. 4.2.5). A 15-fold excess of each of the representative analogues was used to compete for the binding of 150pM ^{125}I-GM-CSF to human neutrophils. Analogues with a significant reduction in biological activity also demonstrated a significantly reduced ability to compete for the

Figure 4.2.5 Binding of GM-CSF first α-helix analogues to the high affinity (GMRαβ$_c$) receptor.

The binding of wild type GM-CSF and the first α-helix analogues to cells expressing the high affinity (GMRαβ$_c$) GM-CSF receptor was compared. Neutrophils (4 x 10^6) were incubated at room temperature for 2.5 hours in the presence of 150pM ^{125}I-GM-CSF and a 15 fold excess of wild type GM-CSF or analogue. Non-specific binding was determined in the presence of a 100-fold excess of unlabelled wild type GM-CSF. The percentage competition of each analogue for ^{125}I-GM-CSF binding was determined from the mean of duplicates and is expressed relative to the competition of wild type GM-CSF taken as 100%. Panel A shows the binding of GM-CSF analogues mutated at solvent exposed residues and panel B the binding of analogues mutated at buried residues.


A
Asn17
Glu21
Arg23
Arg24
Asn27
Leu28
A S T D K E
R
N D
D N
R T S A K
D K
Percent relative competition
100
80
60
40
20
0
B
Ala18
Ile19
Leu25
Leu26
E
G R
E K
E
Percent relative competition
100
80
60
40
20
0

binding of ^{125}I-GM-CSF. Mutation of the surface residue Glu^{21} (Fig. 4.2.5A) and the buried residue Ile^{19} (Fig. 4.2.5B) caused the most significant reduction in ability to compete for receptor-binding.

Representative analogues were titrated for the ability to compete for ^{125}I-GM-CSF binding to the high affinity (GMR$\alpha\beta_c$) receptor of human neutrophils or to the low affinity (GMRα) receptor of the A9/C7 CHO cell line (Hercus *et al.*, 1994b). The competitive binding curves for representative analogues are illustrated (Fig. 4.2.6) and the derived K_d values for all analogues tested, shown in Table 4.2.2. Analogues mutated at positions 17, 23, 24, 27 and 28 bound GMR$\alpha\beta_c$ with essentially wild type affinity although the N27D analogue displayed a modest (3-fold) increase in affinity, consistent with the biological activity observed for this analogue. As previously shown in Chapter 3, mutation of residue 21 reduced binding to GMR$\alpha\beta_c$ without having a significant effect on the binding to GMRα, confirming the selective interaction of this residue with the β_c chain of the GM-CSF receptor. Mutation of residue 19 had a modest effect on binding to both GMR$\alpha\beta_c$ (<2-fold) and GMRα (4-fold) which, along with the buried nature of this residue, suggests that the mutation may lead to structural perturbations.

4.3 Discussion

A new mutagenesis procedure (OCM) was developed to enable the efficient saturation mutagenesis of residues within the first α-helix of human GM-CSF. Once established, the OCM procedure was able to rapidly generate large numbers of single amino acid substitutions within the first α-helix and should be useful in the analysis of other potential receptor binding regions. A logical extension of the oligonucleotide cassette mutagenesis approach uses oligonucleotides with redundancies at multiple sites to generate libraries containing hundreds or even thousands of different GM-CSF analogues (Fig. 4.2.3E).

Figure 4.2.6 Binding of representative GM-CSF analogues to the high ($GMR\alpha\beta_c$) and low ($GMR\alpha$) affinity receptor.

The binding of wild type GM-CSF (●) and the analogues N17E (■), I19G (□), E21R (○), R23D (▲) and R24D (▼) to cells expressing high affinity ($GMR\alpha\beta_c$) GM-CSF receptors was compared (Panel A). The binding of wild type GM-CSF (●) and the analogues I19G (□), E21R (○) and R23D (▲) to cells expressing low affinity ($GMR\alpha$) GM-CSF receptors was compared (Panel B). Binding experiments were performed as described 2.10.17. Unlabelled wild type GM-CSF or analogues were titrated against 100pM ^{125}I-GM-CSF and 4 x 10^6 neutrophils per tube (A) or 500pM ^{125}I-GM-CSF and 6 x 10^5 A9/C7 cells per tube (B). The values are expressed as a percentage of the ^{125}I-GM-CSF bound in the absence of competitor and are the means of duplicate determinations.

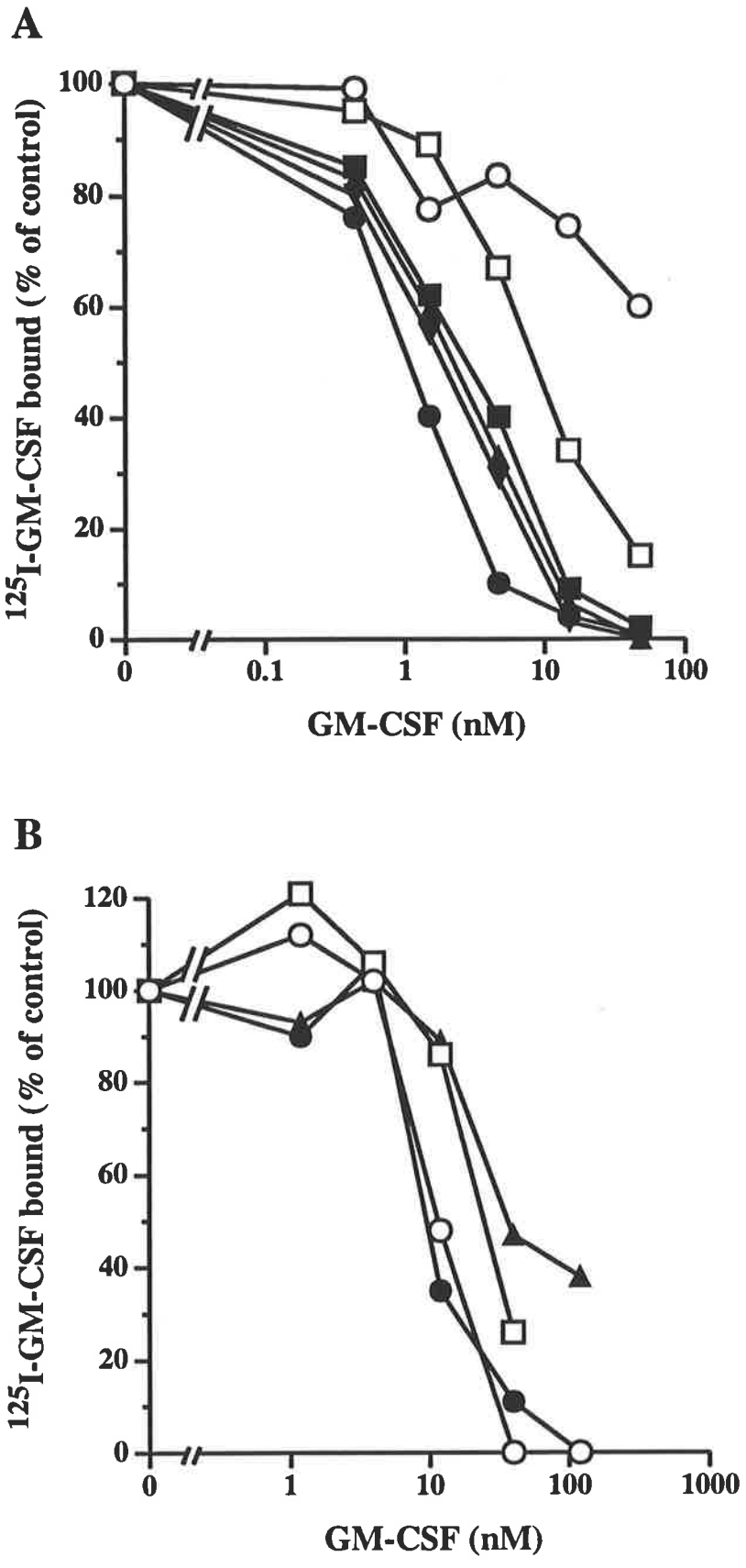

A
100
80
60
40
20
0
^{125}I-GM-CSF bound (% of control)
0
0.1
1
10
100
GM-CSF (nM)
B
120
100
80
60
40
20
0
^{125}I-GM-CSF bound (% of control)
0
1
10
100
1000
GM-CSF (nM)

Table 4.2.2 Binding affinities of GM-CSF first α-helix mutants to GM-CSF receptors.

GM-CSF	Receptor affinity[a]		
	K_d ($\alpha\beta_c$)[b] (pM)	K_d(α)[c] (pM)	ΔK_d[d]
Wild type	60	3,700	60
N17E	30	nd	-
I19G	100	15,700	160
E21R	5,000	7,200	1.4
R23D	70	5,000	70
R24D	40	5,100	130
N27D	20	nd	-
L28D	40	nd	-

[a] Binding affinities from competition experiments were determined using the EBDA/Ligand program (Munson and Rodbard, 1980).
[b] Mean binding affinities derived from multiple competition experiments using 5 x 10^6 human neutrophils (GMR$\alpha\beta_c$) and 200pM ^{125}I-GM-CSF.
[c] Mean binding affinities derived from multiple competition experiments using 6 x 10^5 A9/C7 CHO cells (GMRα) and 500pM ^{125}I-GM-CSF.
[d] ΔK_d, change in K_d from α chain only to $\alpha+\beta_c$

nd, not done

The results presented in this chapter establish the relative, individual contributions to biological activity of residues of the first α-helix of human GM-CSF. Substitution mutagenesis identified residue 21 as the only solvent exposed residue where mutation had a significant effect on GM-CSF biological activity or receptor interaction. These results emphasise the critical role played by Glu^{21} in the interaction of GM-CSF with the β_c chain of its receptor complex and hence the biological activity of GM-CSF. The remaining solvent exposed residues of the first α-helix tolerated a range of substitutions with little or no effect on the biological activity of GM-CSF. Thus individually, the solvent exposed residues of the first α-helix other than Glu^{21}, do not make critical contributions to the interaction of GM-CSF and its receptor.

The absence of a critical contribution does not preclude the majority of the solvent accessible residues in the first α-helix from a role in the interaction with the GM-CSF receptor. Analogues mutated at residue 27 exhibited a reduced level of glycosylation but consistently higher biological activity (Fig. 4.2.4A), suggesting that the presence of N-linked carbohydrate at this position influenced receptor binding. The fact that by itself, the Q20A substitution did not markedly influence GM-CSF activity is in contrast with the influence of this mutation when combined with the E21A substitution (Fig. 3.3.5). In a similar fashion, the wild type activity of the murine GM-CSF analogues, K14A and K20A, suggested no functional role for residues 14 and 20 whereas the marked reduction in activity of the K14A,K20A analogue indicated that both residues do play a role in murine GM-CSF activity (Meropol *et al.*, 1992). Residue 20 of human GM-CSF has been implicated in the interaction of human GM-CSF with the murine β_c chain (Shanafelt *et al.*, 1991b) while residue 23 of human GM-CSF has been implicated in receptor interaction by substitution mutagenesis (Kaushansky *et al.*, 1989) and by the identification of this residue in the binding epitope of a GM-CSF neutralising MoAb (Brown *et al.*, 1990). These observations are consistent with the results reported in this chapter and in most cases, differences are probably attributable to differences in experimental approach. In summary, of the residues located on the surface of the first α-helix of human GM-CSF, only residue 21 appears to play a critical role in the interaction

of GM-CSF with its receptor although it is likely that other residues such as 14, 20 and 23 also make a minor contribution. Although not pursued during the course of this work, it is possible that the mutation of some or all of these residues, in the context of deleterious residue 21 substitutions, may generate GM-CSF analogues devoid of detectable biological activity but retaining interaction with the GMRα chain. Analogues with these properties may be able to function as GM-CSF antagonists.

The buried residues targeted for mutagenesis, Ala^{18}, Ile^{19}, Ala^{22}, Leu^{25} and Leu^{26}, are all absolutely conserved in the species for which the GM-CSF sequence has been determined (Fig. 1.2.2) which suggests a potential structural role for these residues. However, with the exception of Ile^{19}, mutation of the buried residues in the first α-helix had only a minor influence on GM-CSF activity. This finding suggests that the neutral or charged, hydrophilic side chains of the substituted residues where able to be accommodated within the protein core or that mutation induced perturbations were minimal. The analogues A18N and L25E actually exhibited a slightly enhanced biological activity. Surprisingly, the residue 22 analogues which were poorly expressed, perhaps suggestive of some gross defect in protein folding and/or secretion, exhibited only a modest loss of function. In contrast, the sensitivity of Ile^{19} to mutagenesis indicates that the nature of the side chain at this position may be more structurally constrained. Close examination of the side chain environment for Ile^{19}, indicated that the side chain occupies a highly constricted pocket within the protein core. The analysis of analogues mutated at buried residues is complicated by the possibility of the mutations causing structural perturbations leading to a loss of function. Despite absolute conservation of these buried residues amongst the seven cloned species of GM-CSF (Fig. 1.2.2), analogues mutated at four of the five buried residues within the first α-helix, exhibited a relative biological activity equal to at least 10% of wild type (Fig. 4.2.4B). Whilst this represents a significant reduction in biological activity, the level of residual activity indicated a surprising degree of mutational tolerance amongst many of the buried residues. This demonstrates that absolute sequence conservation is not indicative of the functional status

of a residue even though conserved, buried residues are considered to be structurally important.

Analysis of the relative biological activity of all first helix analogues identified 8 residues where at least one analogue displayed biological activity enhanced by 50 to 150% (Fig. 4.2.4). These included all of the solvent accessible residues apart from Gln^{20} and Glu^{21}, and the buried residues Ala^{18} and Leu^{25}. Whilst the influence of the individual substitutions may be minor, it is possible that a single GM-CSF analogue incorporating all 8 enhancing substitutions will give rise to an analogue with significantly enhanced biological activity.

The observation that the E21R analogue retains residual biological activity, presumably mediated through the β_c chain, suggests that additional β_c chain contacts exist. The lack of obvious receptor binding candidates in the first α-helix and the possible role of the fourth α-helix in binding the GMRα chain suggests that alternative sites may be involved with receptor interaction. A number of studies have indicated that a region encompassing the third α-helix of GM-CSF, is involved in the biological activity of human and murine GM-CSF (Kaushansky *et al.*, 1989; Kanakura *et al.*, 1991; Shanafelt *et al.*, 1991a). Intriguingly, the first and third α-helices of human GM-CSF are neighbours in the three-dimensional structure and residues from the third α-helix are in close proximity to Glu^{21} in the first α-helix (Diederichs *et al.*, 1991). Thus residues on the surface of the third α-helix may form part of the β_c chain receptor binding site and analysis of the solvent accessible residues in this region may identify those which are functionally important.

Chapter 5

The expression and purification of GM-CSF residue 21 analogues using an *E. coli* secretion system

5.1 Introduction

The results presented in Chapter 3 of this thesis clearly demonstrated the critical nature of Glu^{21} in the high affinity binding of GM-CSF to its receptor complex $GMR\alpha\beta_c$ and in the biological activities mediated by GM-CSF (Lopez *et al.*, 1992c). Binding experiments suggested that the Glu^{21} residue interacts with the β_c chain, but not the α chain, of the high affinity receptor complex. An alternative explanation is that mutations of Glu^{21} perturb the tertiary structure of GM-CSF leading to an impaired interaction with receptor chains and concomitant loss of function. The aim of the work described in this chapter and Chapter 6 was to generate human GM-CSF residue 21 analogues in *E. coli* to enable more extensive characterisation of their biological and receptor binding properties, particularly receptor binding in the low affinity range. To address the possibility that structural perturbations are responsible for the loss of activity it was intended to implement a preliminary analysis of the biophysical properties of these analogues.

The expression of human GM-CSF analogues in a prokaryotic system offers two clear advantages over the eukaryotic systems used previously. Firstly, *E. coli* represents a cheaper and far more robust expression system that should enable the economical production of large quantities of recombinant human GM-CSF analogues. Secondly, the absence of carbohydrate modifications, which are not required for GM-CSF activity (Kaushansky *et al.*, 1987; Moonen *et al.*, 1987), means that the purified GM-CSF is expected to be a single, homogenous protein which simplifies biophysical characterisation. The three dimensional structure of GM-CSF has been determined by x-ray crystallography using *E. coli*-derived, recombinant human GM-CSF (Diederichs *et al.*, 1991; Walter *et al.*, 1992a).

Naturally occurring human GM-CSF and recombinant human GM-CSF produced in eukaryotic cells such as COS and CHO are extensively modified by the addition of both N- and O-linked carbohydrate (Wong *et al.*, 1985a; Donahue *et al.*, 1986a; Kaushansky *et al.*, 1987; Moonen *et al.*, 1987; Cebon *et al.*, 1990). It has been conclusively demonstrated that this carbohydrate is not required for activity by analysis of a range of human GM-CSF analogues lacking some or all forms of carbohydrate modification. Non-glycosylated GM-CSF has been produced in eukaryotic cells either by enzymatically removing the carbohydrate (Moonen *et al.*, 1987) or by mutagenesis of the sites of carbohydrate addition (Kaushansky *et al.*, 1987), by peptide synthesis (Clark-Lewis *et al.*, 1988) or by expression in prokaryotic cells (Burgess *et al.*, 1987). Analysis of the non-glycosylated GM-CSF indicates a significantly higher specific activity relative to glycosylated GM-CSF (Moonen *et al.*, 1987; Cebon *et al.*, 1990) due to a higher affinity for the GM-CSF receptor (Cebon *et al.*, 1990). Exactly what role the carbohydrate might play in the activity of GM-CSF is not clear although *in vivo* studies indicate that carbohydrate influences pharmacokinetic parameters such as serum clearance times (Donahue *et al.*, 1986a; Denzlinger *et al.*, 1993). It was therefore considered that GM-CSF Glu^{21} mutants produced in *E. coli* and lacking carbohydrate offered a suitable system for more detailed analysis of the effects of these mutations on GM-CSF activity.

5.1.1 Expression of heterologous proteins in *E. coli*

There are many different systems for expressing heterologous proteins in *E. coli* but they can be divided into three categories that differ principally in the form of the expressed protein although are not mutually exclusive.

A. Inclusion body

Expression of heterologous proteins as cytoplasmic inclusion bodies is probably the most common approach and under optimal conditions may yield grams of crude, insoluble, heterologous protein per litre of bacterial culture. Human GM-CSF has been produced as cytoplasmic inclusion bodies, accumulating to 10% of the total cellular protein (Burgess *et al.*, 1987) however other attempts have been unsuccessful (Libby *et*

al., 1987; Greenberg *et al.*, 1988). The heterologous protein usually carries additional residues on the N-terminus, such as an initiating methionine, and must be solubilised and correctly re-folded before use. The high density of inclusion bodies assists the preliminary purification of heterologous protein but final purification relies on conventional or affinity chromatography.

B. Fusion protein

Fusion of heterologous proteins with specific protein marker sequences may yield as much as 100 milligrams of crude, soluble fusion protein per litre of bacterial culture and a variety of systems utilising this approach are commercially available. The most useful feature of these systems is the ability to purify the fusion protein using recognition features endowed by the protein marker sequences. The pGEX system (Pharmacia, Uppsala, Sweden) produces fusion proteins with the 26-kDa glutathione S-transferase of *Schistosoma japonicum* that can then be purified using glutathione agarose beads. The pMAL system (New England Biolabs, Beverly, MA.) produces fusion proteins with the 40-kDa maltose-binding protein of *Escherichia coli* that can be purified using amylose resin. Once purified the fusion protein can be enzymatically cleaved with sequence specific proteases such as thrombin or factor Xa to release the desired protein although the cleavage process tends to be inefficient and expensive.

C. Secretion

Secretion of heterologous proteins is probably the least common approach and may yield optimally only 5 milligrams of crude, soluble, heterologous protein per litre of bacterial culture as observed for human IL-4 (Lundell *et al.*, 1990). Biologically active human GM-CSF has been successfully expressed by secretion expression systems (Libby *et al.*, 1987; Greenberg *et al.*, 1988). High level expression (20% of total cellular protein) of a secreted heterologous protein, β-lactamase, has been reported although the high level of expression leads to an accumulation of insoluble heterologous protein in the periplasm (Ghrayeb *et al.*, 1984). Heterologous proteins are directed to the periplasm or extracellular environment by a bacterial signal peptide, which is cleaved during

translocation releasing the heterologous protein which must be purified by conventional or affinity chromatography. The advantages offered by the secretion system are that the heterologous protein is likely to be soluble and correctly folded and if engineered appropriately the secreted protein will contain an authentic amino terminus.

Generating soluble human GM-CSF analogues offers the possibility of analysing the activity of crude protein preparations as well avoiding potential problems that might be associated with the correct refolding of mutant proteins from inclusion bodies. Thus the expression of soluble human GM-CSF analogues from *E. coli* either as fusion proteins with the advantage of simplified purification protocols or as a secreted protein with an authentic amino terminus, was examined. Production of wild type GM-CSF and E21R using the fusion system, pGEX-2T failed to generate any detectable fusion protein. It is possible that human GM-CSF is refractile to expression in this manner although this seems surprising as ovine GM-CSF (Dr T. Focaretta, personal communication) has been successfully expressed using the pGEX system. However, the production of human GM-CSF in an *E. coli* secretion system was successful. This chapter describes the methods developed for the production and purification of human GM-CSF and mutant analogues from *E. coli* .

5.2 Results

An *E. coli* expression system that can secrete biologically active murine and human GM-CSF (Greenberg *et al.*, 1988; Shanafelt and Kastelein, 1989) was obtained from Drs A.B.Shanafelt and R.A.Kastelein (DNAX Research Institute, Palo Alto, CA). The plasmid pshGM-CSF (Fig. 5.1.1A) contained a synthetic human GM-CSF cDNA (Fig. 5.1.1B) cloned into the *E. coli* expression vector pIN-III-OmpH3 (Lundell *et al.*, 1990), a derivative of pIN-III-OmpA2 (Ghrayeb *et al.*, 1984).

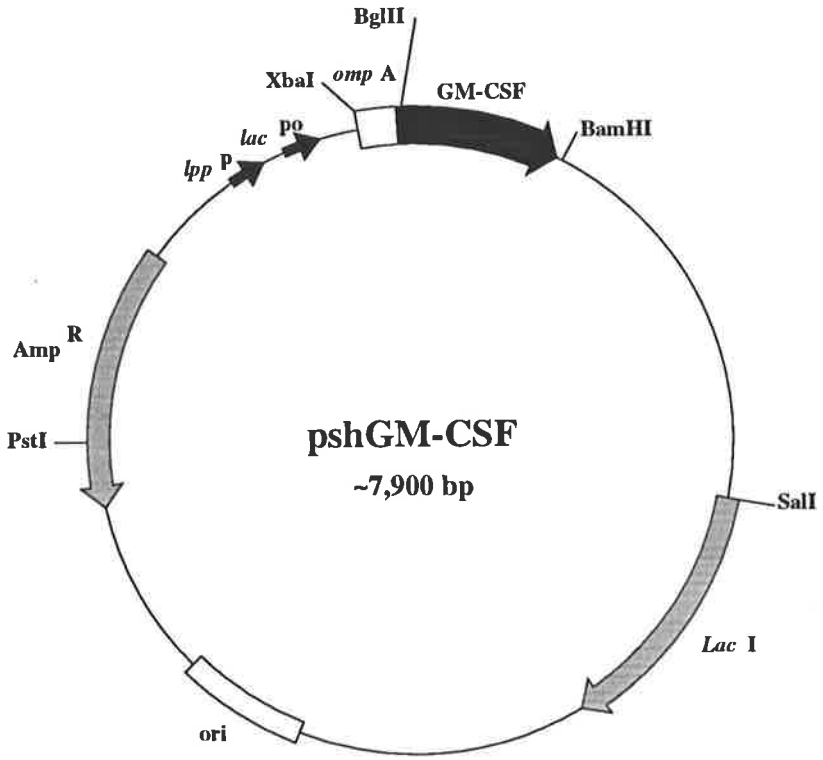
Figure 5.1.1 Map of the expression construct pshGM-CSF and sequence of the GM-CSF cDNA insert.

The pshGM-CSF expression plasmid (A) consists of the vector pIN-III-OmpH3 into which is cloned a synthetic GM-CSF cDNA (B). Expression of the 21 amino acid OmpA signal peptide fused with GM-CSF, is under the control of the lipoprotein promoter (*lpp*^{P}) and the lac promoter-operator (*lac*^{PO}) and is induced by IPTG. The signal peptide is cleaved at a position immediately prior to Ala^{1} of GM-CSF such that the secreted protein has the native sequence.

The synthetic GM-CSF cDNA sequence was cloned between the end-filled HindIII site and the BamHI site of the pIN-III-OmpH3 polylinker. Unique restriction enzyme sites are underlined and the position defined according to the base immediately 5' of the phosphodiester bond cleaved by the enzyme.

Amino acids subjected to mutagenesis are underlined.

A



B

```
          BglII(10)                    NcoI(34)
1       10        20        30        40        50        60
GCTCCGGCTAGATCTCCGTCCCCGTCTACCCAGCCATGGGAACACGTGAATGCTATCCAG
AlaProAlaArgSerProSerProSerThrGlnProTrpGluHisValAsnAlaIleGln
                  10                                        20
                                 SacII(98)
        70        80        90       100       110       120
GAAGCGCGCCGTCTGCTGAACCTGTCTCGTGACACCGCGGCTGAAATGAACGAAACCGTT
GluAlaArgArgLeuLeuAsnLeuSerArgAspThrAlaAlaGluMetAsnGluThrVal
                  30                                        40
                                                MluI(169)     SacI(182)
       130       140       150       160       170       180
GAAGTGATATCTGAAATGTTCGACCTCCAGGAACCGACCTGTCTCCAGACGCGTCTCGAG
GluValIleSerGluMetPheAspLeuGlnGluProThrCysLeuGlnThrArgLeuGlu
                  50                                        60
                SacII(201)                AflII(217)
       190       200       210       220       230       240
CTCTACAAACAGGGTCTCCGCGGTTCCCTGACCAAACTTAAGGGCCCGTTAACCATGATG
LeuTyrLysGlnGlyLeuArgGlySerLeuThrLysLeuLysGlyProLeuThrMetMet
                  70                                        80
  NheI(241)
       250       260       270       280       290       300
GCTAGCCACTACAAACAGCACTGCCCGCCGACTCCGGAAACTTCCTGTGCTACCCAGATC
AlaSerHisTyrLysGlnHisCysProProThrProGluThrSerCysAlaThrGlnIle
                  90                                       100
                                          SpeI(342)
       310       320       330       340       350       360
ATCACCTTCGAATCTTTCAAAGAAAACCTGAAAGACTTTCTACTAGTTATCCCGTTCGAC
IleThrPheGluSerPheLysGluAsnLeuLysAspPheLeuLeuValIleProPheAsp
                 110                                       120
                BamHI(387)
       370       380
TGCTGGGAACCGGTTCAGGAATAATAGGATCC
CysTrpGluProValGlnGluEndEnd
```

5.2.1 Mutagenesis of pshGM-CSF

The plasmid pshGM-CSF encodes wild type GM-CSF with codon usage optimised for *E. coli* expression but incorporating a number of unique restriction enzyme sites. The pshGM-CSF plasmid was mutated to encode a number of GM-CSF mutant analogues. The synthetic GM-CSF cDNA within the pshGM-CSF plasmid contains several unique restriction enzyme sites (Fig 5.1.1) that are suitable for oligonucleotide cassette mutagenesis (OCM). One of the major target regions for mutagenesis was the first helix. Residues of the first helix (13-28) are situated within a 64bp fragment defined by a unique NcoI site (position 34) and one of two SacII sites (position 98). To allow targeting of the NcoI/SacII fragment by OCM, the plasmid pSGMV1 was created in which the second SacII site of pshGM-CSF (position 201) was removed. This was achieved by replacing the 35bp SacI/AflII fragment with the SGMV1.U/L (Table 5.2.1) fragment which alters the codon usage of Leu^{66} and Arg^{67} and thereby eliminates the SacII site (position 201).

OCM has been used to construct a number of plasmids encoding mutated forms of human GM-CSF (Table 5.2.1). The constituent oligonucleotides were 5' phosphorylated and annealed to generate the OCM fragment which was ligated into the appropriately digested plasmid (Table 5.2.1).

1. Plasmid pSGM6.2

This plasmid encodes the P6YY analogue of GM-CSF where Pro^{6} is replaced by two tyrosine residues and was generated by OCM of the 24bp BglII/NcoI fragment (Table 5.2.1). To facilitate receptor binding experiments the P6YY analogue of GM-CSF was designed to enable high specific activity labelling with ^{125}I. This mutation was not expected to influence the activity of GM-CSF and was expected to be accessible for labelling.

Continuation of the work described in Chapter 3 required the generation of mutant analogues in which Glu^{21} was substituted by a range of amino acids that included Arg,

Table 5.2.1 *E. coli* GM-CSF expression plasmids.

#	Plasmid	Mutation/s	Insert[a]	Vector[b]
1.	pshGM-CSF[c]	wild type	synthetic human GM-CSF cDNA	pIN-III-OmpH3
2.	pSGMV1	wild type	V1.U/L	#1 SacI/AflII
3.	pSGM6.2	P6YY	6.2 U/L	#1 BglII/NcoI
4.	pSGM10.1/21.1	T10I/E21R	Nco21.1U/L Sac21.U/L	#2 NcoI/SacII
5.	pSGM21.1	E21R	#4 NcoI/BamHI[d]	#1 NcoI/BamHI
6.	pSGM21.2	E21A	Bgl/Nco.U/L Nco21.2U/L Sac21.U/L	#2 BglII/SacII
7.	pSGM21.3	E21Q	Bgl/Nco.U/L Nco21.34U/L Sac21.U/L	#2 BglII/SacII
8.	pSGM21.4	E21K	Bgl/Nco.U/L Nco21.34U/L Sac21.U/L	#2 BglII/SacII
9.	pSGM21.5	E21H	Bgl/Nco.U/L Nco21.5U/6L Sac21.U/L	#2 BglII/SacII
10.	pSGM21.6	E21F	Bgl/Nco.U/L Nco21.5U/6L Sac21.U/L	#2 BglII/SacII
11.	pSGM100	I100T	Nhe2.U/L 100.U/L Spe2.U/L	#2 NheI/SpeI
12.	pSGM21.1/100	E21R/I100T	Nhe2.U/L 100.U/L Spe2.U/L	#5 NheI/SpeI

[a] The insert DNA was generated by 5' phosphorylating and annealing an equimolar amount of the oligonucleotides that constituted an OCM fragment. The sequence of the oligonucleotides, one encoding the upper strand (U) and the other encoding the lower strand (L), is listed in Appendix A.5.2.1.

[b] The vector describes which of the plasmids (#) listed in this table was the recipient of the OCM fragment and the enzymes used to digest the plasmid.

[c] The details of the parental pshGM-CSF plasmid, including the location of unique restriction enzyme sites, are described in Figure 5.1.1B.

d The insert used to generate the pSGM21.1 plasmid was in fact obtained by NcoI/BamHI digestion of the pSGM10.1/21.1 plasmid.

Lys, His, Gln, Ala and Phe. This range of substitutions included basic, neutral and hydrophobic side chains which should generate analogues with different biological and receptor binding activities.

2a. Plasmid pSGM21.1

This plasmid encodes the E21R analogue of GM-CSF and proved particularly difficult to construct. Initially the NcoI/SacII fragment of pSGMV1 was targeted by OCM and the plasmid pSGM10.1/21.1 carrying the E21R mutation was isolated (Table 5.2.1). This plasmid also harboured an unexpected mutation, T10I, the origins of which are unknown and its location within the BglII/NcoI fragment of the vector, outside the region targeted for mutagenesis, surprising. However, the presence of the unique NcoI site between the T10I and E21R mutations enabled the NcoI/BamHI fragment from pSGM10.1/21.1 to be cloned into NcoI/BamHI digested pshGM-CSF to generate the correct pSGM21.1 plasmid (Table 5.2.1).

2b. Plasmids pSGM21.2 through to pSGM21.6

These plasmids encode various analogues of GM-CSF mutated at residue 21 and were generated by OCM of the 88bp BglII/SacII fragment (Table 5.2.1). This approach was very much more successful than that used to generate the pSGM21.1 plasmid.

3. Plasmids pSGM100 and pSGM21.1/100

During the course of work examining the activity of E21R, it became necessary to examine the role of an Ile/Thr polymorphism at residue 100 (Wong *et al.*, 1985a). The Ile^{100} residue was replaced by Thr^{100} in both wild type GM-CSF and E21R. The plasmids pSGM100 and pSGM21.1/100 encode the I100T analogues of wild type GM-CSF and E21R respectively and were generated by OCM of the 101bp NheI/SpeI fragment (Table 5.2.1).

5.2.2 Expression of wild type GM-CSF and analogues

In pilot expression studies the plasmid pshGM-CSF was transformed into four *E. coli* strains, MC1061, JM101, BB4 and E299 and the level of GM-CSF expression into the periplasm compared by Coomassie blue stained SDS/PAGE and western blots probed with a GM-CSF specific polyclonal antibody. MC1061, BB4 and JM101 are standard laboratory strains although JM101 has been reported to give high level expression of soluble human GM-CSF (Greenberg *et al.*, 1988) while E299 is a *lon* strain, deficient in intracellular protease activity. The conclusion from the pilot study was that GM-CSF was expressed at approximately the same level in all four strains of *E. coli*. In addition to the GM-CSF detected in the periplasm, GM-CSF was also detected in the culture supernatant, particularly from the JM101 and BB4 clones. The presence of GM-CSF in the culture supernatant may be the result of complete translocation across the cell membranes (Sakaguchi *et al.*, 1988) or as a consequence of cell death prematurely releasing the contents of the periplasm.

On the basis of these results, larger scale expression of wild type GM-CSF was attempted with 2 litre cultures of the JM101 and BB4 clones. Periplasmic extracts were prepared from pelleted cells while the culture supernatant was pooled and total protein precipitated with ammonium sulphate. Coomassie stained SDS/PAGE revealed a band of approximately 15kDa consistent with the expected size of non-glycosylated human GM-CSF in both periplasmic and culture supernatant samples (Fig. 5.2.1A). Western blot analysis with a GM-CSF specific polyclonal antibody indicated that there was a significant amount of GM-CSF in the periplasm as well as the pooled culture supernatant (Fig. 5.2.1B). The periplasmic and supernatant forms appeared to be full length as they were the same size as an *E. coli*-derived recombinant human GM-CSF produced in inclusion bodies (Gift from Dr G.Morstyn, Ludwig Institute for Cancer Research, Parkville, Vic.). RIA quantification of the GM-CSF in the periplasm indicated a yield of 3mg per litre with JM101 and 2.5mg per litre with BB4. The culture supernatant contained 0.75mg of GM-CSF per litre. This amount of GM-CSF was considered

Figure 5.2.1 Expression of GM-CSF into the periplasm and culture medium by JM101 and BB4

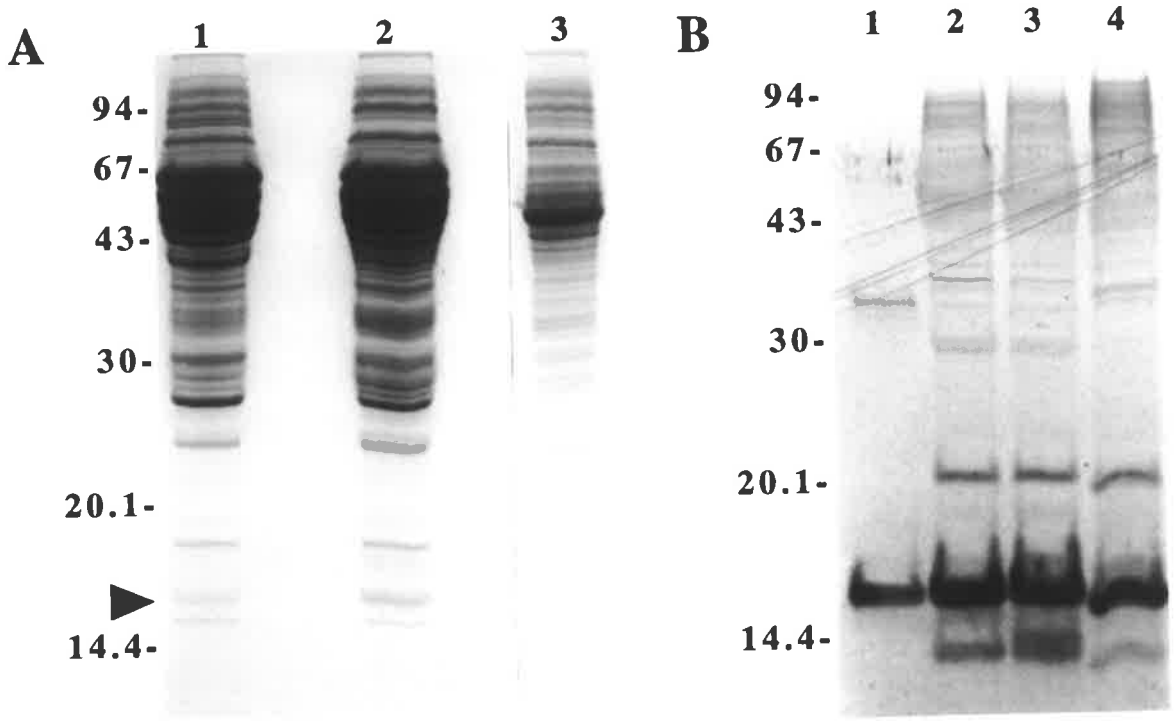
JM101 and BB4 clones harbouring the plasmid pshGM-CSF, were cultured at 37°C in 2L L-broth plus antibiotic, to an OD_{600nm} of 0.9 before adding IPTG to 0.1mM and incubating at room temperature overnight. Cells were pelleted and periplasmic contents isolated by osmotic shock (as described 2.10.8) and concentrated in a Centriprep-10 to a final volume of 7mls. The pooled culture supernatants were subjected to ammonium sulphate precipitation (65% saturation). The pelleted material was resuspended in 60mls MilliQ, dialysed 1:800,000 (final) against PBS, 0.01% (v/v) Tween 20 and 0.02% (w/v) sodium azide and concentrated to a final volume of 4.5mls in a stirred-cell using a 10,000 Mr cutoff membrane.

Panel A) Samples were fractionated by 15% SDS/PAGE and the gel stained with Coomassie brilliant Blue. Molecular weight standards are indicated while the position of GM-CSF migration is indicated "►".

1. 20µl JM101/pshGM-CSF periplasmic contents
2. 20µl BB4/pshGM-CSF periplasmic contents
3. 20µl Pooled culture supernatants (prior to concentration)

Panel B) Samples were fractionated by 15% SDS/PAGE, transferred to nitrocellulose and probed with sheep anti-GM-CSF polyclonal antibody. Molecular weight standards are indicated.

1. 160ng E. coli recombinant human GM-CSF
2. 5µl JM101/pshGM-CSF periplasmic contents
3. 5µl BB4/pshGM-CSF periplasmic contents
4. 1µl Pooled culture supernatants (concentrated)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43-
30-
20.1-
14.4-

insufficient to warrant harvesting as the periplasmic material is much easier to obtain and is more concentrated.

Having established that the secretion expression system was able to produce adequate levels of wild type GM-CSF, the expression of the GM-CSF mutant analogues was examined. Initial expression work centred around obtaining the P6YY analogue for radiolabelling and the E21R analogue for receptor binding studies. The P6YY analogue expressed at about the same level as wild type GM-CSF but the E21R analogue appeared to be particularly refractile to expression. MC1061 harbouring the plasmid pSGM21.1, which encodes E21R, yielded no detectable E21R by either silver stained SDS/PAGE (Fig. 5.2.2A) or western blot analysis with GM-CSF specific polyclonal antibody (Fig. 5.2.2B). This was confirmed by RIA which estimated 1μg E21R per litre of culture which represents a relative yield of 0.02% compared to wild type GM-CSF.

5.2.3 Improving the expression of the E21R analogue

A number of approaches were examined to improve the yield of the E21R analogue. Mutant constructs generated by means of OCM were normally only sequenced through the cassette and flanking restriction sites. However, the entire GM-CSF cDNA of pSGM21.1 was sequenced which confirmed that the E21R mutation was the only mutation present in this plasmid.

The role that culture conditions might play in E21R expression by MC1061 was assessed by varying media composition, media pH, buffering agents and induction conditions. The conclusion was that there was no significant expression of E21R from MC1061 under any of the conditions tested.

The next variable examined was to determine if E21R expression could be detected by using different strains of *E. coli*, particularly protease deficient strains. The strains tested were;

Figure 5.2.2 Expression of E21R in MC1061

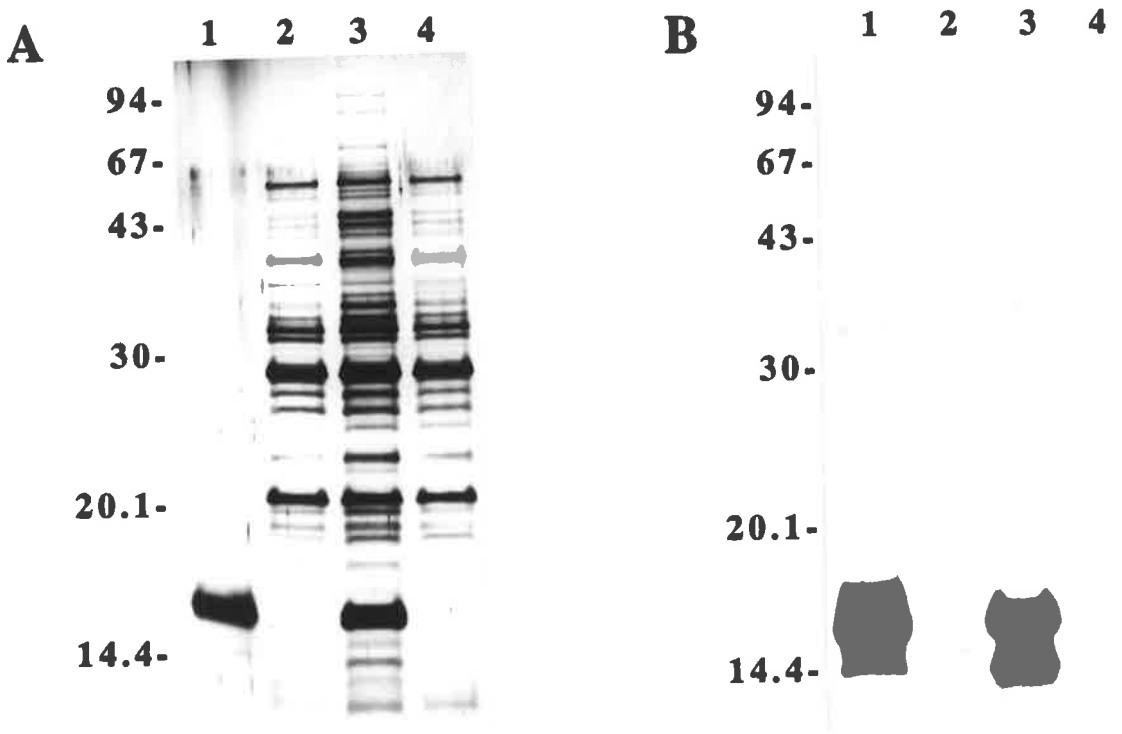
MC1061 clones harbouring the plasmids pIN-III-OmpH3 (expression vector), pSGMV1 (wild type GM-CSF) or pSGM21.1 (E21R) were cultured at 37°C in 1L L-broth plus antibiotic, to an OD_{600nm} of 0.7 before adding IPTG to 0.1mM and incubating at room temperature overnight. Cells were pelleted and periplasmic contents isolated by osmotic shock.

Panel A) Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1. 300ng *E. coli* recombinant human GM-CSF
2. 10µl MC1061/pIN-III-OmpH3 periplasmic contents
3. 10µl MC1061/pSGMV1 periplasmic contents
4. 10µl MC1061/pSGM21.1 periplasmic contents

Panel B) Samples were fractionated by 15% SDS/PAGE, transferred to nitrocellulose and probed with sheep anti-GM-CSF polyclonal antibody. Molecular weight standards are indicated.

1. 100ng *E. coli* recombinant human GM-CSF
2. 10µl MC1061/pIN-III-OmpH3 periplasmic contents
3. 10µl MC1061/pSGMV1 periplasmic contents
4. 10µl MC1061/pSGM21.1 periplasmic contents


A
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94-
67-
43-
30-
20.1-
14.4-
B
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67-
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20.1-
14.4-

1. TOPP strains

The six TOPP strains which are non-K12 strains of *E. coli* that can be useful for the expression of proteins that are otherwise difficult or impossible to express. The genotypes of the TOPP strains have not been characterised.

2. *lon* strains

The E299 and AB1899 *lon* strains are deficient in intracellular protease activity and some eukaryotic proteins are stabilised in these strains.

3. *degP* strain

The B178htrA63 *degP* strain is defective in protease activity associated with the periplasmic space.

4. *ompT* strain

The BL21 *ompT* strain is defective in an outer membrane protease and is probably also deficient in intracellular protease activity (*lon*).

In summary, some twelve strains of *E. coli* were examined for their ability to express the E21R analogue and in no case did the expression of this analogue approach the 3 to 5 milligrams of GM-CSF per litre of culture that was typical for wild type GM-CSF. Western blot analysis with a GM-CSF specific polyclonal antibody, indicated a low level of E21R expression by BL21 (Fig. 5.2.3B). This was confirmed by RIA which estimated 40μg of E21R per litre of culture which represents a yield of 1% compared with wild type GM-CSF. The BL21 strain was subsequently used for expression of all GM-CSF residue 21 analogues as most expressed at a low level when compared to wild type GM-CSF. The lowest yield, as a percentage of wild type expression, was obtained with the charge reversal analogues E21R (1.7%) and E21K (2.1%), while the highest yield was obtained with the more conservative substitutions E21A (21%) and E21Q (30%). The T10I/E21R analogue expressed at consistently higher levels than the E21R analogue with a yield of approximately 10% compared to

Figure 5.2.3 Expression of E21R in the protease deficient strains B178htrA63 and BL21

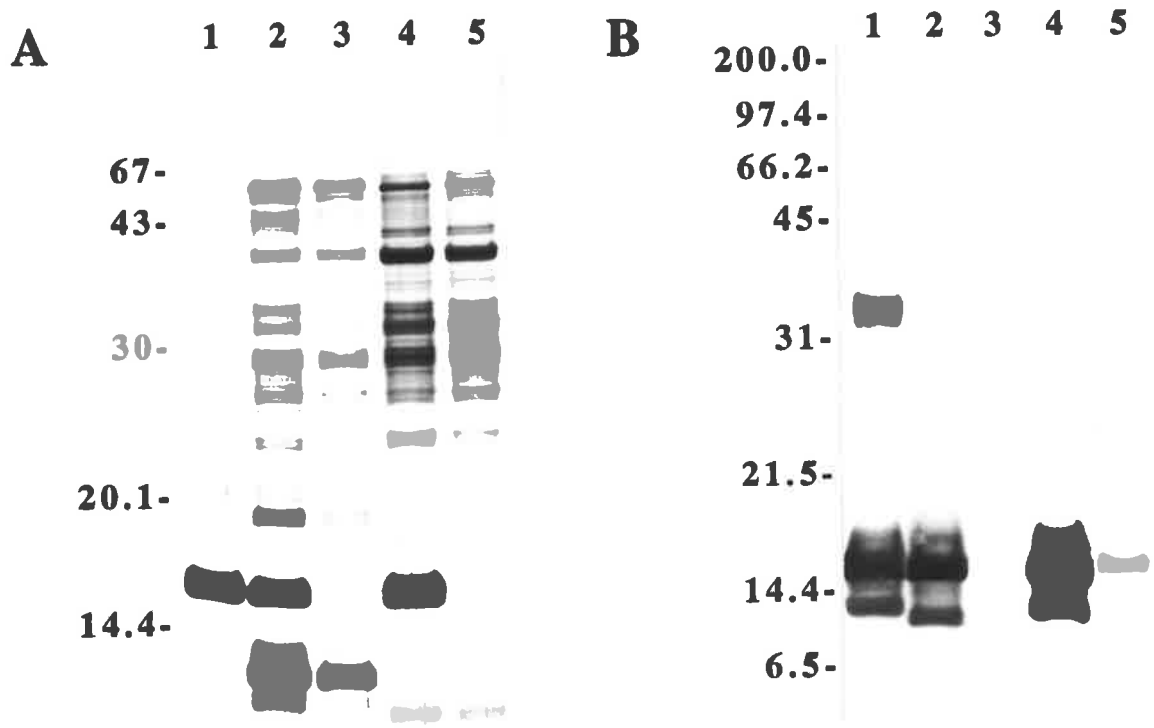
B178htrA63 clones harbouring the plasmids pshGM-CSF (wild type GM-CSF) or pSGM21.1 (E21R) were cultured at room temperature in 1L L-broth plus antibiotic, to an OD_{600nm} of 0.8 before adding IPTG to 0.1mM and incubating for a further 3 hours at room temperature. BL21 clones harbouring the plasmids pshGM-CSF or pSGM21.1 were cultured at 37°C in 1L L-broth plus antibiotic, to an OD_{600nm} of 0.85 before adding IPTG to 0.1mM and incubating for a further 3 hours at room temperature. Cells were pelleted and periplasmic contents isolated by osmotic shock.

Panel A) Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1. 200ng *E. coli* recombinant human GM-CSF
2. 2µl B178htrA63/pshGM-CSF periplasmic contents
3. 2µl B178htrA63/pSGM21.1 periplasmic contents
4. 2µl BL21/pshGM-CSF periplasmic contents
5. 2µl BL21/pSGM21.1 periplasmic contents

Panel B) Samples were fractionated by 15% SDS/PAGE, transferred to nitrocellulose and probed with sheep anti-GM-CSF polyclonal antibody. Molecular weight standards are indicated.

1. 200ng *E. coli* recombinant human GM-CSF
2. 10µl B178htrA63/pshGM-CSF periplasmic contents
3. 10µl B178htrA63/pSGM21.1 periplasmic contents
4. 10µl BL21/pshGM-CSF periplasmic contents
5. 10µl BL21/pSGM21.1 periplasmic contents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45-
31-
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14.4-
6.5-

wild type GM-CSF (data not shown). In addition, batches of wild type GM-CSF and analogues carrying the I100T substitution were expressed in BL21 (discussed in Chapter 6) so that any differences in the activity of analogues relative to wild type, could not be attributed to differences arising as a result of expressing GM-CSF in different strains of *E. coli.*

5.2.4 Purification of GM-CSF

A number of different protocols for the purification of wild type GM-CSF and analogues were investigated and are described below.

A. Gel filtration chromatography of wild type GM-CSF

Analysis of the crude GM-CSF preparations by Coomassie stained SDS/PAGE (Fig. 5.2.1A) indicated that the bulk of the contaminating proteins in both periplasmic and supernatant preparations are significantly larger than GM-CSF. This observation suggested that gel filtration chromatography might provide a significant purification step and in fact has been used to purify human GM-CSF prepared from inclusion bodies (Burgess *et al.*, 1987). Ten milligrams of total protein from the pooled culture supernatant of JM101 and BB4 harbouring pshGM-CSF (Fig. 5.2.1A, lane 3) was fractionated using a Sephadex G-100 gel filtration column and fractions analysed by silver stained SDS/PAGE. A GM-CSF peak was detected eluting at approximately 56.5mls which falls between the calibration standards chymotrypsin, 25kDa (51.5mls) and RNase A, 13.7kDa (58.9mls) (data not shown). This indicated that the GM-CSF was behaving as a monomeric protein as has been reported (Wingfield *et al.*, 1988). Fractions containing GM-CSF were then pooled and fractionated by reversed phase HPLC using a 0-60% gradient of acetonitrile to elute bound proteins. A GM-CSF peak was detected eluting at approximately 45% acetonitrile (data not shown). Figure 5.2.4 shows the results of this purification protocol with the final product judged >95% GM-CSF by silver stained SDS/PAGE.

Figure 5.2.4 Purification of wild type GM-CSF by gel filtration chromatography and reversed phase HPLC

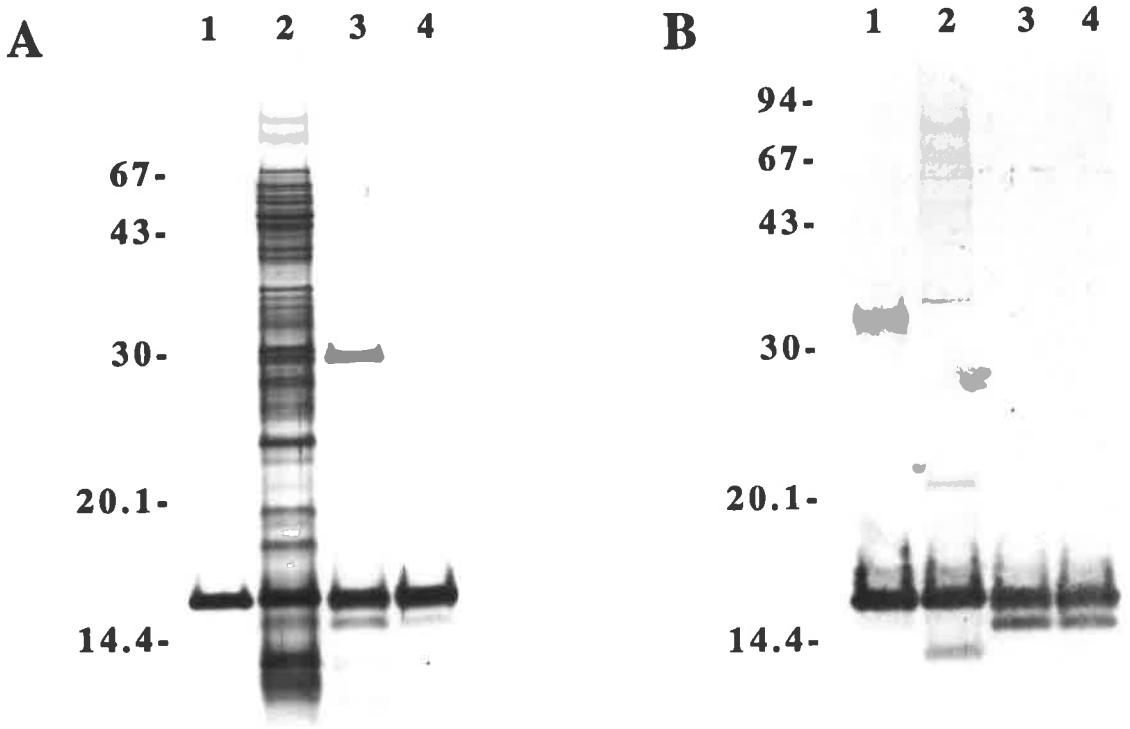
Ten milligrams of total protein from the culture supernatant of JM101 and BB4 clones harbouring the plasmid pshGM-CSF (Fig. 5.2.1) was fractionated on a Sephadex G-100 column (volume=100mls), equilibrated and run in PBS, 0.02% (w/v) sodium azide at 1.8mls per hour. Thirty minute fractions were analysed by silver stained SDS/PAGE and the size-fractionated pool enriched for GM-CSF (volume=8.1mls) was loaded onto a Brownlee Aquapore RP-300 reversed phase HPLC column (4.6x100mm). Protein was eluted at 1ml/min using a 0-60% gradient of acetonitrile containing 0.145% (v/v) TFA and fractions analysed by silver stained SDS/PAGE. The fractions containing purified GM-CSF were pooled, dialysed 1:2,000 (final) against PBS, 0.01% (v/v) Tween 20 and concentrated 10-fold using a Centricon-10.

Panel A) Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1. 320ng *E. coli* recombinant human GM-CSF
2. 0.2µl Crude JM101,BB4 culture supernatants (concentrated)
3. 5µl Sephadex G-100 purified GM-CSF
4. 5µl Reversed phase HPLC purified GM-CSF

Panel B) Samples were fractionated by 15% SDS/PAGE, transferred to nitrocellulose and probed with sheep anti-GM-CSF polyclonal antibody. Molecular weight standards are indicated.

1. 160ng *E. coli* recombinant human GM-CSF
2. 0.2µl Crude JM101,BB4 culture supernatants (concentrated)
3. 1µl Sephadex G-100 purified GM-CSF
4. 1µl Reversed phase HPLC purified GM-CSF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43-
30-
20.1-
14.4-

The amino terminus of this purified GM-CSF was sequenced to confirm that cleavage of the OmpA signal peptide generated the appropriate amino terminus. A sample of purified wild type GM-CSF was fractionated by 15% SDS/PAGE and transferred to Imobilon PVDF membrane in the absence of glycine, stained with Coomassie blue and the GM-CSF band excised and sequenced by Richard Simpson (Ludwig Institute for Cancer Research, Parkville, Vic.). The sequence that was obtained, Ala Pro Ala Arg Ser Pro Ser Pro Ser Thr Gln Pro Trp Glu matched precisely the amino terminus of mature human GM-CSF (Fig. 5.1.1B).

B. Anion exchange chromatography of wild type GM-CSF

A commonly used first step in protein purification is ion exchange chromatography. Therefore conditions for the purification of GM-CSF by ion exchange chromatography were examined. Human GM-CSF prepared in *E. coli* has an experimentally determined p*I* of 5.3, similar to that predicted from the amino acid composition (Schrimsher *et al.*, 1987). Efficient binding of GM-CSF to the anion exchange resin Macro-Prep Q was observed at pH10.5 and was reversible by lowering the pH to 7.0 and increasing the concentration of NaCl (Chris Bagley personal communication). Fourteen milligrams of total protein from the periplasm of MC1061 harbouring pSGMV1 (Fig. 5.2.2, lane 3), was fractionated by anion exchange chromatography. Analysis of the eluted protein by silver stained SDS/PAGE (Fig. 5.2.5A) indicated a GM-CSF peak at fraction 13, eluting in approximately 100mM NaCl (Fig. 5.2.5A lane 11). The fractions containing GM-CSF were then pooled and fractionated by reversed phase HPLC using a 30-50% gradient of acetonitrile to elute bound proteins (Fig. 5.2.5B). The HPLC chromatogram contained a very prominent peak of absorbance at approximately 45% acetonitrile which was co-incident with the peak of GM-CSF eluting from the column as determined by SDS/PAGE analysis of the eluted proteins (data not shown). The final product of this purification protocol was judged >95% GM-CSF by silver stained SDS/PAGE (Fig. 5.2.5C).

Figure 5.2.5 Purification of wild type GM-CSF by anion exchange chromatography and reversed phase HPLC

Fourteen milligrams of total protein from the periplasm of an MC1061 clone harbouring the plasmid pSGMV1 (Fig. 5.2.2) was adjusted to 25mM ethanolamine pH10.5 and loaded onto a 5ml Macro-Prep Q column equilibrated in 25mM ethanolamine pH10.5. The column was washed in 25mM NEM pH7.0 and bound protein eluted at 1ml/min with a 0-500mM gradient of NaCl in 25mM NEM pH7.0. Fractions were analysed by silver stained SDS/PAGE (Panel A) and those containing GM-CSF pooled (fractions 10-17), adjusted to 1% (v/v) acetic acid, 0.1% (v/v) TFA, filtered and loaded onto a Brownlee Aquapore Octyl reversed phase HPLC column (10x100mm). Protein was eluted at 5ml/min using a 30-50% gradient of acetonitrile containing 0.145% (v/v) TFA (Panel B). Fractions were analysed by silver stained SDS/PAGE and those containing GM-CSF were pooled, lyophilised and resuspended in PBS, 0.01% (v/v) Tween 20.

Panel A) Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

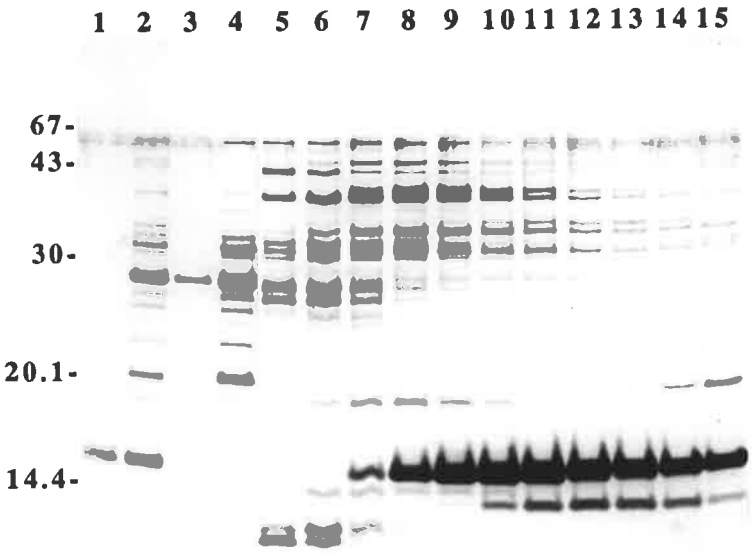
1.	100ng	*E. coli* recombinant human GM-CSF
2.	5µl	Crude MC1061/pSGMV1 periplasmic contents
3.	10µl	Column flow through
4.	10µl	25mM NEM pH7.0 column wash
5.	10µl	NaCl gradient fraction 7
↓		↓
15.	10µl	NaCl gradient fraction 17

Panel B) The trace displays the absorbance recorded at 280nm.

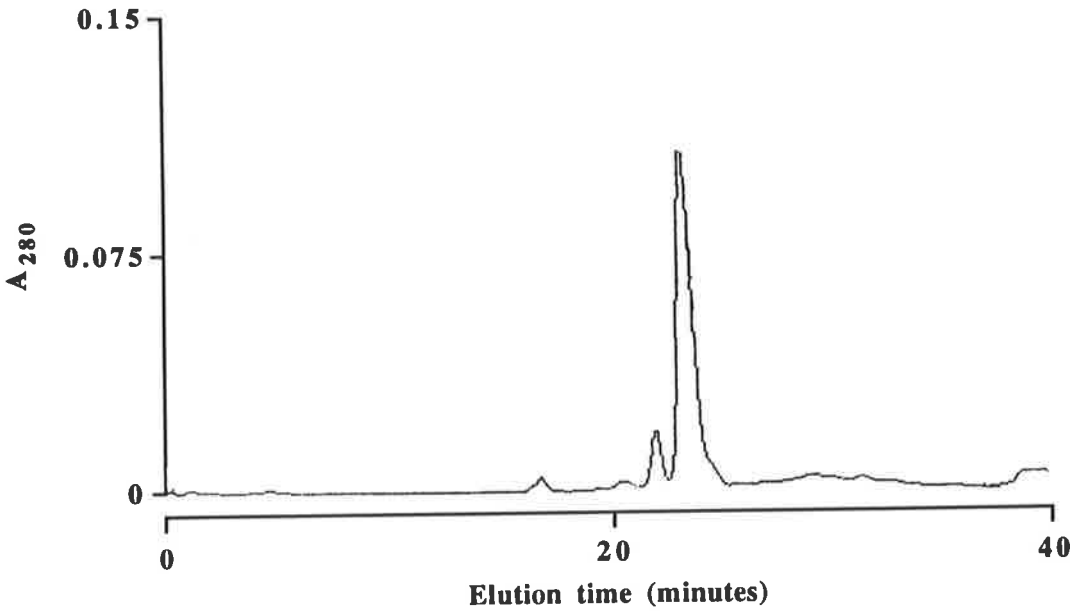
Panel C) Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1.	200ng	*E. coli* recombinant human GM-CSF
2.	10µl	Crude MC1061/pSGMV1 periplasmic contents
3.	5µl	Macro-Prep Q purified GM-CSF
4.	5µl	Reversed phase HPLC purified GM-CSF

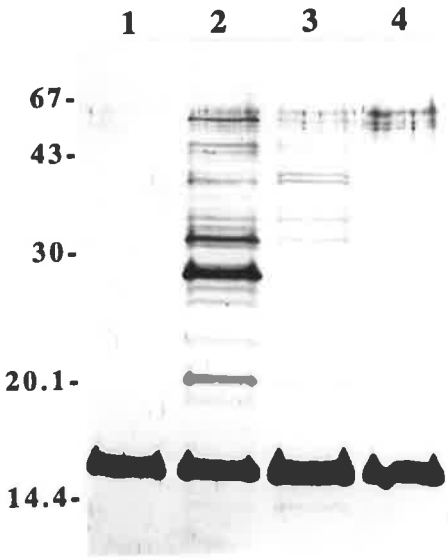
A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
67-
43-
30-
20.1-
14.4-

B


0.15
0.075
0
A_{280}
0
20
40
Elution time (minutes)

C


1 2 3 4
67-
43-
30-
20.1-
14.4-

Comparison of the wild type GM-CSF first purified by gel filtration (Fig. 5.2.4A) or anion exchange (Fig. 5.2.5C) indicated that the final, purified products are identical as determined by silver stained SDS/PAGE. The anion exchange protocol is faster and more robust than the gel filtration protocol and was therefore adopted as a standard protocol for the purification of *E. coli*-derived GM-CSF. The P6YY analogue behaved very much like wild type GM-CSF and was successfully purified using this protocol.

C. Anion exchange chromatography of the E21R analogue

Having identified a strain of *E. coli* in which an acceptable level of E21R was expressed, purification of the E21R analogue was attempted using the anion exchange protocol. Total protein from the periplasm of BL21 harbouring pshGM-CSF or pSGM21.1 (Fig. 5.2.3) was fractionated by anion exchange chromatography and 1ml fractions from the NaCl gradient analysed by silver stained SDS/PAGE (Fig. 5.2.6). Wild type GM-CSF and E21R both eluted at approximately 100mM NaCl (Fig. 5.2.6, Lane 11). The presence of E21R in fractions 13 to 18 (Fig. 5.2.6B, Lanes 8-13) was confirmed by western blot analysis with GM-CSF specific polyclonal antibody (data not shown). Both preparations contained significant levels of a 41 kDa contaminant protein that apparently eluted just prior to, and with GM-CSF. This protein is not present in preparations of GM-CSF produced in MC1061 (Fig. 5.2.5A) which may be explained by the fact that BL21 is an *E. coli* B strain and as such differs quite markedly from *E. coli* K-12 strains such as MC1061.

Fractions containing E21R were pooled (volume=4.8mls) and further purified by reversed phase HPLC using a 30-50% gradient of acetonitrile containing 0.145% TFA (Fig. 5.2.7A). Analysis of the fractions by silver stained SDS/PAGE (Fig. 5.2.7B) indicated that the E21R eluted slightly earlier (~43% acetonitrile) than wild type GM-CSF (Fig. 5.2.5B). However, the E21R analogue co-eluted with the 41 kDa contaminant protein (~43% acetonitrile) along with several other proteins which demonstrated that an alternative purification step was required to achieve a high level of E21R purity. The two

Figure 5.2.6 Purification of wild type GM-CSF and E21R by anion exchange chromatography

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Crude wild type GM-CSF from the periplasm of BL21 harbouring pshGM-CSF and crude E21R from the periplasm of BL21 harbouring pSGM21.1 (Fig. 5.2.3) were purified by anion exchange chromatography. Protein was loaded onto a 5ml Macro-Prep Q column in 25mM ethanolamine pH10.5 and the column washed in 25mM NEM pH7.0. Bound protein was eluted at 1ml/min using a 0-500mM gradient of NaCl in 25mM NEM pH7.0 and 1ml fractions collected. Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

Panel A) Fractionation of 18 milligrams total protein from BL21 harbouring pshGM-CSF.

Panel B) Fractionation of 11 milligrams total protein from BL21 harbouring pSGM21.1.

1.	200ng	*E. coli* recombinant human GM-CSF
2.	5µl	Crude periplasmic contents
3.	10µl	Column flow through
4.	10µl	25mM NEM pH7.0 column wash
5.	10µl	NaCl gradient fraction 10
6.	10µl	NaCl gradient fraction 11
7.	10µl	NaCl gradient fraction 12
↓		↓
16.	10µl	NaCl gradient fraction 21
17.	10µl	NaCl gradient fraction 22
18.	10µl	NaCl gradient fraction 23

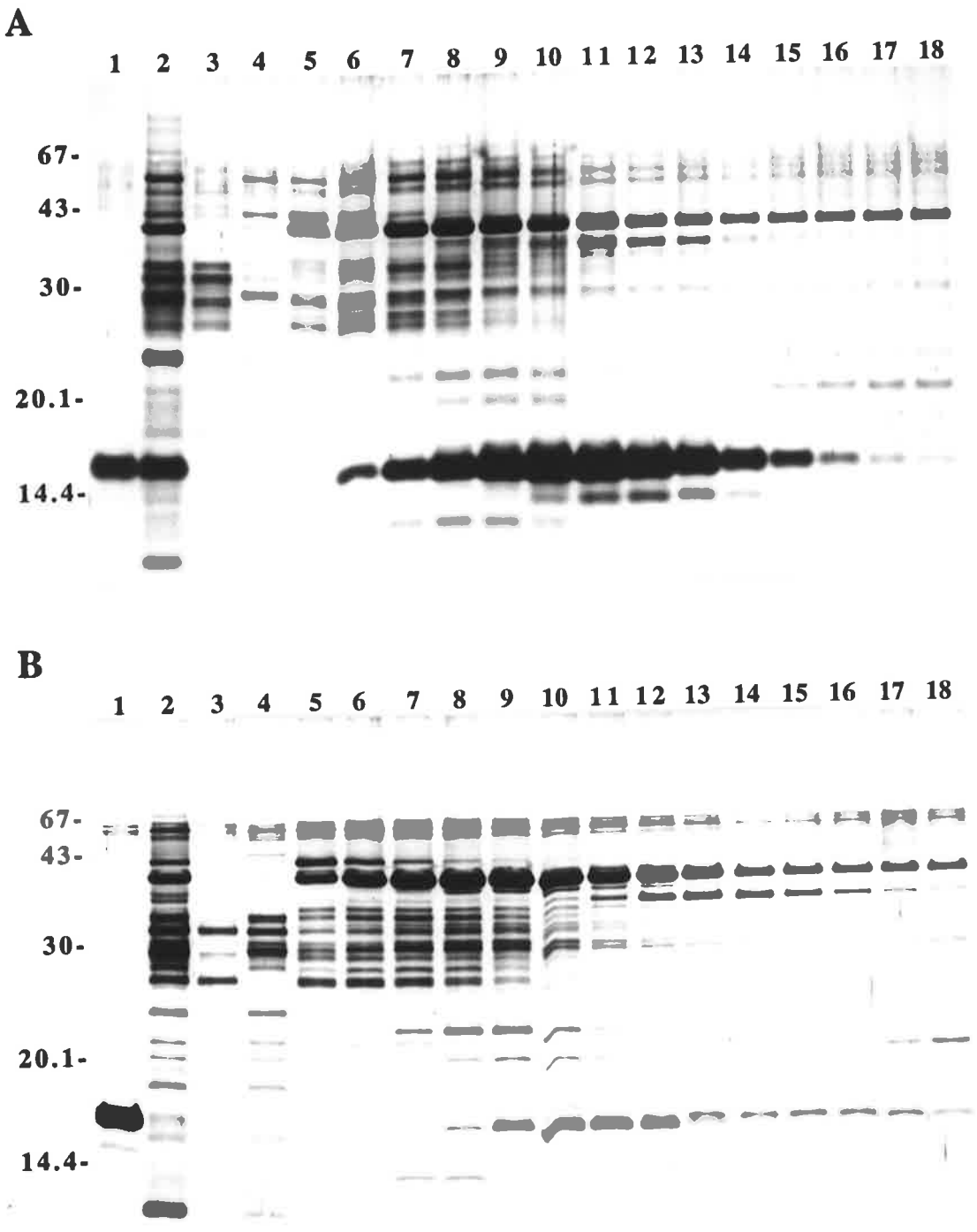

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
67-
43-
30-
20.1-
14.4-
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
67-
43-
30-
20.1-
14.4-

Figure 5.2.7 Reversed phase HPLC of E21R purified by anion exchange chromatography.

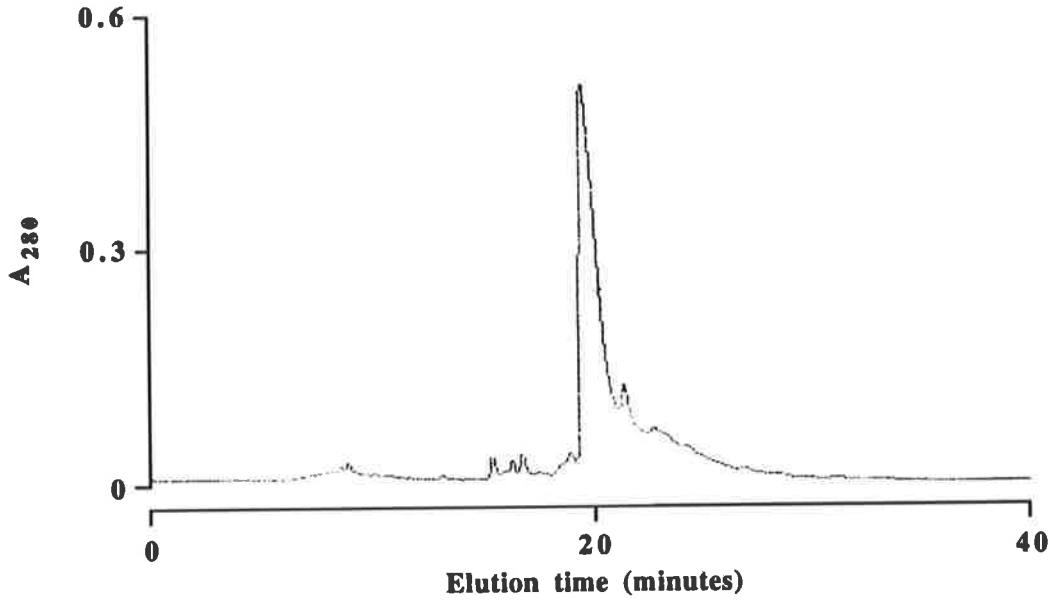
E21R analogue from the periplasm of BL21 harbouring pSGM21.1 and purified by anion exchange chromatography (Fig. 5.2.6) were loaded onto a Brownlee Aquapore RP-300 reversed phase HPLC column (4.6x100mm). Protein was eluted at 1ml/min using a 30-50% gradient of acetonitrile in 0.1% (v/v) TFA.

Panel A) The trace displays the absorbance recorded at 280nm.

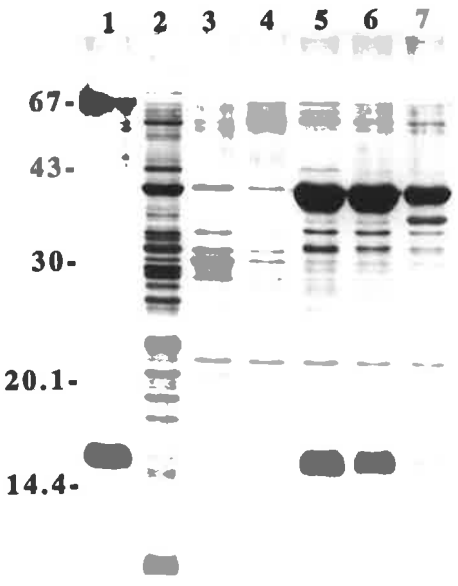
Panel B) Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1. 100ng *E. coli* recombinant human GM-CSF
2. 10µl Crude BL21/pSGM21.1 periplasmic contents
3. 20µl Fraction 9
4. 20µl Fraction 10
5. 20µl Fraction 11
6. 20µl Fraction 12
7. 20µl Fraction 13

A


0.6
0.3
0
A_{280}
0
20
40
Elution time (minutes)

B


1 2 3 4 5 6 7
67-
43-
30-
20.1-
14.4-

options tested were modification of the conditions for RP-HPLC and gel filtration chromatography.

The use of an alternative mobile phase (isopropanol/HFBA) during RP-HPLC was investigated on the basis that GM-CSF and the 41 kDa contaminant may elute under quite different conditions with different mobile phases. Wild type GM-CSF, prepared in BL21 and purified by anion exchange chromatography (Fig. 5.2.6A), was used for trial separations as this material contained large amounts of both GM-CSF and the 41 kDa contaminant. Reversed phase fractionation of the BL21-derived wild type GM-CSF using acetonitrile/TFA was able to separate the 41 kDa contaminant from the wild type GM-CSF (Fig. 5.2.8A,C) unlike the situation observed for E21R (Fig. 5.2.7). This is consistent with the higher concentration of acetonitrile required for the elution of wild type GM-CSF than E21R. Reversed phase fractionation of the BL21-derived wild type GM-CSF using isopropanol/HFBA also separated wild type GM-CSF from the 41 kDa contaminant but reversed the order in which the proteins eluted (Fig. 5.2.8B,C). Examination of the elution times for GM-CSF and the 41 kDa contaminant indicated that the isopropanol/HFBA mobile phase retarded the elution of the 41 kDa protein relative to wild type GM-CSF. This suggested that an isopropanol/HFBA mobile phase may assist in the separation of E21R and the 41 kDa contaminant. However, the modest resolution of the GM-CSF and the 41 kDa contaminant peaks (Fig. 5.2.8B,C) and the vast excess of the 41 kDa contaminant relative to E21R (Fig 5.2.6B), indicated that in addition, gel filtration chromatography may be required to enrich the E21R prior to RP-HPLC.

The periplasmic contents were isolated from 24L of the BL21 clone harbouring pSGM21.1 and fractionated by anion exchange chromatography as described Fig. 5.2.6. Fractions containing E21R (and 41 kDa contaminant) were pooled, concentrated and fractionated at 4°C using a Sephacryl S-200 gel filtration column. Fractions were analysed by silver stained SDS/PAGE and a peak of E21R was detected eluting at approximately 340mls (Fig. 5.2.9 Lanes 7,8). The elution volume of E21R fell between the calibration standards equine myoglobin, 17.5kDa (330mls) and vitamin B12,

Figure 5.2.8 Determining RP-HPLC conditions required to separate GM-CSF from the 41kDa contaminant

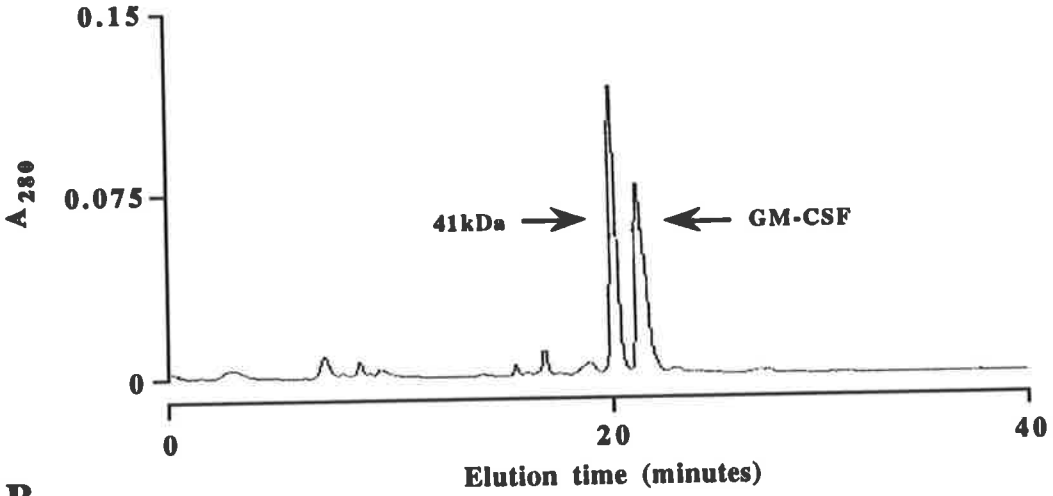
Samples of wild type GM-CSF from the periplasm of BL21 and purified by anion exchange chromatography (Fig. 5.2.6A), were loaded onto a Brownlee Aquapore RP-300 reversed phase HPLC column (4.6x100mm). Protein was eluted at 1ml/min using a 30-50% gradient of acetonitrile in 0.1% (v/v) TFA (A) or a 30-50% gradient isopropanol in 0.13% (v/v) HFBA (B).

Panel A and B) The traces display the absorbance recorded at 280nm. The peaks representing GM-CSF and the 41kDa contaminant are labelled.

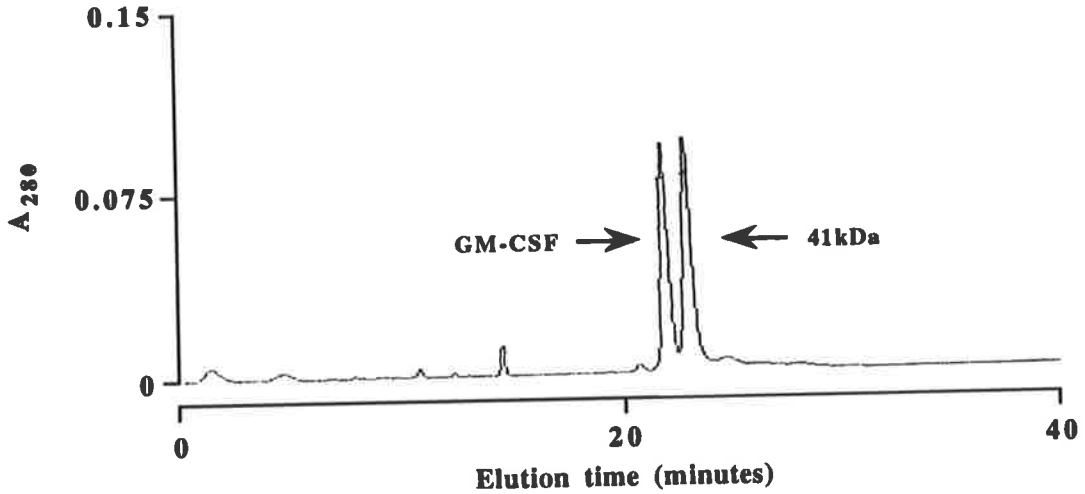
Panel C) Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1.	100ng	*E. coli* recombinant human GM-CSF
2.	5µl	Macro-Prep Q purified GM-CSF
3.	20µl	30-50% acetonitrile gradient, fraction 11
4.	20µl	" fraction 12
5.	20µl	" fraction 13
6.	20µl	" fraction 14
7.	20µl	30-50% isopropanol gradient, fraction 12
8.	20µl	" fraction 13
9.	20µl	" fraction 14
10.	20µl	" fraction 15

A


0.15
0.075
0
A_{280}
41kDa
GM-CSF
0
20
40
Elution time (minutes)

B


0.15
0.075
0
A_{280}
GM-CSF
41kDa
0
20
40
Elution time (minutes)

C

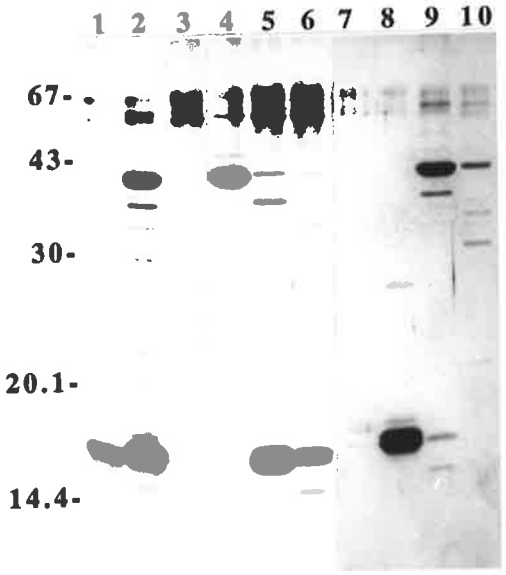

1 2 3 4 5 6 7 8 9 10
67-
43-
30-
20.1-
14.4-

Figure 5.2.9 Purification of E21R by anion exchange and gel filtration chromatography

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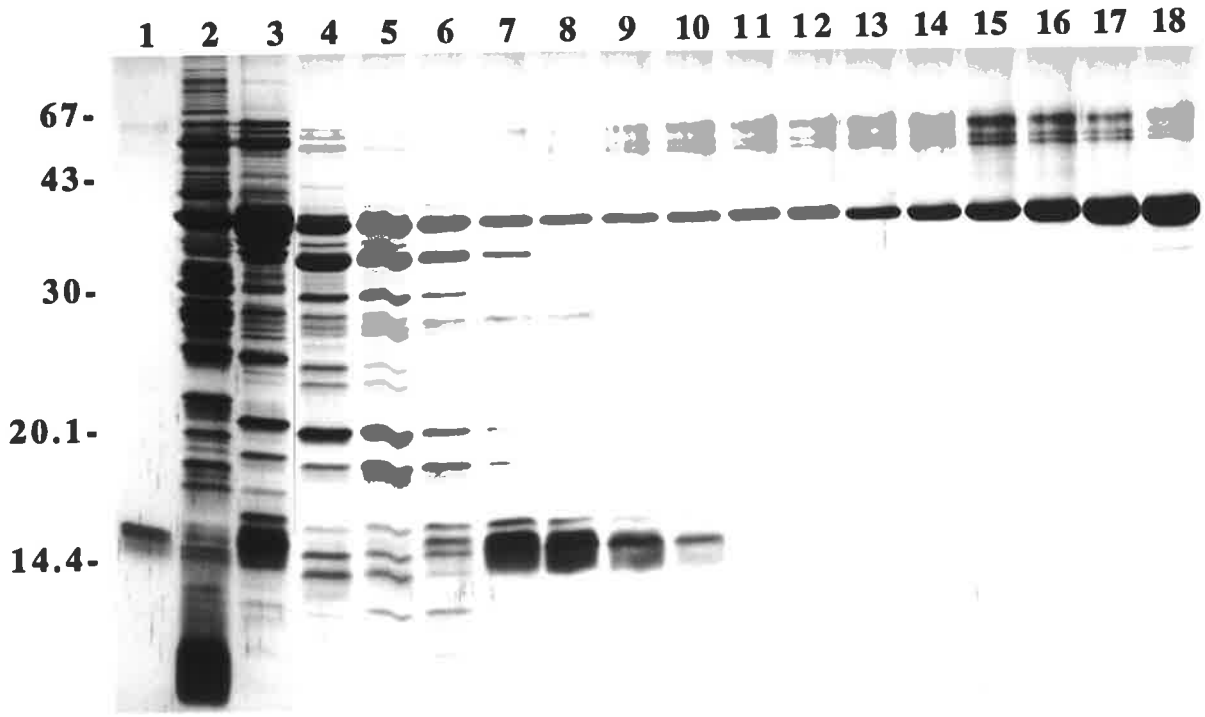
BL21 harbouring pSGM21.1 was cultured at 37°C in 24L L-broth plus antibiotic, to an average OD_{600nm} of 0.75 before adding IPTG to 0.1mM and incubating at room temperature for 3 hours. Cells were pelleted and periplasmic contents isolated by osmotic shock. Crude E21R was initially purified by ion exchange chromatography. Crude protein (total=0.31 grams) was loaded onto a 30ml Macro-Prep Q column in 25mM ethanolamine pH 10.5, washed in 25mM NEM pH 7.0 and bound protein eluted at 4.5ml/min with a 0-500mM gradient of NaCl in 25mM NEM pH7.0. One minute fractions were collected and samples analysed by silver stained SDS/PAGE. Fractions containing E21R were pooled (volume=55mls), concentrated to approximately 5mls and fractionated using a Sephacryl S-200 column (volume=530mls), equilibrated and run at 4°C in PBS, 0.01% (v/v) Tween 20, 0.01% (w/v) sodium azide at 0.4ml/min. Ten minute fractions were collected.

Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1.	100ng	*E. coli* recombinant human GM-CSF
2.	30µl	Crude BL21/pSGM21.1 periplasmic contents
3.	30µl	S-200 column load, Macro-Prep Q purified E21R
4.	30µl	Fraction 308-312mls
5.	30µl	Fraction 316-320mls
6.	30µl	fraction 324-328mls
↓		↓
16.	30µl	Fraction 404-408mls
17.	30µl	Fraction 412-416mls
18.	30µl	Fraction 420-424mls


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
67-
43-
30-
20.1-
14.4-

1.35kDa (490mls) which indicated that E21R was behaving as a monomeric protein (data not shown). Importantly the E21R was effectively separated from the 41 kDa contaminant. The surprising elution volume of 424mls (Fig. 5.2.9 Lane 18) for the 41 kDa protein seemed to be temperature dependent (data not shown) and was consistent with a molecular weight of approximately 2.5kDa rather than the 41kDa determined by SDS/PAGE.

The fractions containing S-200 purified E21R (Fig. 5.2.9, Lanes 6-10) were pooled and fractionated by RP-HPLC using a 30-50% isopropanol gradient containing 0.13% (v/v) HFBA to elute the bound proteins (Fig. 5.2.10A). Fractions were analysed by silver stained SDS/PAGE which showed that E21R had been separated from residual 41 kDa contaminant but still contained a number of other contaminants (Fig. 5.2.10B). To remove the final impurities, fractions containing E21R (Fig. 5.2.10B, Lanes 6-8) were pooled and fractionated by RP-HPLC using a 30-50% acetonitrile gradient containing 0.1% (v/v) TFA to elute the bound proteins (Fig.5.2.10C). Fractions were analysed by silver stained SDS/PAGE to reveal pure E21R (Fig. 5.2.10D, Lanes 6-9). Analysis of the RP-HPLC fractions by RIA demonstrated that the protein eluting just prior to E21R (Fig. 5.2.10D, Lanes 2-5) was also GM-CSF (data not shown). It appeared that some E21R was modified by exposure to isopropanol/HFBA resulting in an increase in apparent molecular weight. Similar modifications were observed during RP-HPLC purification of T10I/E21R using an isopropanol/HFBA mobile phase (Dr C.Bagley personal communication). Preliminary analysis suggested that the modification occurs very rapidly when the E21R analogues are exposed to isopropanol/HFBA.

Samples from the five stages of E21R purification were analysed by silver stained SDS/PAGE (Fig. 5.2.11). The four column procedure was able to extensively purify E21R produced in BL21 with the final product >95% pure E21R as judged by silver stained SDS/PAGE. Unfortunately this purification protocol only yielded 31µg of purified E21R which represents <1.5µg per litre of culture and less than 5% of the 850µg present in the crude periplasmic preparation. There were two factors which were

Figure 5.2.10 Reversed phase purification of E21R

E21R purified from BL21 by anion exchange chromatography and gel filtration chromatography (Fig. 5.2.9) was loaded onto a Brownlee Aquapore RP-300 reversed phase HPLC column (4.6x100mm). Bound protein was eluted using a 30-50% gradient of isopropanol containing 0.13% (v/v) HFBA (Panel A) and the 0.5ml fractions analysed by silver stained SDS/PAGE (Panel B). Fractions containing E21R (Panel B, Lanes 7-9) were pooled, loaded onto the Brownlee Aquapore RP-300 reversed phase column and bound protein eluted using a 30-50% gradient of acetonitrile containing 0.1% (v/v) TFA (Panel C). The 0.5ml fractions were analysed by silver stained SDS/PAGE (Panel D).

Panel A) The trace displays the absorbance recorded at 280nm.

Panel B) Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1.	100ng	*E. coli* recombinant human GM-CSF
2.	10µl	Macro-Prep Q and S-200 purified E21R
3.	5µl	Fraction 22
4.	5µl	Fraction 23
	↓	↓
9.	5µl	Fraction 28
10.	5µl	Fraction 29

Panel C) The trace displays the absorbance recorded at 280nm.

Panel D) Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1.	20ng	*E. coli* recombinant human GM-CSF
2.	10µl	Fraction 19
3.	10µl	Fraction 20
↓		↓
8.	10µl	Fraction 25
9.	10µl	Fraction 26

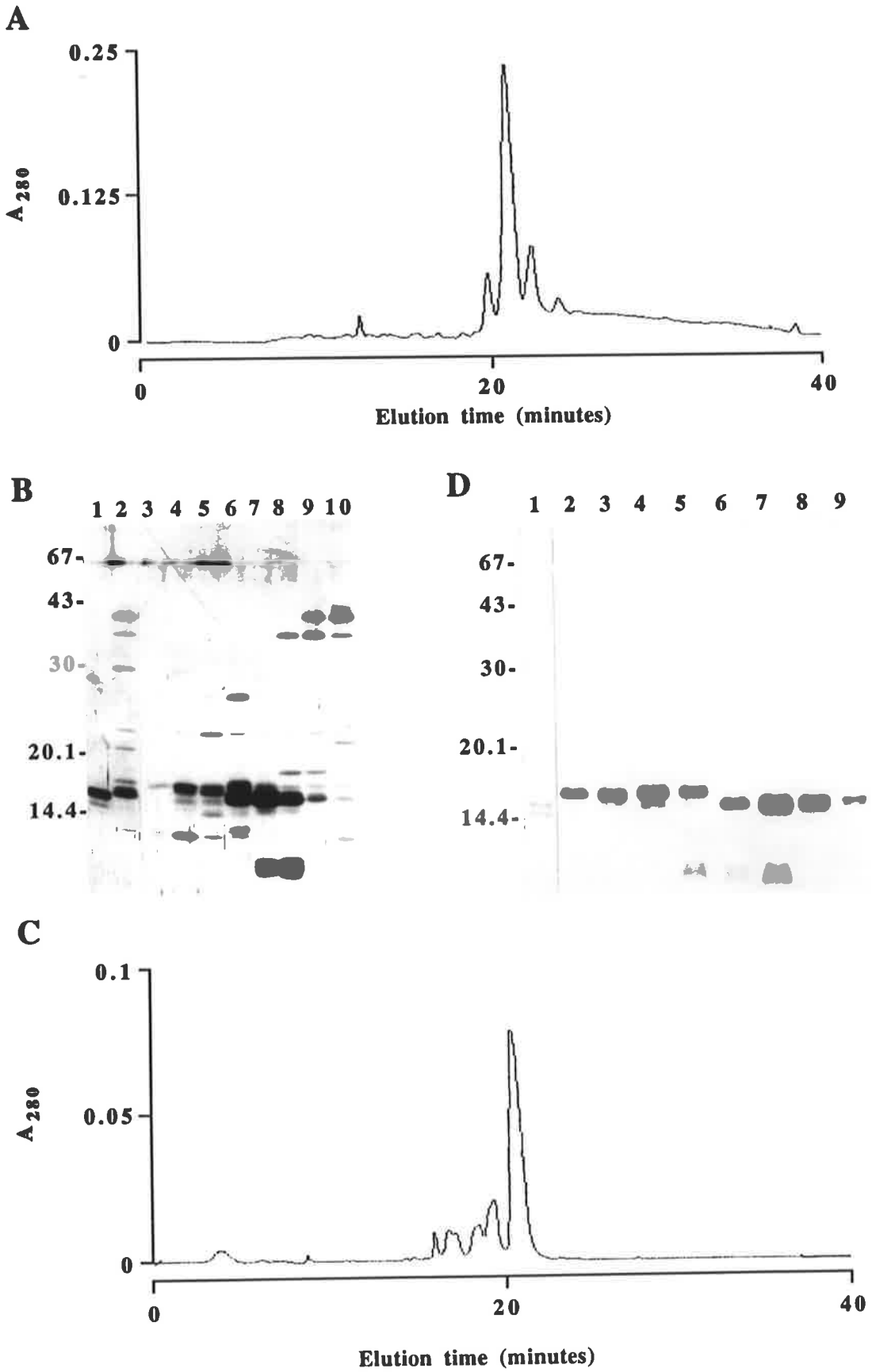
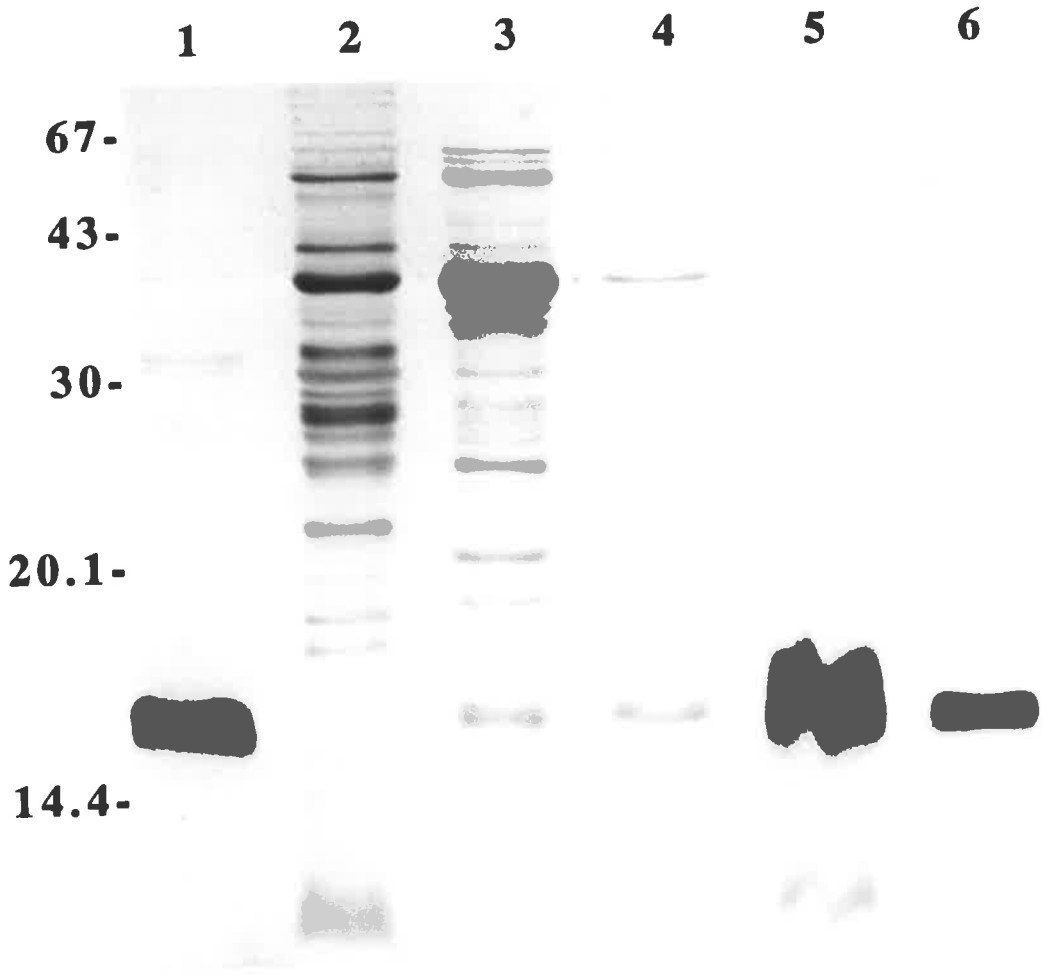

A
0.25
0.125
0
A_{280}
0
20
40
Elution time (minutes)
B
1 2 3 4 5 6 7 8 9 10
67-
43-
30-
20.1-
14.4-
D
1 2 3 4 5 6 7 8 9
67-
43-
30-
20.1-
14.4-
C
0.1
0.05
0
A_{280}
0
20
40
Elution time (minutes)

Figure 5.2.11 Purification of E21R from BL21

E21R was purified from 24L of BL21/pSGM21.1 periplasmic isolate by anion exchange chromatography and gel filtration chromatography (Fig. 5.2.9) and then two rounds of reversed phase purification (Fig. 5.2.10). Samples from each stage of the purification process were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1.	100ng	*E. coli* recombinant human GM-CSF
2.	20µl	Crude BL21/pSGM21.1 periplasmic contents
3.	20µl	Macro-Prep Q purified E21R
4.	20µl	S-200 purified E21R
5.	2µl	Isopropanol/HFBA RP-HPLC purified E21R
6.	2µl	Acetonitrile/TFA RP-HPLC purified E21R


1
2
3
4
5
6
67-
43-
30-
20.1-
14.4-

considered to be responsible for the very low yield of purified E21R. One was the intrinsically low level of E21R expression by BL21 while the second was the inefficient purification protocol with significant losses associated with the anion exchange chromatography and the isopropanol/HFBA RP-HPLC. The BL21 strain has already been shown to give the highest level of E21R expression so it was considered that an improved purification protocol was necessary to generate sufficient E21R for full characterisation.

4. Immunoaffinity purification of the E21R analogue

During the course of this work, a number of monoclonal antibodies (MoAbs) were raised against recombinant human GM-CSF in the lab and were used for immunoaffinity chromatography. Ascites from each of four hybridomas was ammonium sulphate precipitated and the partially purified immunoglobulin titrated against ^{125}I-GM-CSF to determine which preparations exhibited the strongest binding to GM-CSF. These titrations indicated that MoAb 4D4.C5 and MoAb 4A12.B might be suitable reagents for the generation of a GM-CSF specific immunoaffinity matrix (data not shown). Ammonium sulphate precipitated 4D4.C5 and 4A12.B were coupled to CNBr activated Sepharose-4B and the affinity matrix mixed with crude wild type GM-CSF isolated from MC1061. Specifically bound GM-CSF was eluted using 100mM NaCl, 100mM glycine pH4.0 and analysed by silver stained SDS/PAGE. Both immunoaffinity matrices were able to purify GM-CSF in one step from a crude periplasmic preparation with >95% purity (Fig. 5.2.12). After further analysis of the elution conditions for each matrix, MoAb 4D4.C5 was adopted for use as it required milder elution conditions (100mM NaCl, 100mM acetate pH5.0) than MoAb 4A12.B (100mM NaCl, 100mM glycine pH3.0) for recovery of GM-CSF.

The 4D4.C5 immunoaffinity matrix was used in an attempt to purify E21R produced in BL21. Periplasmic contents were isolated from 10L of the BL21 clone harbouring pSGM21.1, concentrated and the crude protein subjected to three rounds of adsorption to 1ml of 4D4.C5 immunoaffinity matrix followed by elution with 100mM

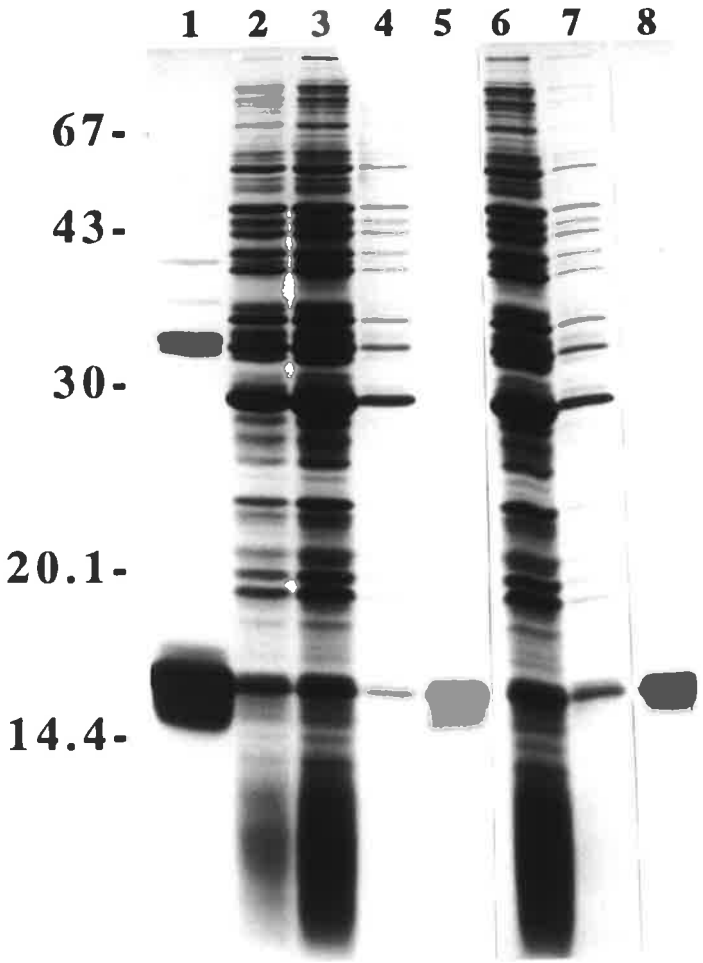
Figure 5.2.12 Analysis of two monoclonal antibodies generated against human GM-CSF for their use in the immunoaffinity purification of GM-CSF

One ml of crude ascites fluid was precipitated with ammonium sulphate (40% saturation) and the pelleted material resuspended in 2mls of PBS and dialysed 1:60,000 (final) against coupling buffer. Five milligrams of the purified MoAbs 4D4.C5 and 4A12.B were coupled to 1ml of swollen, CNBr activated Sepharose-4B according to the manufacturers instructions to generate an immunoaffinity matrix.

MC1061 harbouring the plasmid pshGM-CSF was cultured at 37°C in 2L L-broth plus antibiotics, to an OD_{600nm} of 0.85 before adding IPTG to 0.1mM and incubating at room temperature for 3 hours. Cells were pelleted and periplasmic contents isolated by osmotic shock. Crude wild type GM-CSF was mixed with the affinity matrix at 4°C overnight. Matrix was then collected, washed extensively with PAT buffer and specifically bound protein eluted with 100mM NaCl, 100mM glycine pH4.0 as described 2.10.9.

Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1. 270ng *E. coli* recombinant human GM-CSF
2. 10µl Crude MC1061/pshGM-CSF periplasmic contents
3. 25µl 4A12.B column, flow through
4. 25µl 4A12.B column, wash
5. 25µl 4A12.B column, glycine pH4.0 eluate
6. 25µl 4D4.C5 column, flow through
7. 25µl 4D4.C5 column, wash
8. 25µl 4D4.C5 column, glycine pH4.0 eluate


1 2 3 4 5 6 7 8
67-
43-
30-
20.1-
14.4-

NaCl, 100mM acetate pH5.0. Analysis of the eluates by silver stained SDS/PAGE indicated that the one step purification yielded E21R at >95% purity as was observed with wild type GM-CSF (Fig. 5.2.12). The eluates were pooled and fractionated by RP-HPLC using a 30-50% gradient of acetonitrile. Analysis of the RP-HPLC chromatogram confirmed that the E21R was essentially free of protein contaminants after the immunoaffinity purification (Fig. 5.2.13A). Comparison of the wild type GM-CSF and E21R purifications indicated the final products are both >95% pure as judged by silver stained SDS/PAGE (Fig. 5.2.13B). The two column procedure yielded 112µg of purified E21R which represented 11µg per litre of culture and approximately 50% of the E21R present in the crude periplasmic preparation. It should be noted that although the GM-CSF was >95% pure after affinity chromatography, the final purification by RP-HPLC was essential to remove non-protein *E. coli* contaminants such as LPS. The activation of myeloid cells by factors such as LPS may mask or complicate the analysis of the activity of GM-CSF analogues.

The protocol of immunoaffinity chromatography and RP-HPLC was used to purify all subsequent GM-CSF analogues expressed in the BL21 strain of *E. coli.* Table 5.2.2 summarises the yields for the purification of all GM-CSF analogues from BL21.

5.2.3 Quantification of purified GM-CSF

It is essential that estimates of the concentration of the purified protein be accurate to enable comparisons of the relative activities of wild type GM-CSF and mutant analogues. Throughout this work crude and purified GM-CSF has been quantified by means of a radioimmunoassay (RIA). However as was mentioned in Chapter 3, a potential problem with immunologically based protocols is the assumption that the antibody displays equal recognition of the protein used to generate the standard curve and the protein of unknown concentration. Clearly this may not be the case when quantifying GM-CSF mutant analogues, using wild type GM-CSF to generate a standard curve. To some extent this problem is avoided by using polyclonal antibodies which recognise a

Figure 5.2.13 Immunoaffinity purification of E21R

BL21 harbouring pSGM21.1 was cultured at 37°C in 10L L-broth plus antibiotics, to an OD_{600nm} of 0.84 before adding IPTG to 0.1mM and incubating at room temperature for 3 hours. Cells were pelleted and periplasmic contents isolated by osmotic shock. Periplasmic contents containing crude E21R were concentrated from 350mls to 50mls and mixed with 1ml of the 4D4.C5 immunoaffinity matrix at 4°C overnight. Matrix was collected, washed extensively with PAT buffer and specifically bound protein eluted with 100mM NaCl, 100mM acetate pH5.0. This process was repeated twice with the crude E21R and the eluted protein analysed by silver stained SDS/PAGE. Eluate fractions containing E21R were pooled, loaded onto a Brownlee Aquapore RP-300 reversed phase HPLC column (4.6x100mm) and bound protein eluted using at 1ml/min using a 30-50% gradient of acetonitrile containing 0.1% (v/v) TFA (Panel A). The purified E21R was then lyophilised and resuspended in PBS, 0.01% (v/v) Tween 20.

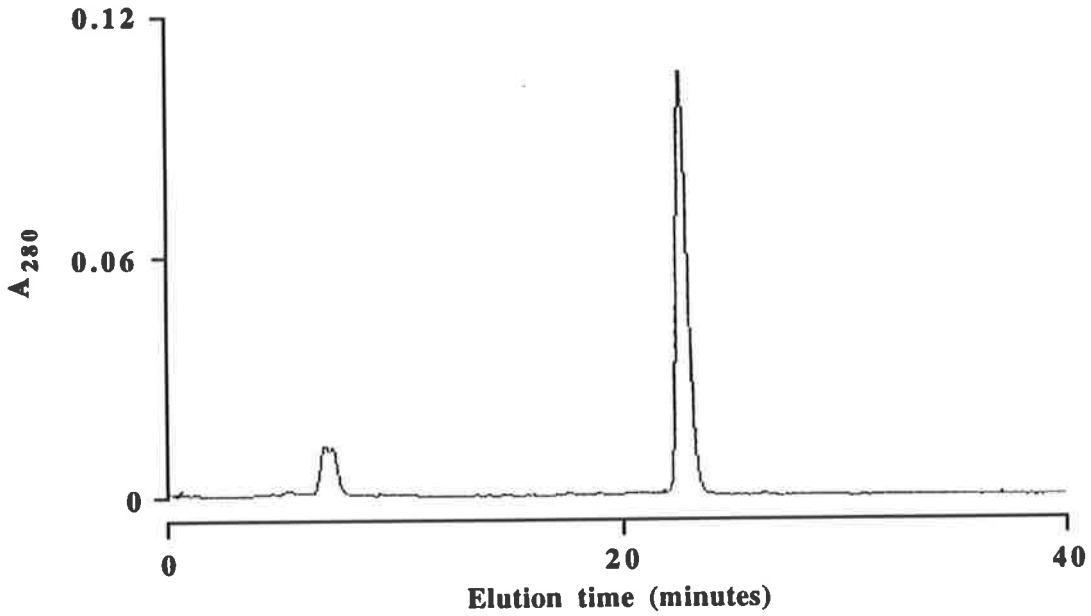
Wild type GM-CSF expressed in MC1061 was purified using the 4D4.C5 immunoaffinity matrix (Fig. 5.2.12), subjected to RP-HPLC and the final, purified material lyophilised and resuspended in PBS, 0.01% (v/v) Tween 20.

Panel A) The trace displays the absorbance recorded at 280nm.

Panel B) Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1.	10µl	Crude MC1061/pshGM-CSF periplasmic contents
2.	5µl	Pooled Glycine pH4.0 eluates from 4D4.C5 matrix
3.	10µl	RP-HPLC purified wild type GM-CSF from MC1061
4.	10µl	Crude BL21/pSGM21.1 periplasmic contents
5.	5µl	Pooled Acetate pH5.0 eluates from 4D4.C5 matrix
6.	5µl	RP-HPLC purified E21R from BL21

A


0.12
0.06
0
A_{280}
0
20
40
Elution time (minutes)

B

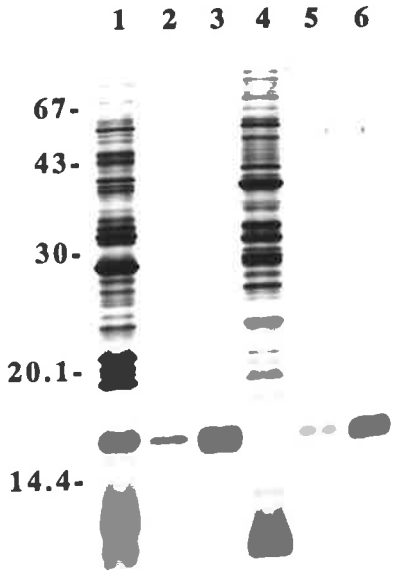

1 2 3 4 5 6
67-
43-
30-
20.1-
14.4-

Table 5.2.2 Yield of purified wild type GM-CSF and analogues from the *E. coli* strain BL21.

GM-CSF analogue	Culture volume (Litres)	Specific yield per litre[a] (μg/L)
wild type	1	170
E21Q	4	50
E21A	5	35
E21H	4	17
E21F	5	13
E21K	10	12
E21R	10	10
I100T	1	220
E21R/100T	10	20

[a] The specific yield for each analogue apart from E21R was determined only once. In the case of E21R the specific yield is the average value obtained from three independent preparations.

number of different epitopes but if a major epitope is modified then quantification may be inaccurate.

Quantification of purified protein can be achieved using a number of different techniques. Previously amino acid analysis was used to quantify GM-CSF analogues purified from CHO cells (Table 3.2.3). However, this approach was not particularly convenient as we do not have the facilities to perform amino acid analysis. High performance size exclusion chromatography (HP-SEC) was investigated as an alternative method for the quantification of purified GM-CSF analogues. The method required that a sample of purified GM-CSF was subjected to HP-SEC and the absorbance at 280nm measured. The area under the peak corresponding to GM-CSF absorbance was then integrated and using the calculated extinction coefficient for GM-CSF (wild type GM-CSF = 0.95 $AU.ml^{-1}.mg^{-1}$), the amount of GM-CSF injected onto the column was determined.

Samples of purified wild type GM-CSF and mutant analogues were analysed by HP-SEC with commercial preparations of BSA and RNase A used as size calibration standards (Fig. 5.2.14). Purified wild type GM-CSF and E21R, both 15.5kDa, eluted at 10mls which was consistent with the elution of BSA, 67kDa, at 8.9mls and RNase A, 13.7kDa, at 10.3mls (Fig. 5.2.14A). HP-SEC confirmed the purity of these protein preparations and enabled quantification of the GM-CSF preparations (Fig. 5.2.14B, C). HP-SEC quantification was highly reproducible as repeated analysis of a preparation of purified wild type GM-CSF indicated a concentration of 1.53mg/ml ± 0.025mg/ml (mean ± SEM). The SEM for these quantifications performed over several weeks was <5% indicating that the protocol was very reliable. Having determined that the method was reliable, all purified GM-CSF analogues were quantified by HP-SEC with the analysis of E21R shown as an example (Fig. 5.2.14C). Analysis of the purified residue 21 analogues by silver stained SDS/PAGE confirmed the purity of these preparations and the fact that there was equivalent amounts of protein for each analogue (Fig. 5.2.15).

Figure 5.2.14 HP-SEC analysis of purified GM-CSF

HP-SEC was used to quantify samples of purified GM-CSF using a Beckman Ultraspherogel SEC3000 (7.5x300mm) run at 0.5mls/min in 0.1M sodium sulphate, 0.1M sodium phosphate pH7.0, 0.05% (w/v) sodium azide. Samples in a total volume of 100µl were loaded and the absorbance at 280nm recorded. Integration of the area under the peak (AU.ml) and use of the calculated GM-CSF extinction coefficient (0.95 $AU.ml^{-1}.mg^{-1}$) enabled the amount of GM-CSF in the original sample to be calculated.

Panel A) The trace displays the absorbance recorded at 280nm for the HP-SEC of a sample containing 75µg BSA and 50µg of RNase A.

Panel B) The trace displays the absorbance recorded at 280nm for the HP-SEC of a sample containing 20µl of purified wild type GM-CSF. Integration of the peak indicated 2µg of GM-CSF.

Panel C) The trace displays the absorbance recorded at 280nm for the HP-SEC of a sample containing 10µl of purified E21R. Integration of the peak indicated 4.6µg of E21R.

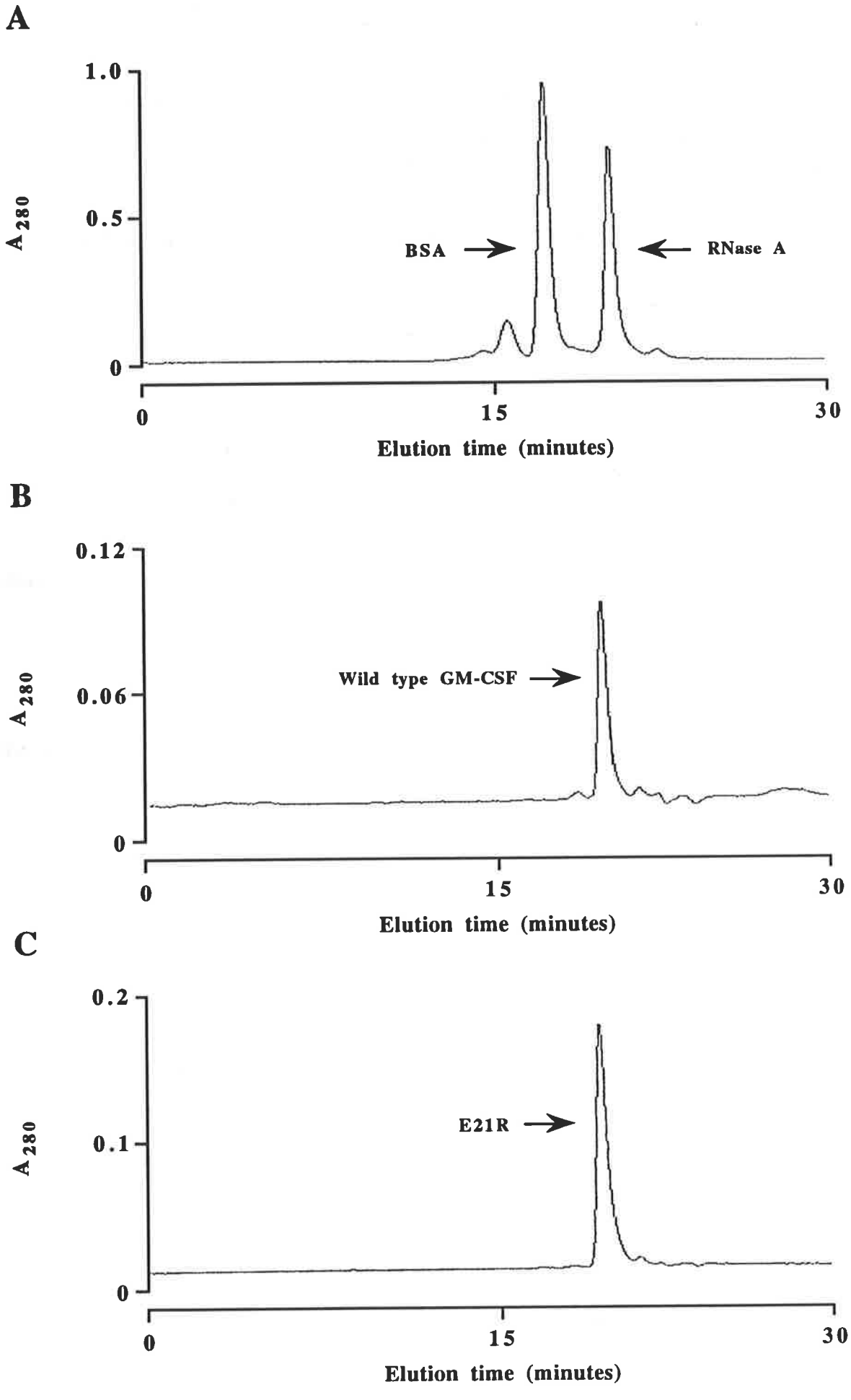
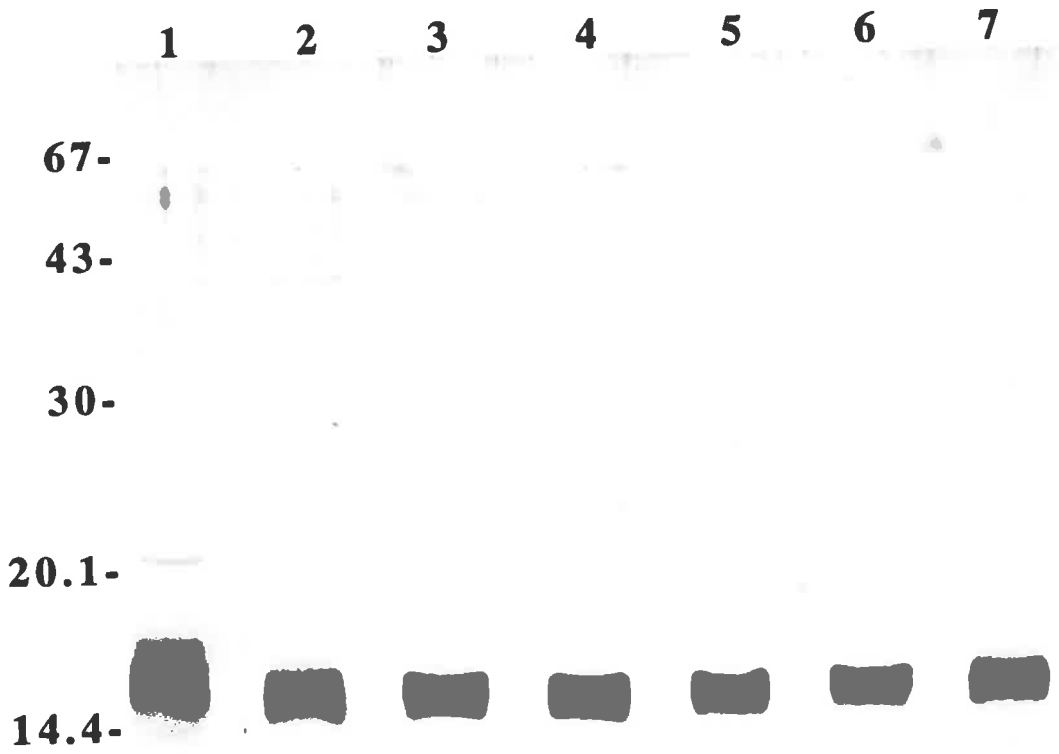

A
1.0
0.5
0
A_{280}
BSA
RNase A
0
15
30
Elution time (minutes)
B
0.12
0.06
0
A_{280}
Wild type GM-CSF
0
15
30
Elution time (minutes)
C
0.2
0.1
0
A_{280}
E21R
0
15
30
Elution time (minutes)

Figure 5.2.15 Purified GM-CSF residue 21 analogues

Human GM-CSF analogues mutated at residue 21 were expressed in BL21, purified by immunoaffinity chromatography and RP-HPLC and quantified by HP-SEC. The wild type GM-CSF was expressed in MC1061 and purified by anion exchange chromatography and RP-HPLC. Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Molecular weight standards are indicated.

1. 340ng Wild type GM-CSF
2. 400ng E21A
3. 410ng E21Q
4. 410ng E21F
5. 415ng E21H
6. 400ng E21K
7. 440ng E21R


1
2
3
4
5
6
7
67-
43-
30-
20.1-
14.4-

Having directly quantified E21R, the RIA was used to examine if there was any difference in the ability of the rabbit anti-GM-CSF polyclonal sera to recognise wild type GM-CSF and the E21R analogue (Fig. 5.2.16). The E21R was recognised by the rabbit anti-GM-CSF antibody as well as wild type GM-CSF which indicated that the RIA was able to accurately measure the concentration of this analogue and probably other analogues mutated at residue 21. Wild type GM-CSF, irreversibly reduced by incubation with DTT and blocked with iodoacetamide, was unable to compete for the binding of ^{125}I-GM-CSF to the rabbit anti-GM-CSF antibody which indicated that the recognition of GM-CSF was probably conformation dependent. The intramolecular disulphide bridges are known to be essential for GM-CSF activity as was discussed in Chapter 3 (Wingfield *et al.*, 1988).

5.3 Discussion

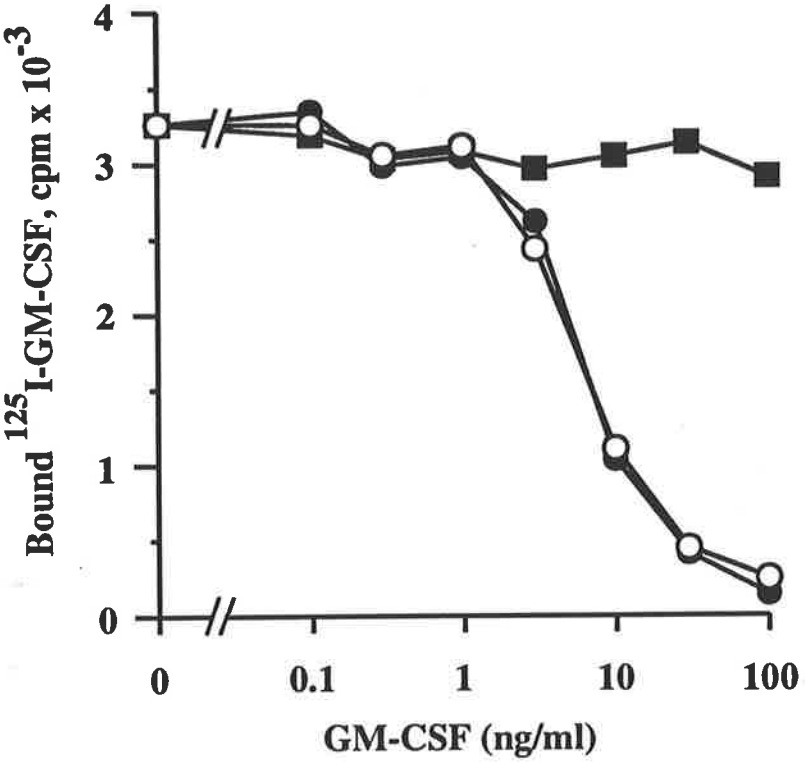
The work presented in this chapter describes the protocols developed for the production of wild type GM-CSF and mutant analogues of GM-CSF in an *E. coli* secretion expression system followed by purification and quantification of the GM-CSF protein.

Secretion expression systems based on the pIN-III-OmpA vectors (Ghrayeb *et al.*, 1984) have been used to express biologically active human GM-CSF (Libby *et al.*, 1987; Greenberg *et al.*, 1988). In both cases correctly processed human GM-CSF was found to accumulate in the periplasmic space of *E. coli*. Unfortunately although the GM-CSF was secreted into the periplasm it could not be released by osmotic shock and was in fact associated with outer membrane components as an insoluble complex. Correctly processed GM-CSF could be recovered from lysed cells by solubilisation with 8M urea (Libby *et al.*, 1987). However using the plasmid pshGM-CSF approximately 3

Figure 5.2.16 Rabbit anti-GM-CSF polyclonal antibody shows equal response to wild type GM-CSF and E21R but does not recognise chemically reduced GM-CSF

A radioimmunoassay using the standard rabbit anti-GM-CSF polyclonal antisera was performed using titrations of wild type GM-CSF (●), E21R (O) and reduced and blocked wild type GM-CSF (■) quantified by HP-SEC. Duplicate titrations of GM-CSF competitor were mixed with rabbit anti-GM-CSF polyclonal antibody (final 1:4,000) and ^{125}I-GM-CSF (0.1ng/tube) and incubated at 4°C overnight. Rabbit antibody was then precipitated using anti-rabbit immunobeads and counted.

The chemically reduced and blocked GM-CSF was prepared as described below. Wild type GM-CSF, purified by anion exchange chromatography and RP-HPLC and resuspended in PBS, was incubated with a 100-fold molar excess of reducing agent (DTT) at 25°C for 30'. A 10-fold molar excess (with respect to DTT) of the blocking agent, fresh iodoacetamide, was added and the incubation continued for 5'. Mixture was then adjusted to 1% (v/v) acetic acid and 0.1% (v/v) TFA, filtered and loaded onto a Brownlee Aquapore Octyl reversed phase HPLC column (10x100mm). Protein was eluted at 5mls/min using a 30-50% gradient of acetonitrile containing 0.1% (v/v) TFA and fractions analysed by silver stained SDS/PAGE. Fractions containing GM-CSF were pooled, lyophilised and resuspended to 130µg/ml in PBS containing a final concentration of 800mM urea, 0.1% (w/v) sodium azide and 0.1% (v/v) Tween 20. The reduced and blocked GM-CSF was quantified by HP-SEC.

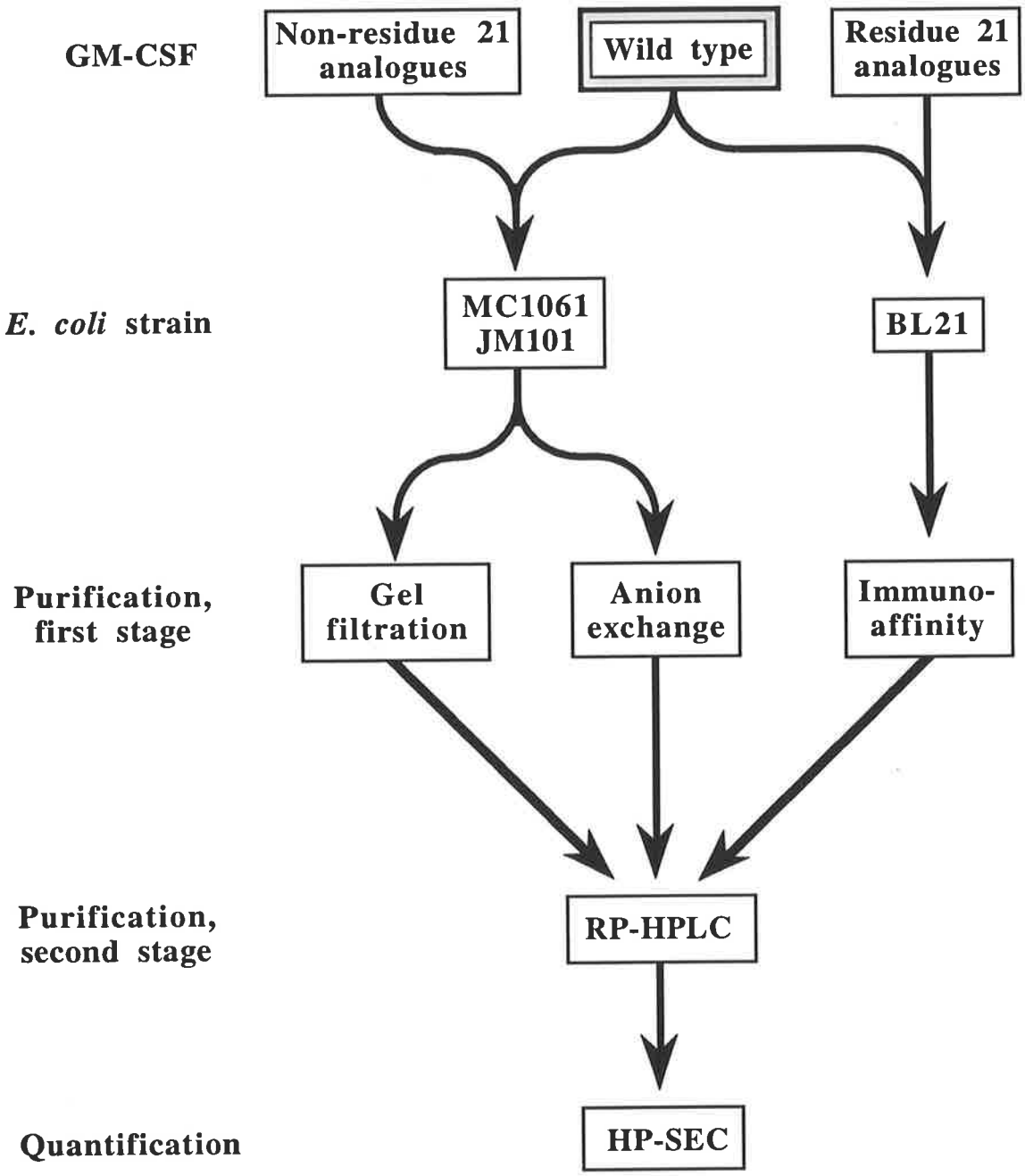

Bound ^{125}I-GM-CSF, cpm x 10^{-3}
4
3
2
1
0
0
0.1
1
10
100
GM-CSF (ng/ml)

milligram of soluble GM-CSF per litre of culture was obtained using a standard osmotic shock protocol (Koshland and Botstein, 1980). Libby *et al.* (1987) reported a yield of 20 milligram of secreted, insoluble GM-CSF per litre of culture so it is quite possible that after osmotic shock releases soluble GM-CSF some 90% of the expressed GM-CSF remains as an insoluble complex with outer membrane components. Cell pellets were not examined for the presence of insoluble GM-CSF as the aim was to generate soluble GM-CSF. Despite the potential loss of insoluble GM-CSF, a yield of 3 milligrams soluble GM-CSF per litre of culture was quite useable. Wild type GM-CSF was purified using either gel filtration chromatography or anion exchange chromatography as the first purification step, followed by reversed phase HPLC with the final product judged >95% pure by silver stained SDS/PAGE (Fig. 5.3.1). Quantification of the purified wild type GM-CSF by HP-SEC indicated a yield of 500μg per litre of culture which represented a recovery of approximately 17%. Amino terminal sequence analysis of the purified GM-CSF indicated that the OmpA signal peptide was appropriately cleaved to leave GM-CSF with the correct amino terminus.

The E21R analogue was of particular interest (see Chapter 3) and so, was the first residue 21 analogue to be expressed in *E. coli*. Major problems were encountered with very low levels of E21R expression and then difficulties with the purification protocol had to be negotiated. During the course of this work, 12 strains of *E. coli* were screened for the ability to express E21R with only one, BL21, expressing any E21R. The highest level of E21R expression amounted to just 1.0% of the expression obtained with wild type GM-CSF. All the residue 21 substitutions examined expressed poorly in BL21 relative to wild type GM-CSF but the more conservative substitutions such as Ala or Gln expressed considerably better than the charge reversal substitutions Arg and Lys. Purification of GM-CSF from BL21 was complicated by the presence of a 41 kDa contaminant that co-purified with GM-CSF at most stages of the conventional purification protocol. Eventually the problems associated with this contaminant were overcome by developing an immunoaffinity purification protocol used to purify all the residue 21 analogues (Fig. 5.3.1).

Figure 5.3.1 Protocols for the purification of secreted, soluble human GM-CSF

Human GM-CSF expressed as a soluble product in the periplasm *E. coli* was purified by either conventional chromatography or affinity chromatography depending on the strain used for expression. The purification was completed by RP-HPLC and the >95% pure GM-CSF quantified by HP-SEC.


GM-CSF
Non-residue 21 analogues
Wild type
Residue 21 analogues
E. coli strain
MC1061 JM101
BL21
Purification, first stage
Gel filtration
Anion exchange
Immuno-affinity
Purification, second stage
RP-HPLC
Quantification
HP-SEC

A key question that remains unanswered is why do residue 21 substitutions in general and charge reversal mutations in particular, reduce the level of GM-CSF expressed. One possible explanation for the low level of E21R expression was that E21R was being proteolysed within the *E. coli*. Multiple strains of *E. coli* were examined for E21R expression with a particular emphasis on strains exhibiting some deficiency in protease activity. The strains examined exhibited deficiencies in intracellular protease activity (*lon*), periplasmic protease activity (*degP*) or outer membrane protease activity (*ompT*). The *ompT* strain, BL21, was able to express the E21R analogue but with a relative yield of only 1% compared with wild type GM-CSF. The fact that none of the protease deficiencies were able to support wild type levels of E21R expression suggested that the problem was not attributable, at least directly, to bacterial protease activity. Alternatively the protease activity associated with low level E21R expression remained active in all the strains of *E. coli* that were examined.

A second and potentially related explanation for the low level of E21R expression was that the mutation was structurally unfavourable and interfered with protein folding. The Glu^{21} residue is conserved in all species of GM-CSF examined (Fig. 1.2.2) and a structurally conserved acidic amino acid is found in the first α-helix of most of the four helix cytokines (Shanafelt *et al.*, 1991b). The fact that this motif appears to play a critical role in the activity of GM-CSF (Shanafelt *et al.*, 1991b; Lopez *et al.*, 1992c), IL-2 (Zurawski and Zurawski, 1989) and IL-3 (Barry *et al.*, 1994) does not preclude it from also having a structural role. Molecular modelling studies suggested that a number of different amino acids may be accommodated at position 21 without generating any destabilising steric clashes although a cluster of potentially destabilising positive charges may result from the introduction of a positive charge at this position (Dr C.Bagley personal communication). Additionally, the analogues E21A and E21R were efficiently expressed using the CHO cell expression system (Table 3.2.3). Despite these observations, the serendipitous mutation T10I enhanced the expression of the E21R analogue. Expression of the double mutant T10I/E21R was about ten-fold higher than the single mutant, E21R. The enhanced expression brought about by a compensatory

mutation at a different location, suggested that there might be a structural basis for the low expression of the E21R analogue. The properties of this analogue will be discussed further in Chapter 6 but so far the only observable effect of the T10I mutation appears to be on the expression level of the E21R analogue.

The observation that secreted GM-CSF can become associated in an insoluble complex with membrane components (Libby *et al.*, 1987; Greenberg *et al.*, 1988) suggested another explanation. Mutation of residue 21 may enhance the association of GM-CSF with membrane components forming an insoluble aggregate with a concomitant loss of soluble GM-CSF from the periplasm. The effectiveness of the *E. coli* B strain, BL21, in the expression of E21R may be the result of a weaker association between E21R and outer membrane components leading to a higher proportion of soluble E21R in the periplasm.

An alternative explanation, not examined during the course of this work, is that the mutations at residue 21 disrupt the transcriptional or translational machinery thereby lowering expression of the various analogues. This explanation does not seem likely given that the single amino acid substitutions involve a residue 41 amino acids downstream of the initiating methionine.

This chapter has described the bacterial expression and purification of wild type GM-CSF and a number of GM-CSF analogues mutated at residue 21. Whilst expression of high yields of the residue 21 analogues was not as successful as wild type, sufficient purified material was obtained and accurately quantified to enable detailed analysis of the properties of these analogues. Chapter 6 will describe the analysis of the biological activity and receptor binding properties of the GM-CSF residue 21 analogues.

Chapter 6

The biological activities and receptor binding properties of *E. coli*-derived GM-CSF residue 21 analogues: Identification of GM-CSF antagonists

6.1 Introduction

The results presented in Chapters 3 and 4 showed that the Glu^{21} residue in the first α-helix of GM-CSF plays a crucial role in biological activity. Alignment of the predicted amino acid sequence of GM-CSF from seven different species illustrated the absolute conservation of the Glu^{21} residue (Fig. 1.2.2). Acidic residues structurally equivalent to the Glu^{21} of human GM-CSF are also present in many of the four α-helix cytokines (Shanafelt *et al.*, 1991b). With at least two of these cytokines, murine IL-2 (Zurawski and Zurawski, 1989) and human IL-3 (Barry *et al.*, 1994), considerable functional significance is attached to the acidic nature of this residue. This evidence suggests that the conserved acidic residue in the four α-helix cytokines may display a conserved functional role in the interaction of ligand and receptor, perhaps through the formation of a salt bridge.

An alternative explanation, perhaps equally consistent with the highly conserved nature of the residue, is that the conserved acidic residue plays an important structural role in the four α-helix cytokines. Mutation of this residue may perturb or destabilise the tertiary structure leading to an impaired interaction with receptor and concomitant loss of function. The alternative explanations for the function of the conserved acidic residue are not mutually exclusive as this residue may serve both structural and functional roles. The results presented in Chapter 5 showed that mutation of Glu^{21} strongly influenced the level of GM-CSF expression using an *E. coli* secretion system. The fact that protease deficient strains of *E. coli* were only partially able to enhance the expression of residue 21 mutants and that a second mutation at residue 10 was also able to enhance expression of residue 21 mutants, suggested that Glu^{21} may be a structurally important residue. Interestingly, mutation of Glu^{21} did not appear to influence GM-CSF expression using the COS or CHO cell expression systems described in Chapter 3.

The aim of the work described in this chapter was to analyse the biological and receptor binding properties of human GM-CSF residue 21 analogues generated using an *E. coli* secretion expression system, as described in Chapter 5. This also enabled the influence of carbohydrate on GM-CSF activity to be examined in the context of GM-CSF analogues with mutations at residue 21. In preliminary experiments designed to determine a possible structural role for Glu^{21}, the influence of residue 21 mutations on the stability of GM-CSF was examined.

6.2 Results

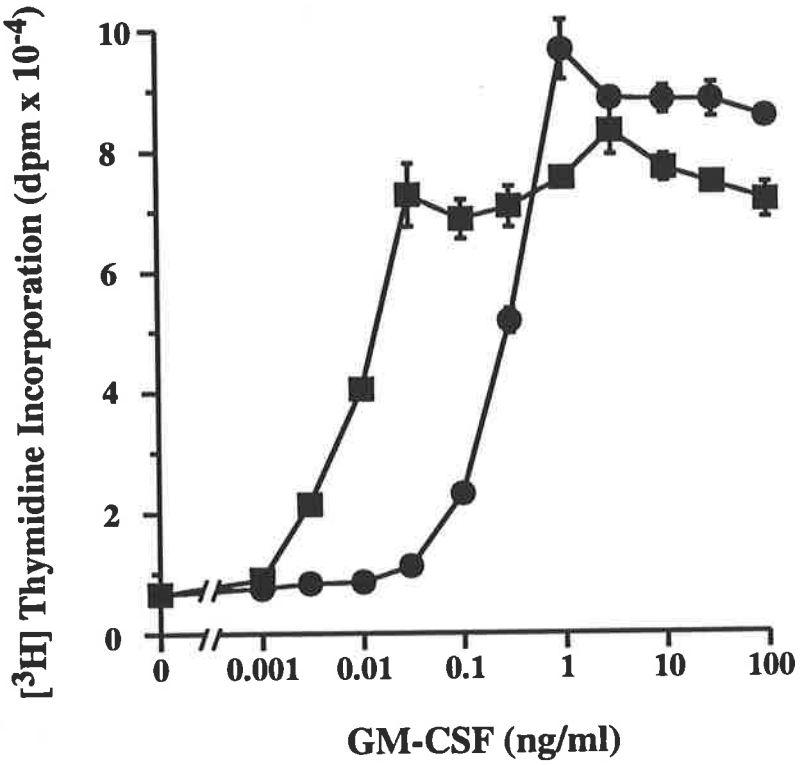
6.2.1 Activity of *E. coli*-derived wild type GM-CSF

Wild type GM-CSF expressed in *E. coli* (eco GM-CSF) was compared with wild type GM-CSF expressed in CHO cells (cho GM-CSF) for the ability to stimulate the proliferation of TF-1 cells. The TF-1 cell line is a stable, erythroleukaemia cell line, dependent upon GM-CSF, IL-3 or erythropoietin for proliferation (Kitamura *et al.*, 1989). GM-CSF from either source was able to stimulate the dose-dependent proliferation of TF-1 cells with ED_{50} values of 0.01ng/ml for the *E. coli*-derived material and 0.3ng/ml for the CHO cell-derived material (Figure 6.2.1). Thus eco GM-CSF is 30-fold more active than cho GM-CSF as previously reported (Moonen *et al.*, 1987). This indicated that GM-CSF expressed by the pIN-III-Omp secretion system in *E. coli* is a fully active form of non-glycosylated GM-CSF.

The analogue designed to facilitate high specific activity labelling with ^{125}I, P6YY, exhibited wild type biological activity in TF-1 proliferation assays (data not shown). As the substitution at residue 6 did not interfere with biological activity, it was considered unlikely that the substitution would influence receptor binding. Importantly, under identical labelling conditions with ^{125}I, the P6YY mutant analogue was consistently labelled to a higher specific activity than wild type GM-CSF, S.A. = 61μCi/μg and 13.5

Figure 6.2.1 Comparison of the biological activity of eco GM-CSF and cho GM-CSF.

Eco GM-CSF (■) and cho GM-CSF (●) were titrated for their ability to stimulate [^{3}H]thymidine incorporation into TF-1 cells as described (2.10.16). Each value represents the mean of triplicate determinations and error bars represent the SEM.


[3H] Thymidine Incorporation (dpm x 10-4)
10
8
6
4
2
0
0
0.001
0.01
0.1
1
10
100
GM-CSF (ng/ml)

μCi/μg, respectively (Dr J.Woodcock, personal communication). On the basis of wild type biological activity and high specific activity labelling, ^{125}I-GM P6YY was used routinely in the receptor binding assays and radioimmunoassays described in this chapter.

6.2.2 Comparison of the activity of eco GM-CSF and eco E21R

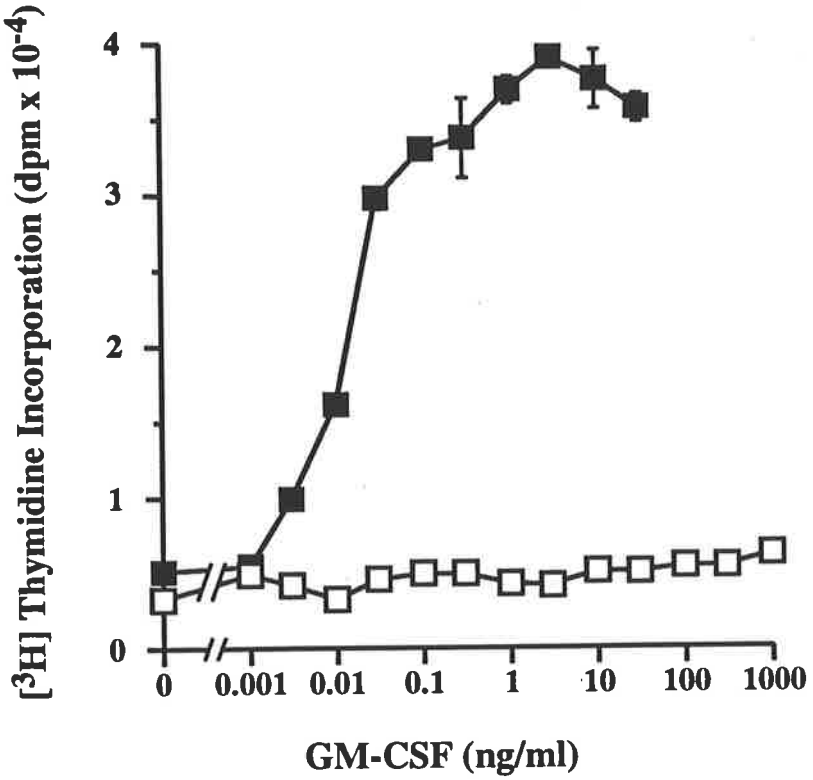
The *E. coli*-derived wild type GM-CSF and E21R analogue were then compared for their ability to stimulate the proliferation of TF-1 cells (Fig. 6.2.2A) or to stimulate the production of superoxide anions by human neutrophils (Fig. 6.2.2B). Quite surprisingly the E21R analogue was devoid of activity in both assays. Wild type GM-CSF stimulated the dose-dependent proliferation of TF-1 cells with an ED_{50} of 0.02ng/ml whereas a 50,000-fold higher concentration of E21R (1,000 ng/ml) was completely ineffective. Similarly, wild type GM-CSF stimulated the dose-dependent release of superoxide anions from neutrophils with an ED_{50} of 0.07ng/ml whereas a 14,000-fold higher level of E21R (1,000ng/ml) was completely ineffective. No activity was detected for eco E21R purified by either conventional chromatographic techniques or by immunoaffinity chromatography (data not shown).

The absence of activity for the *E. coli*-derived E21R analogue (eco E21R) was in contrast to the activity exhibited by the CHO cell-derived E21R analogue (cho E21R) which was able to stimulate the proliferation of CML cells (Fig. 3.3.5), the release of superoxide anions from neutrophils (Fig. 3.3.6A) and the adherence of monocytes (Fig. 3.3.6B) (Lopez *et al.*, 1992c). In all of the activities examined, cho E21R was approximately 300-fold less potent than cho GM-CSF. Why does the eco E21R analogue exhibit a complete loss of biological activity when the cho E21R analogue exhibited reduced, but readily detectable activity?

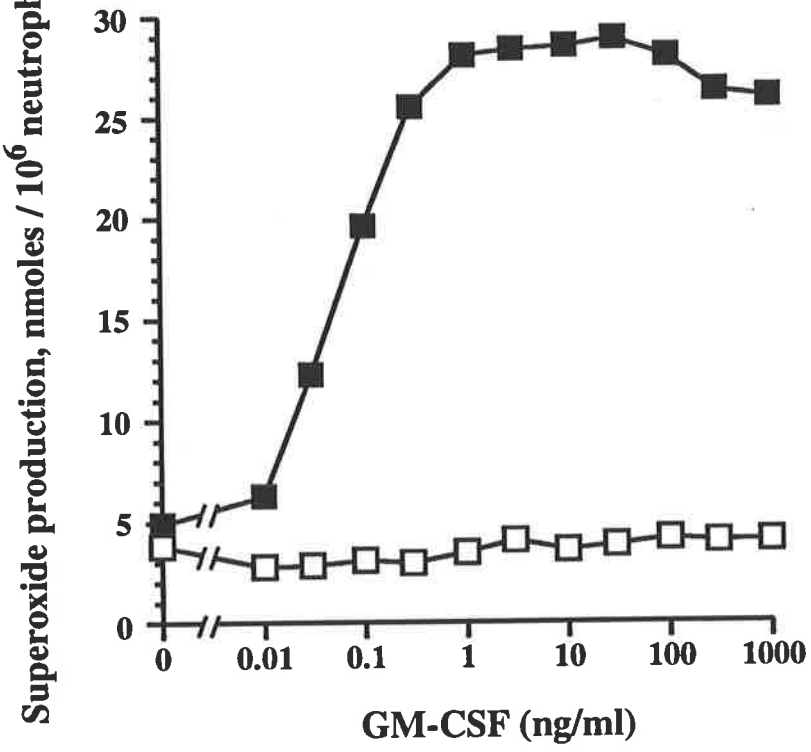
Figure 6.2.2 Comparison of the biological activity of eco GM-CSF and eco E21R.

Eco GM-CSF (■) and eco E21R (□) were titrated for their ability to stimulate [^{3}H]thymidine incorporation into TF-1 cells (Panel A) or to stimulate the production of superoxide anions from human neutrophils as described (2.10.16) (Panel B). Each value represents the mean of triplicate determinations and error bars represent the SEM.

A


[3H] Thymidine Incorporation (dpm x 10-4)
4
3
2
1
0
0
0.001
0.01
0.1
1
10
100
1000
GM-CSF (ng/ml)

B


Superoxide production, nmoles / 10^6 neutrophils
30
25
20
15
10
5
0
0
0.01
0.1
1
10
100
1000
GM-CSF (ng/ml)

6.2.3 Receptor binding characteristics of eco E21R

A. Binding of eco E21R to the high (GMRαβc) and low (GMRα) affinity receptor

One immediate explanation for the lack of activity exhibited by eco E21R was that it was unable to interact with the GM-CSF receptor. Therefore binding of eco E21R to the high affinity receptor (GMRαβc) of human neutrophils (Gasson *et al.*, 1986; Park *et al.*, 1986) or the low affinity receptor (GMRα) of the A9/C7 cell line (Hercus *et al.*, 1994b) was analysed. Competitive binding studies on human neutrophils expressing the GMRαβc complex indicated that eco GM-CSF and eco E21R were able to bind with affinities of 7pM and 770pM respectively (Fig. 6.2.3A). Competitive binding studies on the A9/C7 cell line expressing the GMRα chain indicated that eco GM-CSF and eco E21R were able to bind with affinities of 2,700pM and 1,100pM respectively (Fig. 6.2.3B). Thus the eco E21R exhibited a reduced affinity for the GMRαβc complex on human neutrophils but a slightly enhanced affinity for the cloned GMRα chain.

B. Comparative binding of eco E21R and cho E21R to the high (GMRαβc) and low (GMRα) affinity receptor

Eco E21R and cho E21R were compared in parallel for the ability to bind the high affinity (GMRαβc) or low affinity (GMRα) GM-CSF receptor. The competitive receptor binding curves are shown in Figure 6.2.4 and the derived K_d values in Table 6.2.1. Using human neutrophils both eco E21R (Fig. 6.2.4A) and cho E21R (Fig. 6.2.4B) exhibit an 80- to 100-fold reduction in affinity compared with their respective wild type proteins (Table 6.2.1). In contrast using the CHO cell line A9/C7, both eco E21R (Fig. 6.2.4C) and cho E21R (Fig. 6.2.4D) exhibited affinities similar to the corresponding wild type protein (Table 6.2.1).

These results indicated that eco E21R, like cho E21R, was able to recognise the GMRα chain with wild type affinity but that the interaction with GMRαβc appeared to be reduced.

Figure 6.2.3 Comparison of the binding of eco GM-CSF and eco E21R to human neutrophils and the A9/C7 CHO cell line.

The binding of eco GM-CSF (■) and eco E21R (□) to cells expressing the high (GMR$\alpha\beta_c$, Panel A) or low (GMRα, Panel B) affinity GM-CSF receptors was compared. Binding experiments were performed as described 2.10.17. Unlabelled eco GM-CSF or eco E21R was titrated against 100pM ^{125}I-GM-CSF and 3 x 10^6 neutrophils per tube (A) or 450pM ^{125}I-GM-CSF and 5.5 x 10^5 A9/C7 cells per tube (B). The values are expressed as a percentage of the ^{125}I-GM-CSF bound in the absence of competitor and are the means of duplicate determinations.

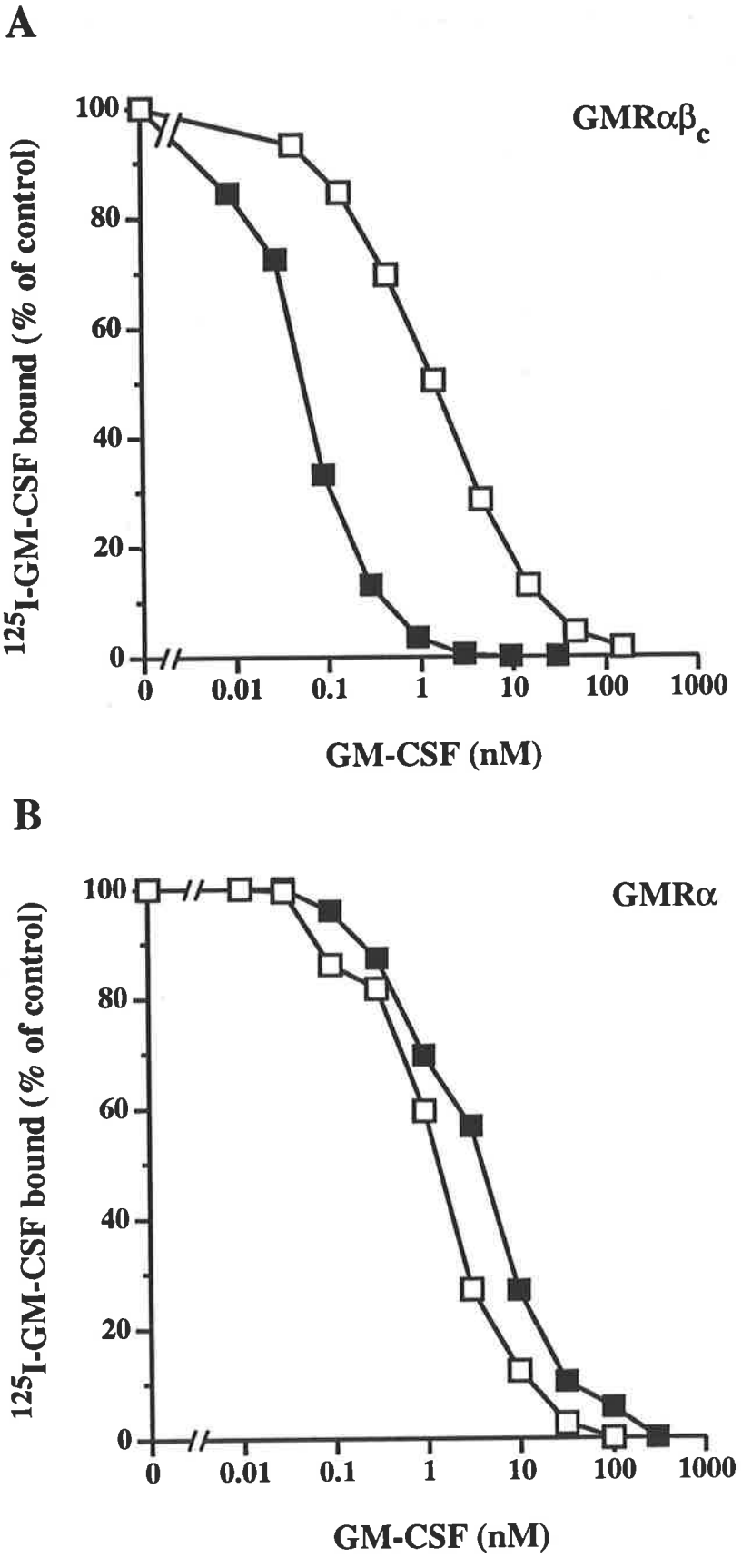

A
GMRαβc
125I-GM-CSF bound (% of control)
100
80
60
40
20
0
0
0.01
0.1
1
10
100
1000
GM-CSF (nM)
B
GMRα
125I-GM-CSF bound (% of control)
100
80
60
40
20
0
0
0.01
0.1
1
10
100
1000
GM-CSF (nM)

Figure 6.2.4 Comparative high and low affinity receptor binding properties of *E. coli*- and CHO cell-derived wild type GM-CSF and E21R.

The binding of *E. coli*- and CHO cell-derived wild type GM-CSF and E21R to cells expressing the high (GMR$\alpha\beta_c$, Panels A and B) or low (GMRα, Panels C and D) affinity GM-CSF receptors was compared. Unlabelled eco GM-CSF (■), eco E21R (□), cho GM-CSF (●) or cho E21R (○) were titrated against ^{125}I-GM-CSF derived from *E. coli* (A,C) or CHO cells (B,D) and mixed with 4 x 10^6 neutrophils (A,B) or 8.3 x 10^5 A9/C7 cells (C,D). The values are expressed as a percentage of the ^{125}I-GM-CSF bound in the absence of competitor and are the means of duplicate determinations.

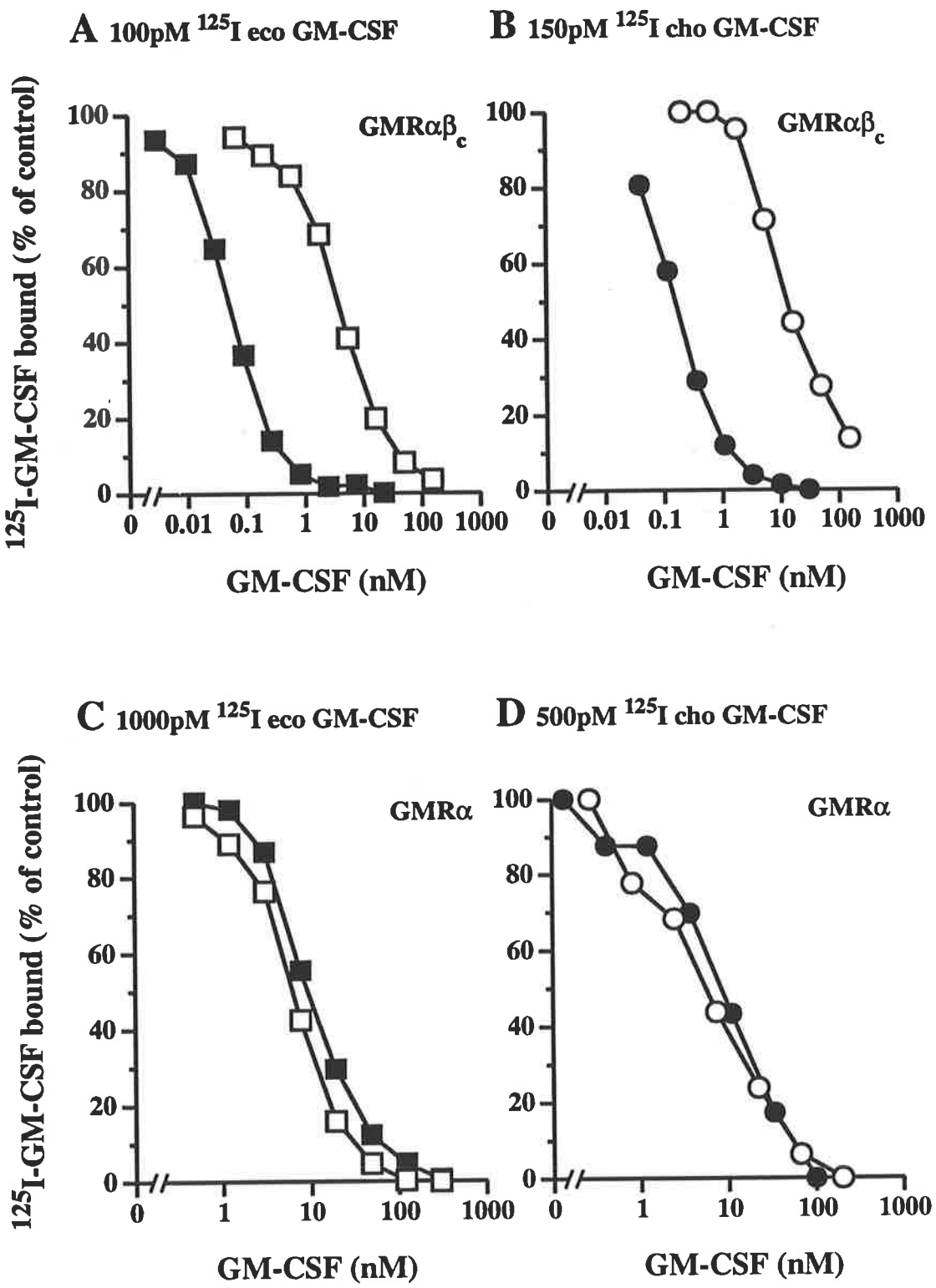

A 100pM 125I eco GM-CSF
B 150pM 125I cho GM-CSF
GMRαβc
GMRαβc
125I-GM-CSF bound (% of control)
100
80
60
40
20
0
0 0.01 0.1 1 10 100 1000
GM-CSF (nM)
C 1000pM 125I eco GM-CSF
D 500pM 125I cho GM-CSF
GMRα
GMRα
0 1 10 100 1000

Table 6.2.1 Binding affinities of *E. coli*- and CHO cell-derived wild type GM-CSF and E21R to GM-CSF receptors.

Radioligand[b]	Competitor	Receptor affinity[a] K_d ($\alpha\beta_c$)[c] (pM)	Receptor affinity[a] K_d(α)[d] (pM)
^{125}I-eco GM-CSF	eco GM-CSF	10	3,000
"	eco E21R	980	1,400
^{125}I-cho GM-CSF	cho GM-CSF	60	10,500
"	cho E21R	5,000	9,500

[a] Binding affinities from competition experiments shown in Figure 6.2.4 were determined using the EBDA/Ligand program (Munson and Rodbard, 1980).
[b] Radioligand was either iodinated, *E. coli*-derived GM P6YY produced within the Division of Human Immunology or iodinated, CHO cell-derived wild type GM-CSF from DuPont.
[c] GMR$\alpha\beta_c$ receptor from human neutrophils (Fig. 6.2.4A, B).
[d] GMRα receptor from human A9/C7 CHO cell line (Fig. 6.2.4C, D).

6.2.4 Comparison of eco E21R and cho E21R

The eco E21R and cho E21R mutant analogues were analysed to try and determine what factors may be responsible for the different biological activities of these analogues. There are two known differences between the eco E21R and cho E21R preparations. The first is a single amino acid change due to a polymorphism at residue 100 (Wong *et al.*, 1985a). The second and more obvious difference is the fact that the CHO cell-derived material is heavily glycosylated (Fig. 3.3.4).

A. The influence of a polymorphism at residue 100 on wild type GM-CSF and E21R activity

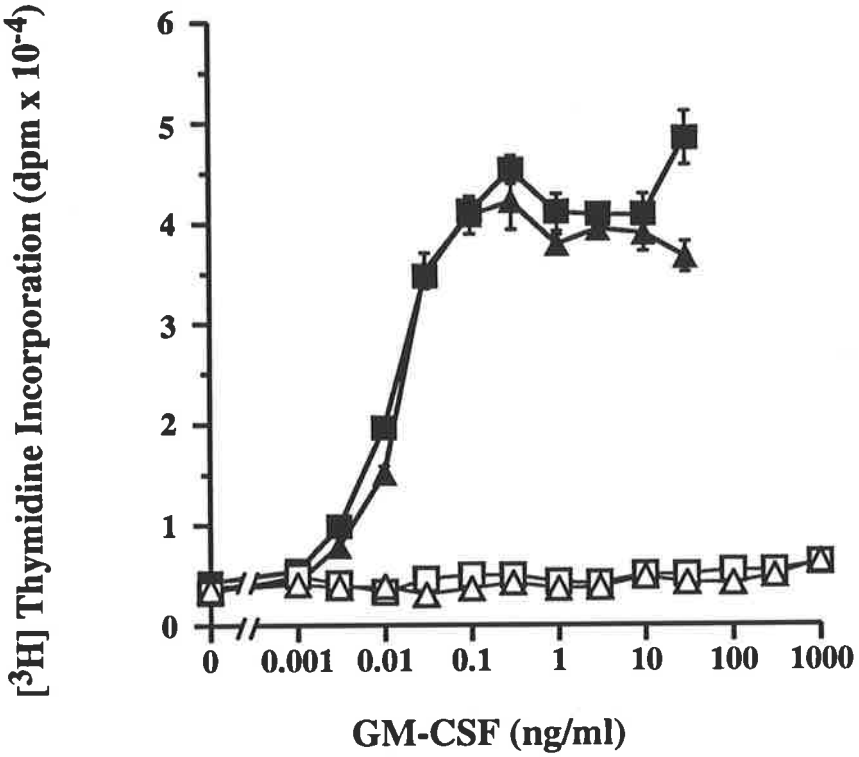
The GM-CSF cDNA clone used for CHO cell expression (GM-CSF(Thr^{100}), Wong *et al.*, 1985a) and *E. coli* expression (GM-CSF(Ile^{100}), Lee *et al.*, 1985) have a single amino acid difference due to a polymorphism at residue 100. The fact that the homologue of residue 100 in IL-3 has been implicated in receptor binding (Lopez *et al.*, 1992b) suggested that this residue might exert some influence on GM-CSF activity. Thus the Thr^{100} substitution was introduced into eco GM-CSF and eco E21R and the biological activity of the resulting analogues tested in a TF-1 proliferation assay (Fig. 6.2.5). Wild type GM-CSF carrying either Ile^{100} or Thr^{100}, stimulated the dose-dependent proliferation of TF-1 cells with an ED_{50} of 0.013ng/ml whereas a 77,000-fold higher level of E21R (1,000ng/ml) carrying either Ile^{100} or Thr^{100}, was completely ineffective. Quite clearly the I100T substitution had no detectable effect on eco GM-CSF or eco E21R activity and therefore this single amino acid difference could not account for the different biological activities of eco E21R and cho E21R.

B. The influence of glycosylation on the biological activity of the E21R analogue

GM-CSF produced in eukaryotic cells, such as COS or CHO cells, is heterogeneously modified by the addition of carbohydrate (Figures 3.3.1A, 3.3.4). The degree of GM-CSF glycosylation has been demonstrated to influence both biological activity and receptor binding with non-glycosylated GM-CSF exhibiting higher specific

Figure 6.2.5 Comparing the effect of the residue 100 polymorphism on eco GM-CSF and eco E21R biological activity.

Eco GM-CSF (■), eco I100T (▲), eco E21R (□) and eco E21R/I100T (△) were titrated for their ability to stimulate [^{3}H]thymidine incorporation into TF-1 cells. Each value represents the mean of triplicate determinations and error bars represent the SEM.


[3H] Thymidine Incorporation (dpm x 10-4)
6
5
4
3
2
1
0
0
0.001
0.01
0.1
1
10
100
1000
GM-CSF (ng/ml)

activity and higher receptor affinity than high Mr, glycosylated GM-CSF (Moonen *et al.*, 1987; Cebon *et al.*, 1990). During the purification of the CHO cell-derived GM-CSF analogues (Fig. 3.3.3), low Mr species were excluded because the CHO cell-derived wild type GM-CSF was devoid of these forms (Fig. 3.3.4). It seemed possible that some of the low Mr species of cho E21R might be devoid of activity as is the non-glycosylated eco E21R.

Cho E21R was affinity purified from the conditioned medium of the CHO cell line, 31.7, expressing the E21R analogue (Table 3.3.2). The affinity purified cho E21R was then fractionated by RP-HPLC and the eluted glycoprotein analysed by silver stained SDS/PAGE. Seven fractions of cho E21R were identified (Fig. 6.2.6A) comprising three size classes of E21R and with molecular weights ranging from 17kDa to 33kDa (Table 6.2.2). The size heterogeneity of CHO cell-derived GM-CSF has been attributed to different degrees of occupancy of the two sites for N-linked carbohydrate (Donahue *et al.*, 1986a). Fractions 1&2 contain high Mr E21R, fractions 3,4&5 contain intermediate Mr E21R while fractions 6&7 contain low Mr E21R. An eighth fraction was created by pooling equal aliquots of fractions 1 to 5 (Fig. 6.2.6A, Lane 9). This pooled fraction displayed a similar degree of molecular weight heterogeneity as the CHO cell-derived wild type GM-CSF obtained from Genetics Institute (Fig. 6.2.6A, Lane 1) and was devoid of the low Mr forms. The fractions of cho E21R were individually quantified by RIA and a total of 200μg identified in the eight fractions. Analysis of the fractions by silver stained SDS/PAGE (Fig. 6.2.6A) suggested that the quantification was probably reliable. Differences may be attributable to silver staining of the carbohydrate, which is most noticeable when comparing fractions containing high Mr GM-CSF (Fig. 6.2.6A, Lane 2) with those fractions containing low Mr GM-CSF (Fig. 6.2.6A, Lane 8).

The fractions of cho E21R were tested for biological activity in a TF-1 proliferation assay (Fig. 6.2.6B). Wild type GM-CSF and the pooled fraction of cho E21R, fraction 8, stimulated the dose-dependent proliferation of TF-1 cells with ED_{50} values of 0.15 and 130 ng/ml, respectively. This demonstrated that cho E21R is 900-fold less active

Figure 6.2.6 Fractionation of cho E21R and biological activity of the variably glycosylated species.

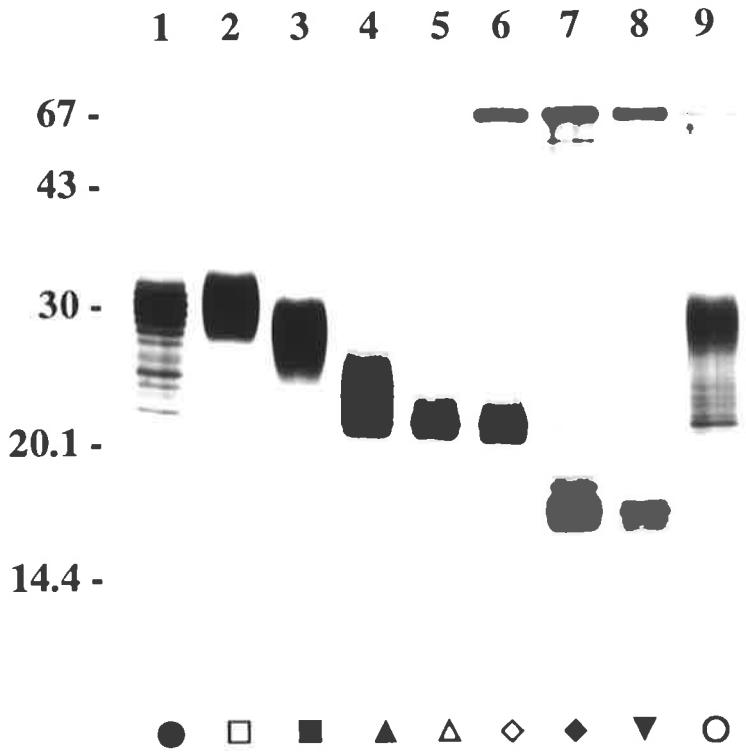
Conditioned medium (volume=4,700mls) from the CHO cell line 31.7, expressing cho E21R, was concentrated to a final volume of 270mls in a stirred-cell using a 10,000 Mr cutoff membrane. Concentrated conditioned medium was mixed with 1ml of fresh 4D4.C5 immunoaffinity matrix at 4°C overnight. Matrix was collected, washed with PAT buffer and specifically bound protein eluted with 100mM NaCl, 100mM sodium acetate, 0.02% (w/v) sodium azide, pH5.0. This process was repeated several times and the eluted protein analysed by silver stained SDS/PAGE. Eluates containing cho E21R were pooled, adjusted to 1% (v/v) acetic acid and 0.1% (v/v) TFA and loaded onto a Brownlee Aquapore RP-300 reversed phase HPLC column (4.6x100mm). Bound protein was eluted using a 30-50% gradient of acetonitrile containing 0.1% (v/v) TFA. Fractions containing cho E21R were lyophilised and resuspended in PBS. Following RIA quantification, the variably glycosylated fractions of cho E21R were analysed by silver stained SDS/PAGE (A) and the biological activities of each compared (B).

Panel A) Samples were fractionated by 15% SDS/PAGE and the gel stained with silver. Approximately 400ng of each GM-CSF sample was loaded per track. Molecular weight standards are indicated.

1. CHO cell-derived recombinant human GM-CSF
2. cho E21R, fraction 1
3. cho E21R, fraction 2
4. cho E21R, fraction 3
5. cho E21R, fraction 4
6. cho E21R, fraction 5
7. cho E21R, fraction 6
8. cho E21R, fraction 7
9. cho E21R, fraction 8 (Pool of fractions 1 to 5)

Panel B) Cho GM-CSF (●) and the cho E21R fractions 1 (□), 2 (■), 3 (▲), 4 (△), 5 (◇), 6 (◆), 7 (▼) and 8 (○) were titrated for their ability to stimulate [^{3}H]thymidine incorporation into TF-1 cells. Each value represents the mean of triplicate determinations and error bars represent the SEM.

A


1 2 3 4 5 6 7 8 9
67 -
43 -
30 -
20.1 -
14.4 -

B

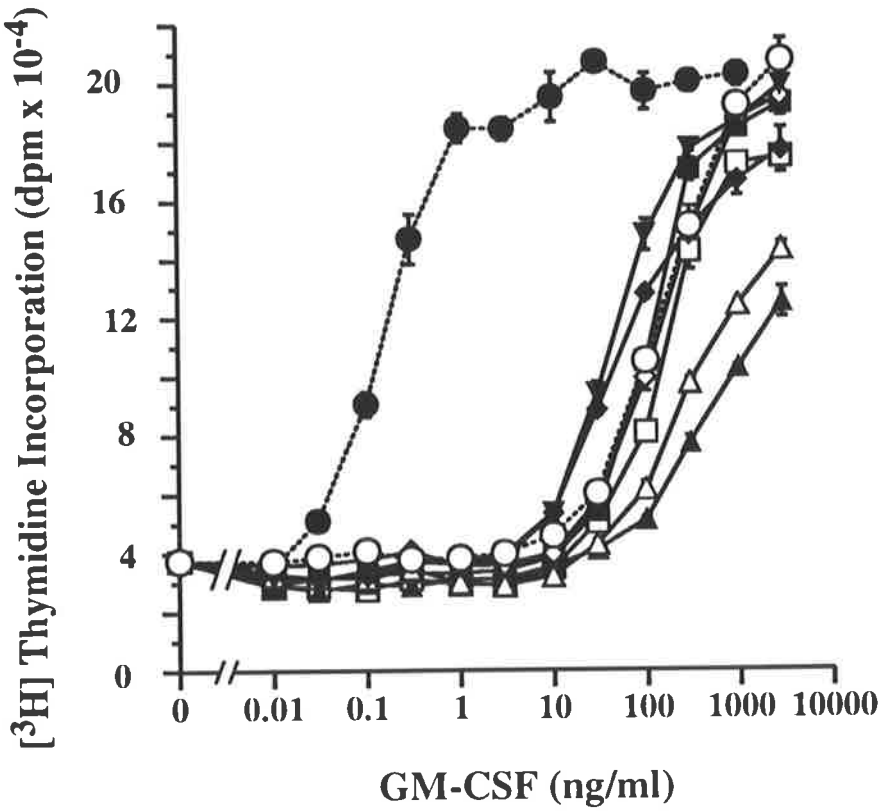

[3H] Thymidine Incorporation (dpm x 10-4)
20
16
12
8
4
0
0
0.01
0.1
1
10
100
1000
10000
GM-CSF (ng/ml)

Table 6.2.2 Molecular weight and relative activity of cho E21R fractions.

GM-CSF	Mr range[a] (kDa)	ED_{50}[b] (ng/ml)
wild type GM-CSF	23.5 - 33	0.15
cho E21R fraction 1	28-33	200
cho E21R fraction 2	26-31	120
cho E21R fraction 3	21-28	2,000
cho E21R fraction 4	21-25	700
cho E21R fraction 5	21-24	130
cho E21R fraction 6	17-23.5	70
cho E21R fraction 7	17-19	50
cho E21R fraction 8	21-33	130

[a] Molecular weight range as determined by silver stained SDS/PAGE (Fig. 6.2.6A).
[b] Concentration of GM-CSF analogue stimulating 50% of the maximal, wild type GM-CSF proliferative response, as measured by [^{3}H]thymidine incorporation by TF-1 cells (Fig. 6.2.6B).

than wild type in agreement with previous estimates of the activity of this mutant (Fig. 3.3.5). The ED_{50} values of the different fractions of cho E21R ranged from 50ng/ml for cho E21R fraction 7 up to 2,000ng/ml for cho E21R fraction 3 (Table 6.2.2). Intermediate Mr E21R, fractions 3&4, exhibited the lowest specific activity (Table 6.2.2). Most importantly the low Mr fraction of cho E21R, fraction 7, which previously had not been analysed, exhibited the highest specific activity (Fig. 6.2.6B, Table 6.2.2). The low Mr of this fraction indicated that it is likely to be devoid of N-linked carbohydrate (Donahue *et al.*, 1986a; Kaushansky *et al.*, 1987). In this respect the cho E21R behaves in the same manner as wild type GM-CSF where the N-linked carbohydrate appears to play a significant role in reducing the specific activity of glycosylated GM-CSF (Moonen *et al.*, 1987: Cebon *et al.*, 1990).

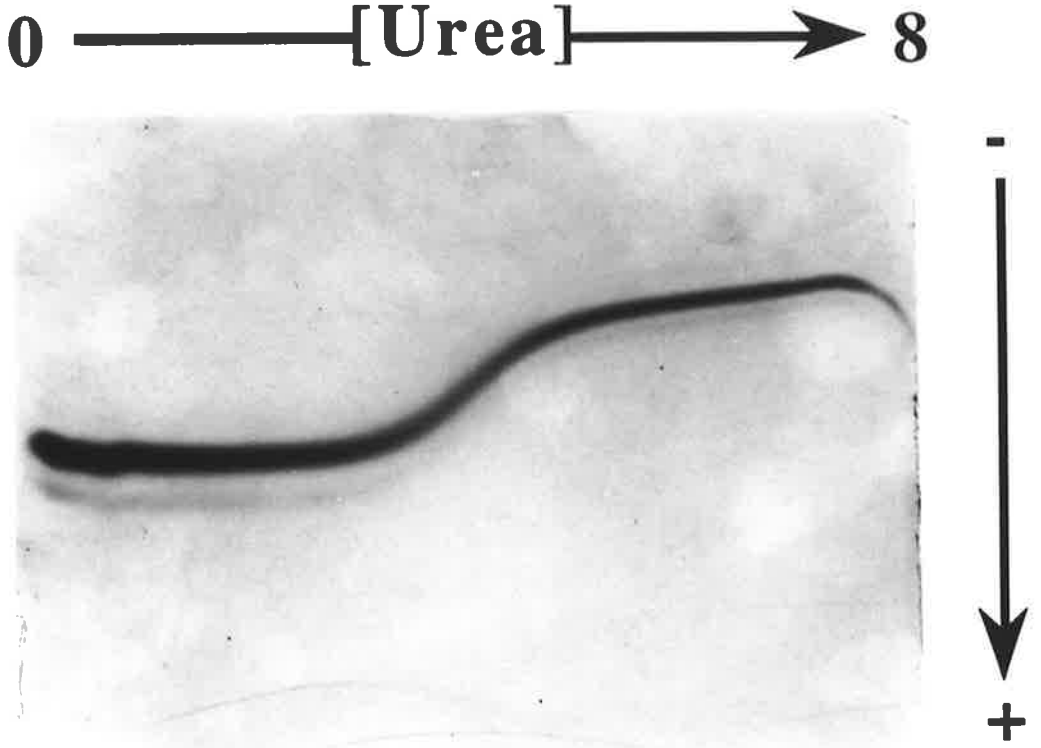
C. Stability of eco GM-CSF and eco E21R

In order to determine what effect the charge reversal has on GM-CSF folding, transverse urea gradient gel electrophoresis was used to analyse eco GM-CSF and eco E21R (Fig. 6.2.7). From these gels it was clear that both eco GM-CSF and eco E21R displayed a reduced electrophoretic mobility as the protein unfolded with increasing concentrations of urea. The smooth mobility transition from the folded to the unfolded state is indicative of a rate of unfolding and folding that is fast compared with the time of electrophoresis (Hollecker and Creighton, 1982). The midpoint of the unfolding transition occurred at about 4M urea for eco GM-CSF and 2.5M urea for eco E21R. The stability of the folded protein ($\Delta G°_{(H_20)}$) was estimated using a procedure described by Hollecker and Creighton (1982) to be -43 kJ/mol for eco GM-CSF and -21 kJ/mol for eco E21R. This observation demonstrated that the folding of eco E21R was significantly less stable than for eco GM-CSF but most importantly that the eco E21R was folded in the absence of urea. This suggested that the highly conserved Glu^{21} residue might play an important role in stabilising the folded state of human GM-CSF, presumably through some form of electrostatic interaction.

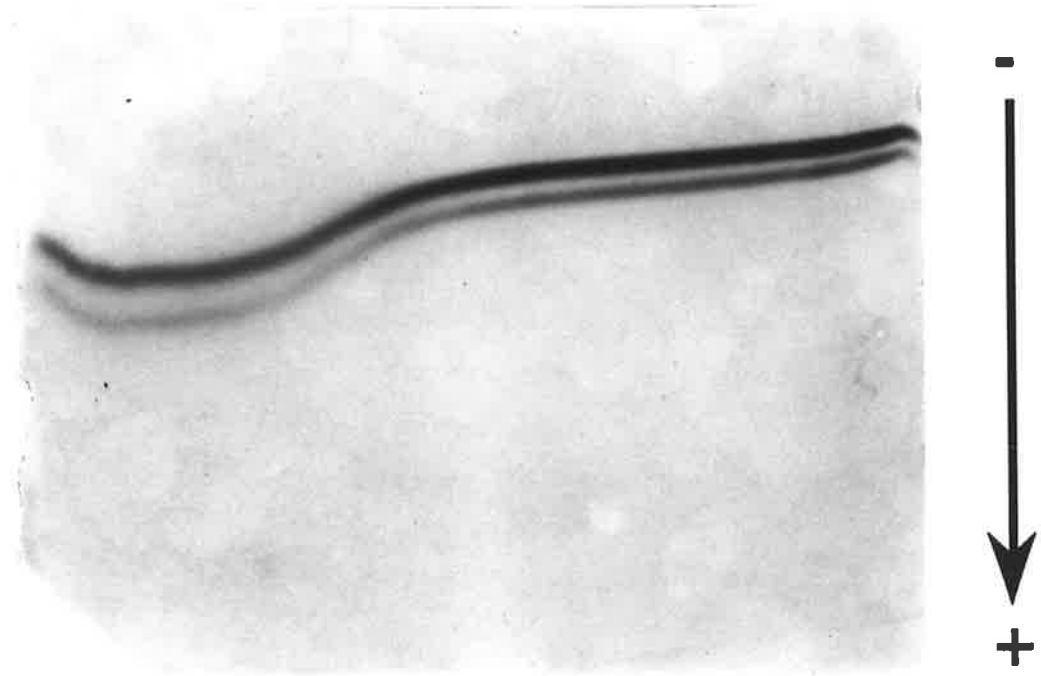
Figure 6.2.7 Comparison of the stability of eco GM-CSF and eco E21R.

Eco GM-CSF (Panel A) and eco E21R (Panel B) were subjected to transverse urea gradient PAGE as described 2.10.12. Fifteen micrograms of purified GM-CSF was fractionated on gels cast as an identical pair and stained with Coomassie blue as described 2.10.13. The direction of electrophoresis (- → +) and orientation of the urea gradient (0 → 8M) are indicated.

A



B



Two distinct bands were observed following transverse urea gradient gel electrophoresis (Fig. 6.2.7) despite the fact that the purified GM-CSF appeared homogenous by SDS/PAGE (Fig. 5.2.15). The two bands displayed essentially identical folding transitions which indicated that they probably represent two different forms of GM-CSF. The fact that two bands were routinely observed following high pH, native gel electrophoresis of purified GM-CSF (data not shown) suggested charge heterogeneity in the GM-CSF preparations.

6.2.5 Analysis of eco E21R for antagonism of GM-CSF mediated proliferation

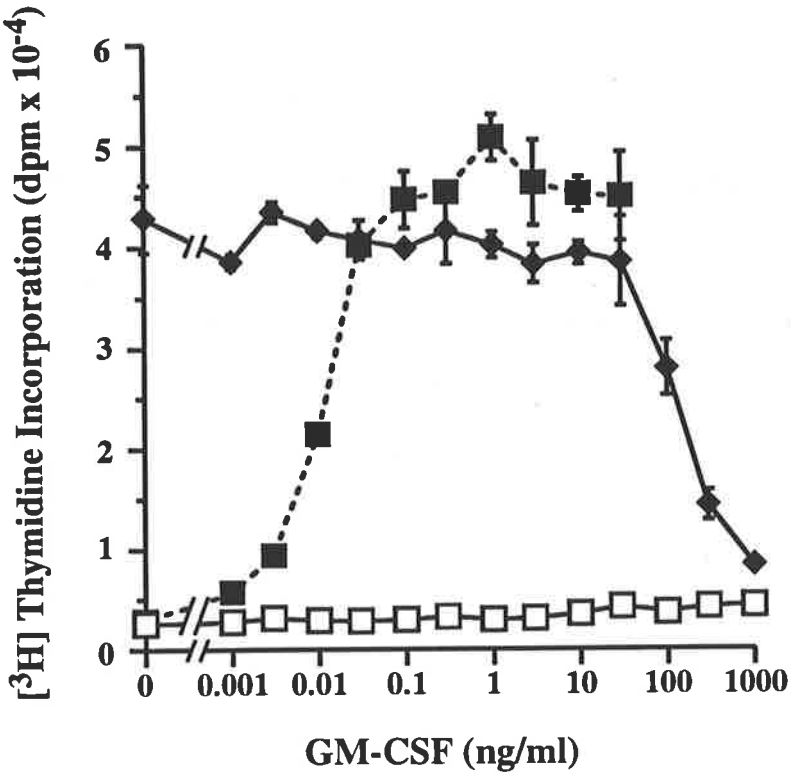
Having established that eco E21R was able to bind with wild type affinity to the GM-CSF receptor α chain (Fig. 6.2.3B and 6.2.4C) but was unable to deliver a biological signal (Fig. 6.2.2), eco E21R was tested for its ability to antagonise the activity of wild type GM-CSF. In a TF-1 cell proliferation assay, eco E21R was titrated against 0.03ng/ml eco GM-CSF, a dose of GM-CSF that stimulates near-maximal TF-1 cell proliferation. Eco E21R completely antagonised the proliferative activity of wild type GM-CSF (Fig. 6.2.8A) and required 200ng/ml to reach 50% inhibition (IC_{50}). To determine the specificity of the antagonism, eco E21R was titrated against 0.3ng/ml IL-3, a dose of IL-3 that stimulates near-maximal TF-1 cell proliferation. There was no detectable antagonism of IL-3 mediated TF-1 cell proliferation (Fig. 6.2.8B).

The ability of eco E21R to antagonise the GM-CSF mediated proliferation of fresh leukaemic cells from patients with AML were examined. Three AML's were selected that exhibited a strong proliferative response to exogenously added GM-CSF. Eco E21R was titrated against 0.3ng/ml of eco GM-CSF. The GM-CSF mediated proliferation of AML 1 was completely antagonised by eco E21R with an IC_{50} of 500ng/ml (Fig. 6.2.9A). All three AML's were antagonised by eco E21R with IC_{50} values of between 300 and 900ng/ml compared with 200ng/ml for TF-1 cells (Fig. 6.2.9B).

Figure 6.2.8 Eco E21R antagonises GM-CSF but not IL-3 mediated proliferation of TF-1 cells.

Eco GM-CSF (■), eco E21R (□) and IL-3 (▲) were titrated for their ability to stimulate [^{3}H]thymidine incorporation into TF-1 cells. In antagonistic experiments, eco E21R (◆) was titrated against 0.03ng/ml eco GM-CSF (Panel A) or 0.3ng/ml IL-3 (Panel B). Each value represents the mean of triplicate determinations and error bars represent the SEM.

A


[3H] Thymidine Incorporation (dpm x 10-4)
6
5
4
3
2
1
0
0
0.001
0.01
0.1
1
10
100
1000
GM-CSF (ng/ml)

B

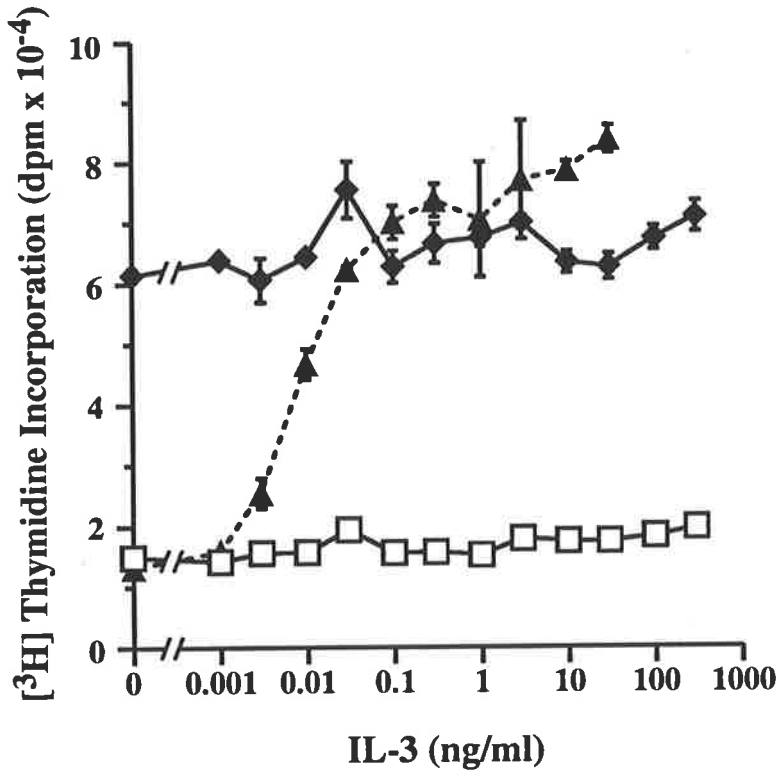
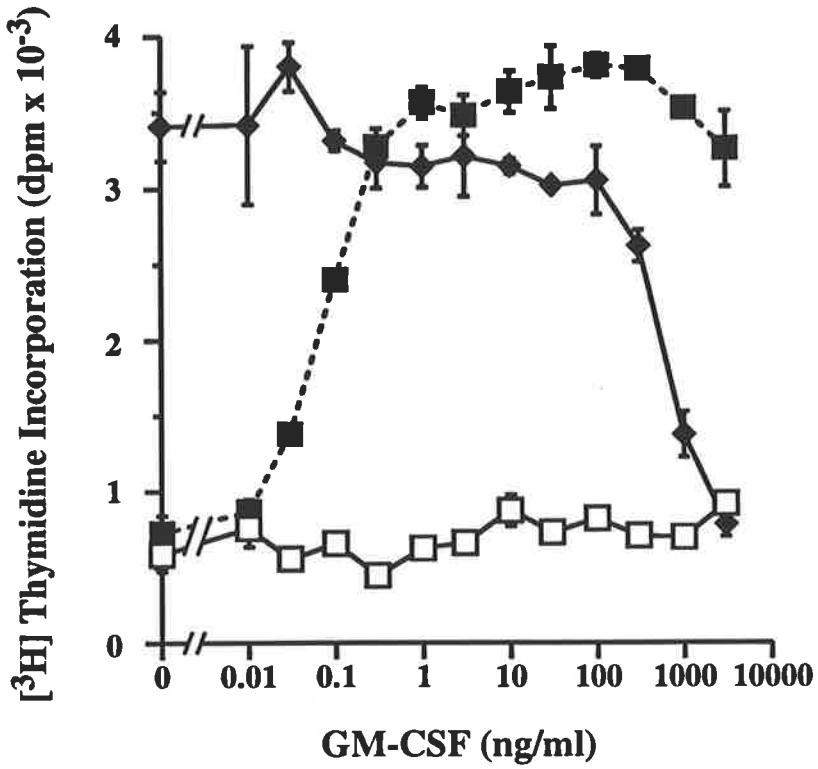

[3H] Thymidine Incorporation (dpm x 10-4)
10
8
6
4
2
0
0
0.001
0.01
0.1
1
10
100
1000
IL-3 (ng/ml)

Figure 6.2.9 Eco E21R antagonises GM-CSF mediated proliferation of AML cells.

Primary AML cells were selected for their ability to incorporate [^{3}H]thymidine in response to GM-CSF. Panel A shows the incorporation of [^{3}H]thymidine by AML #1 in response to eco GM-CSF (■) and eco E21R (□). In antagonistic experiments, eco E21R (◆) was titrated against 0.3ng/ml eco GM-CSF. Each value represents the mean of triplicate determinations and error bars represent the SEM.

In panel B the responsiveness to eco GM-CSF and sensitivity to eco E21R antagonism of 3 AML isolates and the TF-1 cell line are compared.

A



B

Cell	[3H]Thymidine incorporation[a]	ED_{50} GM-CSF[b]	IC_{50} E21R[c]
AML #1	2,900	0.07	500
AML #2	29,000	0.15	300
AML #3	6,400	0.02	900
TF-1	44,000	0.01	200

[a] Maximum GM-CSF stimulated [3H]thymidine incorporation, in dpm.

[b] Concentration of GM-CSF, in ng/ml, stimulating 50% of the maximal proliferative response.

[c] Concentration of E21R, in ng/ml, producing 50% inhibition of cell proliferation stimulated by 0.3ng/ml GM-CSF.

GM-CSF isolated from *in vivo* sources is variably but quite extensively glycosylated (Lusis *et al.*, 1981; Donahue *et al.*, 1986a: Cebon *et al.*, 1990). The biological activity and receptor binding properties of GM-CSF are influenced by the degree of glycosylation (Cebon *et al.*, 1990) so the ability of eco E21R to antagonise the activity of glycosylated GM-CSF produced in yeast or CHO cells was assessed. Eco E21R was titrated against concentrations of the different forms of wild type GM-CSF that stimulate near-maximal TF-1 cell proliferation. Eco E21R antagonised the proliferative activity of all forms of wild type GM-CSF (Fig. 6.2.10) with IC_{50} values of eco GM-CSF, 600ng/ml, yeast GM-CSF, 700ng/ml, and cho GM-CSF, 90ng/ml. The yeast GM-CSF used in this assay, although partially glycosylated, appeared to display the same level of biological activity as the eco GM-CSF (Fig. 6.2.10A and B). This is consistent with the fact that this preparation of yeast GM-CSF lacked N-linked carbohydrate (Cosman *et al.*, 1988) and therefore would be expected to exhibit higher specific activity than GM-CSF modified by the addition N-linked carbohydrate (Moonen *et al.*, 1987; Cebon *et al.*, 1990). Interestingly, the IC_{50} value with cho GM-CSF (90ng/ml) represented a 90-fold excess of eco E21R but the IC_{50} with eco GM-CSF (600ng/ml) represented a 6,000-fold excess.

The effect of the residue 100 polymorphism on eco E21R antagonism was examined in a TF-1 cell proliferation assay. Eco E21R and ecoE21R/I100T were titrated against 0.03ng/ml eco GM-CSF. Both forms of eco E21R were able to completely antagonise the proliferative activity of eco GM-CSF with IC_{50} values of eco E21R, 60ng/ml and eco E21R/I100T, 100ng/ml (Fig. 6.2.11). Thus the I100T substitution has no effect on the antagonistic activity of eco E21R.

6.2.6 Eco E21R antagonism of neutrophil activation

Since GM-CSF is a strong stimulator of inflammatory cells, the ability of eco E21R to antagonise GM-CSF-mediated stimulation of superoxide production by human neutrophils was examined. Eco E21R was titrated against 1.0ng/ml eco GM-CSF, a dose that stimulates near-maximal superoxide release from neutrophils. Eco E21R completely

Figure 6.2.10 Eco E21R antagonises the proliferation of TF-1 cells mediated by glycosylated and non-glycosylated forms of wild type GM-CSF.

Eco GM-CSF (■), yeast-derived GM-CSF (▲), cho GM-CSF (●) and eco E21R (□) were titrated for the ability to stimulate [^{3}H]thymidine incorporation into TF-1 cells. In antagonistic experiments, eco E21R (◆) was titrated against 0.1ng/ml eco GM-CSF (Panel A), 0.3ng/ml yeast GM-CSF (Panel B) or 1.0ng/ml cho GM-CSF (Panel C). Each value represents the mean of triplicate determinations and error bars represent the SEM.

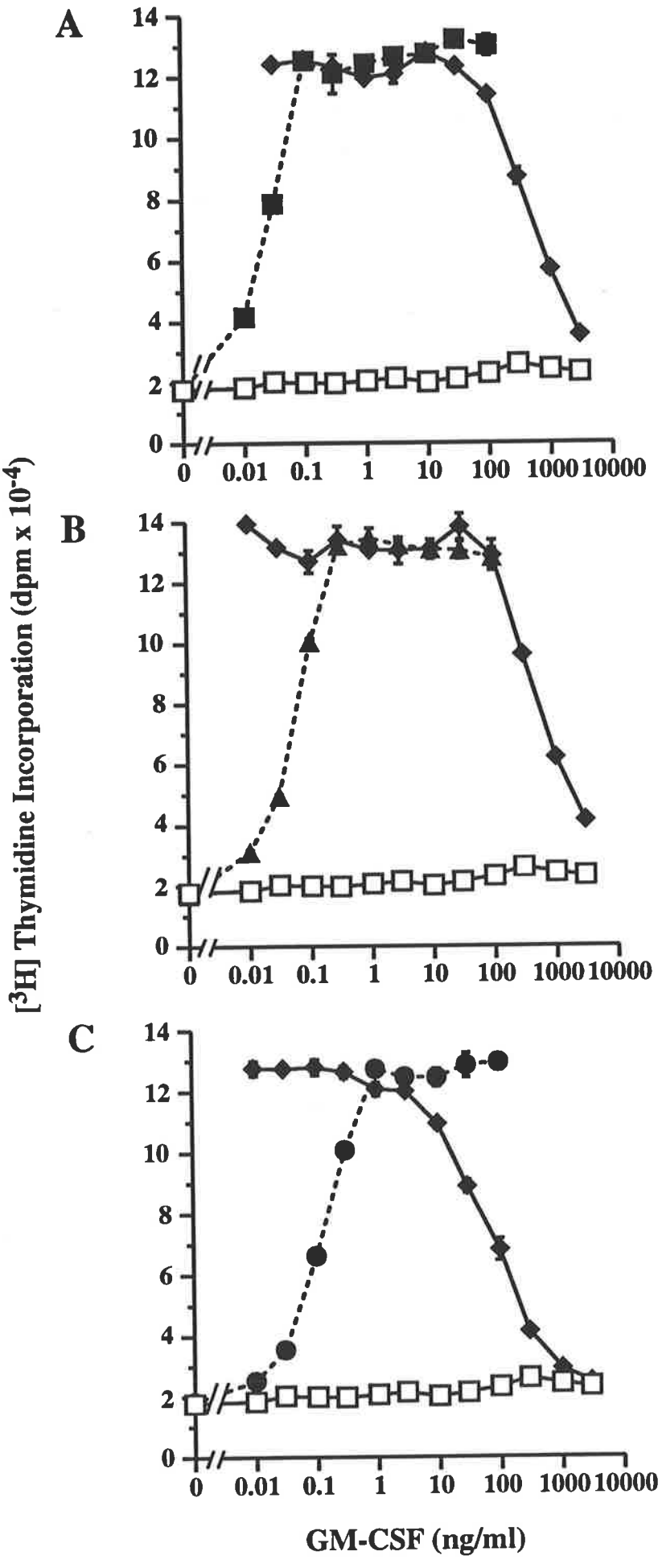
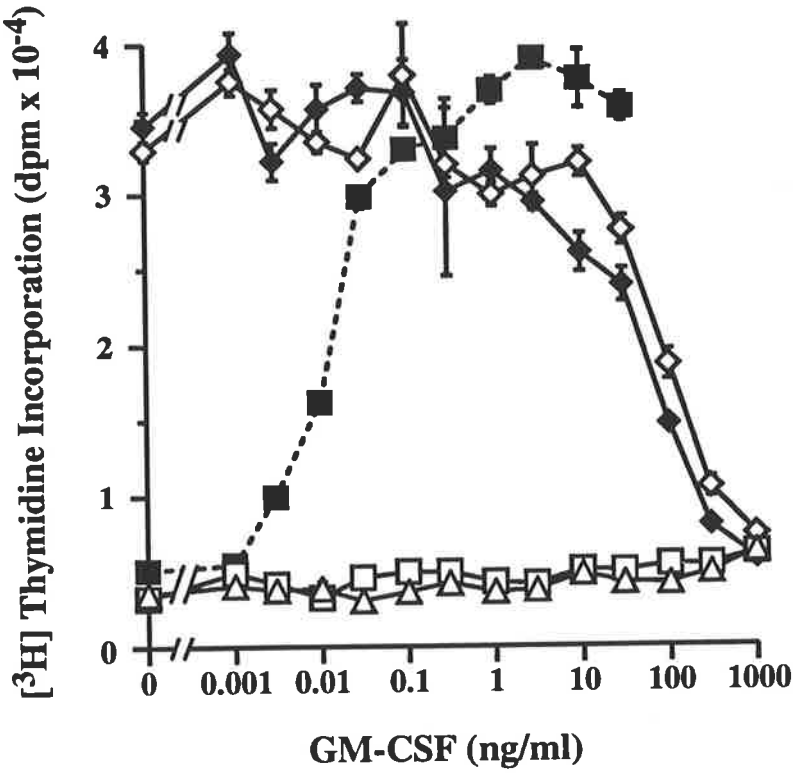

A
B
C
14
12
10
8
6
4
2
0
0
0.01
0.1
1
10
100
1000
10000
[3H] Thymidine Incorporation (dpm x 10-4)
GM-CSF (ng/ml)

Figure 6.2.11 The residue 100 polymorphism has no effect on the ability of eco E21R to antagonise GM-CSF mediated TF-1 cell proliferation.

Eco GM-CSF (■), eco E21R (□) and eco E21R/I100T (△) were titrated for their ability to stimulate [^{3}H]thymidine incorporation into TF-1 cells. In antagonistic experiments, eco E21R (◆) and eco E21R/I100T (◇) were titrated against 0.03ng/ml eco GM-CSF. Each value represents the mean of triplicate determinations and error bars represent the SEM.


[3H] Thymidine Incorporation (dpm x 10-4)
4
3
2
1
0
0
0.001
0.01
0.1
1
10
100
1000
GM-CSF (ng/ml)

antagonised the GM-CSF-mediated release of superoxide from neutrophils (Fig. 6.2.12A) with an IC_{50} of 300ng/ml. To determine the specificity of the antagonism, eco E21R was titrated against 3.0ng/ml TNF-α, a dose that stimulate near-maximal superoxide release from neutrophils. There was no detectable antagonism of TNF-α-mediated release of superoxide from neutrophils (Fig. 6.2.12B).

The ability of eco E21R to antagonise the stimulation of superoxide production by neutrophils mediated by glycosylated GM-CSF was examined. Eco E21R was titrated against 3.0ng/ml cho GM-CSF, a dose that stimulates near-maximal superoxide release from neutrophils. Eco E21R completely antagonised the GM-CSF-mediated release of superoxide from neutrophils (Fig. 6.2.13) with an IC_{50} of 90ng/ml.

6.2.7 Activity of multiple residue 21 analogues

The results presented in this chapter clearly established that the E21R substitution can totally abolish GM-CSF agonism without affecting the binding affinity for the GMRα chain. Previous work, using a COS cell expression system, suggested that other substitutions at position 21 would have less dramatic consequences (Figures 3.3.5 and 4.2.4).

A. Functional activity of residue 21 analogues

Eco GM-CSF and the *E. coli*-derived residue 21 analogues, E21A, E21Q, E21F, E21H, E21K and E21R were compared for their ability to stimulate the proliferation of TF-1 cells. Eco GM-CSF stimulated dose-dependent TF-1 cell proliferation with an ED_{50} of 0.01ng/ml while the eco E21R and E21K analogues were completely ineffective. The other analogues stimulate dose-dependent TF-1 cell proliferation with ED_{50} values of 0.2ng/ml for eco E21A and E21Q, 6ng/ml for eco E21F and 10ng/ml for eco E21H (Fig. 6.2.14). The fold decrease in activity for the eco E21A analogue compared with eco GM-CSF was similar to that observed for the cho E21A analogue compared with cho GM-CSF (Fig. 3.3.5). These results illustrated the fact that many

Figure 6.2.12 Eco E21R antagonises GM-CSF but not TNFα mediated stimulation of human neutrophils.

Eco GM-CSF (■), eco E21R (□) and TNF-α (▲) were titrated for their ability to stimulate the production of superoxide anions from human neutrophils. In antagonistic experiments, eco E21R was titrated against 1.0ng/ml (◆) of eco GM-CSF (Panel A) or 3.0ng/ml (◆) of TNF-α (Panel B). Each value represents the mean of triplicate determinations and error bars represent the SEM.

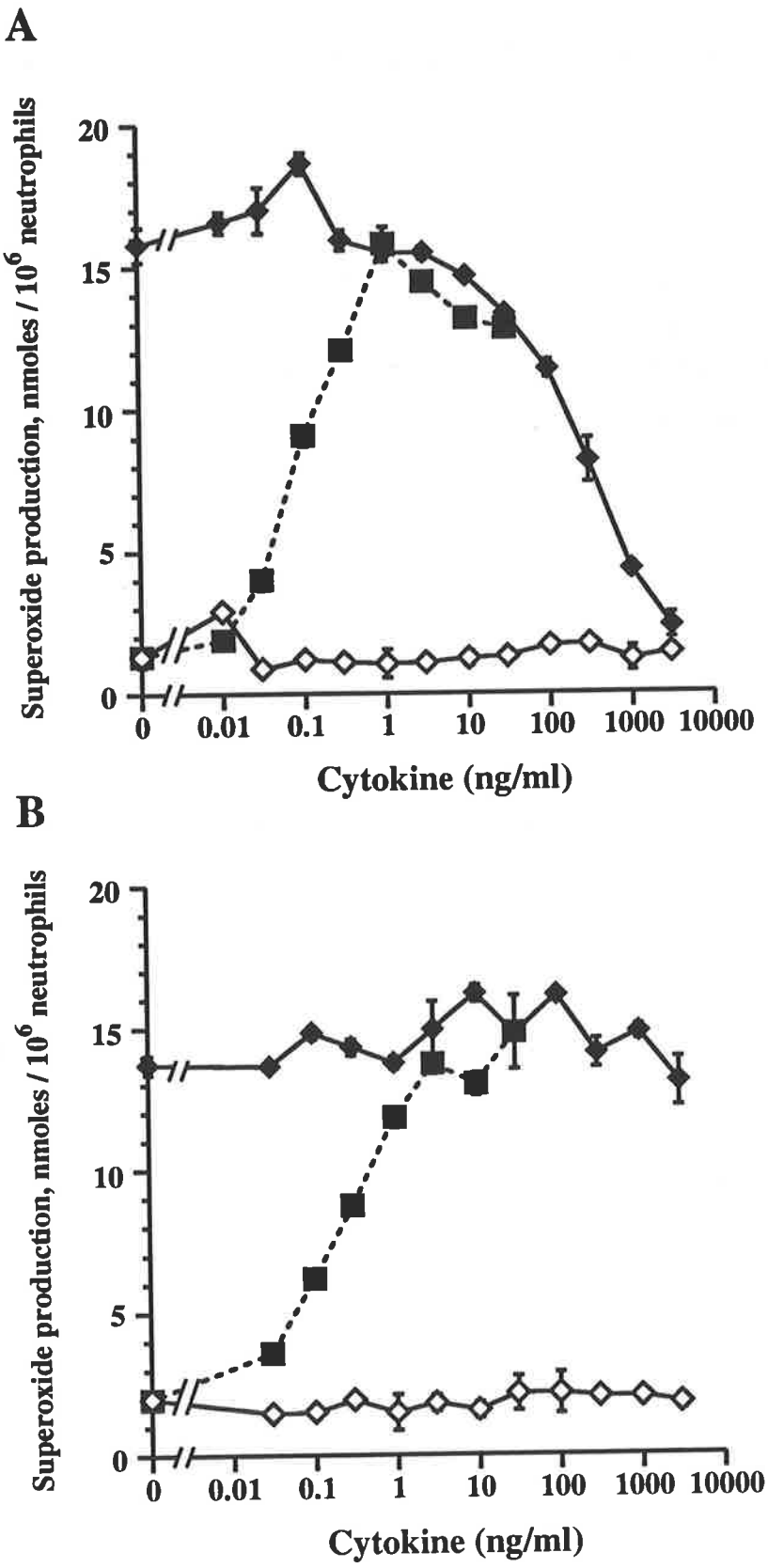

A
Superoxide production, nmoles / 10^6 neutrophils
20
15
10
5
0
0
0.01
0.1
1
10
100
1000
10000
Cytokine (ng/ml)
B
Superoxide production, nmoles / 10^6 neutrophils
20
15
10
5
0
0
0.01
0.1
1
10
100
1000
10000
Cytokine (ng/ml)

Figure 6.2.13 Eco E21R antagonises the activation of human neutrophils mediated by glycosylated wild type GM-CSF.

Cho GM-CSF (●) and eco E21R (□) were titrated for their ability to stimulate the production of superoxide anions from human neutrophils. In antagonistic experiments, eco E21R was titrated against 3.0ng/ml (◆) of cho GM-CSF. Each value represents the mean of triplicate determinations and error bars represent the SEM.

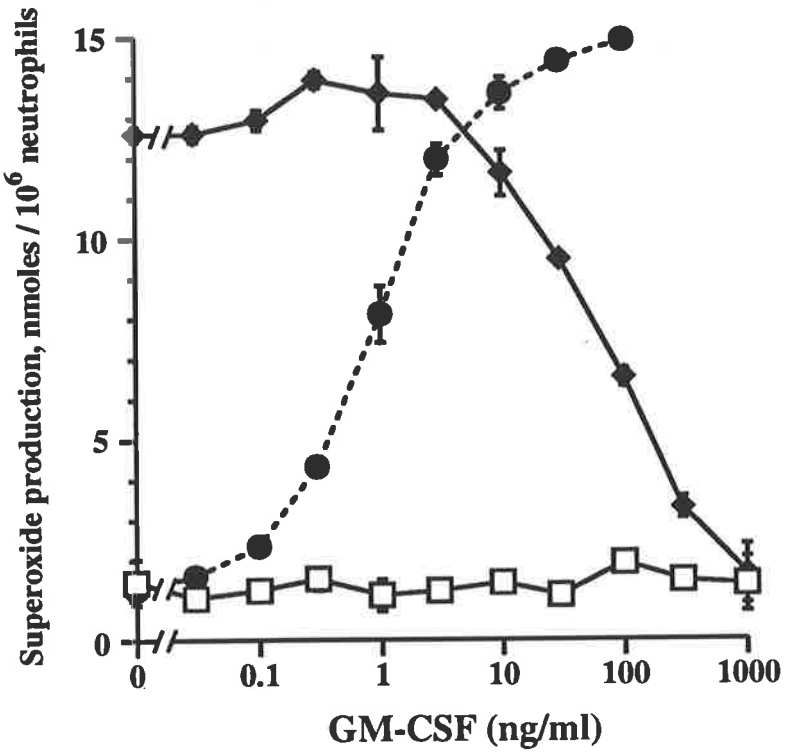
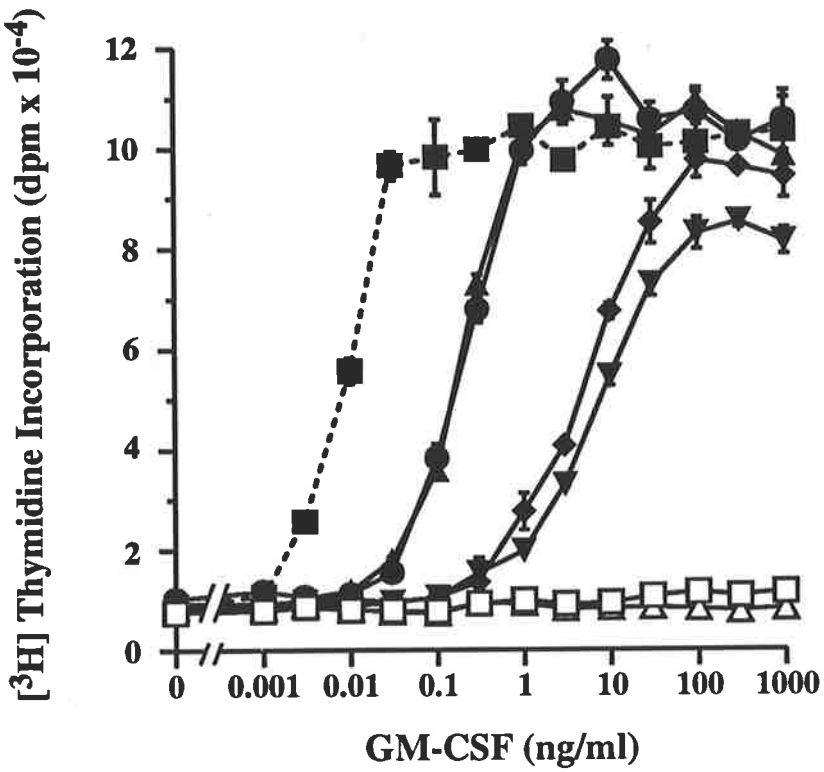

Superoxide production, nmoles / 10^6 neutrophils
15
10
5
0
0
0.1
1
10
100
1000
GM-CSF (ng/ml)

Figure 6.2.14 Comparison of the biological activity of eco GM-CSF and the *E. coli*-derived, residue 21 analogues.

Eco GM-CSF (■), eco E21A (▲), eco E21Q (●), eco E21F (◆), eco E21H (▼), eco E21K (Δ) and eco E21R (□) were titrated for their ability to stimulate [^{3}H]thymidine incorporation into TF-1 cells. Each value represents the mean of triplicate determinations and error bars represent the SEM.


[3H] Thymidine Incorporation (dpm x 10-4)
12
10
8
6
4
2
0
0
0.001
0.01
0.1
1
10
100
1000
GM-CSF (ng/ml)

amino acids are tolerated at residue 21, albeit with a reduction in biological activity, but that charge reversal has a dramatic influence on GM-CSF activity.

B. Receptor binding of residue 21 analogues

The binding of eco GM-CSF and the residue 21 analogues to the high affinity ($GMR\alpha\beta_c$) and low affinity ($GMR\alpha$) GM-CSF receptor was compared. The competitive receptor binding curves are shown in Figure 6.2.15 and the derived Kd values in Table 6.2.3. Using human neutrophils which express the high affinity receptor ($GMR\alpha\beta_c$) all the residue 21 analogues exhibit a 100- to 230-fold reduction in affinity compared to eco GM-CSF (Fig. 6.2.15A and Table 6.2.3). In contrast using the CHO cell line A9/C7 which expresses the low affinity receptor ($GMR\alpha$), eco GM-CSF and the residue 21 analogues all exhibit similar affinities (Fig. 6.2.15B and Table 6.2.3). Thus despite their considerable differences in biological activity (Fig. 6.2.14), the binding of the *E. coli*-derived GM-CSF residue 21 analogues to the high and low affinity receptors are virtually indistinguishable from each other.

6.2.8 Analysis of eco E21K for antagonistic activity

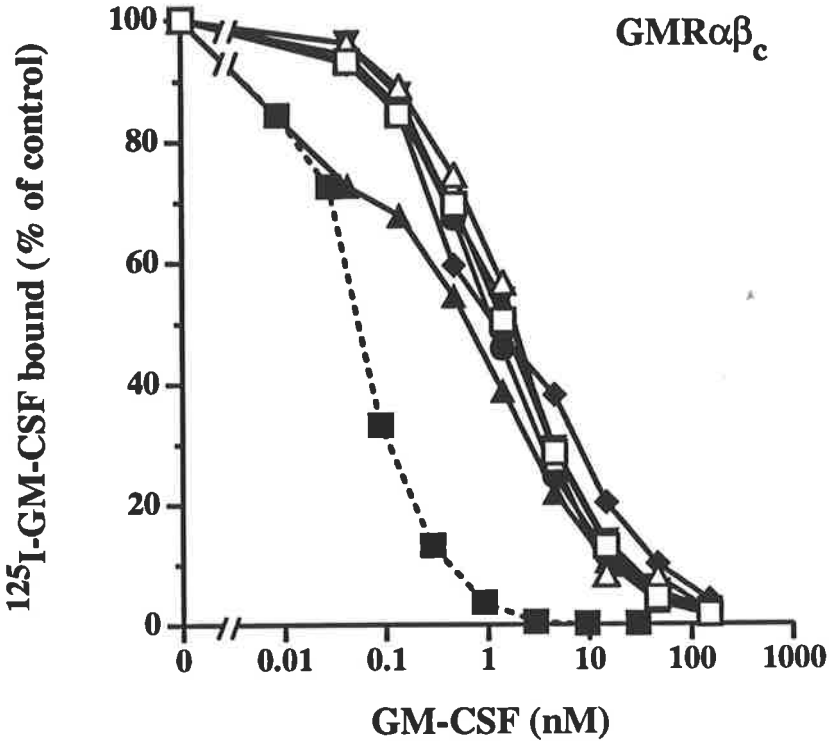
The fact that eco E21K was able to bind with wild type affinity to the GM-CSF receptor α chain (Fig. 6.2.15C) but was unable to deliver a biological signal (Fig. 6.2.14) suggested that it would posses antagonistic activity like the eco E21R analogue. In a TF-1 proliferation assay, eco E21K and eco E21R were titrated against 0.03ng/ml eco GM-CSF and 3.0ng/ml cho GM-CSF. Both analogues completely antagonised the proliferative activity of eco GM-CSF (Fig. 6.2.16A) with IC_{50} values of 150ng/ml for eco E21K and 200ng/ml for eco E21R. Similarly the proliferative activity of cho GM-CSF was antagonised (Fig. 6.2.16B) with IC_{50} values of 500ng/ml for eco E21K and 350ng/ml for eco E21R.

In the neutrophil activation assay, eco E21K and eco E21R were titrated against 1.0ng/ml eco GM-CSF or 3.0ng/ml cho GM-CSF. Both analogues completely antagonised the superoxide release mediated by eco GM-CSF (Fig. 6.2.16C) with IC_{50}

Figure 6.2.15 Comparison of the binding of eco GM-CSF and the *E. coli*-derived residue 21 analogues to human neutrophils and the A9/C7 CHO cell line.

The binding of eco GM-CSF (■), eco E21A (▲), eco E21Q (●), eco E21F (◆), eco E21H (▼), eco E21K (Δ) and eco E21R (□) to cells expressing the high (GMR$\alpha\beta_c$, Panel A) or low (GMRα, Panel B) affinity GM-CSF receptors was compared. Unlabelled eco GM-CSF or analogues were titrated against 100pM ^{125}I-GM-CSF and 3 x 10^6 neutrophils per tube (A) or 450pM ^{125}I-GM-CSF and 5.5 x 10^5 A9/C7 cells per tube (B). The values are expressed as a percentage of the ^{125}I-GM-CSF bound in the absence of competitor and are the means of duplicate determinations.

A


GMRαβc
125I-GM-CSF bound (% of control)
100
80
60
40
20
0
0
0.01
0.1
1
10
100
1000
GM-CSF (nM)

B

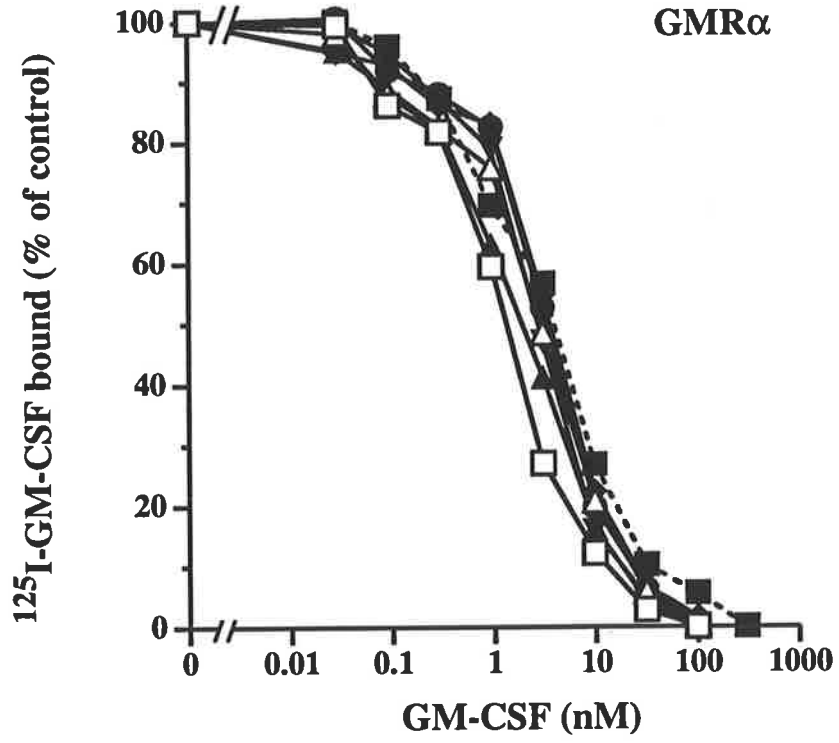

GMRα
125I-GM-CSF bound (% of control)
100
80
60
40
20
0
0
0.01
0.1
1
10
100
1000
GM-CSF (nM)

Table 6.2.3 Binding affinities of eco GM-CSF and the residue 21 analogues.

GM-CSF	Receptor affinity[a]	
	K_d ($\alpha\beta_c$)[b] (pM)	K_d(α)[c] (pM)
Wild type	7	1,800
E21A	730	820
E21Q	940	1,300
E21F	1,600	1,400
E21H	1,060	800
E21K	1,060	1,200
E21R	730	1,000

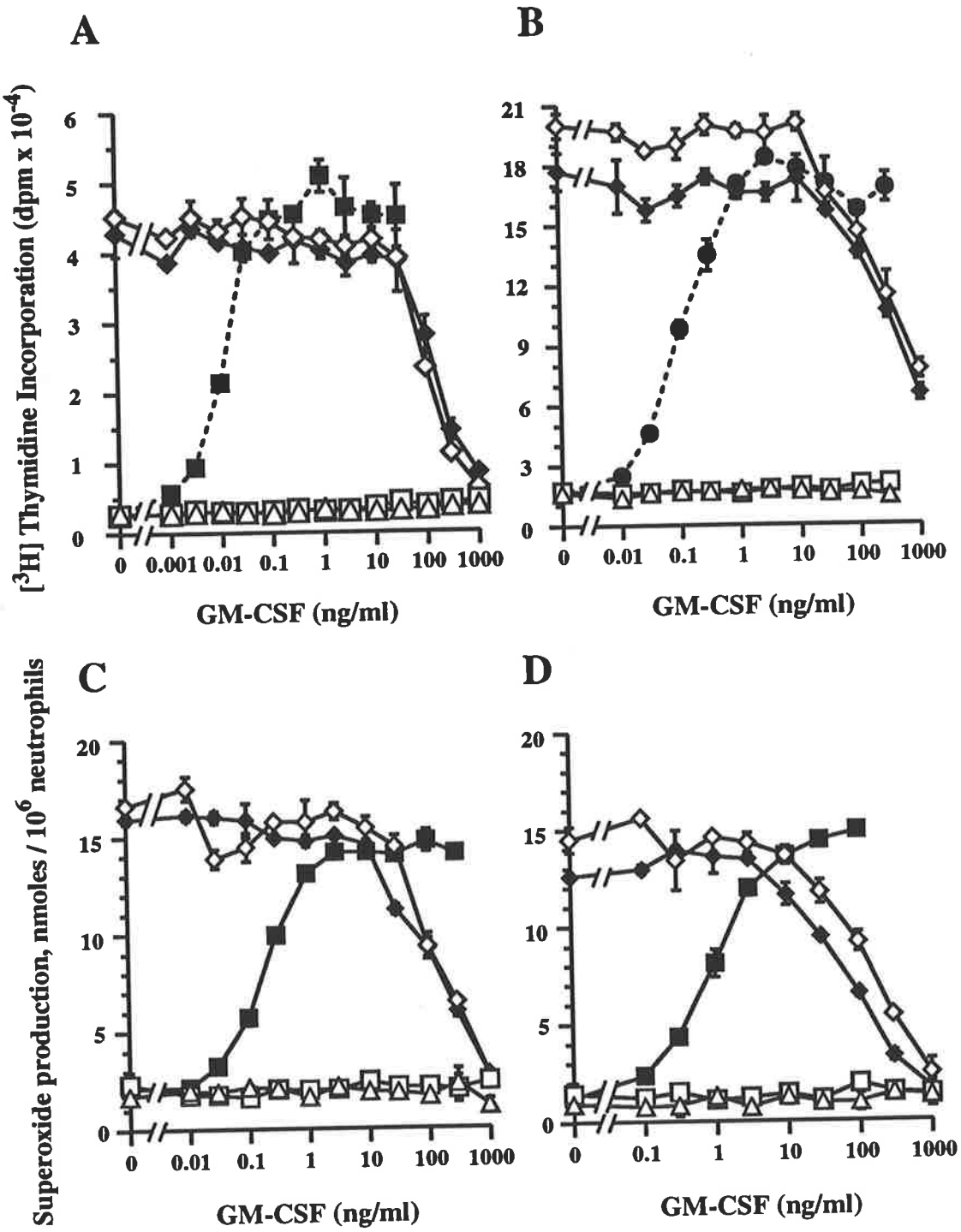
[a] Binding affinities from competition experiments shown in Figure 6.2.15 were determined using the EBDA/Ligand program (Munson and Rodbard, 1980).

[b] Mean binding affinities derived from two competition experiments on human neutrophils (GMR$\alpha\beta_c$) using 100pM ^{125}I-GM-CSF and different concentrations of residue 21 analogues.

[c] Mean binding affinities derived from three competition experiments on the CHO cell line A9/C7 (GMRα) using 500pM ^{125}I-GM-CSF and different concentrations of residue 21 analogues.

Figure 6.2.16 Eco E21K and E21R antagonise the proliferation of TF-1 cells and the activation of human neutrophils mediated by glycosylated and non-glycosylated forms of wild type GM-CSF.

Eco GM-CSF (■), cho GM-CSF (●), eco E21R (□) and eco E21K (△) were titrated for the ability to stimulate [^{3}H]thymidine incorporation into TF-1 cells (Panels A and B) and for the ability to stimulate the production of superoxide anions from human neutrophils (Panels C and D). In antagonistic experiments, eco E21R (◆) and eco E21K (◇) were titrated against 0.03ng/ml eco GM-CSF (Panel A), 3.0ng/ml cho GM-CSF (Panel B), 1.0ng/ml eco GM-CSF (Panel C) or 3.0ng/ml cho GM-CSF. Each value represents the mean of triplicate determinations and error bars represent the SEM.


A
B
C
D
[3H] Thymidine Incorporation (dpm x 10-4)
Superoxide production, nmoles / 10^6 neutrophils
GM-CSF (ng/ml)
GM-CSF (ng/ml)
GM-CSF (ng/ml)
GM-CSF (ng/ml)
0 0.001 0.01 0.1 1 10 100 1000
0 0.01 0.1 1 10 100 1000
0 0.01 0.1 1 10 100 1000
0 0.1 1 10 100 1000

values of 150ng/ml for eco E21K and eco E21R. Similarly eco E21K and eco E21R completely antagonised the superoxide release mediated by cho GM-CSF (Fig. 6.2.16D) with IC_{50} values of 200ng/ml for eco E21K and 80ng/ml for eco E21R.

Thus the eco E21R and E21K GM-CSF analogues, which are devoid of measurable agonistic activity, are equally effective antagonists of GM-CSF mediated activity.

6.3 Discussion

The work presented in this chapter describes the analysis of *E. coli*-derived wild type GM-CSF and analogues mutated at residue 21. The somewhat unexpected finding that the E21R analogue was devoid of activity when the same analogue produced in CHO cells, was biologically active (Chapter 3) was examined in some detail. The absence of biological activity in certain GM-CSF analogues carrying basic amino acids at residue 21 (Fig. 6.2.14) but the retention of wild type binding affinity for the GM-CSF receptor alpha chain (Fig. 6.2.15) resulted in the generation of GM-CSF specific antagonists (Fig. 6.2.16). The potential clinical application of GM-CSF antagonists are discussed.

6.3.1 The properties of the GM-CSF residue 21 analogues

A. Receptor binding and biological activity

The results described in Chapter 3 indicated that residue 21 of human GM-CSF is specifically involved in the binding to the β_c chain of the GM-CSF receptor. The results with the *E. coli*-derived residue 21 analogues support a model of GM-CSF interaction with its receptor that emphasises the role of residue 21 and suggests a possible role for carbohydrate in the β_c chain interaction.

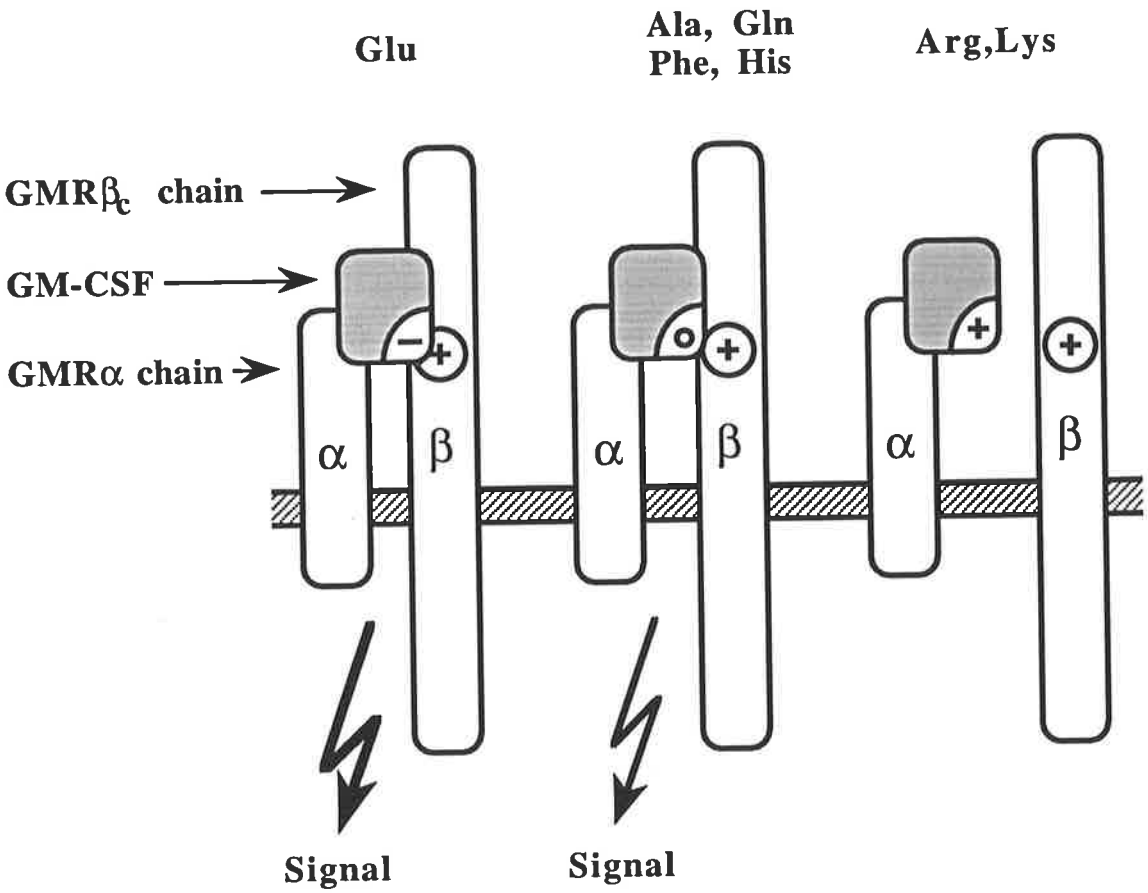
The GM-CSF residue 21 analogues E21A, E21Q, E21F and E21H all exhibited significantly reduced biological activity (Fig. 6.2.14) yet the binding affinity of these analogues for human neutrophils expressing GMR$\alpha\beta_c$ appeared to consist of low affinity binding only, equivalent to the binding affinity observed for the A9/C7 cell line expressing the GMRα chain (Table 6.2.3). Thus despite the fact that the E21A, E21Q, E21F and E21H analogues were able to deliver a biological signal, presumably through interaction with the β_c chain, this interaction did not result in higher affinity binding to GMR$\alpha\beta_c$ than GMRα alone. As the binding affinity of the residue 21 analogues for the GMRα chain was essentially unaltered (Table 6.2.3), the biological activity probably reflects the absolute effect of the mutations on β_c interaction. The possibility that the analogues signal through binding to the GMRα chain alone, does not account for the different biological potencies of these analogues. The data strongly support a model where Glu^{21} of GM-CSF directly interacts with β_c and that mutations at this residue disturb this interaction (Fig. 6.3.1). The lack of a direct correlation between binding affinity and biological activity as observed for the residue 21 analogues is a result of the interaction of residue 21 with the critical signalling component (β_c) rather than the primary binding component (GMRα) of the GMR$\alpha\beta_c$ receptor complex. A region of the β_c chain that specifically includes His^{367}, has been shown to be important for high affinity binding of GM-CSF (Lock *et al.*, 1994). The ability of the β_c chain mutant Y365A/H367A/I368A to partially restore the high affinity binding of eco E21R (Woodcock *et al.*, 1994) provides additional evidence for a direct interaction between GM-CSF Glu^{21} and β_c.

The exact mechanism of GM-CSF receptor activation is not known but appears to involve the low affinity binding of GM-CSF to GMRα and functional association of this complex with β_c, leading to high affinity binding of GM-CSF and signalling (Nicola *et al.*, 1993; Goodall *et al.*, 1993; Hoang *et al.*, 1993; Miyajima *et al.*, 1993). Recent studies indicate that GMRα and β_c may exist as a preformed complex in the absence of ligand (Frank Stomski, personal communication; Ronco *et al.*, 1994), demonstrating that association of GMRα and β_c alone is insufficient for receptor activation. Considerable

Figure 6.3.1 Interaction of *E. coli*-derived GM-CSF residue 21 analogues with the GM-CSF receptor.

The interaction of wild type GM-CSF with the GMR$\alpha\beta_c$ complex is characterised by high affinity binding and maximal biological activity. Substitution of the glutamic acid at residue 21 with neutral amino acids reduced binding to low affinity only and led to a marked reduction in biological activity. Substitution of the glutamic acid at residue 21 with strong basic amino acids reduced binding to low affinity only and eliminated detectable biological activity (N.D.). Binding studies with mutant β_c chains suggested that the region interacting with GM-CSF residue 21 possibly includes residues Tyr^{365}, His^{367} and Ile^{368} (Woodcock *et al.*, 1994). Binding affinity refers to the data described in Table 6.2.3 while the biological activity was determined in a TF-1 cell proliferation assay described in Figure 6.2.14.

Amino acid at position 21 of *E. coli*-derived GM-CSF



Binding affinity	**High**	**Low**	**Low**
Biological activity	**100%**	**5 - 0.1%**	**N.D.**

evidence indicates that the low affinity binding of GM-CSF to a GMR$\alpha\beta_c$ complex is sufficient to trigger a biological response perhaps through an allosteric interaction. Thus, human GM-CSF binds with low affinity to murine cell lines expressing the endogenous murine β_c chain and the transfected human GMRα chain and elicits a proliferative response (Metcalf *et al.*, 1990; Kitamura *et al.*, 1991a). Similarly the cho E21R (Fig. 6.2.6B) and eco E21A, E21Q, E21F and E21H (Fig. 6.2.14) analogues, which lack high affinity binding, were able to elicit a proliferative response. In all these cases the reduced binding affinity was correlated with a reduction in biological potency. In a most unusual example however, the murine GM-CSF E21A analogue binds with low affinity only yet triggers a full biological response with wild type potency (Shanafelt and Kastelein, 1992). In contrast, the eco E21R and E21K analogues were unable to deliver a biological signal yet exhibited binding affinities for the GMR$\alpha\beta_c$ receptor that were indistinguishable from the eco E21A, E21Q, E21F and E21H analogues (Fig. 6.2.15). This demonstrated that low affinity binding does not necessarily lead to receptor activation and suggested that the eco E21R and E21K analogues were completely unable to interact with the β_c chain. This was also demonstrated by the inability of eco E21R to antagonise the IL-3 mediated proliferation of TF-1 cells (Fig. 6.2.8) or to cross-compete for ^{125}I-IL-3 binding (data not shown).

B. Structural properties

Transverse urea gradient gel electrophoresis was used to obtain an estimate of the stability of wild type GM-CSF and the E21R analogue expressed in *E. coli* (Fig. 6.2.7). The stability of the folded protein ($\Delta G°(H_2O)$) was estimated to be -43 kJ/mol for eco GM-CSF and -21 kJ/mol for eco E21R with folding:unfolding transitions at urea concentrations of 4M (eco GM-CSF) and 2.5M (eco E21R). Thus the urea gradient gels provided qualitative evidence that the substitution of arginine at residue 21 reduced the folded stability of GM-CSF. However, it must be emphasised that in the absence of denaturant the E21R analogue did appear to adopt a folded conformation (Fig. 6.2.7). The estimate of eco GM-CSF stability (-43 kJ/mol) differs from that previously reported for *E. coli*-derived wild type GM-CSF where $\Delta G°(H_2O)$ = -24.2kJ/mol (Wingfield *et al.*,

1988). This presumably reflects a difference in data analysis as the 4M folding transition observed for eco GM-CSF is similar to the 4.1M transition reported by Wingfield *et al.* (1988).

The reduced stability of the eco E21R analogue does not imply gross structural differences and is unlikely to account for the dramatic loss of function. Two observations suggested that the tertiary structure of the E21R analogue was closely related to that of wild type GM-CSF. Firstly, all residue 21 analogues bind with wild type affinity to the GMRα chain (Fig. 6.2.15B and Table 6.2.3). Secondly, the conformation sensitive polyclonal antibody used for RIA quantification, exhibited full recognition of the eco E21R analogue (Fig. 5.2.16).

6.3.2 Potential role of carbohydrate in cho E21R

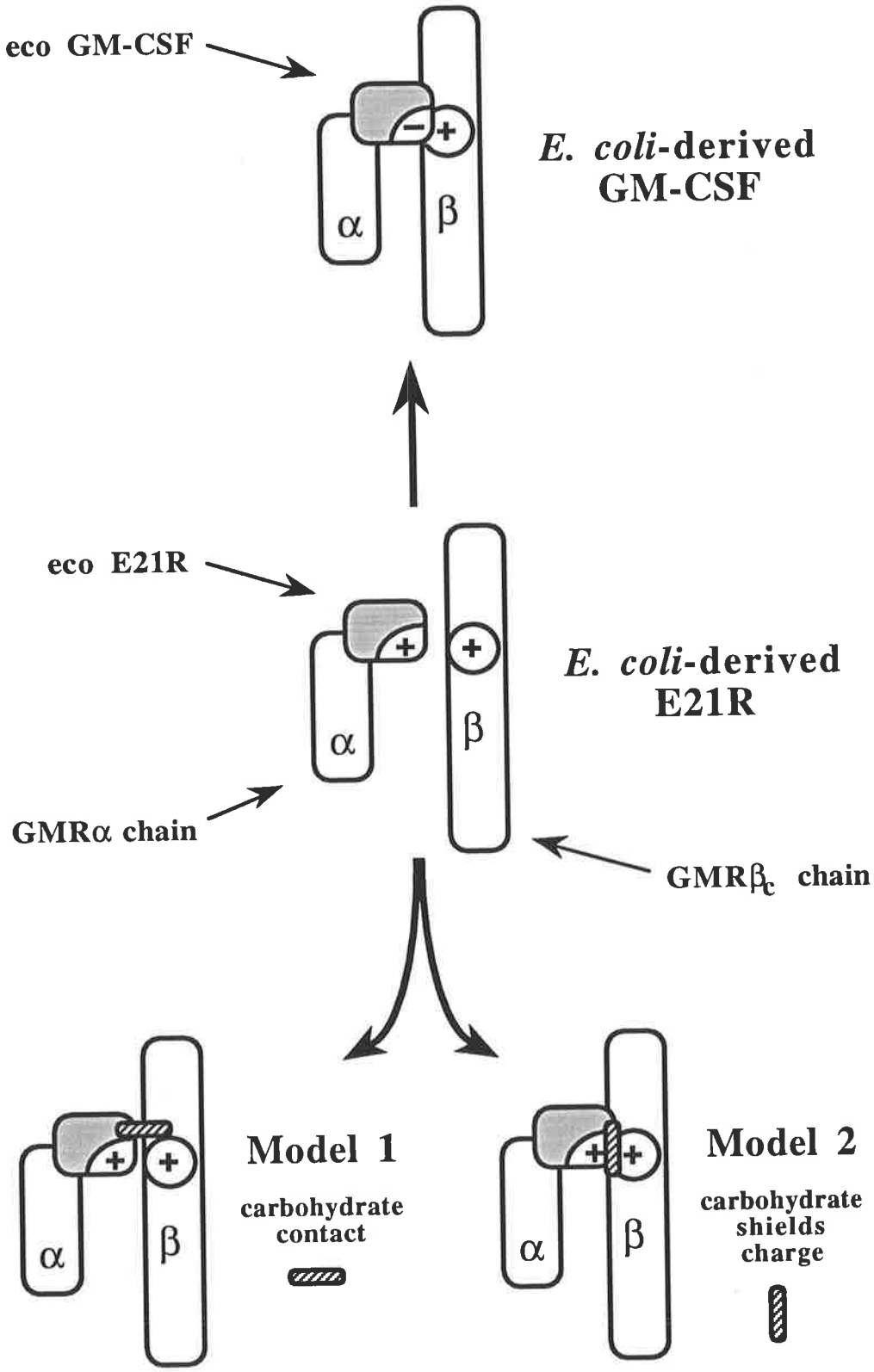
The main difference between the model described in Figure 6.3.1 and the previous model (Fig. 3.4.3) is that the *E. coli*-derived, residue 21 charge reversal mutants were unable to signal. The eco E21R and cho E21R analogues exhibit significant differences in biological activity (Fig. 6.2.2 vs Fig. 6.2.6B) despite essentially identical receptor binding properties (Fig. 6.2.4). The only known difference between the two analogues is the presence of extensive carbohydrate modifications in the cho E21R. Fractionation of cho E21R by reversed phase HPLC was able to separate a number of glycosylation variants of the E21R analogue (Fig. 6.2.6A), all able to stimulate proliferation of TF-1 cells (Fig. 6.2.6B). The fact that cho E21R which apparently lacks N-linked carbohydrate exhibited the highest biological activity (Fig. 6.2.6B, Table 6.2.1) suggested that the presence of O-linked carbohydrate may play a role in the biological activity of cho E21R. If such a role does exist for the O-linked carbohydrate of cho E21R, then its removal either enzymatically or by mutation, should eliminate the biological activity of the cho E21R analogue. Interestingly, in the context of the E21A substitution, the presence of carbohydrate did not appear to influence the relative activity of the analogue as both eco E21A (Fig. 6.2.14) and cho E21A (Fig. 3.3.5) exhibited an activity of approximately 5 to 10% of the respective wild type GM-CSF molecules.

At least two mechanisms can be envisaged to account for the potential role of carbohydrate in the biological activity of the cho E21R analogue (Fig. 6.3.2). The first model proposes that the substitution of basic amino acids at residue 21 removes a critical contact between GM-CSF and the β_c chain. The presence of carbohydrate moieties on cho E21R, possibly O-linked, enables direct contact between GM-CSF and the β_c chain in the absence of an interaction utilising the Glu^{21} of GM-CSF (Opdenakker *et al.*, 1993). A carbohydrate mediated β_c chain contact is clearly not required for the activity of non-glycosylated wild type GM-CSF (Fig. 6.2.1), may or may not occur with glycosylated wild type GM-CSF and is not measurable in binding experiments (Table 6.2.2). In this last respect, direct carbohydrate contact would generate a signal but with no affinity conversion and be similar to the situation described in Figure 6.3.1 for the *E. coli*-derived GM-CSF analogues able to signal but exhibiting only low affinity binding. The second model proposes that the substitution of basic amino acids at residue 21 introduces a charge repulsion between GM-CSF and the β_c chain that completely abolishes β_c chain contact. The presence of carbohydrate may be able to mask the charge repulsion and thereby enable interaction between GM-CSF mutant analogues such as cho E21R and the β_c chain. The potential of carbohydrate to mask charge interactions may also function with wild type GM-CSF to mask favourable charge interactions with the β_c chain and thereby be partially responsible for the reduced activity of glycosylated GM-CSF compared with non-glycosylated GM-CSF (Fig. 6.2.1).

An alternative explanation is that the carbohydrate influences the stability of the E21R analogue. In general, the presence of covalently attached carbohydrate increases the overall dynamic stability of a protein (Opdenakker *et al.*, 1993) although it has been demonstrated that carbohydrate does not increase the stability of wild type human GM-CSF (Wingfield *et al.*, 1988). Eco E21R adopted a folded conformation in the absence of denaturant but was approximately 50% less stable than eco GM-CSF (Fig. 6.2.7). It is possible though, that in the context of the E21R substitution, carbohydrate might exert a stabilising influence on the protein, perhaps by nullifying destabilising electrostatic interactions introduced by the basic amino acid (Hollecker and Creighton, 1982). This

Figure 6.3.2 Models for the role of carbohydrate in the biological activity of the cho E21R analogue.

Models proposed to account for the possible role of carbohydrate in the biological activity of the CHO cell-derived E21R analogue. In model one the carbohydrate moieties on cho E21R are able to bind the β_c chain and this leads to signalling. In model two the carbohydrate moieties on cho E21R mask a charge repulsion of the β_c chain that enables cho E21R to interact with the β_c chain leading to signalling. Fractionation of the various glycosylation species of cho E21R indicates that the relevant carbohydrate moieties may be O-linked (Fig. 6.2.6).



CHO cell-derived E21R

question could be addressed by determining the stability of the cho E21R analogue using the transverse urea gradient gel electrophoresis method.

6.3.3 Clinical relevance of GM-CSF antagonists

In vitro and *in vivo* studies have demonstrated that GM-CSF is a pleiotropic cytokine that stimulates both the production of different haemopoietic lineages (Clark and Kamen, 1987; Gasson, 1991), and the effector function of mature myeloid cells (Lopez *et al.*, 1983, 1986; Grabstein *et al.*, 1986; Metcalf *et al.*, 1986, 1987; Gasson *et al.*, 1984; Sieff *et al.*, 1985; Haak-Frendscho *et al.*, 1988). These properties have lead to the *in vivo* use of GM-CSF to stimulate haemopoiesis following chemotherapy (Antman *et al.*, 1988; Brandt *et al.*, 1988; Socinski *et al.*, 1988), bone marrow transplantation (Nemunaitis *et al.*, 1991; Peters *et al.*, 1993; Rabinowe *et al.*, 1993) and in situations of myelodysplasia (Vadhan-Raj *et al.*, 1987; Ganser *et al.*, 1989). In patients with AIDS GM-CSF stimulates myelopoiesis (Groopman *et al.*, 1987) as well as enhancing neutrophil function (Baldwin *et al.*, 1988).

It is also apparent that GM-CSF may play a role in the pathogenesis of several disease conditions, in particular chronic inflammation and leukaemia. Transgenic mice carrying additional copies of the murine GM-CSF gene display elevated levels of GM-CSF and an accumulation of macrophages in the eyes and striated muscles leading to blindness, muscle damage and premature death possibly as a result of GM-CSF-mediated macrophage activation (Lang *et al.*, 1987). In humans the presence of elevated levels of GM-CSF in the bronchoalveolar lavage of atopic patients (Kato *et al.*, 1992) and in the synovial fluid of patients with rheumatoid arthritis (Williamson *et al.*, 1988; Xu *et al.*, 1989; Alvaro-Gracia *et al.*, 1989) suggest that GM-CSF plays a pathological role in some inflammatory diseases. This idea is supported by the observations that patients with rheumatoid arthritis possess activated neutrophils in the synovial fluid (Emery *et al.*, 1988) and an increase in the level of circulating monocytes expressing the GM-CSF receptor (Field and Clinton, 1993). GM-CSF also stimulates the production of the neutrophil chemoattractant IL-8 (McCain *et al.*, 1993) and, in conjunction with

lipopolysaccharide, the monocyte chemoattractant macrophage inflammatory protein 1α (Kasama *et al.*, 1993) thus regulating the localisation of leukocytes at the site of inflammation.

Studies on AML (Hoang *et al.*, 1986; Begley *et al.*, 1987b), CMMoL (Everson *et al.*, 1989) and ALL (Freedman *et al.*, 1993) have demonstrated a proliferative response to paracrine GM-CSF. In certain cases of AML (Young and Griffin, 1986; Young *et al.*, 1987, 1988) and ALL (Freedman *et al.*, 1993), GM-CSF mRNA or protein has been detected suggesting the possibility of an autocrine response to GM-CSF. The expression of GM-CSF by AML blast cells appears to be regulated by IL-1 (Delwel *et al.*, 1989; Russell, 1992) as is the expression of other cytokines such as G-CSF and IL-6 which are also able to stimulate AML proliferation (Bradbury *et al.*, 1990). In addition, non-haemopoietic tumours such as certain small cell lung carcinomas (Baldwin *et al.*, 1989), osteogenic sarcomas (Dedhar *et al.*, 1988) and certain colon adenocarcinomas (Berdel *et al.*, 1989) aberrantly proliferate in response to GM-CSF.

The ability of the GM-CSF analogues eco E21R and eco E21K to specifically inhibit the GM-CSF mediated activation of neutrophils *in vitro* (Fig. 6.2.17 D) and leukaemic cell proliferation (Fig. 6.2.17B), suggests a potential therapy for diseases exacerbated by GM-CSF. The clinical potential of these GM-CSF antagonists may be enhanced by generating more potent, second generation antagonists, which exhibit higher affinity for the GMRα chain. Phage display has been used to select a variant of human growth hormone with 15 substitutions that exhibits a 400-fold increase in affinity for the growth hormone binding protein (Lowman *et al.*, 1991; Lowman and Wells, 1994). This approach should be suitable for the selection GM-CSF variants with enhanced affinity for the GMRα chain. The results shown in Chapter 3 which identify the fourth α-helix of GM-CSF as a potential GMRα chain binding site (Fig. 3.3.11), suggest a region where appropriate mutations might enhance the affinity of GM-CSF for the GMRα chain.

Chapter 7

Final Discussion

The aim of the work described in this thesis was to study the structure-function properties of the human cytokine GM-CSF and specifically to identify regions involved in the interaction of GM-CSF with its receptor complex. The biological and receptor binding properties of analogues generated by mutagenesis of the GM-CSF cDNA were examined and the functional role of the wild type sequences deduced. Using this approach, two spatially distinct receptor binding sites were identified that are required for maximal biological activity and interaction with the two chains, GMRα and β_c, of the GM-CSF receptor. This information paved the way for the generation of the first GM-CSF specific antagonists.

GM-CSF is a pleiotropic cytokine able to stimulate both the production of different haemopoietic lineages and the effector function of mature myeloid cells (reviewed in Gasson, 1991). *In vivo*, recombinant GM-CSF has been used to stimulate haemopoiesis following chemotherapy (Antman *et al.*, 1988; Brandt *et al.*, 1988; Socinski *et al.*, 1988) or bone marrow transplantation (Nemunaitis *et al.*, 1991) and to enhance neutrophil production and function in AIDS patients (Baldwin *et al.*, 1988). A number of studies have also suggested that endogenous GM-CSF activity may be involved in disease conditions such as chronic inflammation and leukaemia. Thus GM-CSF has been implicated as a pathological agent in diseases such as rheumatoid arthritis (Williamson *et al.*, 1988; Xu *et al.*, 1989; Alvaro-Gracia *et al.*, 1989), allergy (Kato *et al.*, 1992) and asthma (Broide *et al.*, 1992). GM-CSF is also able to stimulate the proliferation of certain leukaemias (Hoang *et al.*, 1986; Begley *et al.*, 1987b) and there is evidence that GM-CSF may function as an autocrine growth factor for leukaemic cell proliferation (Young and Griffin, 1986; Young *et al.*, 1987, 1988; Freedman *et al.*, 1993).

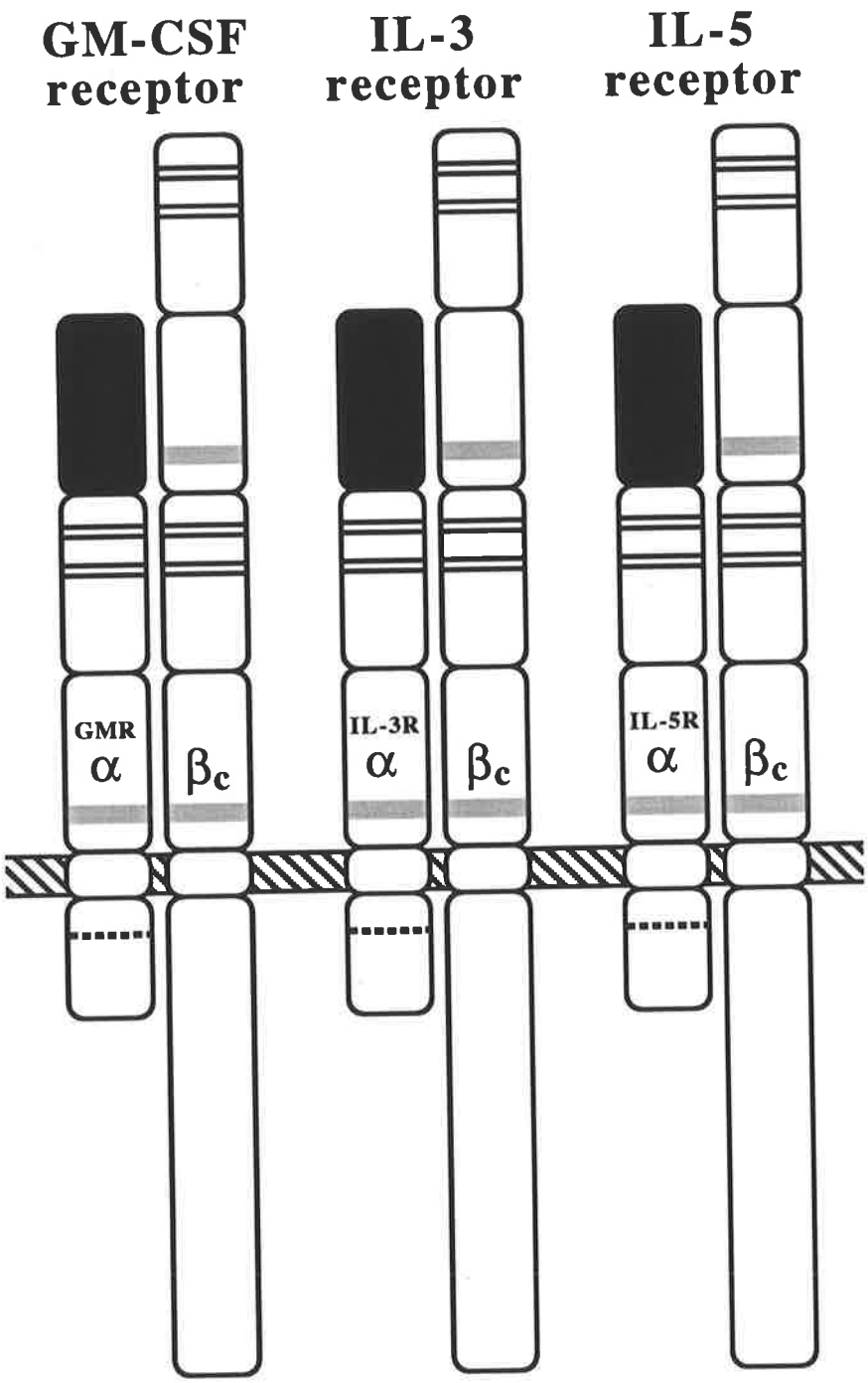
Human GM-CSF functions by binding to receptors located on the surface of responsive cells. Since the commencement of the work described in this thesis, considerable information about the cytokine receptors has become available. The GM-CSF receptor was first cloned from a human placental cDNA library, is expressed in a variety of haemopoietic cells that bind GM-CSF and when expressed in COS cells is able

to bind GM-CSF with low affinity only (Gearing *et al.*, 1989). This chain is the low affinity receptor α chain (GMRα). A soluble form of the human GM-CSF receptor has been cloned from chorio-carcinoma cells and appears to be a splicing variant of the membrane bound GMRα chain (Raines *et al.*, 1991). A second subunit of the GM-CSF receptor was cloned from the TF-1 cell line and while unable to detectably bind GM-CSF when expressed by itself, was able to contribute to the high affinity binding of GM-CSF when co-expressed with the GMRα chain (Hayashida *et al.*, 1990). Cloning of low affinity receptors for IL-3 (IL-3Rα) and IL-5 (IL-5Rα) revealed that the second chain of the GM-CSF receptor was also able to contribute to the high affinity binding of IL-3 and IL-5 when co-expressed with the appropriate receptor α chain (Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991). The presence of a common, affinity converting β chain (β_c) in the GM-CSF (GMR$\alpha\beta_c$), IL-3 (IL-3R$\alpha\beta_c$) and IL-5 (IL-5R$\alpha\beta_c$) high affinity receptors explains the cross-competition observed for these cytokines on human cells (Elliott *et al.*, 1989; Lopez *et al.*, 1989, 1990a, 1991, 1992a). The GM-CSF receptor chains are members of the type I cytokine receptor superfamily which includes the receptors for IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, GM-CSF, G-CSF, EPO, GH, LIF, OSM, CNTF and the β and γ subunits of the IL-2 receptor (reviewed in Miyajima *et al.*, 1993). The extracellular, ligand binding domains exhibit little sequence homology apart from four highly conserved cysteine residues and a Trp-Ser-X-Trp-Ser (WSXWS) motif (Miyajima *et al.*, 1993). Structurally, the extracellular domain is predicted to contain cytokine receptor modules of approximately 200 amino acids that form two discrete domains containing seven β strands folded into anti-parallel β sandwiches with a topology similar to the immunoglobulin constant domains (Bazan, 1990b). Another feature of the cytokine receptors is the lack of sequence homology in the cytoplasmic domain apart from a few motifs such as the RLFP motif (Miyajima *et al.*, 1993) and the absence of intrinsic tyrosine kinase activity (Kaczmarski and Mufti, 1991). Figure 7.1 illustrates some of the features of the type I cytokine receptor structure as found in the GM-CSF, IL-3 and IL-5 receptors.

Figure 7.1 The structure of the receptors for GM-CSF, IL-3 and IL-5.

The structural features of the type I cytokine receptors for GM-CSF, IL-3 and IL-5. The receptor β_c chain comprises two cytokine receptor modules, each containing two anti-parallel β_c sandwich domains while the receptor α chains each contain a single cytokine receptor module together with an N-terminal domain shown in black. Within each cytokine receptor module the four conserved cysteine residues are shown as horizontal lines while the WSXWS motif is represented as a grey box. The receptor α chains contain short cytoplasmic domains with a conserved RLFP motif shown as a dashed horizontal line while the β_c chain contains a significantly larger cytoplasmic domain.

This figure was derived from Miyajima *et al.*, 1993.


GM-CSF receptor
IL-3 receptor
IL-5 receptor
GMR α
βc
IL-3R α
βc
IL-5R α
βc

The three-dimensional structure of several cytokines, including human GM-CSF, was determined during the course of the work described in this thesis. The most striking structural feature of human GM-CSF is the presence of four α-helices with an unusual two-up-two-down topology (Fig. 3.4.1A). Elucidation of the three-dimensional structures for other members of the cytokine family revealed considerable structural similarities with GM-CSF. Cytokines with a GM-CSF-like fold (Diederichs *et al.*,1991; Walter *et al.*, 1992a) are G-CSF (Hill *et al.*, 1993), M-CSF (Pandit *et al.*, 1992), IL-2 (Brandhuber *et al.*, 1987; revised McKay, 1992; Bazan, 1992), IL-4 (Powers *et al.*, 1992; Smith *et al.*, 1992; Walter *et al.*, 1992b), IL-5 (Milburn *et al.*, 1993) and growth hormone (Abdel-Meguid *et al.*, 1987; de Vos *et al.*, 1992). Members of this family contain a core structure composed of four α-helices arranged in a two-up-two-down topology that is unique to this family. Comparison of the structures suggested that, based on the length of their α-helices, the helical cytokines be divided into two sub-families, the short-chain cytokines which include GM-CSF, M-CSF, IL-2, IL-4 and IL-5, and the long chain cytokines which include G-CSF and growth hormone. The alignment of predicted structural features in other cytokines indicates that IL-3, IL-7 and SCF are also likely to be members of the short-chain helical cytokine family (Rozwarski *et al.*, 1994). Comparison of the position of the α-carbon atoms amongst the members of the short chain cytokine family demonstrated that approximately half of the residues of each cytokine are maintained in a common three-dimensional position forming a conserved structural core (Wlodawer *et al.*, 1993; Rozwarski *et al.*, 1994). This structural homology exists despite the very low level of amino acid sequence identity displayed by these cytokines which within the conserved structural core is as low as 11% for GM-CSF and IL-2. The structural homology and unique topology of the short-chain helical cytokines suggests a common ancestry for these proteins. The remarkable similarity in the exon structure of the genes encoding the helical cytokine family of proteins lends support to the hypothesis that the members of this family have diverged from a common ancestral protein (Bazan, 1990a; Rozwarski *et al.*, 1994).

The data presented in this thesis identifies two residues of human GM-CSF, located in the first (Glu^{21}) and fourth (Asp^{112}) α-helices, that are critical for the functional interaction of GM-CSF with either the β_c or GMRα chains, respectively, of the GM-CSF receptor complex. Mutagenesis within the first α-helix of human GM-CSF identified residue 21 as the only solvent exposed residue that was by itself, critical for activity. Receptor binding studies with residue 21 analogues indicated that the Glu^{21} of wild type GM-CSF was essential for binding to the high affinity (GMRαβ_c) but not the low affinity (GMRα) receptor and thus identified Glu^{21} as a β_c chain contact residue. This observation also indicated that a second functional domain must exist which is required for GMRα chain interaction and demonstrated that GM-CSF stimulation of both proliferation and mature cell activation are mediated through high affinity receptors. Mutagenesis within the fourth α-helix identified a role for residue 112 in GM-CSF biological activity. Binding studies showed that Asp^{112} of wild type GM-CSF was important for high (GMRαβ_c) and low (GMRα) affinity binding and thereby identified Asp^{112} as a potential GMRα chain contact residue.

The identification of functionally important residues in the first α-helix of human GM-CSF is in agreement with a number of structure-function studies of human and murine GM-CSF (Clark-Lewis *et al.*, 1988; Kaushansky *et al.*, 1989; Shanafelt and Kastelein, 1989; Shanafelt *et al.*, 1991a, b, 1992; Meropol *et al.*, 1992). Amongst these studies a region that includes residues 20 and 21 is consistently identified as being important for both human and murine GM-CSF function. The work described in this thesis clearly identified the critical nature of residue 21 for human GM-CSF function. Mutagenesis studies with murine GM-CSF indicated that mutation of residue 21 had only a marginal effect on GM-CSF function but that mutation of residue 21 in conjunction with mutations at residue 14 or 20, had a pronounced effect on murine GM-CSF activity and high affinity receptor interaction (Meropol *et al.*, 1992; Shanafelt and Kastelein, 1992). Interestingly, the murine GM-CSF analogue E21A exhibits near wild type biological activity in absence of high affinity binding (Meropol *et el.*, 1992; Shanafelt and Kastelein, 1992). The properties of the murine E21A analogue are quite unlike those

of the human E21A analogue described in this thesis where loss of high affinity binding (Fig. 3.3.7) is paralleled by a loss of biological activity (Fig. 3.3.5). The near wild type biological activity of murine E21A in the absence of high affinity binding prompted the suggestion that ligand binding to GMRα chain induces a conformational change in GMRα that is required for receptor activation but not necessarily required for high affinity binding (Shanafelt and Kastelein, 1992). Such a mechanism is not inconsistent with the properties of GM-CSF analogues described in this thesis however, the only GM-CSF analogue for which the postulation of such a mechanism appears to be necessary is the murine E21A analogue. Functional differences between murine and human GM-CSF analogues with mutations in the first α-helix may also be a result of species specificity. This possibility is at least consistent with the fact that the critical regions in the first α-helix of human and murine GM-CSF were also identified using human:murine GM-CSF chimeras, a method that is expected to reveal regions of species specificity (Kaushansky *et al.*, 1989; Shanafelt *et al.*, 1991b).

The Glu^{21} residue is absolutely conserved in GM-CSF from seven different species (Fig. 1.2.2) and an acidic residue is conserved at a structurally similar position in the first α-helix of many structurally related cytokines (Shanafelt *et al.*, 1991b; Rozwarski *et al.*, 1994). Sequence alignment of the first α-helix of GM-CSF and IL-5 together with the first predicted α-helix of IL-3 (Goodall *et al.*, 1993; Rozwarski *et al.*, 1994) identified glutamic acid residues in the first α-helix of IL-3 (Glu^{22}) and IL-5 (Glu^{13}) that aligned with Glu^{21} of GM-CSF. Mutagenesis of Glu^{22} in IL-3 greatly reduced the biological activity and high affinity receptor (IL-3Rαβ_c) binding of IL-3 but did not affect binding to the low affinity IL-3 receptor (IL-3Rα) (Barry *et al.*, 1994). Direct evidence for the role of Glu^{13} in IL-5 activity is not available although Shanafelt *et al.* (1991b) have demonstrated that the first predicted α-helix of murine IL-5 is able to mediate a functional interaction with the murine β_c chain, AIC2B. Thus GM-CSF, IL-3 and IL-5 interaction with the β_c chain involves residues in the first α-helix and at least in the case of GM-CSF and IL-3, the conserved glutamic acid plays a critical role in this interaction. A critical receptor binding site has also been identified for IL-2 and IL-4 that

includes a conserved acidic residue in the first α-helix (Collins *et al.*, 1988; Zurawski and Zurawski, 1989; Zurawski *et al.*, 1993; Kruse *et al.*, 1993).

Scanning-mutagenesis of residues located on the surface of the first α-helix revealed the unique functional sensitivity of residue 21 (Fig. 4.2.4). Despite tolerating a variety of different amino acids without drastically influencing biological activity, a number of the surface exposed residues within the first α-helix are likely to contribute to GM-CSF activity. Residue 20 was identified as functionally important for human GM-CSF activity but only in the context of analogues mutated at residue 21 (Fig. 3.3.5 & 3.3.6). The observation of a functional role for residue 20 in murine GM-CSF also appears to be dependent upon residue 21 mutation (Meropol *et al.*, 1992). Mutation of residue 20 in murine GM-CSF affects binding to both GMRα and GMRαβ_c, suggesting that this residue is a potential GMRα chain contact (Shanafelt and Kastelein, 1992). Interestingly, mutation of residue 21 in human IL-3, which is structurally homologous to GM-CSF residue 20, reduced the biological activity and binding to both IL-3Rα and IL-3Rαβ_c (Barry *et al.*, 1994). Thus, as was observed for murine GM-CSF, adjacent residues in the first α-helix of IL-3 appear to be required for the interaction with the α and β_c chains of the IL-3 receptor complex. This indicates that residue 20 of human GM-CSF may also contribute to GMRα chain binding although we have no direct evidence for this. The loss of biological activity and receptor binding observed for murine GM-CSF analogues mutated at residues 14 and 21 indicated that residue 14 is also functionally important (Meropol *et al.*, 1992) and contributes to β_c chain binding (Shanafelt and Kastelein, 1992). Thus despite tolerating a variety of substitutions, in the context of analogues mutated at residue 21 a functional role for residue 14 of human GM-CSF is also likely, perhaps via interaction with the GMRα chain. Mutations at residue 23 had only a modest influence on human GM-CSF activity (Fig. 4.2.4; Kaushansky *et al.*, 1989) however, the fact that this residue contributes to the binding epitope of a neutralising MoAb suggests that it is likely to be in close proximity to the receptor binding site (Brown *et al.*, 1990). Glycosylated GM-CSF possess a lower specific activity (Fig. 6.2.1) and receptor affinity (Table 6.2.1) than non-glycosylated

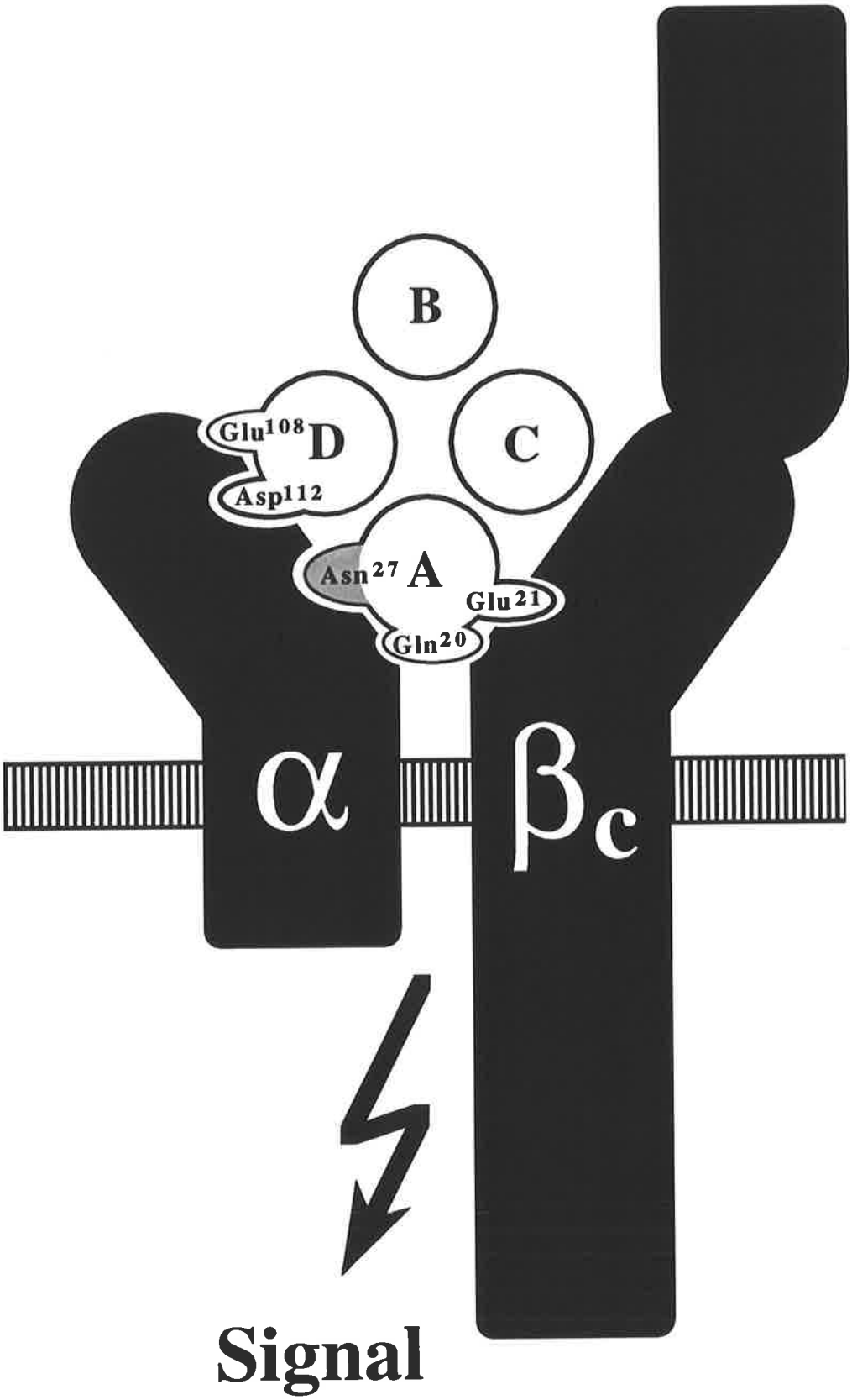
GM-CSF. Analogues mutated at residue 27, one of two N-linked glycosylation sites, exhibit reduced glycosylation and are consistently more active than wild type GM-CSF (Fig. 4.2.4) suggesting that the N-linked carbohydrate associated with residue 27 may influence receptor binding.

Initial examination of residues within the fourth α-helix of human GM-CSF identified two that contribute to GM-CSF activity and one, residue 112, that appears to be important for interaction with the GMRα chain (Fig. 3.3.11). The identification of functionally important residues within the fourth α-helix of human GM-CSF is consistent with a number of studies that have identified this region as being important for the biological activity of both human and murine GM-CSF (Shanafelt and Kastelein, 1989; Nice *et al.*, 1990; Seelig *et al.*, 1990; Kanakura *et al.*, 1991; Shanafelt *et al.*, 1991a). The C-terminus of murine GM-CSF, including the fourth α-helix, has been demonstrated to be important for interaction with the murine GMRα chain (Shanafelt *et al.*, 1991b). Despite being identified as a functionally important region, only one other study has identified a specific residue within the fourth α-helix that contributes to GM-CSF function. Thus residue 102 is important for the activity of murine GM-CSF (Shanafelt *et al.*, 1991a) and is structurally homologous to residue 105 of human GM-CSF which is located on the same face of the fourth α-helix as residue 112 (Fig. 3.1.2). Residues in the fourth α-helix are also important for the activity of the structurally and functionally related cytokines, IL-3 and IL-5. The use of interspecies chimeras demonstrated that residues spanning the fourth α-helix of IL-5 are important for biological activity and receptor binding (McKenzie *et al.*, 1991) while a human IL-3 analogue mutated in the region of the predicted fourth α-helix (D101A,K116V), exhibits increased biological activity and a 15-fold enhanced binding to the IL-3Rα chain (Lopez *et al.*, 1992b).

On the basis of the data presented in this thesis, a model describing the interaction of GM-CSF with its receptor is proposed (Fig. 7.2) that incorporates information presented in earlier models described in this thesis (Figures 3.4.3 and 6.3.1). In the

Figure 7.2 Model of the functionally important receptor contacts on the surface of GM-CSF.

The work presented in this thesis identified residues in the first and fourth α-helices of GM-CSF that are important for the biological activity and receptor binding properties of GM-CSF. Residue 21 is critical for β_c binding while residue 112 and to a lesser extent residue 108, appear likely to be GMRα chain contacts. Residue 20 is also functionally important although the receptor chain through which the influence of this residue is mediated was not identified. The possible influence of carbohydrate at residue 27 (shown in grey) on binding to the GMRα chain is included.


B
Glu108 D
Asp112
C
Asn27 A
Glu21
Gln20
α
βc
Signal

current model, residues in the first and fourth α-helices of GM-CSF interact with the GMRα chain while residues in the first α-helix also interact with the β_c chain. The critical roles of Asp^{112}, for interaction with the GMRα chain, and Glu^{21}, for interaction with the β_c chain, are indicated. In addition residue 108 is likely to contact the GMRα chain although it has only a very modest influence on biological activity while residue 20 may be able to interact with either the GMRα chain or the β_c chain or both. The possible influence of N-linked glycosylation at residue 27 on biological activity and binding to the GMRα chain is also indicated.

Although the work described in this thesis targeted residues in the first and fourth α-helix, a number of other studies have identified the third α-helix of GM-CSF as important for biological activity (Kaushansky *et al.*, 1989; Nice *et al.*, 1990; Brown *et al.*, 1990; Kanakura *et al.*, 1991; Shanafelt *et al.*, 1991a). Within the third α-helix of human GM-CSF, residue 78 has been identified as important for biological activity although only in the context of analogues mutated at residue 80 (Shanafelt *et al.*, 1991a) The location of the third α-helix and its proximity to the first α-helix in the three-dimensional structure indicates that contact with the receptor β_c chain is the most likely function of this region (Fig. 7.2).

Structure-function studies of GM-CSF have utilised a number of different methods such as epitope mapping of neutralising MoAbs, deletion and substitution mutagenesis and interspecies chimeras in an attempt to identify the regions and amino acids that define the functional interaction of GM-CSF with its receptor. Typically the receptor interacting regions are identified by a loss of function or receptor binding, although the loss of function may be a consequence of structural perturbations rather than a direct effect on the receptor interacting regions. Unfortunately, all of the methods used can potentially introduce structural perturbations which may therefore limit the conclusions that can be drawn from the functional data. Thus the binding of a neutralising MoAb may sterically hinder or structurally perturb the receptor binding site when bound at a distant and functionally irrelevant site on the ligand. Deletion or substitution mutagenesis may also

perturb the protein structure and hence influence function although substitution mutagenesis is the method least likely to introduce structural changes. Finally, interspecies chimeric analysis is essentially a multiple substitution approach but as this usually involves substitution of residues buried in the protein core, is considerably more likely to perturb protein folding. An additional problem with chimeric analysis is that functionally important residues that are conserved amongst species specific cytokines will not be identified and Glu^{21} of GM-CSF is an example of this problem. The functionally important regions of human and murine GM-CSF identified using the methods described above are summarised in Table 7.1. The functionally important regions span residues 14-31 and 37-127 and includes 86% of the mature human GM-CSF sequence! At least two factors are likely to contribute to the broad range of residues implicated in GM-CSF function. The first is the lack of precision mapping the binding epitopes of neutralising MoAbs which include residues 23 and 40-127. This is highlighted by the fact that residues 78-94 and 110-127 contain the epitopes for both neutralising and non-neutralising MoAbs (Kanakura *et al.*, 1991). The second factor is that regions identified by loss of biological activity or receptor binding as functionally important, may in fact be structurally important and not part of the receptor binding site.

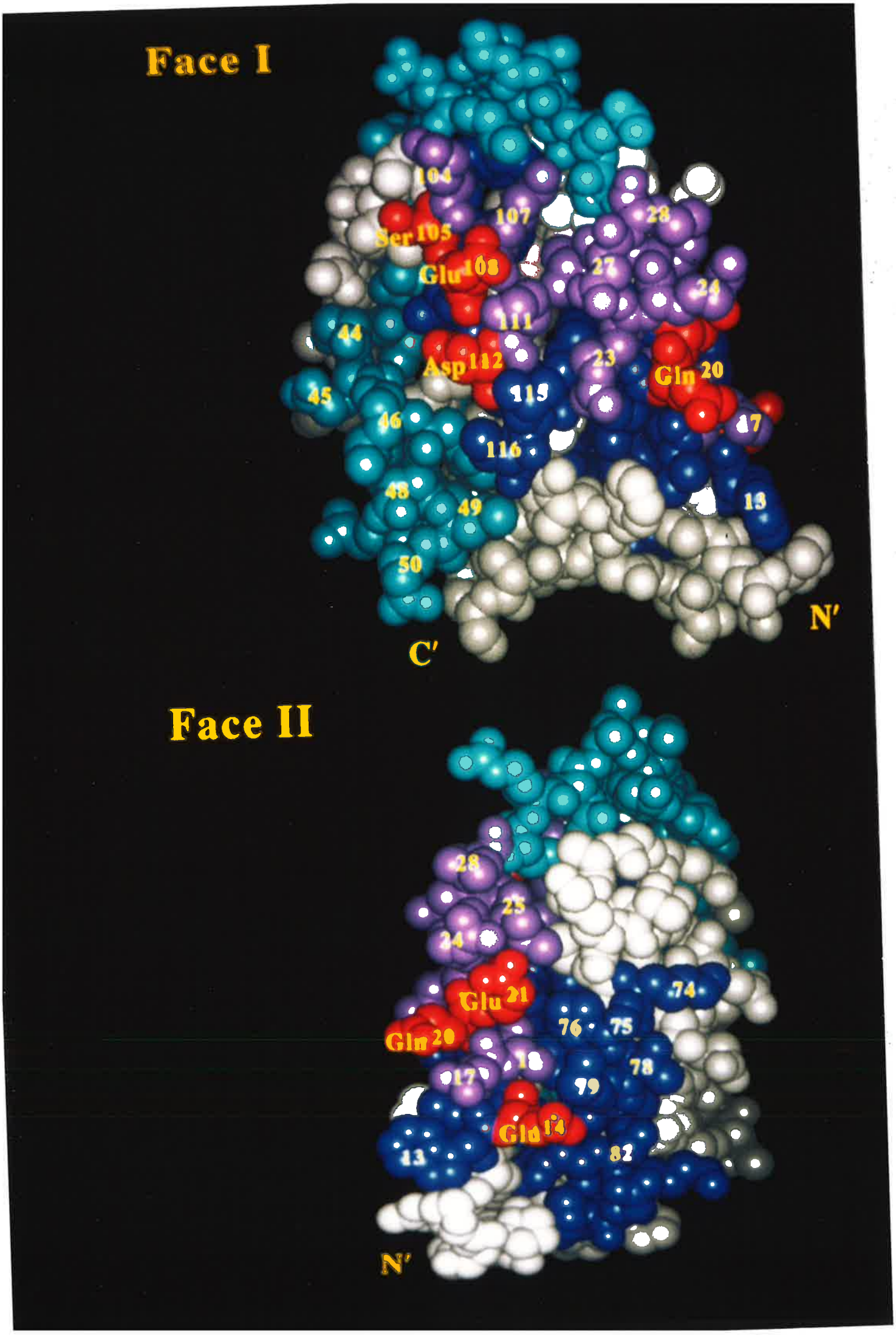
Comparison of all the structure-function data enables a GM-CSF consensus receptor binding site to be identified. The consensus receptor binding surface for GM-CSF contains regions identified as functionally important by at least three different techniques (Table 7.1). Data from human and murine GM-CSF studies was pooled for this comparison. The consensus receptor binding surface comprises residues in the first, third and fourth α-helices and appears likely to represent the minimum receptor binding surface of human and murine GM-CSF (Fig. 7.3). Additional receptor contacts by residues outside of the first, third and fourth α-helix consensus are quite likely (Table 7.1) but will require further investigation before inclusion in a more comprehensive receptor binding surface for GM-CSF. By comparison with the model describing the interaction of GM-CSF and its receptor complex (Fig. 7.2), Face I, comprising residues

Table 7.1 Structure-function studies on GM-CSF

Species	Functionally important residues	Reference
1. Epitope mapping of MoAbs		
Human	86-93, 112-127	Nice *et al.*, 1990
Human	23, 77-94	Brown *et al.*, 1990
Human	110-127	Seelig *et al.*, 1990
Human	40-77, 78-94, 110-127 neutralising	Kanakura *et al.*, 1991
	1-16, 78-94, 110-127 non-neutralising	
2. Deletion analysis		
Murine	11-15, 24-37, 47-49, 81-89	Gough *et al.*, 1987
Human	14-25	Clark-Lewis *et al.*, 1988
Murine	18-22, 34-41, 52-61, 94-115	Shanafelt and Kastelein, 1989
Murine	118-124	LaBranche *et al.*, 1990
Human	20-21, 55-60, 77-82, 89-120	Shanafelt *et al.*, 1991a
Human	20-21	Lopez *et al.*, 1992c
3. Substitution mutagenesis		
Murine	20 β_c contact	Shanafelt *et al.*, 1991b
Murine	21, 56, 60, 63, 107	Altmann *et al.*, 1991
Murine	14, 20, 21	Meropol *et al.*, 1992
Murine	14, 21 GMR$\alpha\beta_c$ binding	Shanafelt and Kastelein, 1992
	20 GMRα and GMR$\alpha\beta_c$ binding	
Human	20, 21	Lopez *et al.*, 1992c
	21 β_c contact	
Human	21 β_c contact	Hercus *et al.*, 1994a
Human	21, 108, 112	Hercus *et al.*, 1994b
	112 GMRα and GMR$\alpha\beta_c$ binding	
4. Interspecies chimeric GM-CSF		
Hum/Mur	21-31, 78-94	Kaushansky *et al.*, 1989
Human	43, 78, 80, 122	Shanafelt *et al.*, 1991a
Murine	92, 98, 102	"
Murine	17-22 β_c contact	Shanafelt *et al.*, 1991b
	103-124 GMRα contact	

Figure 7.3 The consensus receptor binding surface of GM-CSF.

Two views of the consensus receptor binding surface of GM-CSF as identified by structure-function studies of human and murine GM-CSF, can be considered to comprise two adjacent surfaces of GM-CSF. Face I comprises residues from the first and fourth α-helices as well as the loop connecting the first and second α-helices while Face II comprises residues from the first and third α-helices. Residues of the first, third and fourth α-helices which form the consensus receptor binding surface are coloured blue. Residues that appear to make functionally important contact with the receptor are coloured red while those that have not demonstrated a functionally important role are coloured magenta. Residues of the loop connecting the first and second α-helices are coloured cyan. Remaining residues not included in the consensus receptor binding surface of GM-CSF, are coloured white.


Face I
104
107
28
Ser 105
Glu 108
27
24
111
44
Asp 112
23
Gln 20
45
115
46
17
116
48
49
13
50
C′
N′
Face II
28
25
24
Glu 21
74
Gln 20
76
75
19
78
17
79
Glu 14
13
82
N′

of the first and fourth α-helices, is the GMRα chain binding site while Face II, comprising residues of the first and third α-helices, is the β_c chain binding site.

Human growth hormone represents the only member of the cytokine family for which the structure of the hormone:receptor complex has been resolved (de Vos *et al.*, 1992). Analysis of the interaction between growth hormone and its binding protein provides a paradigm for understanding the hormone:receptor interactions of other members of the cytokine family. Growth hormone has a core structure composed of four α-helices and although the helices are longer than those found in the short-chain helical cytokines, the unusual connectivity of the helical cytokines is preserved (Abdel-Meguid *et al.*, 1987; de Vos *et al.*, 1992). The extracellular portion of the growth hormone binding protein consists of two domains, each composed of seven β strands in an antiparallel sandwich and with a topology identical to the D2 domain of CD4 and the chaperone protein PapD (de Vos *et al.*, 1992). The structure of the growth hormone binding protein is very similar to that previously predicted for members of the cytokine receptor family including the GHbp (Bazan, 1990b).

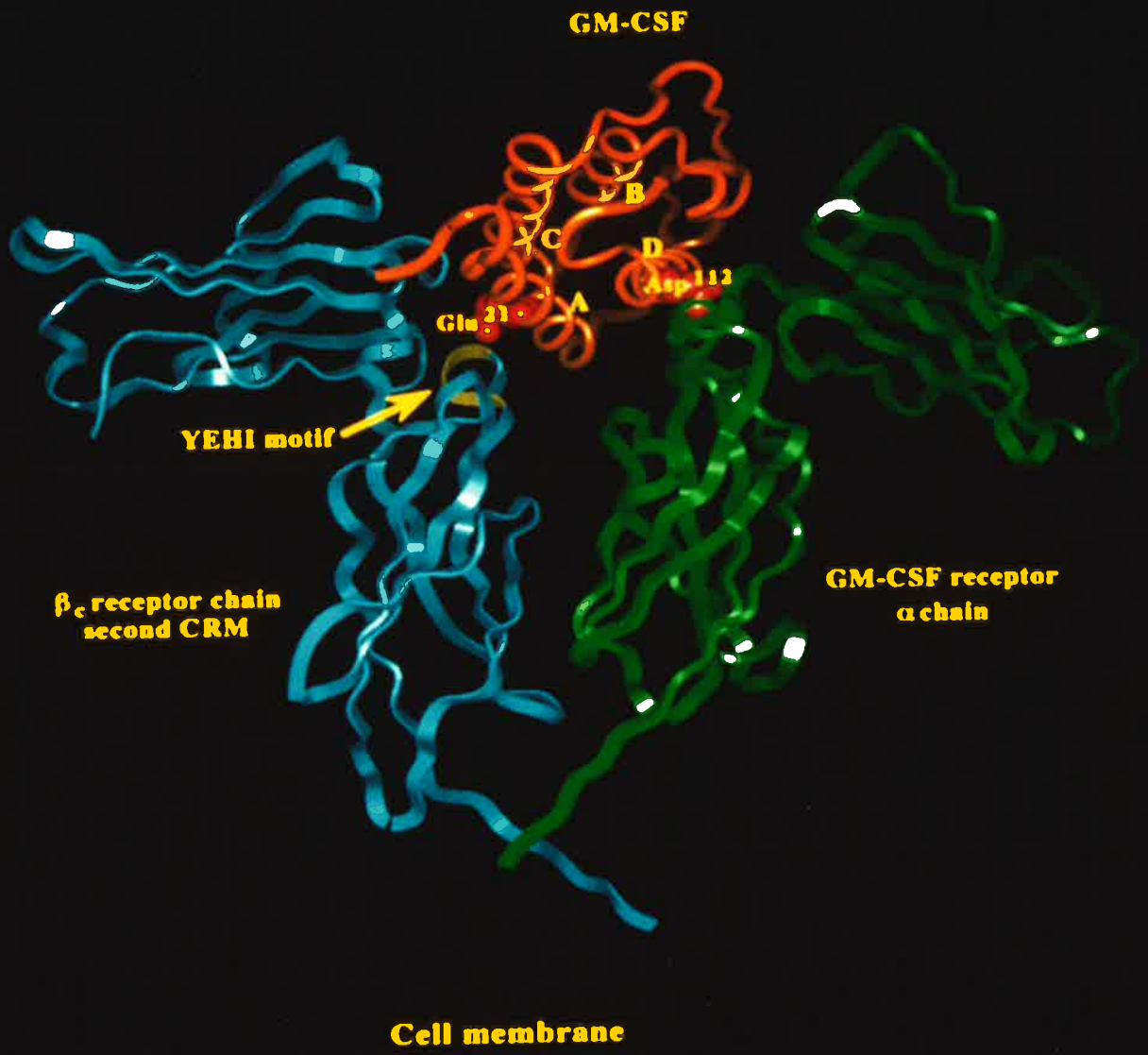
Biophysical measurements and mutagenesis studies identified two receptor binding sites on GH with site 1 comprising residues from the first and fourth α-helices together with residues from the loop between the first and second α-helices and site 2 comprising residues from the first and third α-helices (Cunningham *et al.*, 1989; Cunningham and Wells, 1989; Cunningham *et al.*, 1991). The only receptor binding region identified on growth hormone but not included in the receptor binding surface of GM-CSF (Fig. 7.3) is the loop between the first and second α-helices (residues 28-55). The binding epitope of a neutralising MoAb maps to residues 40-77 of human GM-CSF (Kanakura *et al.*, 1991), overlapping the loop between the first and second α-helices so that this region may also form part of the receptor binding surface for GM-CSF. These studies also demonstrated that growth hormone binds to two molecules of the GHbp to form a GH:$(GHbp)_2$ complex (Cunningham *et al.*, 1991). Analysis of the crystallised GH:$(GHbp)_2$ complex, demonstrated that the binding of growth hormone to its receptor

buried 1230 Å^2 through the site 1 interaction and 900 Å^2 through the site 2 interaction (de Vos *et al.*, 1992). Interaction between the membrane-proximal domains of the two receptor molecules buried a further 500 Å^2. The majority of amino acids identified in GH or the GHbp as functionally important by mutagenesis studies, are located in the hormone:receptor interface and are classified as direct binding determinants (de Vos *et al.*, 1992). Interestingly all of the amino acids specifically identified as important or at least potentially involved in GM-CSF receptor binding and biological activity, residues 14, 20, 21, 23, 27, 78, 105, 108 and 112, aligned with amino acids in growth hormone that contact the two molecules of the growth hormone binding protein (Goodall *et al.*, 1993). Formation of the GH:$(GHbp)_2$ complex occurs through a sequential binding mechanism (Cunningham *et al.*, 1991). Thus growth hormone binds molecule 1 of GHbp through site 1 and this complex is then able to bind molecule 2 of GHbp through site 2 and an interaction of the membrane proximal domains of GHbp1 and GHbp2. It has been proposed that the binding of growth hormone to the second receptor molecule through site 2 is stabilised by the interaction of the membrane-proximal domains of the two receptor molecules (de Vos *et al.*, 1992). An analogue of growth hormone in which the second receptor binding site is mutated but the first binding site remains active, was unable to dimerise the GHbp and functioned as an antagonist of wild type growth hormone activity (Fuh *et al.*, 1992). The properties of the GH antagonist closely resemble the properties of the GM-CSF antagonists described in Chapter 6.

A model illustrating the interaction of GM-CSF with the high affinity GM-CSF receptor complex (Fig. 7.4) has been generated using the GH:$(GHbp)_2$ complex as a starting point for molecular modelling (model generated by Dr C.Bagley). In this model GM-CSF is located in a binding groove formed by the juxtaposition of the GMRα and β_c chains and with residues from both domains of the cytokine receptor modules contributing to the ligand binding sites. By comparison with the interaction of growth hormone and its binding protein, Face I of GM-CSF binds the GMRα chain and is the equivalent of the site 1 interaction with GHbp1 while Face II of GM-CSF binds the β_c chain and is the equivalent of the site 2 interaction with GHbp2. The model illustrates the

Figure 7.4 Model for the interaction of GM-CSF with the GM-CSF receptor complex ($GMR\alpha\beta_c$).

A model illustrating the proposed interaction of GM-CSF with the high affinity GM-CSF receptor complex, $GMR\alpha\beta_c$, based on the interaction of growth hormone and its binding protein (model generated by Dr C.Bagley). Ribbon traces of the membrane proximal cytokine receptor modules from both the GMRα chain, shown in green and the β_c chain, shown in blue, were modelled using the co-ordinates for the growth hormone binding protein to define the structurally conserved β strands (de Vos *et al.*, 1992). A region of the β_c chain (Tyr^{365}-Ile^{368}) that is important for the high affinity binding of GM-CSF and appears to directly interact with the adjacent residue 21 of GM-CSF, is shown in yellow (Woodcock *et al.*, 1994). The GM-CSF structure was aligned to the position occupied by growth hormone and is shown in orange. The heavy atoms of residues Glu^{21} and Asp^{112} which are critical for the interaction of GM-CSF with the receptor chains are shown as red spheres.


GM-CSF
B
C
D
Asp 113
Glu 21
YEHI motif
β_c receptor chain
second CRM
GM-CSF receptor
α chain
Cell membrane

relative proximity of residues within GM-CSF to the receptor chains and also indicates which regions of the receptor molecules might directly participate in ligand binding. Features of the receptor chains that cannot be studied using this model are the influence of the N-terminal domain of the GMRα chain or the second cytokine receptor module of the β_c chain on GM-CSF binding.

A region of the GM-CSF receptor β_c chain including His^{367}, has been identified as critical for the high affinity binding of GM-CSF as well as for the mediation of maximal biological activity (Lock *et al.*, 1994). This region of the β_c chain is located adjacent to Glu^{21} of GM-CSF (Fig. 7.4) and also participates in the high affinity binding of IL-3 and IL-5 to their respective receptor complexes while mutations at residues Tyr^{365}, His^{367} and Ile^{368} of the β_c chain are able to complement the binding defects of the GM-CSF E21R analogue and partially restore high affinity binding (Woodcock *et al.*, 1994). Thus it appears likely that residue 21 of human GM-CSF directly interacts with the β_c chain of the GMR$\alpha\beta_c$ receptor complex through residues in the vicinity of His^{367}.

GM-CSF expressed from cultured human lymphocytes (Donahue *et al.*, 1986a; Cebon *et al.*, 1990) and recombinant GM-CSF expressed from eukaryotic cells (Kaushansky *et al.*, 1987) is extensively glycosylated. Glycosylation is not required for the activity of wild type GM-CSF and in fact reduces the specific biological activity (Moonen *et al.*, 1987; Fig. 6.2.1) and the high- (GMR$\alpha\beta_c$) and low-affinity (GMRα) receptor binding (Cebon *et al.*, 1990; Table 6.2.1) of GM-CSF. In general, the relative biological activity and receptor binding properties of glycosylated and, *E. coli*-derived, non-glycosylated GM-CSF residue 21 analogues were comparable. However, the non-glycosylated E21R and E21K analogues were devoid of activity and yet were able to bind the GMRα chain with wild type low affinity. This demonstrated that low affinity binding can be dissociated from receptor activation and biological signalling. The lack of activity for the non-glycosylated E21R analogue was in contrast to the reduced but measurable activity observed for the glycosylated E21R analogue. It has not been determined why the two analogues exhibit different levels of biological activity although

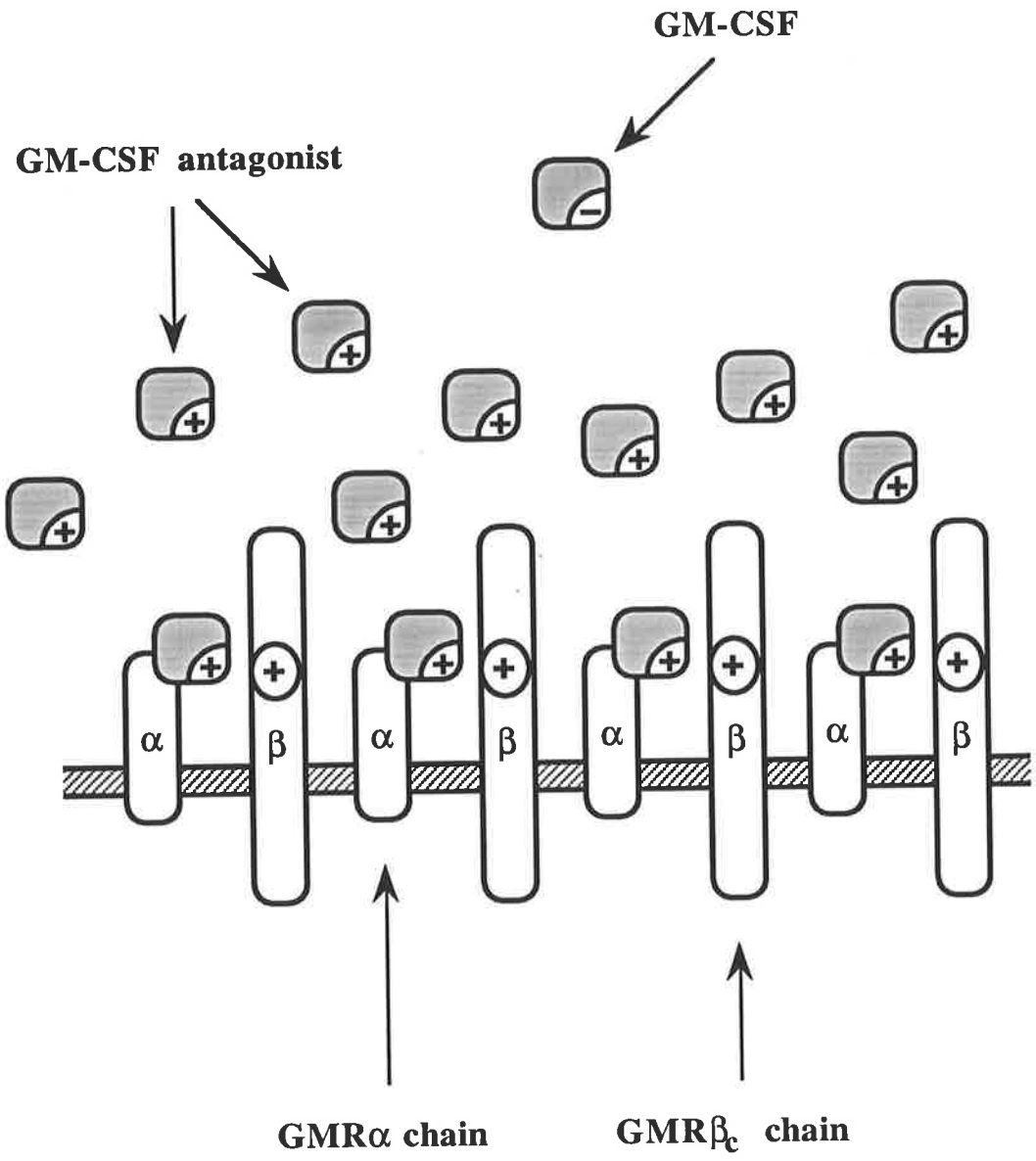
it appears that the O-linked carbohydrate may be sufficient to impart agonistic activity to the glycosylated E21R analogue (Fig. 6.2.6).

The work presented in Chapter 6 described the properties of the first GM-CSF antagonist (Hercus *et al.*, 1994a). The *E. coli*-derived GM-CSF analogues, E21R and E21K, were devoid of biological activity and yet exhibited wild type binding affinity for the low affinity receptor (GMRα). These analogues were demonstrated to be effective antagonists of the GM-CSF-mediated proliferation of leukaemic cells and the GM-CSF-mediated stimulation of neutrophil superoxide release (Fig. 6.2.16). The specificity of this antagonism was illustrated by the fact that eco E21R was unable to antagonise the IL-3 mediated proliferation of TF-1 cells (Fig. 6.2.8B) or the TNF-α-mediated stimulation of neutrophil superoxide release (Fig. 6.2.12B). Importantly, the eco E21R and E21K analogues were also effective antagonists of glycosylated GM-CSF species (Fig. 6.2.17) suggesting the potential use of these antagonists *in vivo* to block the activity of endogenous, glycosylated GM-CSF. The receptor binding properties of the GM-CSF antagonists (Table 6.2.3) suggested that these analogues block wild type GM-CSF activity by sequestration of all of the available GMRα chains into complexes that are unable to interact with the β_c chain (Fig. 7.5). In the absence of unoccupied GMRα chains, the wild type GM-CSF is prevented from binding and hence eliciting a biological response. The structural and functional similarities displayed by GM-CSF, IL-3 and IL-5 suggests that functionally similar antagonists of both IL-3 and IL-5 may be generated by charge-reversal mutation of the conserved acidic residue in the first α-helix. The fact that the IL-3 E22R analogue exhibited biological activity, albeit at a 20,000-fold reduced potency, demonstrated that in the case of IL-3, residues other than the conserved acidic residue make functionally relevant contacts with the β_c chain and may need to mutated in conjunction with residue 22 to generate an antagonistic analogue (Barry *et al.*, 1994).

Cytokine analogues with antagonistic properties have also been described for other members of the cytokine family. Thus an analogue of human IL-6 with wild type affinity for the IL-6Rα chain but impaired binding to gp130, which is functionally equivalent to

Figure 7.5 Model for GM-CSF antagonist action.

The model proposed to account for the activity of the GM-CSF antagonists. The GM-CSF antagonists, eco E21R and E21K, retain wild type binding affinity for the GMRα chain but are apparently unable to interact with the β_c chain. Exposure of cells expressing the GM-CSF receptor complex (GMR$\alpha\beta_c$) to saturating levels of these analogues, occupies the GMRα chain in a complex that is unable to interact with the β_c chain and therefore signal. In the presence of such an excess of GM-CSF antagonist, wild type GM-CSF at a concentration normally able to stimulate a maximal biological response, is unable to bind and is therefore prevented from stimulating a biological response.



No biological signal

the GM-CSF receptor β_c chain, is able to antagonise wild type IL-6 function (Savino *et al.*, 1994). In a similar manner, analogues of murine IL-2 (Zurawski *et al.*, 1990; Zurawski and Zurawski, 1992) or human IL-4 (Kruse *et al.*, 1992) with impaired binding to the γ_c chain shared by the receptors for IL-2 and IL-4 (Kondo *et al.*, 1993; Russell *et al.*, 1993), are specific antagonists of the appropriate wild type cytokine function. The recurring theme amongst the cytokine antagonists is one of selective interaction with the ligand binding but not the receptor activation components of a multi-subunit receptor complex.

A feature of the GM-CSF antagonists was that the typical IC_{50} values of around 200ng/ml, were considerably higher than the 15ng/ml (1nM) required to achieve 50% occupancy of the GMRα chain (Fig. 6.2.15). The IC_{50} value presumably reflects the level of GMRα chain occupancy by antagonist required to block 50% of the wild type GM-CSF signalling. The relatively high IC_{50} values for the GM-CSF antagonists indicates that approximately 90% of the GMRα chain needs to be occupied by antagonist for effective blocking of the signal from wild type GM-CSF. The required excess of antagonist was consistent with reports that maximum biological effects of colony stimulating factors can be exerted with steady state receptor occupancy levels as low as 10% (Nicola *et al.*, 1988). Although the antagonists and wild type GM-CSF exhibit similar affinities for the GMRα chain (Table 6.2.3), the ability of wild type GM-CSF to interact with the β_c chain and form a high affinity complex is probably critical for the biological signalling mediated by low concentrations of wild type GM-CSF. GM-CSF antagonists with enhanced GMRα chain binding, may function as better antagonists and exhibit lower IC_{50} values by competing more effectively with wild type GM-CSF for the GMRα chain.

The effectiveness of the GM-CSF antagonists is dependent upon their affinity for the GMRα chain and it seems likely that β_c chain defective antagonists for IL-3 and IL-5 would be similarly influenced. The affinity of IL-3 for the IL-3Rα chain (K_d=30nM) is considerably lower than the affinity of GM-CSF for the GMRα chain (K_d=1nM). This

suggests that an IL-3 antagonist using the mechanism described for GM-CSF (Fig. 7.5) would possibly require significantly higher concentrations of IL-3 antagonist to achieve equivalent IC_{50} responses. Thus a typical IC_{50} value for the IL-3 antagonists might be expected to be 6,000ng/ml compared with 200ng/ml for the GM-CSF antagonists. The somewhat higher affinity of IL-5 for the IL-5Rα chain (K_d=0.5nM) suggests that an IL-5 antagonist using the mechanism described for GM-CSF (Fig. 7.5) might exhibit IC_{50} values of around 100ng/ml.

The excess of antagonist over agonist required for 50% inhibition of biological activity exhibited considerable variation. The values range from 5,000-fold excess to achieve 50% inhibition of eco GM-CSF mediated TF-1 cell proliferation to 50-fold excess to achieve 50% inhibition of cho GM-CSF mediated neutrophil superoxide release (Table 7.2). This probably reflects differences in the ED_{50} values of wild type GM-CSF in these biological assays which range from 0.017ng/ml for eco GM-CSF stimulation of TF-1 cell proliferation to 0.53ng/ml for cho GM-CSF stimulation of neutrophil superoxide release (Table 7.2). The difference can partially be accounted for by the effect of the carbohydrate on GM-CSF activity as eco GM-CSF exhibited a 5- to 10-fold higher potency (lower ED_{50}) than cho GM-CSF. However, there are real differences in the concentration of GM-CSF required to trigger receptor activation in different cell types as there is a 3- to 5-fold difference in the ED_{50} values of neutrophil activation and TF-1 cell proliferation (Table 7.2) using the same form of GM-CSF. One possible explanation for this difference in the ED_{50} values is that the proliferative response of TF-1 cells and the release of superoxide anions by neutrophils occur through different pathways, utilising different signalling components and with different receptor occupancy thresholds. Alternatively, the presence of different forms of the receptor molecules with cytoplasmic domain variants (Crosier *et al.*, 1991) or the presence of additional signalling chains in the GM-CSF receptor complex of certain cell types, might explain the differences in ED_{50} values. The presence of a third receptor chain has been demonstrated for the IL-2 receptor complex (Takeshita *et al.*, 1992).

Table 7.2 **Comparison of ED_{50} values for wild type GM-CSF and the antagonist excess at IC_{50}, for TF-1 cells and neutrophils.**

	Neutrophil superoxide release	TF-1 cell proliferation
A) ED_{50} GM-CSF (ng/ml)[a]		
eco GM-CSF	0.09 ± 0.02[b] n=10	0.017 ± 0.004 n=13
cho GM-CSF	0.53 ± 0.074 n=12	0.16 ± 0.05 n=4
B) Fold excess of antagonist (E21R or E21K)[c]		
eco GM-CSF	514 ± 330 n=5	4,950 ± 1,120 n=6
cho GM-CSF	50[d] n=2	120 ± 39 n=3

[a] The ED_{50} values for wild type GM-CSF derived from *E. coli* (eco GM-CSF) or CHO cells (cho GM-CSF) was determined in the TF-1 cell proliferation assay and the neutrophil superoxide anion release assay.

[b] Error values are the standard error of the mean (SEM).

[c] The antagonist excess for eco E21R and eco E21K was determined at the IC_{50} value against both eco GM-CSF and cho GM-CSF in the TF-1 cell proliferation assay and the neutrophil superoxide anion release assay. The values are expressed as an excess of antagonist over agonist and the data from both antagonists pooled as the two analogues are essentially equipotent GM-CSF antagonists.

[d] No SEM calculated because only two data points.

Apart from the clinical potential, the generation of GM-CSF analogues devoid of interaction with the β_c chain but retaining wild type affinity for the GMRα chain provides an opportunity to specifically examine the functional role of the GMRα chain. It has recently been demonstrated that GM-CSF binding to the human GMRα chain expressed on *Xenopus laevis* oocytes, caused activation of 2-deoxyglucose transport through endogenous glucose transporters (Ding *et al.*, 1994). Importantly, GM-CSF is also able to stimulate 2-deoxyglucose uptake in neutrophils under conditions that prevented the tyrosine phosphorylation normally associated with GM-CSF receptor activation thereby directly implicating the GMRα chain in glucose transport. The GM-CSF antagonist provides the ideal reagent for specifically testing the role of the GMRα chain in activating glucose transport in cell types such as neutrophils that also express the β_c chain.

It is now evident that GM-CSF activity is not essential for steady state haemopoiesis as mice homozygous for a disrupted GM-CSF gene develop a normal haemopoietic population (Dranoff *et al.*, 1994; Stanley *et al.*, 1994). However, GM-CSF deficient mice do develop a non-fatal lung condition, similar to forms of alveolar proteinosis, characterised by an accumulation of lipids and surfactant protein (Dranoff *et al.*, 1994) and the presence of opportunistic bacterial and fungal infections (Stanley *et al.*, 1994) within the alveoli. M-CSF modulates the consequences of GM-CSF deficiency as mice deficient in both GM-CSF and M-CSF develop a much more severe lung disease that is often fatal (Lieschke *et al.*, 1994). The *in vivo* role of the GMRα chain might be better understood by exposing GM-CSF-deficient mice to GM-CSF analogues that specifically bind to the GMRα chain only. It is therefore of great interest that a murine GM-CSF antagonist has been developed that is functionally similar to the human GM-CSF antagonists described in this thesis (R. Kastelein personal communication). Thus the functional role of the GMRα chain in GM-CSF-deficient mice might be studied by intra venous administration of the murine GM-CSF antagonist or by generating transgenic mice, deficient in wild type GM-CSF but expressing the murine GM-CSF antagonist.

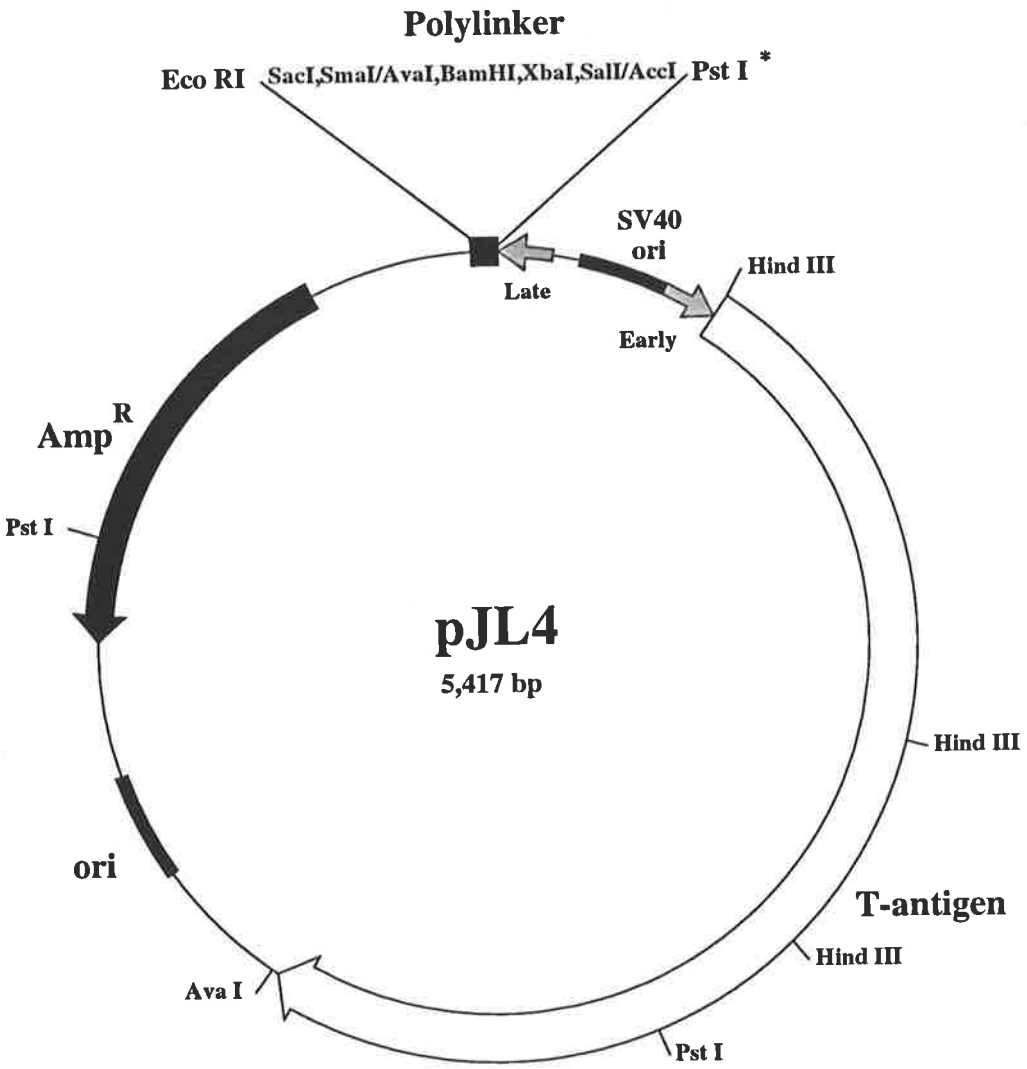
The work discussed in this chapter describes some of the structure-function properties of GM-CSF and other members of the cytokine family. It is now quite apparent that the principal features of cytokine structure and function are remarkably similar amongst a family of proteins exhibiting very little amino acid homology and a vast range of biological activities. This functional similarity amongst the helical cytokines should assist in the identification of functionally important regions and enable the generation of antagonistic analogues for all the members of the helical cytokine family.

Appendices

Appendix A.3.2.1 Map of the pJL4 expression vector.

The pJL4 expression vector (Gough *et al.*, 1985) was used to transiently express wild type GM-CSF and analogues from COS cells. COS cells are a monkey kidney cell line transformed by SV40. The presence of the SV40 origin of replication enables the pJL4 plasmid to replicate in COS cells. Expression of GM-CSF sequences cloned into the polylinker, is under the control of the SV40 late promoter.

Restriction sites in the polylinker that are not unique are marked with an asterisk.


Polylinker
Eco RI
SacI,SmaI/AvaI,BamHI,XbaI,SalI/AccI
Pst I *
SV40
ori
Late
Early
Hind III
Amp R
Pst I
pJL4
5,417 bp
Hind III
ori
T-antigen
Hind III
Ava I
Pst I

Appendix A.3.3.1 GM-CSF mutagenic oligonucleotides

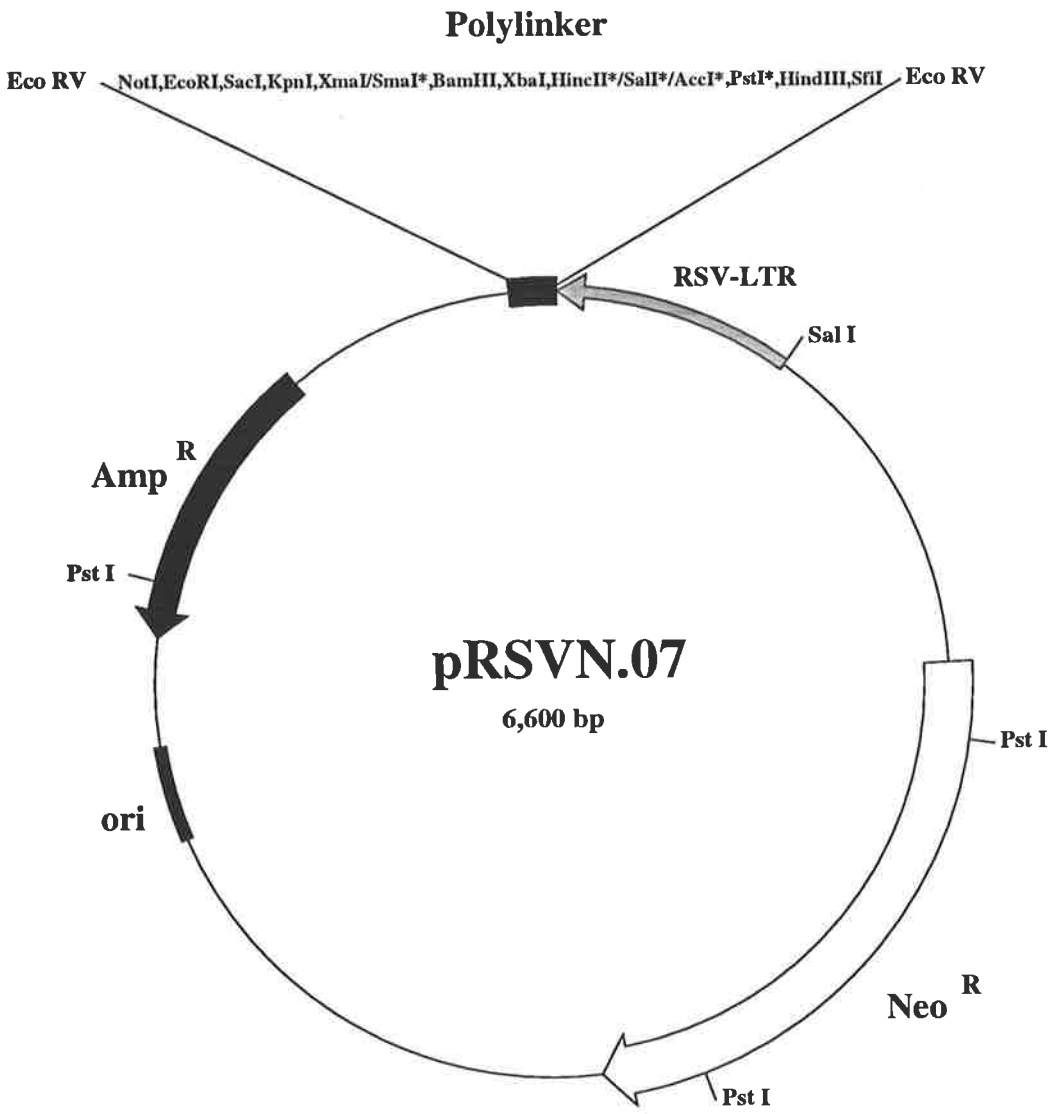
Hyphen = deleted region, **lower case** = mutated residues

Name	Mutation	Size	Sequence
OL-9	Δ1-24	32	5' GCCTGCAGCATCTCT -- CTCCTGAACCTGAGTAG 3'
OL-10	Δ7-24	31	5' CCCGCCCGCTCGCCC -- CTCCTGAACCTGAGTA 3'
OL-11	Δ14-18	30	5'AGCACGCAGCCCTGG -- ATCCAGGAGGCCCGG 3'
OL-12	Δ14-24	32	5' AGCACGCAGCCCTGG -- CTCCTGAACCTGAGTAG 3'
OL-45	Δ20-21	20	5' GAATGCCATC -- GCCCGGCGTC 3'
OL-42	Ala^{20}	18	5' TGCCATC**gcc**GAGGCCCG 3'
OL-43	Ala^{21}	18	5' CATCCAG**gcc**GCCCGGCG 3'
OL-31	Arg^{21}	18	5' CCATCCAG**cgt**GCCCGGC 3'
OL-44	$Ala^{20,21}$	24	5' AATGCCATC**gccgcc**GCCCGGCGT 3'
OL-32	$Ala^{20}Arg^{21}$	21	5' TGCCATC**gcccgt**GCCCGGCG 3'
OL-37	Asp^{107}	18	5' AAGTTTC**gac**GAGAACCT 3'
OL-38	Arg^{108}	18	5' TTTCAAA**cgt**AACCTGAA 3'
OL-39	Asp^{111}	18	5' AGAACCTG**gac**GACTTTC 3'
OL-40	Arg^{112}	18	5' CCTGAAG**cgt**TTTCTGCT 3'
OL-51	Ala^{54}	15	5' CCGACC**gcc**CTACAG 3'
OL-52	Ala^{88}	15	5' CAGCAC**gcc**CCTCCA 3'
OL-53	Ala^{96}	15	5' ACTTCC**gcc**GCAACC 3'
OL-54	Ala^{121}	21	5' CCCTTTGAC**gcc**TGGGAGCCA 3'
GM-55	Ser^{88}	19	5' AAGCAGCAC**agc**CCTCCAA 3'
GM-56A	Ser^{121}	22	5' ATCCCCTTTGAC**agc**TGGGAGC 3'

Appendix A.3.3.2 Map of the pRSVN.07 expression vector.

The pRSVN.07 expression vector (Dr A.Robbins, Department of Biochemistry, University of Adelaide) was used to express GM-CSF analogues from permanently transfected CHO cell lines. CHO cells are a chinese hamster ovary cell line. The presence of the neomycin resistance gene enables CHO cells harbouring pRSVN constructs to be selected with geneticin. Expression of GM-CSF sequences cloned into the polylinker, is under the control of the RSV-LTR.

Restriction sites in the polylinker that are not unique are marked with an asterisk.

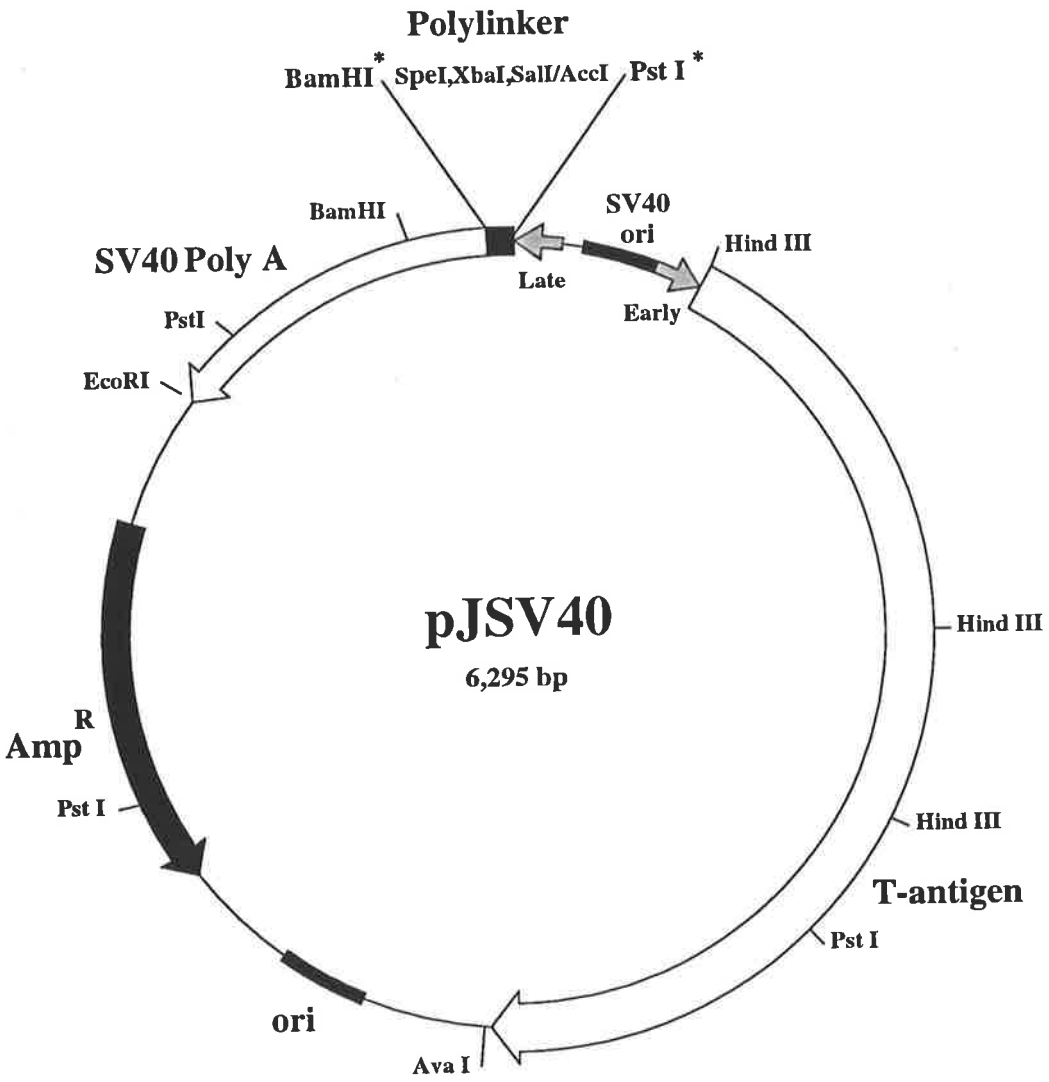

Polylinker
Eco RV
NotI,EcoRI,SacI,KpnI,XmaI/SmaI*,BamHI,XbaI,HincII*/SalI*/AccI*,PstI*,HindIII,SfiI
Eco RV
RSV-LTR
Sal I
Amp R
Pst I
pRSVN.07
6,600 bp
Pst I
ori
Neo R
Pst I

Appendix A.3.3.3 Map of the pJSV40 expression vector.

The pJSV40 expression vector was created by cloning an SV40 poly (A)-addition into the pJL4 expression vector (A.3.2.1) and was used to transiently express wild type GM-CSF and analogues from COS cells. The properties of this plasmid are essentially the same as for the pJL4 expression vector.

An EcoRI/HpaI fragment containing the SV40 polyadenylation sequence from the pSV2CAT plasmid (Gorman *et al.*, 1982), was subcloned into SmaI/EcoRI digested pBluescript SK(-) (Phillips *et al.*, 1989). Subsequently, the SV40 polyadenylation sequence was isolated as an XbaI/EcoRI fragment and cloned into XbaI/EcoRI digested pJL4 to generate the pJSV40 expression vector.

Restriction sites in the polylinker that are not unique are marked with an asterisk.


Polylinker
BamHI *
SpeI,XbaI,SalI/AccI
Pst I *
BamHI
SV40 Poly A
PstI
EcoRI
SV40
ori
Late
Early
Hind III
pJSV40
6,295 bp
Hind III
R
Amp
Pst I
Hind III
T-antigen
Pst I
ori
Ava I

Appendix A.4.2.1 GM-CSF mutagenic oligonucleotides

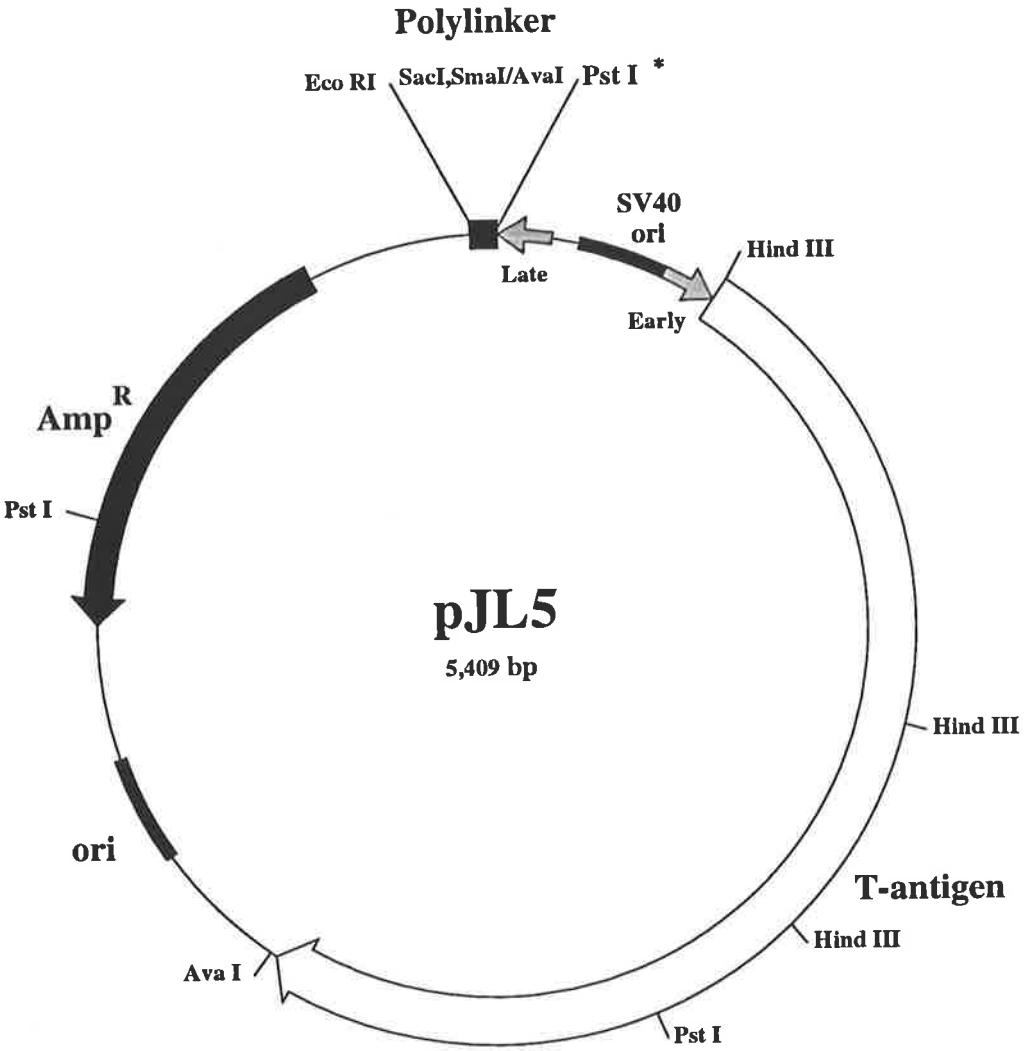
Lower case = mutated residues, underline = restriction enzyme sites

Name	Mutation	Size	Sequence
OL-256	Hairpin minus	26	5' GGCCCGGCGTCT**gt**TGAACCTGAGTA 3'
MI-2	SalI$^{(90)}$	22	5' GCCCAGCCC**gtcg**ACGCAGCCC 3'
MI-3	SalI$^{(134)}$	22	5' GGAGGCCCGtCGaCTCCTGAAC 3'
MI-5	XbaI$^{(150)}$	22	5' GTTGAACCTG**tc**TAGAGACACT 3'
OL-36	Asp^{24}	18	5' AGGCCCGG**gac**CTCCTGA 3'

Appendix A.4.2.2 Map of the pJL5 expression vector.

The pJL5 expression vector was created by digestion of the pJL4 expression vector (Appendix A.3.2.1) with BamHI and SalI, incubated with Klenow fragment to blunt-end the restriction sites and religation. Thus pJL5 is a $BamHI^-$, $XbaI^-$ and $SalI^-$ version of pJL4 with the two plasmids showing essentially the same properties. The GM-CSF cDNA's mutated to carry unique SalI or XbaI sites flanking the first α-helix, were cloned into the SmaI site to create the pJGMV series of plasmids used for OCM and the transient expression GM-CSF mutant analogues from COS cells.

Restriction sites in the polylinker that are not unique, are marked with an asterisk.


Polylinker
Eco RI
SacI,SmaI/AvaI
Pst I *
SV40
ori
Late
Early
Hind III
Amp R
Pst I
pJL5
5,409 bp
Hind III
ori
T-antigen
Hind III
Ava I
Pst I

Appendix A.4.2.3 Plasmid constructs generated by OCM, for the expression of GM-CSF first helix mutants in COS cells.

The sequence of the OCM oligonucleotides is listed in Appendix A.4.2.4.

Code	Plasmid	Mutation/s	OCM oligonucleotides
M91	pJGM14.1	E14Q	14.12U/14.34L
M92	pJGM14.2	E14K	14.12U/14.34L
M93	pJGM14.3	E14A	14.12U/14.34L
M94	pJGM14.4	E14T	14.12U/14.34L
M81	pJGM17.1	N17E	17.1U/L
M82	pJGM17.2	N17D	17.1U/L
M83	pJGM17.3	N17A	17.1U/L
M84	pJGM17.4	N17K	17.4U/L
M85	pJGM17.6	N17S	17.4U/L
M86	pJGM17.7	N17T	17.4U/L
M46	pJGM18.1	A18N	18.123U/L
M47	pJGM18.2	A18D	18.123U/L
M48	pJGM18.3	A18K	18.123U/L
M49	pJGM18.4	A18E	18.123U/L
M50	pJGM19.1	I19Q	19.123U/L
M51	pJGM19.2	I19E	19.123U/L
M52	pJGM19.3	I19R	19.123U/L
M53	pJGM19.4	I19G	19.123U/L
M54	pJGM19.5	I19K	19.123U/L
M55	pJGM20.1	Q20K	20.12U/L
M56	pJGM20.2	Q20E	20.12U/L
M32	pJGM21.1	E21K	21.1U/L
M33	pJGM21.2	E21Q	21.2U/L
M34	pJGM21.3	E21S	21.3U/L
M35	pJGM21.4	E21D	21.4U/L
M58	pJGM21.5	E21N	21.56U/L
M59	pJGM21.6	E21T	21.56U/L
M60	pJGM22.1	A22N	22.123U/L
M61	pJGM22.2	A22D	22.123U/L
M62	pJGM22.3	A22K	22.123U/L
M39	pJGM23.1	R23L	23.1U/L
M63	pJGM23.2	R23S	23.23U/L + 35.U/L
M64	pJGM23.3	R23E	23.23U/L + 35.U/L
M65	pJGM23.4	R23K	23.23U/L + 35.U/L
M66	pJGM23.5	R23G	23.23U/L + 35.U/L
M67	pJGM23.6	R23D	23.67U/L + 35.U/L
M68	pJGM23.7	R23N	23.67U/L + 35.U/L
M36	pJGM24.1	R24S	24.1U/L + 35.U/L
M37	pJGM24.2	R24N	24.2U/L + 35.U/L
M38	pJGM24.3	R24E	24.3U/L + 35.U/L

Appendix A.4.2.3 (continued)

Code	Plasmid	Mutation/s	OCM oligonucleotides
M69	pJGM25.1	L25Q	25.123U/L + 35.U/L
M70	pJGM25.2	L25E	25.123U/L + 35.U/L
M71	pJGM25.3	L25K	25.123U/L + 35.U/L
M40	pJGM26.1	L26Q	26.1U/L
M41	pJGM26.2	L26E	26.2U/L
M42	pJGM26.3	L26K	26.3U/L
M43	pJGM27.1	N27D	27.1U/L
M44	pJGM27.2	N27K	27.2U/L
M87	pJGM27.3	N27S	27.3U/L
M88	pJGM27.4	N27R	27.3U/L
M89	pJGM27.5	N27A	27.5U/L
M90	pJGM27.6	N27T	27.5U/L
M72	pJGM28.2	L28K	28.1U/L
M73	pJGM28.3	L28M	28.1U/L
M74	pJGM28.4	L28G	28.1U/L
M75	pJGM28.6	L28V	28.1U/L
M76	pJGM28.7	L28Q	28.1U/L
M77	pJGM28.8	L28W	28.1U/L
M78	pJGM28.9	L28D	28.9U/L
M79	pJGM28.10	L28H	28.9U/L
M80	pJGM28.11	L28N	28.9U/L

Appendix A.4.2.4 GM-CSF oligonucleotides for OCM in the COS expression vector

Underline = restriction enzyme sites, **lower case** = mutated codons,
double underline = 5' overhang used for ligation of oligonucleotide fragments

Name	Mutation	Size	Sequence
14.12U	Gln^{14} Lys^{14}	44	5' TCGACGCAGCCCTGG**(ca)ag**CATGTGAATGCCATCCAGGAG GCCCG 3'
14.34L	Ala^{14} Thr^{14}	44	5' TCGACGGGCCTCCTGGATGGCATTCACATG**gg(ct)**CCAGGG CTGCG 3'
17.1U	Glu^{17} Asp^{17}	44	5' TCGACGCAGCCCTGGGAGCATGTG**g(ac)(gc)**GCCATCCAG GAGGCCCG 3'
17.1L	Ala^{17}	44	5' TCGACGGGCCTCCTGGATGGC**(cg)(tg)c**CACATGCTCCCA GGGCTGCG 3'
17.4U	Lys^{17} Arg^{17}	44	5' TCGACGCAGCCCTGGGAGCATGTG**a(agc)(gc)**GCCATCCAG GAGGCCCG 3'
17.4L	Ser^{17} Thr^{17}	44	5' TCGACGGGCCTCCTGGATGGC**(gc)(tcg)t**CACATGCTCCCA GGGCTGCG 3'
18.123U	Asn^{18} Asp^{18}	44	5' TCGACGCAGCCCTGGGAGCATGTGAAT**(ag)a(cg)**ATCCAG GAGGCCCG 3'
18.123L	Lys^{18} Glu^{18}	44	5' TCGACGGGCCTCCTGGAT**(gc)t(tc)**ATTCACATGCTCCCA GGGCTGCG 3'
19.123U	Gln^{19} Glu^{19} Arg^{19}	44	5' TCGACGCAGCCCTGGGAGCATGTGAATGCC**(cga)(ag)g**CAG GAGGCCCG 3'
19.123L	Gly^{19} Lys^{19}	44	5' TCGACGGGCCTCCTG**c(tc)(gct)**GGCATTCACATGCTCCCA GGGCTGCG 3'
20.12U	Lys^{20} Glu^{20}	44	5' TCGACGCAGCCCTGGGAGCATGTGAATGCCATC**(ag)ag**GAG GCCCG 3'
20.12L		44	5' TCGACGGGCCTC**ct(tc)**GATGGCATTCACATGCTCCCAGGG CTGCG 3'
21.1U	Lys^{21}	44	5' TCGACGCAGCCCTGGGAGCATGTGAATGCCATCCAG**aag**GCC CG 3'
21.1L		44	5' TCGACGGGC**ctt**CTGGATGGCATTCACATGCTCCCAGGGCTG CG 3'
21.2U	Gln^{21}	44	5' TCGACGCAGCCCTGGGAGCATGTGAATGCCATCCAG**cag**GCC CG 3'
21.2L		44	5' TCGACGGGC**ctg**CTGGATGGCATTCACATGCTCCCAGGGCTG CG 3'
21.3U	Ser^{21}	44	5' TCGACGCAGCCCTGGGAGCATGTGAATGCCATCCAG**agc**GCC CG 3'
21.3L			5' TCGACGGGC**gct**CTGGATGGCATTCACATGCTCCCAGGGCTG CG 3'

Appendix A.4.2.4 (continued)

Name	Mutation	Size	Sequence
21.4U	Asp^{21}	44	5' TCGACGCAGCCCTGGGAGCATGTGAATGCCATCCAGgacGCC CG 3'
21.4L		44	5' TCGACGGGCgtcCTGGATGGCATTCACATGCTCCCAGGGCTG CG 3'
21.56U	Asn^{21} Thr^{21}	44	5' TCGACGCAGCCCTGGGAGCATGTGAATGCCATCCAGa(ac)cGCC CG 3'
21.56L		44	5' TCGACGGGCg(gt)tCTGGATGGCATTCACATGCTCCCAGGGCTG CG 3'
22.123U	Asn^{22} Asp^{22}	44	5' TCGACGCAGCCCTGGGAGCATGTGAATGCCATCCAGGAG (ag)a(cg)CG 3'
22.123L	Lys^{22} Glu^{22}	44	5' TCGACG(gc)t(tc)CTCCTGGATGGCATTCACATGCTCCCAGGG CTGCG 3'
23.1U	Leu^{23}	61	5' TCGACGCAGCCCTGGGAGCATGTGAATGCCATCCAGGAG GCCctgCGTCTGTTGAACCTGT 3'
23.1L		61	5' CTAGACAGGTTCAACAGACGcagGGCCTCCTGGATGGCATT CACATGCTCCCAGGGCTGCG 3'
23.23U	Ser^{23} Glu^{23} Asp^{23} Gly^{23} Lys^{23}	26	5' GGAGGCC(ag)(ga)(cg)CGTCTGTTGAACCTGT 3'
23.23L	Asn^{23}	25	5' CTAGACAGGTTCAACAGACG(gc)(ct)(tc)GG 3'
23.67U	Asp^{23}	26	5' GGAGGCC(ag)acCGTCTGTTGAACCTGT 3'
23.67L	Asn^{23}	25	5' CTAGACAGGTTCAACAGACGgt(tc)GG 3'
24.1U	Ser^{24}	26	5' GGAGGCCCGGagcCTGTTGAACCTGT 3'
24.1L		25	5' CTAGACAGGTTCAACAGgctCCGGG 3'
24.2U	Asn^{24}	26	5' GGAGGCCCGGaacCTGTTGAACCTGT 3'
24.2L		25	5' CTAGACAGGTTCAACAGgttCCGGG 3'
24.3U	Glu^{24}	26	5' GGAGGCCCGGgagCTGTTGAACCTGT 3'
24.3L		25	5' CTAGACAGGTTCAACAGctcCCGGG 3'
25.123U	Gln^{25} Glu^{25}	26	5' GGAGGCCCGGCGT(cga)agTTGAACCTGT 3'
25.123L	Lys^{25}	25	5' CTAGACAGGTTCAAct(gct)ACGCCGGG 3'

Linker	Size	Sequence
35.U	35	5' TCGACGCAGCCCTGGGAGCATGTGAATGCCATCCA 3'
35.L	36	5' CCTCCTGGATGGCATTCACATGCTCCCAGGGCTGCG 3'

Appendix A.4.2.4 (continued)

Name	Mutation	Size	Sequence
26.1U	Gln^{26}	17	5' TCGACTGcagAACCTGT 3'
26.1L		17	5' CTAGACAGGTTctgCAG 3'
26.2U	Glu^{26}	17	5' TCGACTGgagAACCTGT 3'
26.2L		17	5' CTAGACAGGTTctcCAG 3'
26.3U	Lys^{26}	17	5' TCGACTGaagAACCTGT 3'
26.3L		17	5' CTAGACAGGTTcttCAG 3'
27.1U	Asp^{27}	17	5' TCGACTGTTGgacCTGT 3'
27.1L		17	5' CTAGACAGgtcCAACAG 3'
27.2U	Lys^{27}	17	5' TCGACTGTTGaagCTGT 3'
27.2L		17	5' CTAGACAGcttCAACAG 3'
27.3U	Arg^{27}	17	5' TCGACTGTTGag(gc)CTGT 3'
27.3L	Ser^{27}	17	5' CTAGACAG(gc)ctCAACAG 3'
27.5U	Ala^{27}	17	5' TCGACTGTTG(ga)ccCTGT 3'
27.5L	Thr^{27}	17	5' CTAGACAGgg(ct)CAACAG 3'
28.1U	Arg^{28} Lys^{28} Met^{28} Glu^{28} Gly^{28} Val^{28} Gln^{28}	17	5' TCGACTGTTGAAC(agct)(gat)gT 3'
28.1L	Trp^{28}	17	5' CTAGAc(cta)(tcga)GTTCAACAG 3'
28.9U	Asp^{28} His^{28}	17	5' TCGACTGTTGAAC(gca)acT 3'
28.9L	Asn^{28}	17	5' CTAGAgt(cgt)GTTCAACAG 3'

Appendix A.5.2.1 GM-CSF oligonucleotides for OCM in *E. coli* expression vector

Underline = restriction enzyme sites, **lower case** = mutated codons,
double underline = 5' overhang used for ligation of oligonucleotide fragments

Name	Mutation	Size	Sequence
V1.U	$\Delta SacII_{(201)}$	35	5' CTACAAACAGGGTCT**gcgtgg**TTCCCTGACCAAAC 3'
V1.L		43	5' TTAAGTTTGGTCAGGGAA**ccacgc**AGACCCTGTTTGTAGAGCT 3'
6.2.U	$Tyr^{6,7}$	27	5' GATCC**tactac**TCCCCGTCTACCCAGC 3'
6.2.L		27	5' CATGGCTGGGTAGACGGGGA**gtagta**G 3'
Nco21.1.U	Arg^{21}	37	5' CATGGGAACACGTGAATGCTATCCAG**cgt**GCGCGCCG 3
Nco21.1.L		38	5' GCAGACGGCGCGC**acg**CTGGATAGCATTCACGTGTTCC 3'
Nco21.2.U	Ala^{21}	37	5' CATGGGAACACGTGAATGCTATCCAG**gcg**GCGCGCCG 3'
Nco21.2.L		38	5' GCAGACGGCGCGC**cgc**CTGGATAGCATTCACGTGTTCC 3'
Nco21.34.U	Lys^{21}	37	5' CATGGGAACACGTGAATGCTATCCAG**(ca)a(ga)**GCGCGCCG 3'
Nco21.34L	Gln^{21}	38	5' GCAGACGGCGCGC**(ct)t(gt)**CTGGATAGCATTCACGTGTTCC 3'
Nco21.5.U	His^{21}	37	5' CATGGGAACACGTGAATGCTATCCAG**cac**GCGCGCCG 3'
Nco21.6.L	Phe^{21}	38	5' GCAGACGGCGCGC**gaa**CTGGATAGCATTCACGTGTTCC 3'
100.U	Thr^{100}	29	5' AAACTTCCTGTGCTACCCAG**acc**ATCACC 3'
100.L		29	5' **ggt**CTGGGTAGCACAGGAAGTTTCCGGAG 3'

Linker	Size	Sequence
Bgl/Nco.U	24	5' GATCTCCGTCCCCGTCTACCCAGC 3'
Bgl/Nco.L	24	5' CATGGCTGGGTAGACGGGGACGGA 3'
Sac21.U	27	5' TCTGCTGAACCTGTCTCGTGACACCGC 3'
Sac21.L	20	5' GGTGTCACGAGACAGGTTCA 3'
Spe2.U	36	5' TTCGAATCTTTCAAAGAAAACCTGAAAGACTTTCTA 3'
Spe2.L	46	5' CTAGTAGAAAGTCTTTCAGGTTTTCTTTGAAAGATTCGAAGGTGAT 3'
Nhe2.U	36	5' CTAGCCACTACAAACAGCACTGCCCGCCGACTCCGG 3'
Nhe2.L	26	5' TCGGCGGGCAGTGCTGTTTGTAGTGG 3'

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Publications

Publications arising from this thesis

Lopez, A.F., Shannon, M.F., **Hercus, T.**, Nicola, N.A., Cambareri, B., Dottore, M., Layton, M.J., Eglinton, L. and Vadas, M.A. (1992c). Residue 21 of human granulocyte-macrophage colony-stimulating factor is critical for biological activity and for high but not low affinity binding. EMBO J. 11, 909-916.

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Hercus, T.R., Cambareri, B., Dottore, M., Woodcock, J., Bagley, C.J., Vadas, M.A., Shannon, M.F. and Lopez, A.F. (1994b). Identification of residues in the first and fourth helices of human GM-CSF involved in biological activity and in binding to the α and β chains of its receptor. Blood 83, 3500-3508.

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Specific human granulocyte–macrophage colony-stimulating factor antagonists

(cytokines/inflammation/leukemia/receptors)

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ABSTRACT **Human granulocyte–macrophage colony-stimulating factor (GM-CSF) is a pleiotropic hemopoietic growth factor and activator of mature myeloid cell function. We have previously shown that residue 21 in the first helix of GM-CSF plays a critical role in both biological activity and high-affinity receptor binding. We have now generated analogues of GM-CSF mutated at residue 21, expressed them in *Escherichia coli*, and examined them for binding, agonistic, and antagonistic activities. Binding experiments showed that GM E21A, E21Q, E21F, E21H, E21R, and E21K bound to the GM-CSF receptor α chain with a similar affinity to wild-type GM-CSF and had lost high-affinity binding to the GM-CSF receptor α-chain–common β-chain complex. From these mutants, only the charge reversal mutants E21R and E21K were completely devoid of agonistic activity. Significantly we found that E21R and E21K antagonized the proliferative effect of GM-CSF on the erythroleukemic cell line TF-1 and primary acute myeloid leukemias, as well as GM-CSF-mediated stimulation of neutrophil superoxide production. This antagonism was specific for GM-CSF in that no antagonism of interleukin 3-mediated TF-1 cell proliferation or tumor necrosis factor α-mediated stimulation of neutrophil superoxide production was observed. *E. coli*-derived GM E21R and E21K were effective antagonists of both nonglycosylated and glycosylated wild-type GM-CSF. These results show that low-affinity GM-CSF binding can be dissociated from receptor activation and have potential clinical significance for the management of inflammatory diseases and certain leukemias where GM-CSF plays a pathogenic role.**

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a glycoprotein with an apparent molecular weight of 23,000–28,000 and is produced by a variety of cells including activated T cells, macrophages, and endothelial cells (1). *In vitro* and *in vivo* studies have demonstrated that GM-CSF is a pleiotropic cytokine that stimulates both the production of different hemopoietic cell lineages and the effector function of mature myeloid cells (1–7). These properties have led to the *in vivo* use of GM-CSF to stimulate hemopoiesis and the effector function of neutrophils and monocytes (8, 9).

GM-CSF may also play a role in the pathogenesis of several disease conditions, in particular chronic inflammation and leukemia. Transgenic mice carrying the murine GM-CSF gene display elevated levels of GM-CSF and an accumulation of macrophages in the eyes and striated muscles, leading to blindness, muscle damage, and premature death, possibly as a result of GM-CSF-mediated macrophage activation (10). In humans the presence of elevated levels of GM-CSF in the bronchoalveolar lavage of atopic patients (11) and in the synovial fluid of patients with rheumatoid arthritis (12), along with the activated phenotype of neutrophils in this fluid (13), suggests that GM-CSF plays a pathological role in some inflammatory diseases. GM-CSF also stimulates the production of the neutrophil chemoattractant interleukin (IL) 8 (14) and, in conjunction with lipopolysaccharide, the monocyte chemoattractant macrophage inflammatory protein 1α (15), thus regulating the localization of leukocytes at the site of inflammation. In terms of leukemia, GM-CSF has been shown to be necessary for the survival and continued proliferation of some leukemic cells *in vitro* (1). Certain acute myeloid leukemias (AMLs) (16, 17) and lymphoblastic (18) leukemias have been shown to exhibit disregulated growth in response to autocrine (16) or paracrine (19) GM-CSF.

GM-CSF exerts its effects through binding to its high-affinity receptor (20). This is made up of a GM-CSF-specific, low-affinity receptor α chain (GMRα) (21) and a receptor β chain, which is shared with the IL-3 and IL-5 receptors. This common β chain (β_c) does not by itself detectably bind GM-CSF but confers high-affinity binding when coexpressed with the α chain and is required for signal transduction (22). The exact mechanism of receptor activation is not known, but it is thought to require binding of GM-CSF to GMRα followed by association with β_c, leading to the formation of a high-affinity receptor complex (23, 24). However, signaling can also be observed following low-affinity binding of GM-CSF (25–28), implying that GM-CSF binding to GMRα is sufficient for receptor activation.

The three-dimensional structure of human GM-CSF determined by x-ray crystallography (29) revealed the presence of a four-α-helix bundle with a fold common to many of the cytokines including IL-2 (30), IL-4 (31), IL-5 (32), and growth hormone (33). GM-CSF, IL-5, and IL-3 have been demonstrated to interact with their β_c through residues in their first helix (34) and more specifically with the conserved glutamate residue in this helix (27, 28, 35, 36). Using a CHO cell expression system, we previously showed that a GM-CSF mutant, E21R, had lost high-affinity binding while retaining wild-type affinity for GMRα and exhibited a 100- to 300-fold loss of biological activity (27, 36). We have now expressed several analogues of human GM-CSF mutated at residue 21 in an *Escherichia coli* secretion system and found that while all mutants bound the GM-CSF receptor with similar low affinities, they exhibited differences in their agonistic activities. Two mutants, E21R and E21K, were devoid of measurable agonistic activity and significantly were able to specifically antagonize GM-CSF-dependent proliferation of leukemic cells as well as GM-CSF activation of neutrophils.

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Abbreviations: AML, acute myeloid leukemia; GM-CSF, granulocyte–macrophage colony-stimulating factor; GMRα, GM-CSF receptor α chain(s); β_c, common β chain; IL, interleukin; TNF-α, tumor necrosis factor α; rh, recombinant human.

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The development of specific GM-CSF antagonists has important clinical implications and suggests that similar antagonists may be constructed for GM-CSF-related cytokines such as IL-3 and IL-5.

MATERIALS AND METHODS

Site-Directed Mutagenesis of Human GM-CSF. The plasmid pshGM-CSF containing a synthetic human GM-CSF cDNA cloned into the *E. coli* expression vector pIN-III-OmpH3, a derivative of pIN-III-OmpA2 (37), was kindly provided by A. B. Shanafelt and R. A. Kastelein (DNAX Research Institute, Palo Alto, CA). Oligonucleotide cassette mutagenesis was used to introduce residue 21 substitutions between unique *Nco* I and *Sac* II sites (64 bp). Plasmid constructs were sequenced through the entire oligonucleotide cassette (38) using a Sequenase kit (United States Biochemical).

Expression and Purification of *E. coli*-Derived GM-CSF and Analogues. *GM-CSF expression.* GM-CSF was expressed in logarithmic phase *E. coli* cultures after a 3-hr induction by 0.1 mM isopropyl β-D-thiogalactoside. Soluble GM-CSF was recovered from the periplasmic space by osmotic shock (39) with a typical yield of GM-CSF for wild type and most analogues of 1 mg of GM-CSF per liter of cultured cells as assessed by RIA (27). Certain of the residue-21 analogues proved difficult to express, and a number of *E. coli* strains were screened for their ability to express these analogues. Eventually the strain BL21 (40) (a gift from R. Morona, Department of Microbiology and Immunology, University of Adelaide) was found to be the most suitable, but expression was still poor, notably for E21R, with an expression level of only 1% of wild-type GM-CSF.

GM-CSF purification. Wild-type GM-CSF and analogues were purified from crude osmotic shock supernatants using the 4D4 anti-GM-CSF monoclonal antibody affinity column (36). Affinity-purified GM-CSF was then loaded onto a Brownlee Aquapore RP-300 reverse-phase column (4.6 × 100 mm) and was eluted using a 30–50% gradient of acetonitrile containing 0.1% trifluoroacetic acid. The resulting GM-CSF, typically at >95% purity, was lyophilized and resuspended in phosphate-buffered saline containing 0.02% (vol/vol) Tween 20.

GM-CSF quantitation. Purified GM-CSF and analogues were quantified by high-performance size-exclusion chromatography. Samples were chromatographed on a Beckman Ultraspherogel SEC3000 (7.5 × 300 mm) using a 0.1 M sodium phosphate, pH 7.0/0.1 M sodium sulfate mobile phase. The area under the GM-CSF peak was integrated by using the calculated extinction coefficient of 0.95 absorbance units·ml^{-1}·mg^{-1}. The yield of purified wild-type GM-CSF was typically 500 μg/liter of culture but was as little as 5–10 μg/liter of culture for purified E21R.

Radioiodination of GM-CSF. An *E. coli*-derived GM-CSF analogue containing two tyrosine residues inserted in place of a proline at residue six (GM P6YY) was radioiodinated by the iodine monochloride method (41) to a specific activity of ≈60 μCi/μg (1 Ci = 37 GBq). Iodinated protein was processed as described (27).

Recombinant Human (rh) Cytokines. Wild-type GM-CSF derived from CHO cells and *E. coli*-derived IL-3 were kindly provided by S. Clark (Genetics Institute, Cambridge, MA). *E. coli*-derived tumor necrosis factor α (TNF-α) was obtained from Genentech.

GM-CSF Functional Assays. *Hemopoietic cell proliferation assay.* The proliferative activity of GM-CSF mutants was assayed using the GM-CSF-dependent erythroleukemia cell line TF-1 (42) or primary AML cells selected for the ability to incorporate [^{3}H]thymidine in response to GM-CSF. Proliferation assays were performed essentially as described (42). Briefly cells (5 × 10^4 cells per well) were incubated with growth factors in 96-well plates for 48 hr (TF-1) or 72 hr (AML) before being pulsed with [^{3}H]thymidine (ICN) at 1 μCi per well for 5 hr. The cells were then harvested, and cell-associated radioactivity was determined in a Packard TriCarb liquid scintillation counter.

Superoxide anion production assay. This assay was carried out using purified human neutrophils as described (6).

Receptor Binding Studies. Competition for binding to high-affinity receptors (GMRαβ$_c$) used freshly purified human neutrophils, which express only high-affinity receptors (20). Competition for binding to low-affinity receptors (GMRα) used the stably transfected A9/C7 CHO cell line, which expresses 2–5 × 10^5 GMRα per cell (36). Binding experiments were performed as described (27). Data from receptor binding experiments were analyzed using the LIGAND program (43) obtained from Biosoft (Cambridge, U.K.).

RESULTS

Differential Biological Activity and Receptor Binding Properties of GM-CSF Analogues Mutated at Residue 21. Wild-type human GM-CSF and analogues with substitutions of the Glu-21 residue were produced by using an *E. coli* expression system and purified by immunoaffinity chromatography and reversed-phase HPLC. Dose-dependent stimulation of TF-1 cell proliferation was observed with the GM-CSF analogues E21A, E21Q, E21F, and E21H, although they exhibited ED_{50} values increased by 40- to 3000-fold compared with wild-type GM-CSF (Table 1). In contrast, the E21R and E21K analogues were unable to stimulate TF-1 cell proliferation even at concentrations 100,000-fold higher than the concentration of wild-type GM-CSF required to stimulate half-maximal proliferation of TF-1 cells (Table 1).

To study the relationship between biological activity and receptor binding, we tested the GM-CSF residue-21 analogues for their ability to compete for the binding of wild-type GM-CSF to the high-affinity (GMRαβ$_c$) or low affinity (GMRα) GM-CSF receptor. We found that using human neutrophils, which express only the high-affinity receptor (20), all mutants showed a 100- to 200-fold reduction in affinity (Fig. 1*A* and Table 1). In contrast, using the stably transfected CHO cell line A9/C7, which expresses large numbers of GMRα only, we found that all mutants exhibited an affinity similar to wild-type GM-CSF (Fig. 1*B* and Table 1).

GM E21R and E21K Antagonize GM-CSF-Mediated Proliferation of Leukemic Cells. Having established that E21R and E21K were able to bind with near wild-type affinity to the

Table 1. Biological activity and receptor binding characteristics of GM-CSF analogues mutated at residue 21

GM-CSF	ED_{50},* ng/ml	K_d (αβ$_c$),† pM	K_d (α)‡, pM
Wild type	0.01	7	1800
E21A	0.4	730	820
E21Q	0.6	940	1300
E21F	8	1600	1400
E21H	30	1060	800
E21K	—§	1060	1200
E21R	—§	730	1000

*Concentration of GM-CSF analogue stimulating 50% of the maximal, wild-type GM-CSF proliferative response, as measured by [^{3}H]thymidine incorporation by TF-1 cells.

†Mean binding affinities derived from two competition experiments on human neutrophils using 100 pM ^{125}I-labeled rhGM-CSF (^{125}I-rhGM-CSF) and different concentrations of E21 analogues.

‡Mean binding affinities derived from three competition experiments on the CHO cell line A9/C7 using 500 pM ^{125}I-rhGM-CSF and different concentrations of E21 analogues.

§No activity detected up to a concentration of 3000 ng/ml.

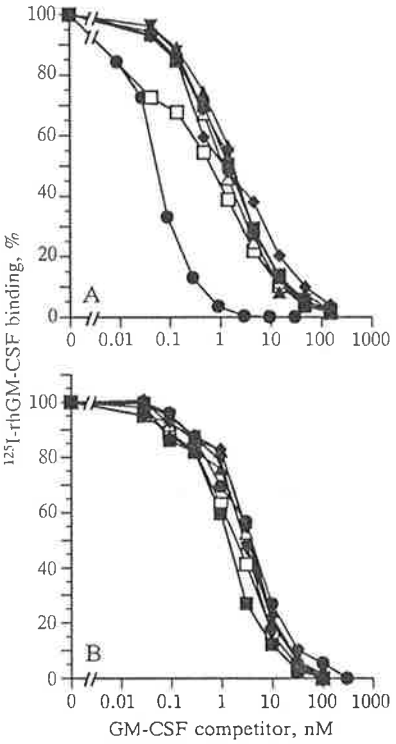

FIG. 1. Comparative binding of wild-type GM-CSF and residue-21 analogues to cells expressing high (GMR$\alpha\beta_c$) (*A*) or low (GMRα) (*B*) affinity GM-CSF receptors. Unlabeled GM-CSF, wild type or mutant protein, was titrated against 100 pM ^{125}I-labeled rhGM-CSF (^{125}I-rhGM-CSF) and 3×10^6 neutrophils per tube in *A* or 500 pM ^{125}I-rhGM-CSF and 5.5×10^5 A9/C7 cells per tube in *B*. Symbols represent competition by wild-type GM-CSF (●), E21A (□), E21Q (△), E21F (◆), E21H (▼), E21K (▲), and E21R (■). The values are expressed as percent ^{125}I-rhGM-CSF bound and are the means of duplicate determinations.

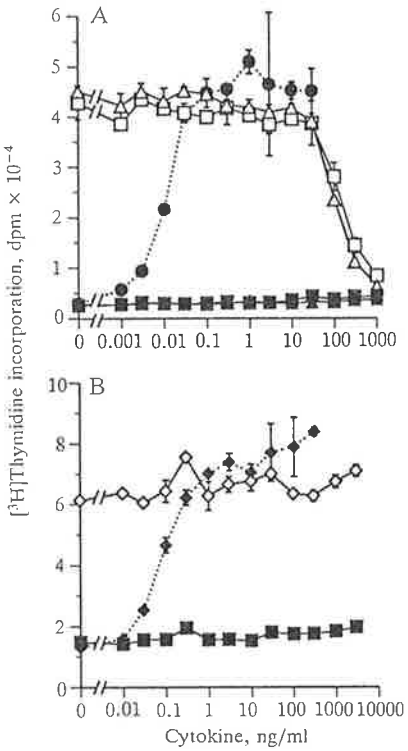

FIG. 2. E21R and E21K antagonize GM-CSF (*A*)- but not IL-3 (*B*)-mediated proliferation of TF-1 cells. Titrations of GM-CSF (●), IL-3 (◆), E21R (■), and E21K (▲) are shown. (*A*) In antagonistic experiments, E21R (□) and E21K (△) were titrated against GM-CSF at 0.03 ng/ml. (*B*) Specificity was determined by titration of E21R (◇) against IL-3 at 0.3 ng/ml. Each value represents the mean of triplicate determinations, and error bars represent the SEM.

GMRα but were unable to deliver a biological signal, we tested these analogues for their ability to antagonize the activity of wild-type GM-CSF. In a TF-1 cell proliferation assay, E21R and E21K were titrated against GM-CSF at 0.03 ng/ml, a dose that stimulates near-maximal TF-1 cell proliferation. We found that E21R and E21K completely antagonize the activity of GM-CSF, requiring a 7000-fold (200 ng/ml) and 5000-fold (150 ng/ml) excess, respectively, to reach 50% inhibition (Fig. 2*A*). When E21R was titrated against IL-3 at 0.3 ng/ml, as a specificity control, we found no detectable antagonism of IL-3 activity by E21R (Fig. 2*B*).

We next examined the ability of GM E21R to antagonize the GM-CSF-mediated proliferation of fresh leukemic cells from patients with AML by titrating GM E21R against GM-CSF at 0.3 ng/ml. The results summarized in Table 2 show that E21R was able to antagonize the GM-CSF-mediated proliferation of all three AMLs tested with a 2000- to 3000-fold (600–900 ng/ml) excess required to reach 50% inhibition.

GM E21R and E21K Antagonize GM-CSF Activation of Neutrophils. Since GM-CSF is a strong stimulator of inflammatory cells, we examined the ability of E21R and E21K to antagonize GM-CSF stimulation of superoxide production by human neutrophils. GM E21R was titrated against GM-CSF at 1 ng/ml or TNF-α at 3 ng/ml as a specificity control. We found that E21R antagonized the activity of GM-CSF, requiring a 300-fold (300 ng/ml) excess to reach 50% inhibition (Fig. 3*A*). However, there was no detectable antagonism of TNF-α even at a 10,000-fold excess.

To determine whether this antagonism could also be observed on glycosylated GM-CSF, which is the form that prevails *in vivo* (44), we tested E21R and E21K for their ability to antagonize glycosylated GM-CSF stimulation of superoxide production by neutrophils. A titration of GM E21R and E21K against glycosylated GM-CSF at 3 ng/ml produced in CHO cells showed that a 30-fold excess (90 ng/ml) of E21R and a 70-fold excess (200 ng/ml) of E21K were required to reach 50% inhibition of GM-CSF stimulation (Fig. 3*B*).

DISCUSSION

We describe here the discovery of two GM-CSF analogues, GM E21R and GM E21K, which are antagonists of GM-CSF function. *E. coli*-derived E21R and E21K completely antagonized the proliferative activity of GM-CSF on leukemic cells and the activation of human neutrophils by GM-CSF. These effects were specific in that IL-3-mediated cell proliferation or TNF-α-mediated neutrophil activation was not inhibited. These antagonists should help our understanding of the basic mechanism of GM-CSF receptor activation and have clinical potential for therapeutic intervention in inflammatory diseases and leukemias where GM-CSF may play a pathogenic role.

The exact mechanism of GM-CSF receptor activation is not known, but it appears to involve the low affinity binding of GM-CSF to GMRα and association of this complex with β_c, leading to high-affinity binding and signaling (23, 24).

Table 2. E21R antagonizes the GM-CSF-dependent proliferation of primary human myeloid leukemias

Cell	[^{3}H]Thymidine incorporation,* dpm	ED_{50} (GM-CSF)†, ng/ml	IC_{50} (E21R)‡, ng/ml
AML 1	6,400	0.02	900
AML 2	29,000	0.15	300
AML 3	2,900	0.07	500
TF-1	44,000	0.01	200

*Maximum GM-CSF-stimulated [^{3}H]thymidine incorporation.
†Concentration of GM-CSF stimulating 50% of the maximal proliferative response.
‡Concentration of E21R producing 50% inhibition of cell proliferation stimulated by GM-CSF at 0.3 ng/ml.

Considerable evidence indicates, however, that the low-affinity binding of GM-CSF to an $\alpha\beta_c$ receptor complex is sufficient to trigger a biological response, perhaps through an allosteric interaction. Thus, human GM-CSF binds with low affinity to murine cell lines expressing the endogenous murine β_c chain and the transfected human GMRα chain and elicits a proliferative response (25, 26). Similarly CHO cell-derived human GM-CSF E21R lacks high-affinity binding yet elicits a biological response (27). In all these cases the reduced binding affinity correlated with a reduced biological potency. In a most extreme example, the murine GM-CSF mutant E21A binds with only low affinity yet triggers a full biological response with wild-type potency (28). We show

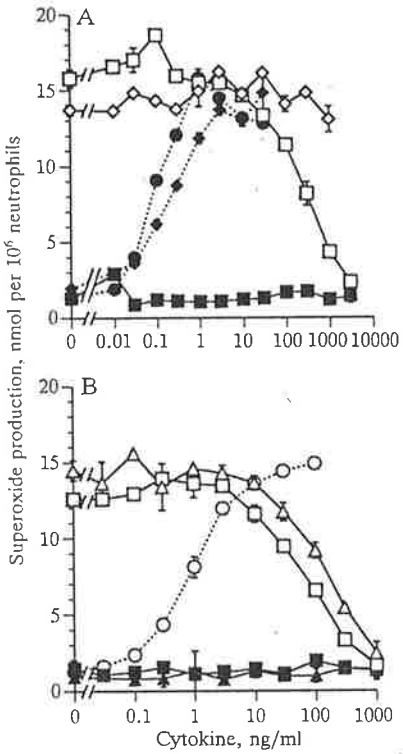

FIG. 3. E21R antagonizes GM-CSF- but not TNF-α-mediated stimulation of human neutrophils (*A*), and both E21R and E21K also antagonize neutrophil stimulation by CHO cell-derived GM-CSF (*B*). (*A*) Titrations of *E. coli*-derived wild-type GM-CSF (●), TNF-α (◆), and E21R (■) are shown. In antagonistic experiments, E21R was titrated against *E. coli*-derived GM-CSF (□) at 1 ng/ml or TNF-α (◇) at 3 ng/ml. (*B*) Titrations of CHO cell-derived wild-type GM-CSF (○), E21R (■), and E21K (▲) are shown. In antagonistic experiments, E21R (□) or E21K (△) was titrated against CHO cell-derived GM-CSF at 3 ng/ml. Each value represents the mean of triplicate determinations, and error bars represent the SEM.

here that low-affinity binding does not necessarily lead to receptor activation. The *E. coli*-derived analogues E21R and E21K fully bound the GMRα but did not stimulate leukemic cell proliferation or neutrophil activation. This shows that GMRα binding can be dissociated from receptor activation and illustrates the feasibility of constructing cytokine antagonists that can interact with and sequester the major binding chain of a receptor complex.

The lack of agonistic activity of E21R and E21K suggests that these mutations have abolished the interaction of GM-CSF with β_c. This is also supported by the inability of E21R to cross-compete for ^{125}I-labeled IL-3 binding (unpublished data) or to antagonize IL-3 activity (Fig. 2*B*). However, E21A, E21Q, E21F, and E21H exhibited agonistic activity and stimulated function with different potencies despite binding to GMRα with essentially the same affinity as E21R and E21K. This suggests a weak residual interaction with β_c, which is unable to enhance the measurable affinity of ligand binding to GMR$\alpha\beta_c$ over binding to GMRα alone. These data suggest that Glu-21 of GM-CSF directly interacts with β_c and that a charge reversal at this position has the most deleterious effect on the interaction with β_c. A direct interaction between GM-CSF Glu-21 and β_c is also supported by the ability of β_c mutants Y365A, H367A, and I368A to partially restore high-affinity binding of E21R (unpublished data).

The lack of agonistic activity exhibited by *E. coli*-derived E21R was surprising in view of the weak agonism exhibited by CHO cell-derived E21R (27, 36). One possibility is that the folding of *E. coli*-derived E21R differs from CHO cell-derived E21R. Although the possibility of gross structural alterations is unlikely given that both E21R and E21K bind fully to GMRα (Fig. 1*B*), we have observed a reduced stability for *E. coli*-derived E21R compared to *E. coli*-derived wild-type GM-CSF (unpublished data). A second possibility is that the carbohydrate of CHO cell-derived GM-CSF provides contact with β_c (45), which is not measurable by binding experiments. However, glycosylated GM-CSF exhibits lower specific activity than nonglycosylated GM-CSF (44, 46). Last, the carbohydrate on CHO cell-derived E21R might prevent an active repulsion of β_c caused by the charge reversal, or it may allow a conformational change in the α chain or the β_c chain to occur by masking the positive charge of the arginine group.

The observed IC_{50} values of the E21R and E21K antagonists are typically around 200 ng/ml, which is 10-fold higher than the concentration required to achieve 50% occupancy of the neutrophil $\alpha\beta_c$ receptor (Fig. 1*A*). This presumably reflects the degree of GMRα that must be occupied by antagonist to block wild-type signaling. This is consistent with previous reports that maximum biological effects of colony-stimulating factors can be exerted with steady-state receptor occupancy levels as low as 10% (47). The IC_{50} values of E21R and E21K exhibit a considerable variation in terms of excess of antagonist over agonist required for 50% inhibition, ranging from 7000-fold (Fig. 2*A*) to 30-fold (Fig. 3*B*). This probably reflects differences in the ED_{50} values of wild-type GM-CSF in these biological assays, which range from 0.01 ng/ml (Fig. 2*A*) to 1 ng/ml (Fig. 3*B*). The difference can partially be accounted for by the effect of carbohydrate on wild-type GM-CSF activity (44) but also appears to reflect differences in the concentration of GM-CSF required to trigger receptor activation in different cell types.

Potential applications of GM-CSF antagonists *in vivo* are in the management of inflammatory conditions exacerbated by abnormal levels of GM-CSF and inhibition of tumor cell growth. The detection of elevated mRNA for GM-CSF but not other cytokines in the bronchoalveolar lavage of atopic patients (11) suggests a role for GM-CSF antagonists in the treatment of allergic reactions. In rheumatoid arthritis, the presence of GM-CSF in synovial fluid (12) and the activated phenotype of the neutrophils in this fluid (13) provide another

scenario for therapeutic intervention with GM-CSF antagonists. The demonstration that E21R and E21K inhibit the GM-CSF-mediated growth of leukemic cells *in vitro* raises the possibility that these analogues may also be effective antagonists of leukemic cell proliferation mediated by GM-CSF produced in either a paracrine or autocrine manner *in vivo* (16, 19).

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(EMBO J. in press, 1994)

Three residues in the common β chain of the human GM-CSF, IL-3 and IL-5 receptors are essential for GM-CSF and IL-5 but not IL-3 high affinity binding and interact with Glu^{21} of GM-CSF.

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ABSTRACT

The β subunit of the receptors for human granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-3 and IL-5 is essential for high affinity ligand binding and signal transduction. An important feature of this subunit is its common nature, being able to interact with GM-CSF, IL-3 and IL-5, hence being termed β common (β_c). Analogous common subunits have also been identified in other receptor systems which include gp130 and the IL-2 receptor gamma subunit. It is not clear however, how a common receptor subunit is involved in binding to all ligands, in particular whether a single region exists which can bind to all ligands or distinct regions exist which are specific for each ligand. We have used site-directed mutagenesis and binding assays with radiolabelled GM-CSF, IL-3 and IL-5 to identify the residues in the β_c subunit involved in affinity conversion for each ligand. Using paired alanine scanning mutagenesis we found that the region Tyr^{365}-Ile^{368} in β_c was essential for high affinity binding of GM-CSF and IL-5 but not IL-3 high affinity binding. Single alanine substitutions in this region showed that Tyr^{365} was required for GM-CSF and IL-5 high affinity binding, Glu^{366} was unimportant, whereas His^{367} and Ile^{368} were essential for GM-CSF but had an intermediate effect on IL-5 high affinity binding. In contrast, single alanine substitutions of each of these four residues only marginally reduced the conversion of IL-3 binding to high affinity by β_c. To identify likely contact points in GM-CSF involved in binding to the 365-368 β_c region we used the GM-CSF mutant eco E21R which is unable to interact with wild type β_c whilst retaining full GM-CSF receptor a chain binding. We found that eco E21R exhibited higher binding affinity to receptor αβ complexes composed of mutant β chains Y365A, H367A and I368A than those composed of wild type β_c or mutant E366A. These results i) identify the residues Tyr^{365}, His^{367} and Ile^{368} as critical for affinity conversion by β_c, ii) show that high affinity binding of GM-CSF and IL-5 can be dissociated from IL-3, and iii) suggest that Tyr^{365}, His^{367} and Ile^{368} in β_c interact with Glu^{21} of GM-CSF.

INTRODUCTION

The human cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and interleukin-5 (IL-5) are important regulators of haemopoiesis and inflammation (reviewed in Metcalf, 1991 and Lopez *et al.*, 1992b). The regulatory effects of these cytokines are mediated by high affinity cell surface receptors that are known to comprise at least an α chain and a β chain. The β chain (Hayashida *et al.*, 1990) and α chains of the GM-CSF (Gearing *et al.*, 1989), IL-3 (Kitamura *et al.*, 1991b) and IL-5 (Tavernier *et al.*, 1991, Murata *et al.*, 1992) receptors have been cloned and the receptor complexes reconstituted and characterized (Hayashida *et al.*, 1990, Kitamura *et al.*, 1991b and Tavernier *et al.*, 1991). These experiments showed that whilst both the α and β receptor subunits are required for signalling (Kitamura *et al.*, 1991a, Kitamura *et al.*, 1992, Takaki *et al.*, 1993) they subserve distinct functions in terms of binding. Thus the α chains are ligand-specific and bind their cognate ligand with low affinity only (Kd = 10^{-9}-10^{-7}M). On the other hand the β subunit is common (β_c) to the GM-CSF, IL-3 and IL-5 receptors and although it does not detectably bind ligand by itself, it acts as an affinity converter allowing high affinity (10^{-10}M) binding when co-expressed with an α chain.

The role of β_c in mediating affinity conversion and signal transduction by different ligands is similar to that of gp130 and the IL-2 receptor γ chain. Thus, gp130 provides high affinity binding for leukaemia inhibitory factor, IL-6, IL-11, oncostatin M and ciliary neurotrophic factor (Akira *et al.*, 1993) and is important for function. Similarly, the γ chain of the IL-2 receptor increases the binding affinity of IL-2 (Takeshita *et al.*, 1992), IL-4 (Russell *et al.*, 1993) and IL-7 (Noguchi *et al.*, 1993) when co-expressed with the respective ligand specific receptor chains and contributes to signalling. It is not clear however, how a single subunit in all these receptor complexes can increase the binding affinity of several different ligands. Interestingly, the β_c subunit increases the affinity of GM-CSF, IL-3 and IL-5 binding to different degrees when co-expressed with the respective α chain. Thus β_c increases IL-5 binding affinity approximately 2-5 fold (Tavernier *et al.*, 1991 and Tavernier *et al.*, 1992), GM-CSF binding affinity 20-100 fold (Hayashida *et al.*, 1990) and IL-3 binding affinity 500-1000 fold (Kitamura *et al.*, 1991b and Kitamura *et al.*, 1992) raising the possibility that more than one region is involved in conferring high affinity binding on GM-CSF, IL-3 and IL-5.

Structurally the β_c and α chains of the GM-CSF, IL-3 and IL-5 receptors belong to the family of cytokine receptors that includes receptors for growth hormone (GH), erythropoietin, IL-2, IL-4, IL-6 and IL-7 (Gearing *et al.*, 1989, Bazan, 1990 and D'Andrea *et al.*, 1989). Members of this family share conserved structural motifs in the extracellular portion which can provide useful information when appropriately aligned. Growth

hormone (GH) binds to a homodimeric receptor. The soluble form of this receptor, growth hormone binding protein (GHbp) has been crystallised in a complex with GH and the three dimensional structure elucidated (de Vos *et al.*, 1992). We have aligned the amino acid sequence of β_c to the GHbp and used information from the GH·$(GHbp)_2$ crystals to hypothesize regions in β_c which may be involved in ligand binding (Goodall *et al.*, 1993). Of these regions we have targetted the region between residues 361-370 because it aligns with a region in GHbp (163-171) that is known to bind GH (de Vos *et al.*, 1992). This region also aligns with residues of the mouse IL-3 receptor β chain AIC2A, and the human IL-2 receptor β chain that are known to be involved in ligand binding (Wang *et al.*, 1992 and Imler *et al.*, 1992). In addition recent studies (Lock *et al.*, 1994) showed that His^{367} of β_c is important for GM-CSF high affinity. It is not clear however, whether this region is involved in IL-3 and IL-5 as well as GM-CSF binding, nor the identity of complementary regions in the ligands involved.

A candidate common motif for the binding of human GM-CSF, IL-3 and IL-5 to β_c is represented by the conserved acidic residue Glu^{21} in GM-CSF, Glu^{22} in IL-3 and Glu^{13} in IL-5. The mutation of this Glu selectively impairs high affinity binding for human GM-CSF (Lopez *et al.*, 1992a, Shanafelt *et al.*, 1992), IL-3 (Barry *et al.*, 1994) and IL-5 (unpublished observations). In particular, substitution of Glu^{21} of human GM-CSF for Arg and expression in *E.coli* causes complete loss of high affinity binding as well as signalling and results in a specific antagonist (Hercus *et al.*, 1994a). We have now used this molecule to test the hypothesis that the conserved Glu in GM-CSF, IL-3 and IL-5 interacts with the 361-370 region of β_c.

We show here using alanine mutagenesis in conjunction with binding studies with all three known ligands, that residues Tyr^{365}, His^{367} and Ile^{368}, located in the membrane proximal domain of the extracellular portion of the β_c subunit are involved in high affinity binding. Importantly, these residues are essential for GM-CSF and IL-5 but not for IL-3 high affinity binding implying that an additional region may be involved in IL-3 high affinity binding and suggesting a structural explanation for the large effect of the β_c subunit in increasing IL-3 binding affinity. Finally, by using the GM-CSF mutant eco E21R that is deficient in β_c interaction (Hercus *et al.*, 1994a), we propose a model where the β_c region 365-368 interacts with Glu^{21} in the first helix of GM-CSF.

RESULTS

Identification of residues of the human β chain critical for high affinity receptor formation; differential effects of alanine-scanning mutants on GM-CSF, IL-5 and IL-3 high affinity receptor formation

In order to identify residues of β_C which may play a role in affinity conversion we focussed on a region (358-373) which aligns with a region in the membrane proximal domain of GHbp that is involved in GH binding (de Vos *et al.*, 1992). The two subunits of GHbp bind GH in a non-equivalent manner and are therefore denoted GHbpI and GHbpII. The region 163-171 of GHbp is particularly attractive since it is involved in the binding of GH to GHbpII (de Vos *et al.*, 1992). In this respect GHbpII is functionally analogous to the β_C in that GH does not bind to GHbpII unless it is already bound to GHbpI (Cunningham and Wells, 1989). In addition, the region 358-373 in β_C aligns with regions in the mouse IL-3 receptor AIC2A and the human IL-2 receptor β chain which have been shown to be important in ligand binding (Fig. 1) (Wang *et al.*, 1992 and Imler *et al.*, 1992). This region of β_C is predicted to lie in or close to a loop between two β strands (Bazan, 1990).

Alanine-scanning mutagenesis was carried out across the region 361-370 in order to identify residues of the β chain required for affinity conversion and in particular their involvement with each ligand. Alanine substitutions were used to minimize conformational effects of mutation across this region (Cunningham and Wells, 1989). Initially, residues were substituted with alanines in pairs (Fig. 1). The mutant β chains M361A/K362A, M363A/R364A, Y365A/E366A, H367A/I368A and D369A/H370A were co-expressed on COS cells with either the GM-CSF, IL-3 or IL-5 receptor α chain and binding of the appropriate ligand determined in saturation binding studies. In the absence of β_C the binding affinities of the transfected α chains alone ranged from 2-12 nM for GM-CSF, 20-100 nM for IL-3 and 0.8-1.0 nM for IL-5 (Tables I and II). These values are consistent with previously determined affinities (Gearing *et al.*, 1989, Kitamura *et al.*, 1991b, Tavernier *et al.*, 1991 and Murata *et al.*, 1992). The β chain mutants M361A/K362A, M363A/R364A and D369A/H370A had no significant effect on GM-CSFor IL-3 high affinity binding (Fig. 2 and Table I). However, the mutants Y365A/E366A and H367A/I368A had a profound effect on high affinity conversion by β_C (Fig. 2 and Table I). Both mutants completely abolished GM-CSF high affinity binding. In contrast, the mutant Y365A/E366A did not significantly reduce IL-3 high affinity binding whilst the mutant H367A/I368A caused a reduction in high affinity from 110 pM (wild type β_C) to 530 pM which was statistically significant (Table I). In the case of IL-5, the mutant M361A/K362A did not affect high affinity binding whilst the mutants M363A/R364A and

D369A/H370A exhibited a small loss of IL-5 high affinity binding (Fig. 2 and Table I). The mutants Y365A/E366A and H367A/I368A on the other hand caused the greatest loss of IL-5 high affinity binding (Fig. 2 and Table I).

The loss of high affinity binding associated with mutant β chains Y365A/E366A and H367A/I368A was not due to lack of expression on the transfected cells. The cell surface expression of mutant β chains was monitored by flow cytometry using anti-β chain monoclonal antibodies 3D7 and 4F3 (see Materials and Methods), and CRS-1 (Watanabe *et al.*, 1992). Antibodies 3D7 and 4F3 both specifically precipitate a protein of 120 Kd from permanently transfected surface-labelled CHO cells corresponding in size to the β chain (Fig. 3A). Fig. 3B shows a typical result where the anti-β chain antibody 4F3 stained COS cells transfected with mutant β chain H367A/I368A as well as cells transfected with wild type β chain. Fig. 3B also shows staining for GM-CSF receptor α chain expression on these transfectants with monoclonal antibody 8G6. In IL-5 studies surface expression of mutant β chains was confirmed by affinity crosslinking. Fig. 3C shows the result of crosslinking ^{125}I-IL-5 to COS transfectants expressing IL-5 receptor α chain alone and with wild type or mutant β chain Y365A/E366A. The protein bands detected by the irreversible crosslinking of ^{125}I-IL-5 correspond in size, after subtraction of the molecular weight of an IL-5 monomer (the gel was run under reducing conditions), to the IL-5 receptor α chain (60 kD) and β_c (120 kD).

In order to elucidate the contribution of individual residues to high affinity binding, single alanine substitutions were made at positions 365-368. In each case the effect of these substitutions was determined after cotransfection with either GM-CSF, IL-3 or IL-5 receptor α chain in saturation and competition binding studies. As above, cell surface expression of mutant β chains was confirmed by flow cytometry (data not shown). Results showed that alanine substitution of residues Tyr^{365}, His^{367} and Ile^{368} led to the complete loss of high affinity GM-CSF binding, whereas alanine substitution of Glu^{366} had no significant effect on high affinity GM-CSF binding (Fig. 4, Table II). In contrast to GM-CSF, the effects of individual substitutions at residues 365-368 on IL-3 high affinity binding were small, resulting in only a four fold decrease in affinity relative to the wild-type receptor in each case (Table II). Alanine substitutions at Tyr^{365} , His^{367} and Ile^{368} caused significant reductions in IL-5 high affinity binding whereas substitution at Glu^{366} had no significant effect.

Partial restoration of binding of the GM-CSF analogue eco E21R to mutant β chains.

We have previously shown that the GM-CSF analogue eco E21R is deficient in binding to β_c and signalling while retaining wild type binding to the GM-CSF receptor α chain (Hercus *et al.*, 1994a). In order to ascertain whether the conserved Glu motif of

GM-CSF, IL-3 and IL-5 is involved in direct interaction with the 365-368 region of the β chain we examined the ability of the eco E21R to compete for wild-type GM-CSF binding on mutant β chains. Competition binding studies were carried out on COS cells cotransfected with GM-CSF receptor α chain and wild-type or mutant β chain. Radio-iodinated wild-type GM-CSF binding was determined using 100 pM ^{125}I-GM-CSF in the presence of an increasing amount of eco E21R analogue. Characterization of eco E21R binding showed that this mutant was able to compete with wild-type GM-CSF with equal potency for binding to low affinity α chain receptors but exhibited a 100 fold reduction in its ability to compete for binding to high affinity αβ receptors (Fig. 5A). Significantly, eco E21R competed more effectively on transfectants expressing β chains with alanine substitutions at position 365, 367 and 368 compared to the wild-type β chain or the β chain mutant E366A (Fig. 5B). This suggests that the mutant β chains Y365A, H367A and I368A are able to interact with eco E21R and that the substitutions made in β_c complement in part the charge reversal substitution made in GM-CSF. Comparison of the effective dose for 50% competition (ED_{50}) exhibited by eco E21R showed that eco E21R bound to GM-CSF receptor α chain plus Y365A, H367A and I368A complexes 5-7 times better than to E366A or wild-type β_c complexes (Table III).

DISCUSSION

We show here that the common β chain of the GM-CSF, IL-3 and IL-5 receptors contains three residues, Tyr^{365}, His^{367} and Ile^{368} which are involved in GM-CSF and IL-5 high affinity binding. However, whilst alanine substitution of these three residues abolishes GM-CSF and IL-5 high affinity binding, they affect only marginally IL-3 high affinity binding. These findings raise the possibility that other common subunits of receptor complexes such as gp130 and the IL-2/IL-4/IL-7 receptor gamma chain may also have common regions and unique regions recognized by different ligands. In addition, using the human GM-CSF antagonist eco E21R which has selectively lost its ability to bind to β_c and signal (Hercus *et al.*, 1994a) we show partial binding complementation on β_c mutants Y365A, H367A and I368A suggesting that the conserved Glu in the first helix of GM-CSF directly interacts with the 365-368 region of β_c.

By using alanine substitution mutagenesis we have defined the region 365-368 in β_c and more specifically Tyr^{365}, His^{367} and Ile^{368} as being essential for GM-CSF high affinity binding. The region between Tyr^{365} and Ile^{368} aligns with a region in the second cytokine receptor domain of the GHbp (Goodall *et al.*, 1993) which has been shown to contact GH after GH binds to GHbpI (de Vos *et al*, 1992), and with a region in AIC2A (Wang *et al.*, 1992) and IL-2R β (Imler *et al.*, 1992) important for mouse IL-3 and IL-2 binding respectively. By analogy with these molecules and from structural predictions (Bazan, 1990 and Goodall *et al.*, 1993) this region is predicted to lie in the loop between the B' and C' beta strands of the second cytokine receptor module (CRM) of β_c (Fig. 6). This suggests that rather than forming a discrete contact point for interaction, this region forms a binding surface on which several residues contribute to ligand interaction. It is noteworthy that Glu^{366} does not appear to contribute to ligand interaction suggesting that the side chain of this residue is orientated away from the proposed binding surface.

The loss of affinity converting activity associated with mutant β chains Y365A, H367A and I368A is neither the result of the lack of surface expression nor due to gross conformational changes as judged by the following criteria. Firstly, transfected cells were all stained with monoclonal anti-β chain antibodies. Three different antibodies were used and the staining seen with each antibody was equivalent in all cases confirming cell surface expression and suggesting that the mutations did not introduce gross conformational changes. Secondly, affinity cross-linking with ^{125}I-IL-5 to cells expressing receptor α and Y365A/E366A β_c which bind IL-5 with reduced affinity resulted in cross-linking of two proteins which corresponded in size to the IL-5 receptor α and β chains. Thirdly, mutant β chains which were deficient in affinity converting GM-CSF and IL-5 binding still conferred high affinity binding on IL-3.

In order to identify corresponding residues in GM-CSF likely to interact with the 365-368 region of β_c we focussed on the first helix of GM-CSF which has been shown to be important for high affinity binding (Shanafelt *et al.*, 1991, Lopez *et al.*, 1992a). Of several GM-CSF helix A mutants we have produced, we used the GM-CSF mutant eco E21R because mutations at Glu^{21} lead to a selective loss of GM-CSF-β_c interaction (Lopez *et al.*, 1992a and Shanafelt and Kastelein, 1992) and Glu^{21} is the main residue in the first helix of human GM-CSF that interacts with β_c (Hercus *et al.*, 1994b). We found that the GM-CSF mutant eco E21R competed better for ^{125}I-GM-CSF binding on cells expressing the mutant β chains that affect high affinity GM-CSF binding, namely Y365A, H367A and I368A. No effect was observed on E366A consistent with the lack of effect of this substitution in direct binding studies. These results raise the possibility that residues Tyr^{365}, His^{367} and Ile^{368} directly interact with GM-CSF and more specifically with Glu^{21} (Fig. 6). It is interesting to note that individual substitution of each residue, Tyr^{365}, His^{367} and Ile^{368} is sufficient to abrogate GM-CSF high affinity binding. The mechanism by which these residues contribute to GM-CSF high affinity binding is not exactly known. It is possible that the mutations alter the overall structure of the high affinity binding site, however this is unlikely in view of the fact that all the mutant receptors retained high affinity conversion for IL-3. This argues against severe structural alterations. It is more likely, however, that residues 365 - 368 act cooperatively to recognize helix A of GM-CSF. In this case the contribution of each residue to the ligand-binding determinant is dependent on the presence of the appropriate neighbouring residue. This cooperativity is also likely to explain the slight differences in affinity between the double and single mutants in IL-3 and IL-5 high affinity binding. Our results are consistent with the recent report of His^{367} of β_c being important for high affinity GM-CSF binding (Lock *et al.*, 1994). Interestingly, in contrast to our findings, Tyr^{365} was not found to be important in that study. This difference may be due to the nature of the substitution made; phenylalanine was substituted for Tyr^{365}. This subsitution retains the hydrophobicity of this residue and therefore may not interfere with salt bridge formation. Residue Ile^{368} was not investigated in that paper (Lock *et al.*, 1994).

In the case of IL-5 the results were less clear than with GM-CSF and IL-3 due to the relatively small (2-5 fold) increase in binding provided by β_c over the IL-5 receptor α chain alone. Nevertheless alanine substitutions revealed that Tyr^{365} was essential for affinity conversion by β_c. In contrast, the substitution of Glu^{366} alone did not reduce IL-5 binding and may have slightly attenuated the effect of Y365A in the double mutant. Individual alanine substitutions at positions 367 and 368 resulted in modest decreases in binding but together resulted in the abolition of detectable high affinity binding. Thus, as with GM-CSF binding, residues Tyr^{365}, His^{367} and Ile^{368} are important for the high affinity binding of IL-5.

We found that in contrast to GM-CSF and IL-5, high affinity binding of IL-3 was not abolished by mutation of residues Tyr^{365}, His^{367} and Ile^{368}. In fact only a four fold reduction in affinity was observed (Fig. 2 and 4, Tables I and II) which is a relatively small effect when taking into account the approximate 500 fold increase in IL-3 binding affinity provided by β_c (Kitamura *et al.*, 1991b and Table II). This suggests that an additional or alternative region in β_c contributes to IL-3 high affinity binding which may explain why β_c is able to confer such a large increase in affinity on the IL-3 receptor α chain relative to GM-CSF and IL-5. This notion is supported by two other sets of evidence. Firstly, whilst the GM-CSF eco E21R mutant has completely lost its ability to interact with β_c and signal, behaving as an antagonist (Hercus *et al*, 1994a), the analogous IL-3 mutant eco E22R is a weak agonist (Barry *et al.*, 1994) suggesting residual interaction with β_c. Secondly, we found that the monoclonal antibody 4F3 partially inhibits IL-3 but not GM-CSF high affinity binding and stimulation of TF-1 cell proliferation (unpublished results). Alignment of β_c with the GHbp suggest that residues 309-315, 333-343 and 414-424 in the loops between β strands E and F, G and A', and F' and G' respectively, may be involved and it would be of interest to test these hypotheses experimentally.

Finally, our results illustrate that GM-CSF and IL-5 high affinity binding can be dissociated from IL-3 high affinity binding by discrete mutations in β_c raising the possibility that differences in ligand specificity may also be found in other common receptor subunits such as gp130 and the IL-2 receptor γ chain. The identification of ligand binding regions and determination of their specificity in these common subunits may allow the selective blocking of cytokines with appropriately designed compounds.

MATERIALS AND METHODS

Mutagenesis of β chain and expression plasmid constructs.

The cDNA for the human β chain was cloned by PCR from cDNA prepared from the KMT-2 cell line (Barry *et al.*, 1994). The 2.8 Kb cDNA encoding the β chain was subcloned as an EcoR1-XbaI fragment into pSelect (Promega, Sydney, NSW). Mutations were introduced into the β chain cDNA using oligonucleotide-directed mutagenesis (Altered-sites, Promega). Using this method, a second oligonucleotide which restores ampicillin resistance to template DNA was used in combination with the mutagenic oligonucleotide and mutants selected for ampicillin-resistance in a repair deficient strain of *E. coli.* The mutagenic oligonucleotides used were 30-mers and were synthesised on an Applied Biosystems 381A DNA Synthesiser (Burnwood, VIC). The mutations were confirmed by nucleotide sequencing and the mutant β chain cDNAs subcloned into the eukaryotic expression vector pcDNA1 (Invitrogen, San Diego, CA).

The IL-3 receptor α chain was cloned by PCR from KMT-2 cells (Barry *et al.*, 1994). The GM-CSF receptor α chain was kindly provided by Dr. N. Nicola (Walter and Eliza Hall Institute, Melbourne Victoria) and the IL-5 receptor α chain was obtained as described previously (Tavernier *et al.*, 1991). All the receptor α chains were cloned into the eukaryotic expression vector pCDM8 (Invitrogen) for transfection.

COS cells and DNA transfection.

COS cells were maintained in RPMI-1640 medium supplemented with 5% v/v fetal calf serum (FCS). DNA constructs were introduced into COS cells by electroporation using a Bio-Rad Gene Pulser (North Ryde, NSW). Routinely 2 x 10^7 cells in 0.8 ml ice-cold PBS were co-transfected with 10 g of GM-CSF or IL-3 receptor α chain DNA in pCDM8 and 25 mg of pcDNA1 vector containing the wild type or mutated β chain cDNA at 500 F and 300 V. For IL-5 studies cells were transfected using DEAE-dextran as described previously (Tavernier *et al.*, 1991). Binding assays and antibody staining were carried out on cells 2-3 days after transfection.

GM-CSF, IL-3 and IL-5

Recombinant human GM-CSF and IL-3 were produced in *E. coli.* Wild-type GM-CSF and the analogue eco E21R were expressed in the periplasmic space of *E. coli.* from a synthetic human GM-CSF cDNA in the expression vector pIN-III-OmpH3 and purified as described elsewhere (Hercus *et al.*, 1994a). Recombinant IL-3 was expressed similarly from a native cDNA carrying the mutation F133Y to improve radio-iodination. The cDNA was cloned into the pFLAG vector (International Biotechnologies inc., Newhaven, CT) which adds an octapeptide sequence to the N-terminus to facilitate affinity

purification (Barry *et al.*, 1994). Recombinant human IL-5 was produced in Sf9 cells as detailed (Tavernier *et al.*, 1989).

Radiolabelling cytokines

Recombinant human GM-CSF and hIL-3 were radio-iodinated by the iodine monochloride method (Contreras *et al.*, 1983) to a specific activity of approximately 10 mCi/mg and 36 mCi/mg respectively. Iodinated protein was separated from iodide ions on a Sephadex G-25 column (Pharmacia, North Ryde, NSW) eluted with PBS containing 0.02% v/v Tween 20 and stored at 4°C for up to 4 weeks. Prior to use, radio-iodinated protein was buffer exchanged on a carboxymethyl-Sepharose CL-6B column loaded at pH 2.6 in 10 mM citrate-phosphate buffer and eluted in binding medium consisting of RPMI 1640 supplemented with 10 mM HEPES, 0.5 % w/v bovine serum albumin and 0.1 % w/v sodium azide. Recombinant hIL-5 was iodinated with the Iodogen reagent (Pierce Chemicals, Rockford, IL), as described (Plaetinck *et al.*, 1990).

Binding assays

For binding studies, transfected COS cells were detached by treatment with RPMI containing 40mM EDTA, 200 g/ml chondroitin sulphate and 0.1% w/v sodium azide and resuspended in binding medium (as above). Typically binding assays were performed with 0.5-1 x 10^6 cells in 150 l of binding medium containing radioligand in the presence or absence of unlabelled cytokine in a glass tube under shaking conditions at 24°C for 2-3 hrs. In saturation studies cells were incubated in the presence of increasing concentrations of radioligand. Routinely a concentration range of 10 pM - 8 nM was used of ^{125}I-GM-CSF or ^{125}I-IL-3 and 10 pM - 5 nM of ^{125}I-IL-5. Non-specific binding was determined in assays in which high concentrations of radioligand were used by adding 1 M of the unlabelled cytokine. The non-specific binding component for data points obtained at lower radioligand concentrations was obtained by interpolation. For competition studies cells were incubated with 100 pM radioligand in the presence of increasing concentration of competitor. After incubation cell-associated radioligand was separated from free radioligand by overlaying the cell suspension on a 0.2 ml cushion of FCS or phthalate oil (Plaetinck *et al.*, 1990) and centrifuging for 10 sec at maximum speed in a micro-centrifuge. The visible cell pellet was removed by cutting and radioactivity was determined using the Cobra 5010 gamma counter (Packard, Meriden, CT). Dissociation constants were calculated using the EBDA and LIGAND programs (Munson and Rodbard, 1980, Biosoft, Cambridge, U.K.). Where indicated multiple data files were co-analysed to obtain more accurate estimates. For GM-CSF and IL-3 data files were modelled on one-site and two-site fits and the better fit determined from the LIGAND program. Only where significantly preferred ($p<0.05$) was a two-site model used to determine K_D values. In order to obtain more reliable estimates of the high affinity binding component of the IL-3 binding

isotherms, a fixed value (50nM) was introduced for the dissociation constant for the low affinity binding site. In the case of IL-5 only a single class of receptor affinity was identified in all binding experiments. Statistical significance between K_D values obtained with wild type β and mutant β_c was determined using the non-paired "t" test. ED_{50} values were obtained using the Fig.P program (Biosoft, Cambridge, U.K.).

Ligand-independent monitoring of receptor α and β chain expression

To monitor cell surface expression of transfected GM-CSF and IL-3 receptor α chains and mutant β chains in a ligand-independent manner we generated specific monoclonal antibodies (MAb) and examined their binding to COS cell transfectants. Anti-β chain monoclonal antibodies 4F3 and 3D7 were raised in a mouse immunized with COS cells co-transfected with IL-3 receptor α chain and β chain. The splenocytes from this mouse were fused with NS-1 myeloma cells and the resulting hybridoma supernatants screened on CHO cells permanently expressing GM-CSF receptor α chains and β chains (A8s). Antibodies 4F3 and 3D7 both specifically precipitated a protein of 120 kD from surface labelled A8 cells (Fig. 3A). These two monoclonals and CRS-1 (Watanabe *et al.*, 1992), a rat monoclonal antibody reactive against the human β chain (kindly donated by Drs. R. Kastelein and A. Miyajima, DNAX, Palo Alto, CA) were used to stain β chain transfected COS cells by flow cytometry. Anti-GM-CSFR and anti-IL-3R α chain monoclonal antibodies were obtained essentially in the same way except that hybridoma supernatants were screened on permanently transfected CHO cells; A9/C7 for GM-CSF receptor α chain and F6 for IL-3 receptor α chain (Korpelainen *et al.*, 1993). The appropriate anti-α chain monoclonal antibodies were used to stain COS transfectants for receptor α chain expression; 8G6 for GM-CSF receptor α chain and 6H6 for IL-3 receptor α chain. These antibodies both precipitated a cell surface protein of the appropriate size from the relevant CHO cell line (data not shown). In IL-5 studies cell surface expression of the IL-5 receptor α chain and mutant β chains was confirmed by affinity cross-linking (Plaetinck *et al.*, 1990).

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Figure Legends

Fig 1 Identification of residues in the human β chain important for high affinity GM-CSF, IL-3 and IL-5 binding by alanine scanning mutagenesis. **Top:** Schematic representation of the extracellular domain of the human β chain showing two cytokine receptor modules (CRM1 & 2) (Goodall *et al.*, 1993) and the conserved features of the cytokine receptor family (Bazan 1990). **Bottom:** The amino acid sequences corresponding to the proposed B'- C' loop of CRM2 of the human β chain (β_C) (Hayashida *et al.*, 1990), growth hormone binding protein (GHbp) (Leung *et al.*, 1987), AIC2A (Itoh *et al.*, 1990) and hIL2Rβ (Imler *et al.*, 1992) are aligned and previously identified regions that play a role in ligand binding are underlined (de Vos *et al.*, 1992, Wang *et al.*, 1992, Imler *et al.*, 1992). Mutant forms of the human β chain which were made to identify ligand binding sites are also shown either as double alanine substitutions (left) or single alanine substitutions (right).

Fig 2 Double alanine substitution in the β_C region 365-368 abolishes high affinity binding of GM-CSF and IL-5 but not of IL-3. Scatchard transformation of saturation binding studies with ^{125}I-GM-CSF (top), ^{125}I-IL-3 (middle) and ^{125}I-IL-5 (bottom) were carried out on COS cells expressing either the GM-CSFRα, IL-3Rα or IL-5Rα chains together with wild type () or mutant (•) β_C. The range of ligand concentrations was; 10 pM - 8 nM for ^{125}I-GM-CSF and ^{125}I-IL-3, and 10 pM - 5 nM for ^{125}I-IL-5. In each case specific binding was determined in the presence of 1M unlabelled ligand. Each point is the mean of two replicates using 1x 10^6 COS cells. The broken line indicates the high and low affinity binding components using the wild type β_C, and the solid line represents the line of best fit for the different β_C mutants as determined using the LIGAND programme (Biosoft, Cambridge, U.K.). A representative experiment is shown and the Kd values derived from these and other similar experiments are shown in Table I.

Fig 3 Ligand-independent determination of β_C expression on transfected COS cells. (A) Characterization of anti-β_C MAbs by immuno-precipitation of radio-iodinated surface proteins from A8 CHO cells expressing β chain with MAbs 4F3 and 3D7 or no MAb (-ve). Briefly, 10^7 CHO cells were surface-labelled with I^{125} by the lactoperoxidase method and after washing incubated in lysis buffer containing protease inhibitors for 60 min. at 4°C on a rotating mixer. The mixture was then centrifuged at 10,000 g for 15 min. at 4°C. and the lysate supernatant "precleared" with 100l anti-mouse Ig-Sepharose beads for 18h at 4°C. Following centrifugation the lysate supernatant was mixed with 5g of MAb for 1h at 4°C followed by 100l of anti-mouse Ig Sepharose beads. The beads were then washed, boiled, and the supernatants run on a 7.5% SDS-PAGE. The gel was visualized by Phosphorimager (Molecular Dynamics, Sunnyvale, CA). (B) COS cells transfected with GM-CSF receptor α chain and wild-type or mutant β chain cDNAs were stained with anti-α

(8G6) and anti-β chain (4F3) monoclonal antibodies and cell surface expression of these proteins detected by flow cytometry. (C) Chemical cross-linking of ^{125}I-IL-5 to COS cells expressing IL-5 receptor α chain alone and with wild-type or Y365A/E366A mutant β chain.

Fig.4 The effect of single alanine substitution of residues Tyr^{365}, Glu^{366}, His^{367} and Ile^{368} of the human β chain on high affinity GM-CSF, IL-3 and IL-5 binding. Scatchard transformation of saturation binding studies with ^{125}I-GM-CSF, ^{125}I-IL-3 and ^{125}I-IL-5 were carried out as described in Fig. 2. A representative experiment is shown and the derived Kd values of these and other experiments shown in Table II.

Fig. 5 Alanine substitution at Tyr^{365}, His^{367} and Ile^{368} in β_c improve the ability of eco E21R to compete for wild-type ^{125}I-GM-CSF binding to COS cells expressing GM-CSF receptor α and mutant β chains. (A) Competition of eco E21R and wild-type GM-CSF binding for ^{125}I-GM-CSF at 100 pM on COS cells expressing GM-CSF receptor α chain alone (α) or α and wild type β chains (αβ). (B) Competition of eco E21R for ^{125}I-GM-CSF binding at 100 pM on COS cells expressing GM-CSF receptor α chain and mutant or wild type β chains.

Fig. 6 Schematic diagram of the interaction between GM-CSF and the extracellular portions of the α- and β-chains of its receptor. GM-CSF is represented as a four helix bundle in which the helices are labelled A, B, C, and D. The seven β-strands of each receptor domain are viewed as barrels. The cytokine receptor modules (CRM1 & 2) are each comprised of two domains (I & II). The loops of domain II of CRM2 project towards helix A of GM-CSF. The insert shows the close positioning and putative interaction between Glu^{21} in helix A of GM-CSF and the B'- C' loop on domain II of CRM2. Residues Tyr^{365} (Y), His^{367} (H) and Ile^{368} (I) which are predicted to be involved in interaction with GM-CSF Glu^{21} are outlined in bold circles. The scheme is based on a superposition of receptor sequences onto the homologous GHbp structure and the GM-CSF structure onto that of GH . In particular, residue Glu^{21} of GM-CSF is aligned with residue Arg^{19} of GH, a residue that interacts with the B'- C' loop of GHbpII (de Vos *et al.*, 1992).

Table I. Dissociation constants for GM-CSF, IL-3 and IL-5 binding to COS cells expressing each receptor α chain alone and with wild type or mutant receptor β chains.

COS CELLS EXPRESSING:	LIGANDS											
	GM-CSF				IL-3				IL-5			
α chain only[a]	Kd = 2 - 12 nM				Kd = 20 - 100 nM				Kd = 0.8 - 1 nM			
	Number of sites detected	$Kd_{site\ 1}$		$Kd_{site\ 2}$	(# expts.)	Number of sites detected	$Kd_{site\ 1}$		(# expts.)	Kd		(# expts.)
+ wild type β_c	2 p<0.001	132 pM ± 27%[b]		7 nM ± 33%	(10)	2 p<0.001	112 pM ± 18%[c]		(10)	289 pM ± 6%[d]		(4)
+ M361A/K362A	2 p<0.001	142 pM ± 33%	ns[e]	17 nM ± 30%	(4)	2 p<0.001	33 pM ± 35%	ns	(2)	333 pM ± 16%	ns	(2)
+ M363A/R364A	2 p<0.001	220 pM ± 61%	ns	6 nM ± 53%	(4)	2 p<0.001	104 pM ± 26%	ns	(2)	367 pM ± 8%	p<0.05	(2)
+ Y365A/E366A	1	-[f]		8 nM ± 9%	(10)	1[g]	118 pM ± 31%	ns	(3)	649 pM ± 8%	p<0.001	(3)
+ H367A/I368A	1	-		5 nM ± 12%	(7)	2 p<0.001	531 pM ± 28%	p<0.001	(4)	966 pM ± 6%	p<0.001	(2)
+ D369A/H370A	2 p<0.001	175 pM ± 36%	ns	4 nM ± 59%	(5)	2 p<0.001	158 pM ± 23%	ns	(2)	358 pM ± 8%	p<0.05	(2)

a. Range of affinities observed for ligand binding to receptor α chain only.

b. Binding data from separate experiments (expts.) with GM-CSF were co-analysed and the affinity constants are shown ± the standard error (S.E.) expressed as a percentage (Munson and Rodbard, 1980).

c. Analysis of data was performed as above except that given the extreme low affinity of IL-3 binding to IL-3R α chain, a constant value of 50 nM was used to allow estimation of high affinity ($Kd_{site\ 1}$) sites.

d. Analysis of data was performed as above. A single receptor class was detected with IL-5.

e. Significant differences with p values or not significant (ns) differences between c mutants and wild type c.

f. No high affinity binding sites were detected.

g. Only high affinity binding was detected.

Table II. Dissociation constants for GM-CSF, IL-3 and IL-5 binding to COS cells expressing each receptor α chain alone and with wild type or mutant receptor β chains.

COS CELLS EXPRESSING:	LIGANDS										
	GM-CSF				IL-3				IL-5		
α chain only[a]	Kd = 2 - 12 nM				Kd = 20 - 100 nM				Kd = 0.8 - 1 nM		
	Number of sites detected	$Kd_{site\ 1}$	$Kd_{site\ 2}$	(# expts.)	Number of sites detected	$Kd_{site\ 1}$		(# expts.)	Kd		(# expts.)
+ wild type β_c	2 p<0.001	132 pM ± 27%	7 nM ± 33%	(10)	2 p<0.001	112 pM ± 18%		(10)	289 pM ± 6%		(4)
+ Y365A	1	-[a]	4 nM ± 15%	(4)	2 p<0.001	490 pM ± 39%	p<0.01	(3)	836 pM ± 11%	p<0.001	(2)
+ E366A	2 p<0.001	191 pM ± 31% ns[b]	9 nM ± 21%	(3)	2 p<0.001	435 pM ± 34%	p<0.01	(3)	295 pM ± 12%	ns	(2)
+ H367A	1	-	8 nM ± 21%	(4)	2 p<0.001	476 pM ± 17%	p<0.001	(5)	455 pM ± 16%	p<0.025	(2)
+ I368A	1	-	2 nM ± 19%	(3)	1[c]	432 pM ± 27%	p<0.001	(4)	400 pM ± 11%	p<0.025	(2)

Binding experiments were analysed as per Table I.

a. No high affinity binding sites were detected.

b. Significant differences with p values or not significant (ns) differences between c mutants and wild type c.

c. Only high affinity binding was detected.

Table III. The GM-CSF mutant E21R shows improved binding to COS cells co-expressing the GM-CSF receptor α chain and the β_c mutants Y365A, H367A and I368A.

	EXPT. #1		EXPT. #2		FOLD
COS CELLS EXPRESSING α CHAIN +	**ED_{50}[a]** (nM) ± SEM	**RATIO** Mutant ED_{50}/ Wt ED_{50}	**ED_{50}** (nM) ± SEM	**RATIO** Mutant ED_{50}/ Wt ED_{50}	**IMPROVEMENT**
wild type-β_c	13.93 ±3.24	1	12.35 ±2.45	1	1
Y365A	2.20 ±0.32	0.16	3.06 ±0.62	0.25	5.12
E366A	16.38 ±3.75	1.18	24.40 ±10.58	1.97	0.68
H367A	1.92 ±0.49	0.14	1.97 ±0.25	0.16	6.70
I368A	1.55 ±0.36	0.11	2.93 ±0.68	0.24	6.63

a. Concentration of GM-CSF E21R causing 50% inhibition of wild type ^{125}I-GM-CSF binding to COS cells co-expressing the GM-CSF receptor α chain and different β chain mutants as described in Materials and Methods.

Figure 1

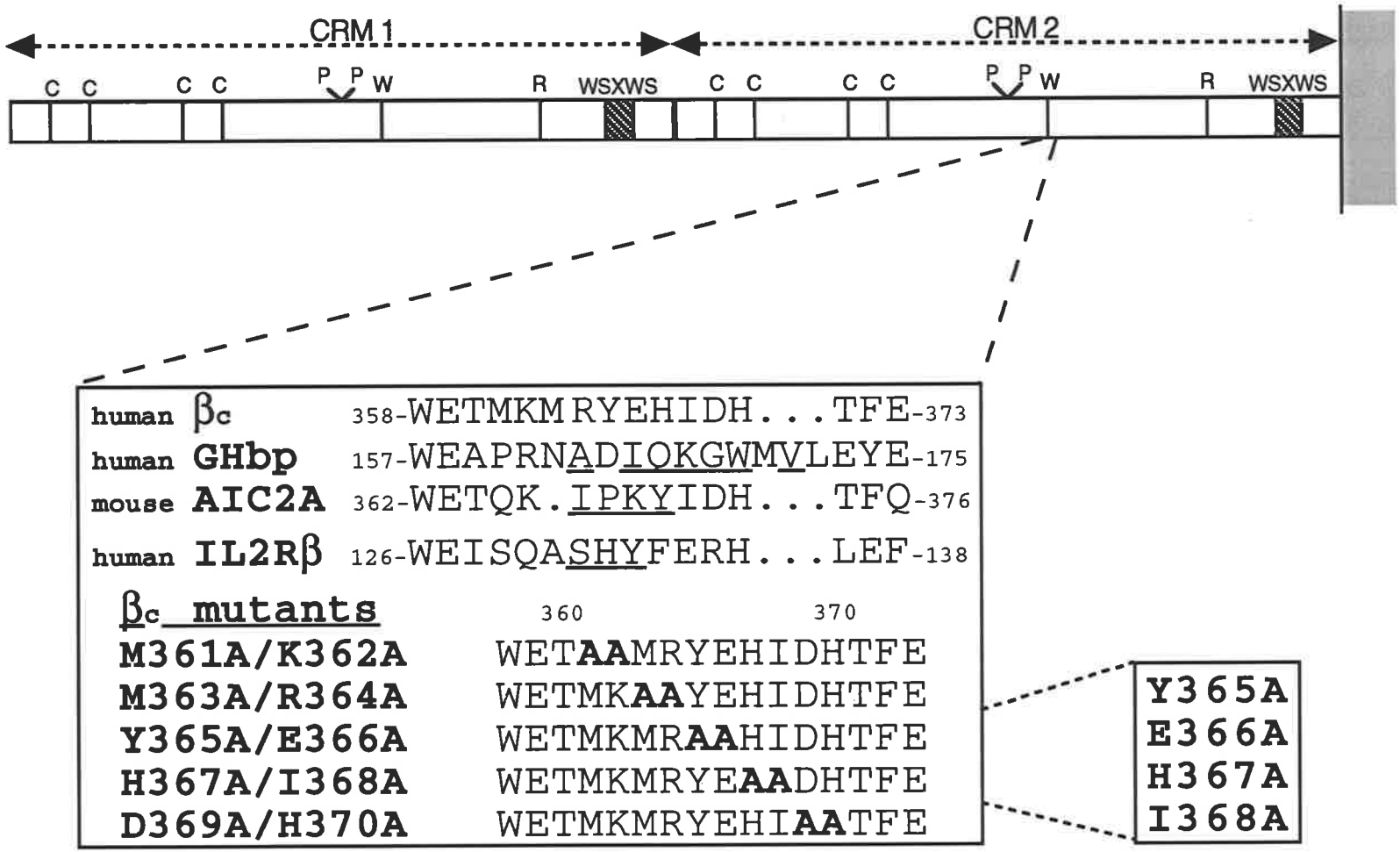

CRM 1
CRM 2
C C C C P P W R WSXWS C C C C P P W R WSXWS
human βc 358-WETMKMRYEHIDH...TFE-373
human GHbp 157-WEAPRNADIQKGWMVLEYE-175
mouse AIC2A 362-WETQK.IPKYIDH...TFQ-376
human IL2Rβ 126-WEISQASHYFERH...LEF-138
βc mutants
360
370
M361A/K362A WETAAMRYEHIDHTFE
M363A/R364A WETMKAAYEHIDHTFE
Y365A/E366A WETMKMRAAHIDHTFE
H367A/I368A WETMKMRYEAADHTFE
D369A/H370A WETMKMRYEHIAATFE
Y365A
E366A
H367A
I368A

Figure 2

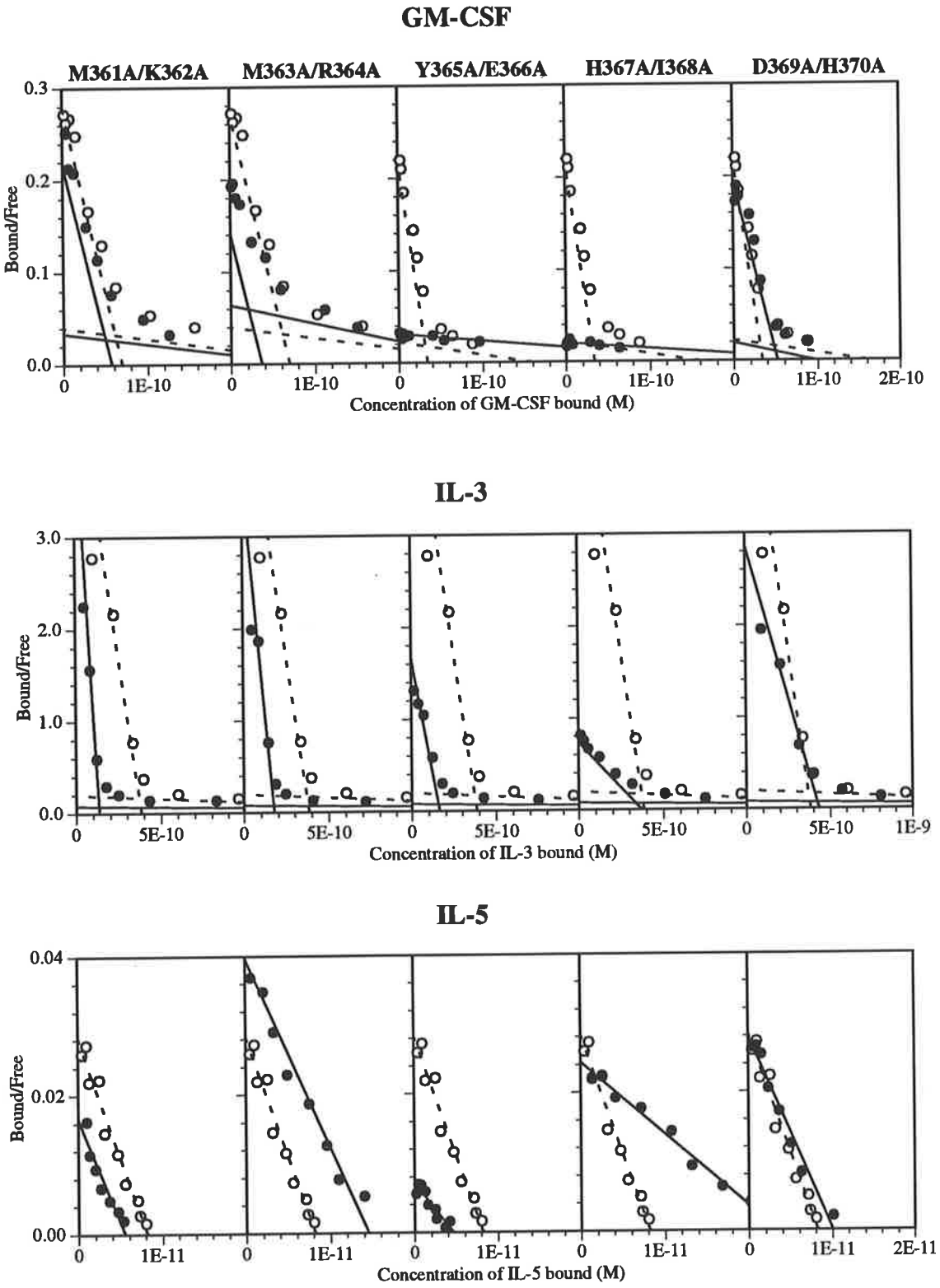

GM-CSF
M361A/K362A
M363A/R364A
Y365A/E366A
H367A/I368A
D369A/H370A
Bound/Free
0.3
0.2
0.1
0.0
0
1E-10
2E-10
Concentration of GM-CSF bound (M)
IL-3
3.0
2.0
1.0
0.0
5E-10
1E-9
Concentration of IL-3 bound (M)
IL-5
0.04
0.02
0.00
1E-11
2E-11
Concentration of IL-5 bound (M)

Fig. 3

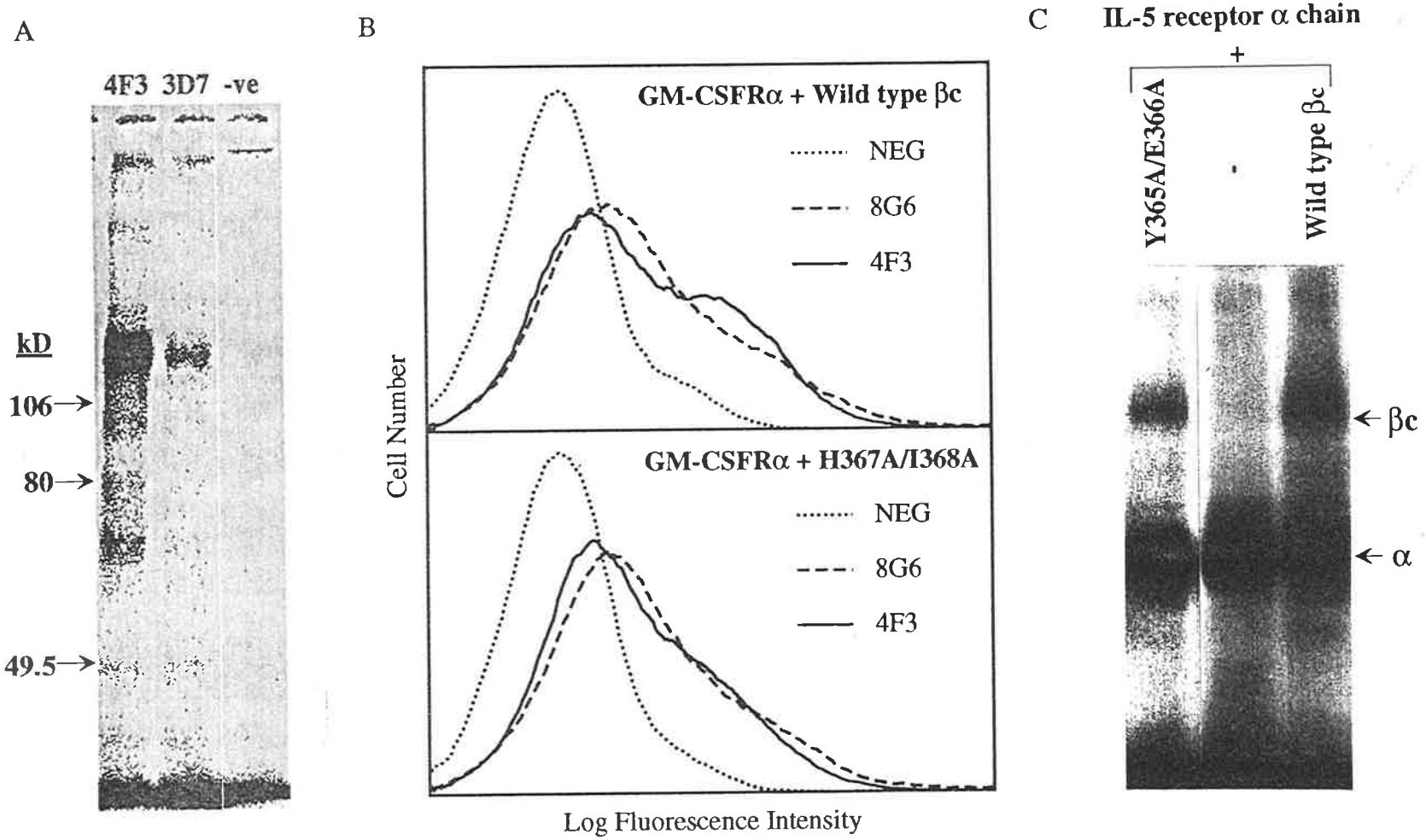

A
4F3 3D7 -ve
kD
106
80
49.5
B
GM-CSFRα + Wild type βc
NEG
8G6
4F3
GM-CSFRα + H367A/I368A
NEG
8G6
4F3
Cell Number
Log Fluorescence Intensity
C
IL-5 receptor α chain
+
Y365A/E366A
Wild type βc
βc
α

Figure 4

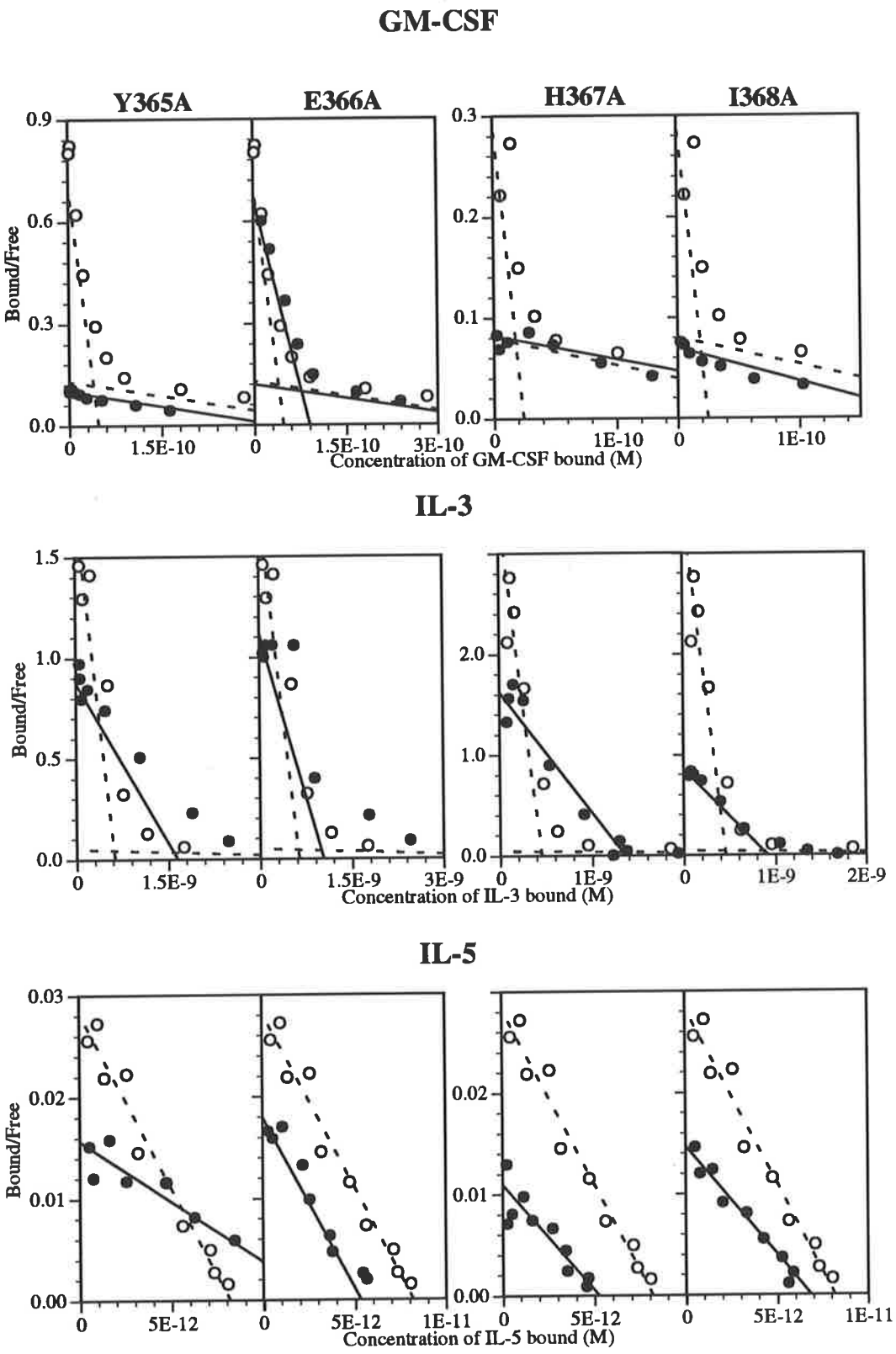
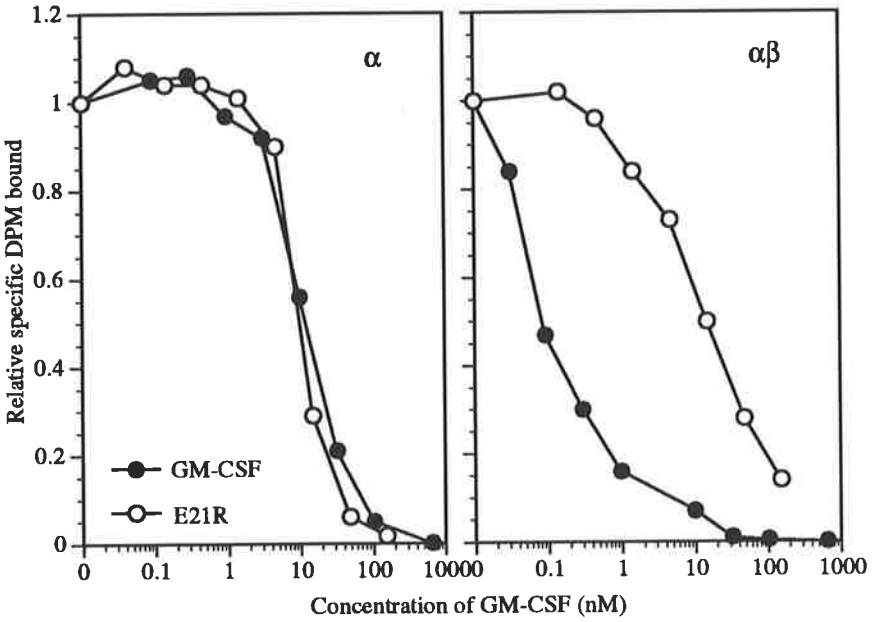

GM-CSF
Y365A
E366A
H367A
I368A
Bound/Free
0.9
0.6
0.3
0.0
0.3
0.2
0.1
0.0
0
1.5E-10
3E-10
1E-10
Concentration of GM-CSF bound (M)
IL-3
1.5
1.0
0.5
0.0
2.0
1.0
1.5E-9
3E-9
1E-9
2E-9
Concentration of IL-3 bound (M)
IL-5
0.03
0.02
0.01
0.00
5E-12
1E-11
Concentration of IL-5 bound (M)

Figure 5

A


α
αβ
Relative specific DPM bound
GM-CSF
E21R
Concentration of GM-CSF (nM)

B

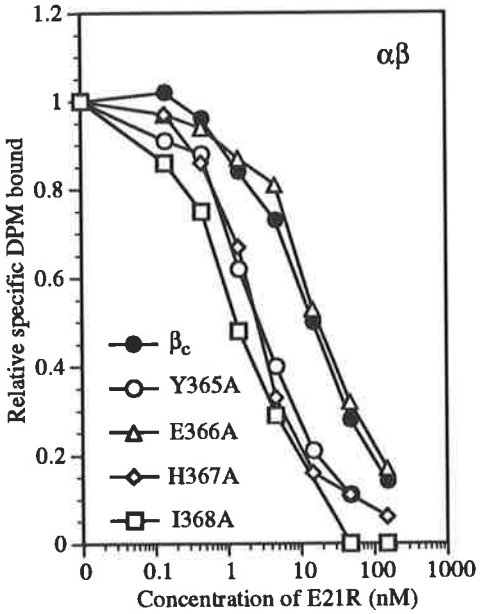
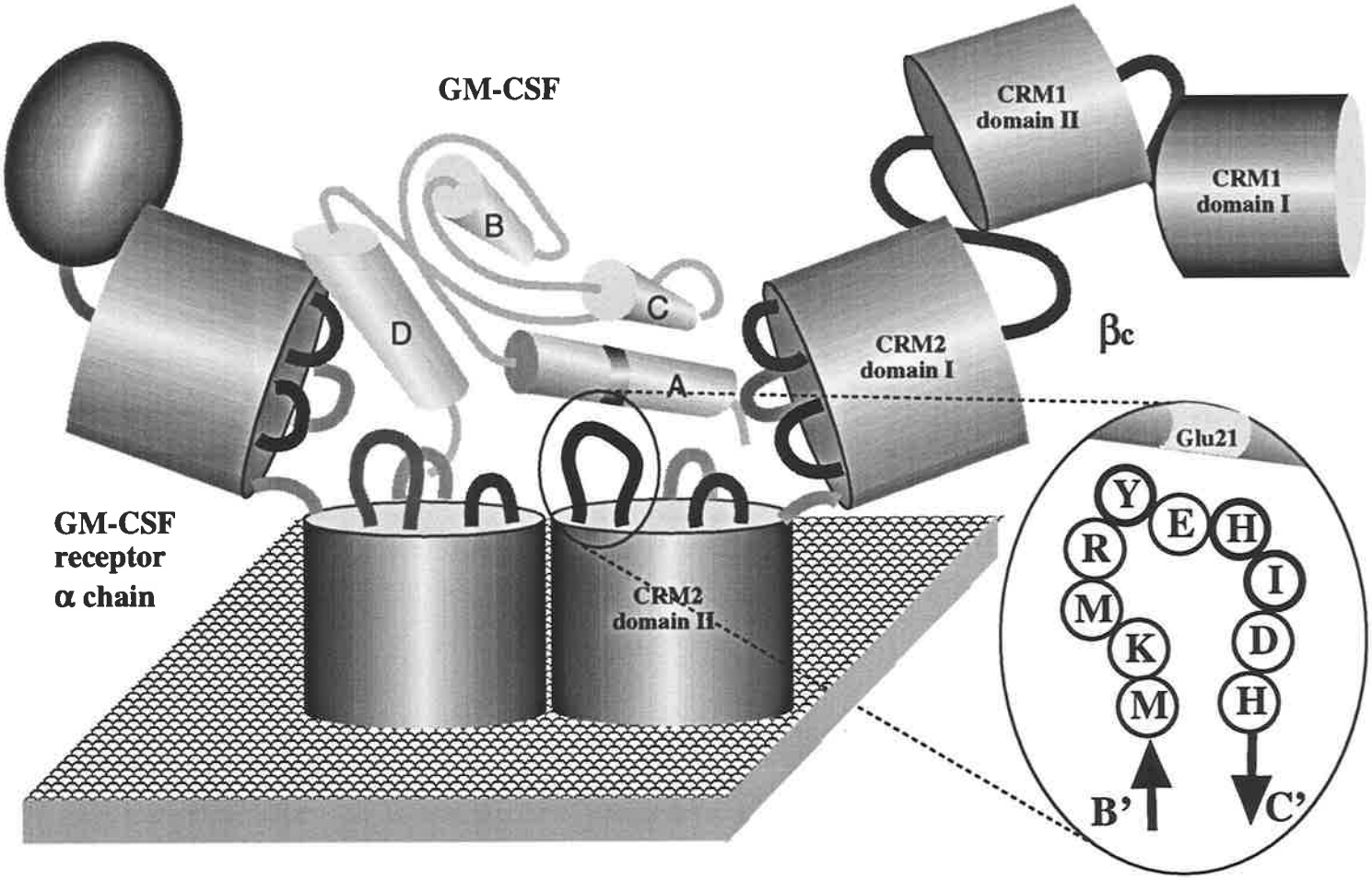

αβ
Relative specific DPM bound
βc
Y365A
E366A
H367A
I368A
Concentration of E21R (nM)

Figure 6


GM-CSF
CRM1
domain II
CRM1
domain I
B
C
D
A
CRM2
domain I
βc
Glu21
GM-CSF
receptor
α chain
CRM2
domain II
Y
E
H
R
I
M
D
K
M
H
B'
C'