



# **THE MOLECULAR BASIS OF IL-3, IL-5 AND GM-CSF RECEPTOR ACTIVATION**

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## ABSTRACT

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The Molecular Basis of IL-3, IL-5 and GM-CSF Receptor Activation

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GM-CSF, IL-3 and IL-5 receptors form a receptor subfamily of the cytokine receptor superfamily. These receptors consist of heterodimers comprising a ligand-specific  $\alpha$  chain and a common  $\beta$  chain. The  $\beta$  chain confers high affinity binding and is essential for signal transduction. This receptor subfamily is predominantly expressed on haemopoietic cells, and is also present on some non haemopoietic and neoplastic cells. The IL-3, IL-5 and GM-CSF subfamily of receptors have many overlapping activities which include regulation of cellular proliferation, differentiation, survival and activation. Receptor activation is initiated through ligand binding the receptor, which in turn produces a number of measurable intracellular responses that include tyrosine phosphorylation of the common  $\beta$  chain and several cytoplasmic proteins. This leads to activation of various signalling molecules that include Ras and the JAK-STAT pathways

The events that lead to the assembly of the ligand-induced receptor complex and type of complex that is formed which is necessary for receptor activation is poorly understood. This thesis addresses the extracellular events that lead to cellular activation. These molecular events are investigated through biochemical studies and mutagenesis of the common  $\beta$  chain. The focus of this thesis has been to provide a model system for receptor activation for this receptor subfamily. It is shown that a common theme has emerged in the GM-CSF/IL-3/IL-5 cytokine receptor subfamily.

Firstly that ligand binding to its respective receptor triggers heterodimerization of a ligand binding  $\alpha$  subunit to a common  $\beta$  subunit. Secondly, two types of heterodimers are formed, one being non covalently-linked as in the case of the IL-6 receptor and the other being disulphide-linked heterodimer which is unique to this receptor subfamily. Thirdly, disulphide-linked heterodimerization is essential for receptor activation but not for high affinity binding. Lastly, that a common motif in the  $\beta_c$  subunit is responsible for the disulphide bonding to the  $\alpha$  subunits. Cys residues in the N-terminal end of  $\beta_c$  at positions 86 and 91 make up this unique motif that is essential for disulphide-linked heterodimerization. This motif is restricted to this subfamily of cytokine receptors and therefore suggests a mechanism unique to this subfamily.

The GM-CSF receptor is shown to have unique features which are not shared with the IL-3 and IL-5 receptors. One being that it exists as a  $GMR\alpha/\beta_c$  preformed complex and secondly, that it becomes activated not only by the binding GM-CSF but is also capable of being transphosphorylated by IL-3 and IL-5. These observations suggest that that the GM-CSF receptor may have a unique biological significance. From the experimental data presented in this thesis, combined with molecular modelling, a hexameric model of active IL-3,IL-5 and GM-CSF receptor complexes is proposed here.

## DECLARATION

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This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institute 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 thesis being made available for loan and photocopying if accepted for the award of the degree

Frank C Stomski

*28/11* November 1997

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## ABBREVIATIONS

<b><math>\beta_c</math></b>	common $\beta$ chain
<b>BSA</b>	bovine serum albumin
<b>cDNA</b>	complimentary DNA
<b>CNTF</b>	ciliary neurotrophic factor
<b>CNTFR</b>	ciliary neurotrophic factor receptor
<b>CRM</b>	cytokine receptor module
<b>DPM</b>	disintegrations per minute
<b>EPO</b>	erythropoietin
<b>FCS</b>	foetal calf serum
<b>FACS</b>	fluorescence activated cell sorting
<b>G-CSF</b>	granulocyte colony-stimulating factor
<b>GM-CSF</b>	granulocyte-macrophage colony-stimulating factor
<b>GH</b>	growth hormone
<b>GHR</b>	growth hormone receptor
<b>IL-</b>	interleukin
<b>JAK</b>	Janus Kinase
<b>kD</b>	Kilodalton
<b>LIF</b>	leukaemia inhibitory factor
<b>MAb</b>	monoclonal antibody
<b>MAPK</b>	mitogen-activated protein kinases
<b>min</b>	minute
<b>mRNA</b>	messenger RNA
<b>Neo<sup>R</sup></b>	neomycin resistance gene
<b>PCR</b>	polymerase chain reaction
<b>pac</b>	puromycin resistance gene
<b>PBS</b>	phosphate buffered saline
<b>-R</b>	receptor

<b>sGMR<math>\alpha</math></b>	soluble form of the human GMR $\alpha$
<b>STAT</b>	signal transducer and activator of transcription
<b>TPOR</b>	thrombopoietin receptor
<b>vol/vol</b>	volume of one constituent as a percentage of the total volume
<b>wt</b>	wild type

## Publications Arising from this Thesis

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**Stomski, F. C.**, Q. Sun, C. J. Bagley, J. M. Woodcock, G. J. Goodall, R. K. Andrews, M. C. Berndt, and A. F. Lopez. (1996). Human interleukin-3 (IL-3) induces disulphide-linked receptor  $\alpha$  and  $\beta$  chain heterodimerization which is required for receptor activation but not high affinity binding. *Mol. Cell. Biol.* 16:3035-3046.

**Stomski, F. C.**, J. M. Woodcock, B. Zacharakis, C. J. Bagley, Q. Sun, and A. F. Lopez. (1998). Identification of a Cys motif in the common beta chain of the IL-3, GM-CSF and IL-5 receptors essential for disulfide-linked receptor heterodimerization and activation of all three receptors. *J. Biol. Chem.* (In Press, Jan 1998)

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Barry, S. C., E. I. Korpelainen, Q. Sun, **F. C. Stomski**, P. A. Moretti, H. Wakao, R. J. D'Andrea, M. A. Vadas, A. F. Lopez, and G. J. Goodall. (1997). Roles of the N and C terminal domains of the interleukin-3 receptor alpha chain in receptor function. *Blood* 89:842-852.

Iversen, P. O., T. R. Hercus, B. Zacharakis, J. M. Woodcock, **F. C. Stomski**, S. Kumar, B. H. Nelson, A. Miyajima, and A. F. Lopez. (1997). The apoptosis-inducing GM-CSF analogue E21R functions through specific regions of the heterodimeric GM-CSF receptor, and requires ICE-like proteases. *J. Biol. Chem.* 15:9877-9883.

# *Chapter I*

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## **INTRODUCTION**



## 1.1 The cytokine receptor superfamily

Cytokine receptors (-R) are cell-surface glycoproteins that bind with a high degree of specificity to their cognate ligands and transduce their signals. These receptors provide a means for cells to communicate with the extracellular environment by responding to stimuli generated in the vicinity or in other parts of the organism. The stimuli is provided by soluble factors, known collectively as cytokines, which are polypeptides that can modulate cell renewal, differentiation and stimulate mature cell functions. Cytokines are secreted by several cell types including activated T-cells and macrophages and are able to induce a wide range of functional responses on a variety of cells either locally or at a distance from the site of production. They act as molecular go-betweens and the term interleukins (IL-) has been assigned to many of the cytokines. The binding of cytokines to their respective receptors is a key event which occurs rapidly, at very low cytokine concentrations, is virtually irreversible and leads to intracellular changes resulting in a biological response. The biological responses are diverse and these vary depending on the type of cytokine receptor and cell type but in general it involves gene expression, changes in the cell cycle and release of mediators such as cytokines themselves.

Cytokine receptors function as highly organised oligomeric complexes which are formed by the association of two to six receptor chains that may be the same or different. In single subunit receptors the identical subunits provide a dual role of binding to cytokines and signalling. Receptors that only use a single type of subunit are those for growth hormone (GH)(Cunningham et al.1991), erythropoietin (EPO)(Miura et al.1993a) and granulocyte colony-stimulating factor (G-

CSF)(Hiraoka et al.1994). In multi-subunit receptors the different subunits can be involved in specific functions either binding ligand or acting as signal transducers. Multi-subunit receptors may consist of two subunit types such as the receptors for granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-3 and IL-5 where an  $\alpha$  subunit is specific for each ligand and a  $\beta$  subunit is common to all three ( $\beta_c$ ), with both chains participating in signalling(Hayashida et al.1990; Gearing et al.1989; Kitamura et al.1991b; Tavernier et al.1991). The IL-6 receptor utilizes two subunit types, IL-6R $\alpha$  and gp130(Taga et al.1989). However, in this case each receptor chain has a more specific role, with IL-6R $\alpha$  being primarily involved in binding IL-6 with no direct role in signalling, and gp130 being the signal transducer(Taga et al.1989). Other receptor complexes contain three different subunits and these include the ciliary neurotrophic factor receptor (CNTFR), formed by the CNTFR $\alpha$  chain, gp130 and the leukaemia inhibitory factor (LIF) receptor(Davis et al.1993), and the IL-2R which consists of the IL-2R $\alpha$  chain, IL-2R $\beta$  and IL-2R $\gamma$  with the latter two being the signalling molecules(Nakamura et al.1994).

Numerous cytokine receptors have been cloned in the last ten years and molecular characterisation has revealed a significant structural and functional conservation which has justified their distinct grouping into the cytokine receptor superfamily. Structural analysis has isolated subsets within this superfamily, that are structurally similar subfamilies, whereby some receptors or receptor subunits are more related to each other than to other members of the receptor superfamily. The conservation of structural motifs was recognised before the three dimensional structure of any of the

receptors was known (Bazan,1990b) and the crystallisation and X-ray structure determination of two cytokine receptors, GH receptor (De Vos et al.1992) and prolactin receptor (Somers et al.1994) has confirmed the overall predicted tertiary structure of receptors in the family.

Within the different receptor subfamilies certain subunits subserve similar functions. For example, the  $\beta_c$  subunit shared by the GM-CSF, IL-3 and IL-5 receptors is functionally analogous to gp130, which is the common subunit of the IL-6, CNTF, cardiotrophin, oncostatin M, LIF and IL-11 receptors; to IL-2R $\gamma$  which is shared by the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15; and to the common subunit of the IL-4 and IL-13 receptors (Zurawski et al.1993; Hilton et al.1996). These common subunits have the dual function of affinity-converting the initial cytokine binding into a high affinity state, and of being the major signal transducer in each of these receptor systems. The sharing of certain subunits by receptors helps explain much of the overlapping activities of the different cytokines in each receptor system.

## **1.2 Structural features of haemopoietic cytokine receptors**

### **1.2.1 Extracellular regions**

The haemopoietic cytokine receptors share a conserved sequence homology of approximately 200 amino acid residues referred to as the cytokine receptor module (CRM). This region was proposed to consist of two  $\beta$  barrel structural domains (Bazan,1990b). A number of sequence motifs may be recognised as typical of this family of receptors. Each domain contains a conserved Trp near its *N*-terminus, the

first domain contains four conserved cysteine residues thought to be involved in disulphide bonds, while the second domain has two or three Pro residues near its *N*-terminus, an alternating pattern of hydrophobic residues (YXVXVRVR consensus) and an especially well-conserved WSXWS motif near its *C*-terminus. The elucidation of the structure of the growth hormone receptor (GHR) complexed with GH (De Vos et al.1992) has provided some explanation for the role of these conserved residues. Each domain of the GHR consists of two beta pleated sheets containing the A, B and E strands and the D, C, F and G strands respectively. The general topology of these strands can be seen in a  $\beta_C$  model of CRM2 ((Bagley et al.1997) and Figure 1.1). The conserved *N*-terminal Trp residues lie in the central, B, strand of the first sheet and constitute part of the hydrophobic cores of the proteins. These residues interact with conserved hydrophobic residues from the central C and F strands of the opposing sheets including the YXVXVRVR motif in the second domain. The WSXWS motif exists as a beta bulge facilitating interdigitation of the two Trp residues between the surface-orientated Arg side-chains of the YXVXVRVR motif in the F strand. Although the GHR has Tyr and Phe residues in place of the more usual Trp in the WSXWS motif, the aromatic rings of these residues superimpose on the Trp heterocycles in the structure of the closely-related prolactin receptor (Somers et al.1994). The elucidation of the structure of the GH/GHR complex has provided a structural paradigm on which our understanding of the structure and function of other cytokine receptors can be built.

Within the CRMs, each structural domain is related to the archetypal fibronectin type III domain but they are not closely related to each other. Even though the amino acid

sequences are very variable, is a common cytokine receptor structural motif seems to have been conserved throughout mammalian evolution and has been found in other vertebrates such as birds (Tanaka et al.1992). This conservation is firstly shown through the presence of sequence motifs such as the WSXWS motifs, disulphide pairs and several other residues (eg. Trp in B strand of both domains). Secondly, almost all of cytokine receptors, have pairs of cytokine receptor domains each constituting a CRM. In addition to CRM, some of the receptors have recruited extra domains into their extracellular regions such as classical type III fibronectin domains, and immunoglobulin domains.

### **1.2.2 Cytoplasmic regions**

There is no structural information on the tertiary structure of the cytoplasmic domains of members of the cytokine receptor family. In general the intracellular portions of the cytokine receptors are highly variable, however a membrane-proximal region of approximately 50 amino acids typically contains proline motifs and is encoded by an exon of conserved structure. This region has been implicated in signal transduction via association with members of the JAK family of protein tyrosine kinases (Quelle et al.1994; Nelson et al.1996). The sequence similarities in the membrane-proximal cytoplasmic regions are denoted as box 1 and box 2 (Murakami et al.1991) (Figure 1.2). In  $\beta_c$  confirmation of the importance of box 1 came from deletion analysis which defined a region comprising residues 456 to 487, encompassing box 1, as being critical for mediating growth response when transfected into Ba/F3 cells (Sakamaki K et al.1992). Sequences corresponding to box 1 are found in a number of cytokine receptors where they probably serve a

common function in the recruitment of kinases of the JAK family. In addition to box 1 residues, a region of  $\beta_C$  comprising residues 517 to 542, that includes box 2 sequences, was found to be required for the full sensitivity of the biological response (Sakamaki K et al.1992).

Extrapolating information from the  $\beta_C$  deletion analysis the same regions are involved in the function of related receptors as well. For example, in the  $\beta$ -chain of the IL-2 receptor, a 13 amino acid deletion of the box 1 region resulted in a 50-fold decrease in the ability to signal in a transient proliferation assay (Goldsmith et al.1994). Furthermore box 2 in IL-2R $\beta$  is essential for variety of activities stimulated by IL-2 (Goldsmith et al.1994). The cytoplasmic region of  $\beta_C$  contains a number of tyrosines that are phosphorylated following cytokine binding. Tyr at position 750, is essential for phosphorylation of  $\beta_C$  (Inhorn et al.1995) and is associated with viability. Tyr at position 577 on the other hand is essential for Shc phosphorylation (Itoh et al.1996; Pratt et al.1996). The role of phosphorylated receptor tyrosines is becoming recognised for being involved in interacting with recruiting proteins and linking receptors to cellular functions is becoming a common theme in the cytokine receptor family (Ihle et al.1995). More tyrosine-based motifs are been identified in signalling receptor subunits that couple the receptor to specific substrates (Stahl et al.1995).

### **1.3 Cytokine receptor assembly and signalling**

In the overall picture of cytokine receptor activation the cytokines firstly bind to their cell surface receptors followed by receptor dimerization/oligomerization

(Heldin,1995). This is a dynamic multi-step process which leads to intracellular signalling and requires intimate interactions between the different receptor components or subunits and the ligand. Ligand-dependent receptor dimerization or oligomerization appears to be a common feature of cytokine receptor superfamily receptor(Heldin,1995). Receptor dimerization/oligomerization can be seen in cases where the receptors are constituted by single chains as with c-kit (Lev et al.1992), the GH receptor (Cunningham et al.1991), EPO receptor (Miura et al.1993a) and G-CSF receptor (Hiraoka et al.1994), as well as in multi-subunit receptors such as the IL-6 (Hibi et al.1990). Receptor dimerization is in most cases induced by ligand. In the cytokine receptor superfamily, GH has been shown to induce GH receptor dimerization (Cunningham et al.1991) and IL-6 induces dimerization of its receptor (Hibi et al.1990). On the other hand the EPOR is not influenced by EPO to become dimerized, as the EPO receptor itself exists as a dimer (Miura et al.1993a). An in between example is the G-CSF receptor where G-CSF induces the conversion of a dimeric receptor into a tetrameric one (Hiraoka et al.1994).

The mechanism of receptor dimerization involves either two identical receptor subunits (homodimerization) or two different subunits of the receptor (heterodimerization). The GH, G-CSF, and EPO receptors have been shown to homodimerize which then interact with appropriate tyrosine kinases and which in turn induce signalling. In multi-subunit receptors such as the IL-6 receptor both heterodimerization and homodimerization have been shown to occur. In the case of the IL-6 receptor, IL-6 triggers the heterodimerization of IL-6R $\alpha$ , the major binding subunit, with gp130, the signalling subunit (Murakami et al.1991). This in turn

allows the homodimerization of gp130 to a second gp130 molecule (Murakami et al.1993). In the case of the related CNTF receptor, CNTF heterodimerizes with gp130 whilst gp130 also heterodimerizes with the LIFR to initiate signalling (Davis et al.1993). In the GM-CSF , IL-3, IL-5 receptor family homodimerization of  $\beta_C$  (Muto et al.1996) as well as heterodimerization after stimulation with GM-CSF, leads to heterodimerization of  $GMR\alpha$  with  $\beta_C$  (Eder et al.1994). Whether ligand induces further homodimerization of  $\beta_C$  after ligand binding is still not resolved.

Dimerization of cytokine receptor subunits has been shown to occur by covalent and non covalent means. The dimerization of single chain receptors has been shown to be non covalent such as with the GHR (De Vos et al.1992) but also covalent involving disulphide bonds as with the EPOR (Miura et al.1993b). In the case of the IL-6R and the CNTFR heterodimerization of the ligand binding  $\alpha$  subunits to the signalling subunit gp130 is non covalent, however, the association between the signalling subunits involves disulphide linkage (Davis et al.1993; Murakami et al.1993).

Disulphide-linked and non disulphide linked dimers are associated with IL-6, LIF, CNTF and EPO receptor activation as measured by phosphotyrosine reactivity of the dimers. Hexameric IL-6:IL-6R complexes have already been demonstrated by analytical centrifugation (Ward et al.1994) and IL-6 mutagenesis and IL-6 receptor immunoprecipitation studies (Paonessa et al.1995). The hexameric complex contains two molecules of IL-6, two molecules of IL-6R $\alpha$  and two molecules of gp130 (Ward

et al.1994). This stoichiometry is facilitated by the ability of IL-6 to interact with its receptor through three distinct binding sites, one for IL-6R $\alpha$  and two for gp130. The ability of gp130 to homodimerize completes and stabilises the hexamer.

In the case of the LIF receptor, activation involves heterodimerization of two different signalling subunits one of which is also able to bind ligand. LIF receptor binds LIF and associates with gp130 to form an active heterodimer. The stoichiometry of this complex has been recently reported and found to be 1:1:1 (Zhang et al.1997) in contrast to the 2:2:2 stoichiometry of the IL-6 receptor complex (Ward et al.1994). This different arrangement is probably due to LIFR being both a signalling and binding subunit.

In the case of the GM-CSF, IL-3, IL-5 receptors, the stoichiometry of the ligand-receptor complex has not yet been defined. This system appears to be different to the IL-6 receptor system as GM-CSF has been found to bind its receptor through two distinct sites; one comprising helix D (Hercus et al.1994b; Seelig et al.1994) and probably also helix C (Brown et al.1994) that interacts with the specific receptor  $\alpha$  chains, and a second one involving mainly the conserved Glu motif in Helix A (Lopez et al.1992b; Shanafelt et al.1992; Hercus et al.1994b; Hercus et al.1994a) that interacts with the B'-C' and F'-G' loops of domain 4 in  $\beta_C$  (Woodcock et al.1994; Lock et al.1994; Woodcock et al.1996).

Ligand-induced oligomerization of cytokine receptor chains not only unites the extracellular but also the intracellular parts of the proteins, a process that initiates a

signalling cascade within the cell. Although haemopoietic cytokine receptors lack intrinsic kinase activity, this is compensated for by kinases of the “Janus” family (JAKs) that are associated with the receptors. JAKs activate both the ras pathway and signal transducers and activators of transcription (STAT).

Activation of the ras pathway has been shown to occur in response to a variety of ligands and studies are beginning to identify the mechanisms by which ligand binding activates this pathway. EPO and IL-3 activate the ras pathway by initially inducing tyrosine phosphorylation of Shc. Following Shc phosphorylation, Grb2 associates with Shc, and subsequently with Sos; there are increases in ras-bound GTP; activation of raf-1; tyrosine phosphorylation of mitogen-activated protein kinases(MAPK); and induction of immediate early genes such as pim-1, c-myc, and c-fos(Carroll et al.1991; Satoh et al.1991; Sakamaki K et al.1992; Sato et al.1993; Cutler et al.1994; Damen et al.1993; Carroll et al.1990). The direct links which are involved in the pathway are still controversial and the requirement for JAKs in activation of the ras pathway is unclear. One theory is that activated JAKs phosphorylate the receptors and create a Shc docking site. Once recruited to the receptor complex, the JAKs may then phosphorylate Shc and initiate a series of events associated with activation of the ras pathway.

Within the JAK family of molecules, four members of the JAK family have been identified, JAK1, JAK2, JAK3 and TYK2(Firnbach-Kraft,1990; Wilks et al.1991; Harpur et al.1992; Witthuhn et al.1994). A region near the N-terminus of the JAK proteins is required for receptor-binding. In many cases, the JAKs associate

constitutively with several different cytokine receptors. For instance, JAK2 associates with  $\beta_c$  (Quelle et al.1994), EPO receptor (Witthuhn et al.1993; Miura et al.1994) and GH receptor (Argetsinger et al.1993) whereas JAK1 associates with IL-2R $\beta$  and JAK3 with IL-2R $\gamma$  (Miyazaki et al.1994). JAKs bind to receptors via conserved proline-rich motifs in the membrane-proximal cytoplasmic regions. In the resting state, kinase activity is suppressed by both an intramolecular mechanism involving the pseudo-kinase domain of JAK, and by phosphatases such as SHP-1 (Yi et al.1993). Juxtaposition of the JAKs attached to receptor chains leads to their activation by a mechanism that involves transphosphorylation of the JAK molecules (Guschin et al.1995; Gauzzi et al.1996).

The role of phosphatases in signalling is poorly understood although two phosphatases have been shown to be closely involved in cytokine signalling. SHP-1 is a phosphatase primarily expressed in haemopoietic tissues that appears to serve as a general down-regulator of cellular tyrosine phosphorylation. SHP-1 has been demonstrated to inactivate signalling in response to EPO (Klingmuller et al.1995) by specifically associating with and dephosphorylating JAK2 (Jiao et al.1996). SHP-2 has been shown to bind to JAKs (Yin et al.1997) and play a positive role in signalling (Ali et al.1996), presumed to be able to recruit Grb2 (Fukada et al.1996) which then activates the ras pathway. Grb2 is also directly recruited to phospho Tyr targets in activated receptors or to phosphorylated Shc which itself recognises Tyr-phosphorylated receptors (de Koning et al.1996).

Once activated, JAKs are able to phosphorylate several additional cellular targets including the receptors to which they are bound and molecules that are, or become, associated with them. JAKs are then able to phosphorylate a family of latent transcription factors known as STATs. They are found mainly free in the cytoplasm but, at least in the case of STATs 1, 2 and 3, they may be also found pre-associated with receptors (Stancato et al.1996). The phosphorylated STAT proteins then dimerize which then allows them to translocate to the nucleus where they may bind to transcriptional control regions within genes. The JAK/STAT pathway seems to be more specific than the ras/MAPK pathway, which may be activated by a large variety of stimuli. The JAK/STAT pathway is more restricted to signalling in response to growth factors and cytokines.

The molecular basis for signal specificity is complex, since the actions of a great number of cytokines are channelled through the activities of only four JAKs and approximately six types of STAT. The precise transcriptional responses to a given cytokine in a specific cell-type will depend on the abundance of receptors, cognate JAKs and STATs, the activities of modulators, such as phosphatases and Ser/Thr kinases as well as the DNA-sequence specificity of the STAT and its cooperative interactions with other transcription factors at the level of the target gene.

#### **1.4 The IL-3, GM-CSF and IL-5 cytokine receptor subfamily**

The receptors consist of heterodimers comprising a ligand-specific  $\alpha$ -chain and a common  $\beta$  chain. The cytokines bind their cognate receptor  $\alpha$ -chains with low

affinity, whereas the  $\beta_c$  alone does not show any detectable binding to any of these cytokines. The  $\beta_c$  is required, in association with the ligand-bound  $\alpha$ -chain, to confer high affinity binding (Hayashida et al.1990; Gearing et al.1989; Tavernier et al.1991; Kitamura et al.1991b). The high affinity conversion varies between the individual receptors; in the case of the IL-3R the  $\beta_c$  increases IL-3 affinity 500~1000 fold (Kitamura et al.1991b; Kitamura et al.1992), In the case of the GM-CSF receptor  $\beta_c$  increases binding affinity 20~100 fold (Hayashida et al.1990), and in the case of the IL-5 receptor  $\beta_c$  increases IL-5 binding affinity 2~5 fold (Tavernier et al.1991; Tavernier et al.1992). The involvement of the  $\beta_c$  not only alters the affinity of the receptor but it is essential for signal transduction (Kitamura et al.1991b; Kitamura et al.1992; Sakamaki K et al.1992).

#### **1.4.1 Receptor expression and distribution**

The receptors for IL-3, GM-CSF and IL-5 are expressed and distributed extensively on a variety of cells covering both haemopoietic and non-haemopoietic cells. Traditionally receptor investigation was carried out by radiolabelled ligand binding assays. It has been shown that eosinophils express high affinity receptor for all three ligands, while monocytes only have GM-CSF and IL-3 receptors, and neutrophils, have only high affinity receptor for GM-CSF (Lopez et al.1988), except that when stimulated with GM-CSF, they also express high affinity IL-3 receptor (Smith et al.1995).

Through the use of monoclonal antibody (MAb), ligand-binding studies and receptor mRNA assays, it has also been revealed that endothelial cells express IL-3 receptor

(Colotta et al.1993; Korpelainen et al.1993) and some breast cancer cells express IL-3 and GM-CSF receptors (Douglas et al.1997). GM-CSF receptors have been detected on a variety of neoplastic cells including melanoma cells and small cell, colon and prostate carcinoma cell lines (Baldwin et al.1989; Baldwin et al.1991; Hirsch et al.1995; Rokhlin et al.1996). Although the significance of these phenomena still needs to be clarified, it extends the potential functions of IL-3, GM-CSF from haemopoiesis to inflammation and possibly to oncogenesis as well.

The GM-CSF/IL-3/IL-5 subfamily of receptors has many overlapping activities, in particular when all three receptors are co-expressed on cells. It is possible to elicit similar functions with any one of the ligands, as is the case with eosinophils and basophils. IL-3 is able to stimulate the development of a number of different lineages of haematopoietic cells, through interaction with immature multipotential haematopoietic progenitors as well as lineage committed progenitors. The lineage committed cells that IL-3 is able to stimulate include granulocytes, macrophages, eosinophils, mast cells, megakaryocytes and erythroid cells (Metcalf,1992; Arai et al.1990). GM-CSF was originally defined as a factor that stimulates colony formation of granulocyte and macrophages. Since these initial studies it has been shown to have a much broader effect ( Table 1.1)(Metcalf,1992; Arai et al.1990; Gasson,1991). IL-5 is more specific, its major role is in the development of eosinophils (Takatsu et al.1988). IL-3, GM-CSF and IL-5 elicit a similar effect on eosinophils and basophils (Sanderson,1992).

Table 1 Major Cellular Targets of IL-3, GM-CSF and IL-5

Biological Activities	IL-3	GM-CSF	IL-5
Blast cell colony formation	+	+	-
Mixed cell colony formation	+	+	-
GM colony formation	+	+	-
Activation of monocytes	+	+	-
Induction of megakaryocyte differentiation	+	+	-
Eosinophil colony formation	+	+	+
Stimulation of eosinophil degranulation	+	+	+
Stimulation of histamine release from basophils	+	+	+
Activation of neutrophils	-	+	-

A phenomena which is unique to this subfamily, is that of GM-CSF, IL-3 and IL-5 cross competing on cells that express more than one type of receptor . In addition GM-CSF, IL-3 and IL-5 cross competition occurs on the cell surface in a hierarchical nature. On eosinophils that express all three receptors, the binding hierarchy is GM-CSF > IL-3 > IL-5, where GM-CSF is the most effective competitor(Lopez et al.1992a). The sharing of the  $\beta_c$  chain by the three  $\alpha$  chains may explain the cross competition but there is no definitive explanation at present for the hierarchy observed.

### 1.4.2 Mechanisms of receptor activation

The  $\alpha$  and  $\beta$  chain heterodimerisation is an essential step for the activation of IL-3, GM-CSF and IL-5 receptors. It is not known if the receptors form only heterodimers or larger oligomers. Following ligand binding and receptor dimerisation, the activation of the receptor complex is initiated. The signalling is mediated through the  $\beta_c$  in association with tyrosine kinases. One of the earliest events after receptor activation is the induction of tyrosine phosphorylation of the  $\beta_c$  chain and several cytoplasmic proteins. Although neither the  $\alpha$  nor  $\beta_c$  in the IL-3, GM-CSF and IL-5 receptors has intrinsic tyrosine kinase activity, rapid tyrosine phosphorylation of many cellular proteins has been described in human cells stimulated by IL-3 or GM-CSF (Miyajima et al.1993). Phosphorylation of various cellular proteins then leads to the activation of various signalling molecules that include Ras (Satoh et al.1992; Duronio et al.1992) and the JAK-STAT (Darnell,1996) pathways. The Ras pathway has been shown to be responsible for anti-apoptotic signals (Wang et al.1992), while the JAK-STAT pathways lead to DNA synthesis (Muli et al.1996). Regions of the  $\beta_c$  receptor cytoplasmic domain have been mapped which that are involved in activating specific stimuli. Initially it was shown that a region between amino acids 518 and 626 was responsible for the tyrosine phosphorylation of intracellular proteins while a membrane proximal region of the receptor between amino acids 456 and 487 is essential for proliferation (Sakamaki K et al.1992). Recently more specific mapping of the  $\beta_c$  chain has determined more precise functional regions. Regions to 541 have been shown to be sufficient to signal clonal suppression, induction of differentiation markers and JAK 2 phosphorylation(Smith et al.1997). Regions between 472 and 583 are sufficient to initiate short term proliferation (Sakamaki K et al.1992; Sato et

al.1993), while regions between 559 and 626 appear to be required for cell survival and proliferation (Kinoshita et al.1995; Smith et al.1997). Regions 626 to 783 induce macrophage migration activity and regions between 642 and 778 are required for induction of the Shc-ras-raf MAP kinase and PI-3 kinase pathway (Sato et al.1993; Smith et al.1997). Other than JAK2 activation it has been shown that other cytoplasmic kinases such as c-fos, p56<sup>lyn</sup>, p62<sup>yes</sup> and Bcr-abl are activated but the specific region of  $\beta_c$  responsible for this activation has not been identified (Corey et al.1994; Hanazono et al.1993; Li et al.1995; Wilson-Rawls et al.1996)

### **1.5 Aims of this Thesis**

Although many residues and motifs in the IL-3, GM-CSF and IL-5 receptors  $\alpha$ - and  $\beta$ - chains have been reported to be important in ligand binding and function the overall pattern of ligand/receptor and receptor/receptor interaction for IL-3, GM-CSF and IL-5 is only just emerging. The process involving formation of ligand-induced receptor assembly and type of complex that is required for receptor activation is still poorly understood .

The focus of thesis is mostly on the extracellular events that lead to activation of the GM-CSF, IL-3 and IL-5 receptors and does not address the cytoplasmic activation pathways and signalling molecules associated with this receptor family following receptor activation. Instead it addresses a number of molecular events, important in receptor activation which in turn activates the signalling pathways. This is addressed through biochemical studies and mutagenesis of  $\beta_c$ . These studies were done with

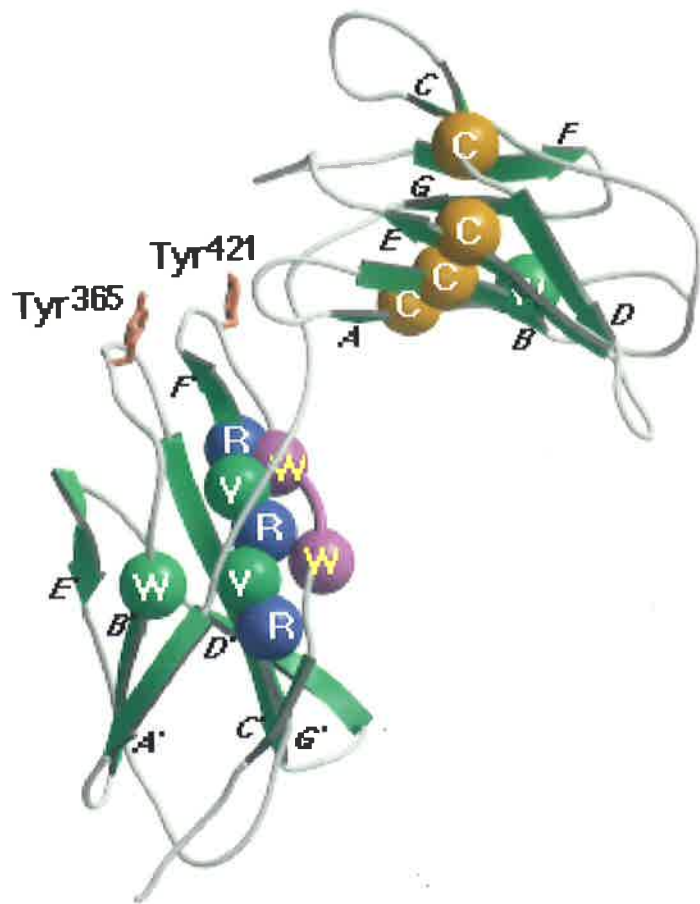
the aim to provide a model system on which receptor activation can be based within this receptor subfamily.

In particular, the identification of key events that control receptor assembly and receptor activation after binding of GM-CSF, IL-3 and IL-5 to their respective receptors are examined. There are a number of paradigms for receptor assembly. Examples of other receptor system models include firstly, that of the GHR where upon stimulation with GH, one GHR subunit binds the ligand, the ligand has a second binding site, which docks in another GHR subunit noncovalently, leading to the formation of a signalling homodimer (Wells et al.1993). The EPOR is another example of homodimerization except it is thought that EPO does not actively induce receptor dimerization, but instead the EPOR exists as a preformed complex (Miura et al.1993a). An example of heterodimerization that is ligand dependent, is the LIF receptor, where the LIFR binds LIF, triggering LIFR to heterodimerize with gp130, producing an active complex. In this complex the LIFR is both a binding and signalling subunit and acts in conjunction with gp130, which is only a signalling subunit (Zhang et al.1997). gp130 is also involved in a number of other receptor complexes, including the IL-6R and CNTF receptor. IL-6 triggers the heterodimerization of the IL6R $\alpha$ , the major binding subunit, with gp130, the signalling unit. This in turn proceeds to homodimerization of gp130 to a second gp130 molecule in a covalent manner (Murakami et al.1993). In the case of the CNTF receptor, CNTF heterodimerizes with gp130 non covalently, while gp130 heterodimerizes with the LIFR covalently. CNTFR is only a binding unit, gp130 and LIFR are the signalling subunits (Davis et al.1993). A common feature of all the

signalling subunits is that upon dimerization and activation they all become tyrosine phosphorylated.

This thesis focuses on the events that are involved in assembling the GM-CSF/IL-3/IL-5 receptor complex, receptor activation and initiation of signalling. It is known that dimerization is required for activation. That both an  $\alpha$  chain and a  $\beta$  chain must come in contact with a ligand. Signalling members of the receptor complex become tyrosine phosphorylated. A number of receptors show covalent association of the signalling partners. The exact cascade of events leading to signalling of the GM-CSF, IL-3 and IL-5 receptors is still poorly understood. This thesis not only makes a comparison between this cytokine subfamily and the other cytokine subfamilies, it also makes a comparison between the three receptors within this subfamily and common and unique features are revealed.

**Figure 1.1** Ribbon diagram of the third and fourth domains of  $\beta_C$ . Ribbon diagram of the third and fourth domains of  $\beta_C$  with conserved residues indicated by CPK spheres. Conserved residues are coloured as follows; Cys, yellow, buried hydrophobics, green, Arg, blue; Trp of WSXWS sequence, purple. The two Tyr residues involved in ligand-binding are drawn in red stick form. The strands are labelled in close proximity to the arrow illustrating the direction of the relevant strand.



**Figure 1.2** Sequences of the immediate cytoplasmic regions of various receptor signalling subunits. The positions of intron/exon boundaries are indicated by (∇). In some cases, these have been inferred from the mouse genes. Conserved residues are boxed with a consensus indicated below; aliphatic (*f*), hydrophobic ( $\Omega$ ), hydrophilic ( $\diamond$ ) or the conserved residue.

▽
← box 1 →
▽

βc	RFGCIYGYR	LRRK	WEEK	IPN	PSK	..	SHLFQNGSAE	..	LW	PPGSMSAFTS
TPOR	RWQFP	ALWPS	LPDL	HRV	LGQY	LRDTA	ALSPP	KATV	S	SDTCEE
EPOR	HRRAL	KQKI	WPG	IPSP	ESEF	EGLFT	THKGN	FQLW	LYQ	NDGCLWW
IL2β	NCRNT	GPKK	VLCN	TPDP	SKFF	SQLS	SEHGG	DVQK	WLS	SPFPSSSF
gp130	NKRDL	IKKH	IWP	VPDP	SKSH	IA	.....	QW	SPHT	PPRHNF
LIFR	RKREW	IKET	FYPD	IPNP	ENC	.....	.....	KALQ	FQKS	VCEGSSA
GCSFR	SPNR	KNPL	WPS	VPDP	AHSS	LGSW	PTIME	EDAF	QLP	GLGTPPI
OBR	SHQR	MKKL	FWED	VPNP	KNCS	WAQGL	..	NFQK	PETF	EHLFIKHTAV
IL4β	KIKKE	WWDQ	IPNP	ARSRL	VAI	IIQDA	QGSQ	WE	KRSR	RGQEPA
conservation:		f K Ω	W	f P P					W	

▽
← box 2 →

βc	GSPPHQGPWGS	RFP	ELEGV	FPV	GGFDSE	V	SPL	TIE	DPKH	VCDP
TPOR	.....	.....	.....	.....	.....	.....	VEPS	LLET	LPKSS	ERTPL
EPOR	SPCTPFTE	.....	.....	.....	.....	.....	DPPAS	LEVL	SERC	WGTM
IL2β	SPGGLA	.....	.....	.....	.....	.....	PEISP	LEVLER	DKVT	QL
gp130	NSKDQMYSDGNF	.....	.....	.....	.....	.....	TDVSV	VEIE	ANDK	KPPEDLKSLD
LIFR	LKTLEMNP	.....	.....	.....	.....	.....	CTPNN	VEVLE	TRS	AFFKIEDTEII
GCSFR	.....	.....	.....	.....	.....	.....	TKLTV	LEEDE	KKPP	WESHN
OBR	T	.....	.....	.....	.....	.....	CGPLL	LEPET	ISED	ISVDTSWKNK
IL4β	KCPHWKNCLTK	.....	.....	.....	.....	.....	LLPC	FLEH	NMKR	DEDPHK
conservation:							f	f E	◇◇◇	

## *Chapter II*

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### **MATERIALS AND METHODS**

## **2.1 Cells**

### **2.1.1 RG cells**

Primary cells obtained from the blood of a patient with chronic myeloid leukaemia (RG cells) after separation on Ficoll-Paque (Pharmacia). RG cells were T and B cell antigen negative, CD33 and CD34 positive, and IL-3R $\alpha$ , GM-CSFR $\alpha$  and  $\beta_c$  positive, as judged by flow cytometry using specific MAb followed by FITC-conjugated goat anti-mouse Ig.

### **2.1.2 COS cell transfectants**

COS cells were maintained in RPMI-1640 supplemented with 10 % v/v fetal calf serum (FCS) and transfected by electroporation. Routinely  $2 \times 10^7$  COS cells were co-transfected in 0.8 ml PBS at 0°C with 25  $\mu$ g of wild type or mutated  $\beta_c$  cDNA together with 10  $\mu$ g of GM-CSFR and 10  $\mu$ g IL-3R  $\alpha$ -chain cDNA at 500  $\mu$ F with 300 V. After electroporation cells were centrifuged through a 1 ml cushion of FCS and cells were plated in either 25 ml of medium per 150 cm<sup>2</sup> flask or 24 well plates for binding analysis. Transfectants were incubated for 2 days prior to cytokine treatment (Woodcock et al.1994).

### **2.1.3 HEK293T cell transfectants**

The HEK293T cell line derived from the adenovirus type transformed human embryonic kidney 293 cell line, containing the simian virus 40 large tumour antigen (DuBridge,1987) were maintained in RPMI-1640 medium supplemented with 10% v/v FCS. On the day before transfection,  $1.4 \times 10^6$  cells were plated into 6 cm tissue culture dishes to adhere overnight. Four hours after a medium change, 6  $\mu$ g of wild

type or mutated  $\beta_c$  cDNA together with 4  $\mu\text{g}$  of GM-CSFR  $\alpha$  or 4  $\mu\text{g}$  IL-3R  $\alpha$  or 4  $\mu\text{g}$  IL-5R  $\alpha$ -chain cDNAs and 0.5  $\mu\text{g}$  of JAK2 cDNA were added to cells in the form of a calcium phosphate precipitate (Graham et al.1973), and the cells were placed in an incubator for 4 h to permit the uptake of the DNA-calcium phosphate precipitate. The cells were then washed, replated in 4 plates/150  $\text{cm}^2$  and placed in the incubator for 48h prior to cytokine treatment.

#### **2.1.4 Mo7e cell line**

A human megakaryoblastic cell line was obtained from Dr P Crozier, Auckland , New Zealand. The cells were maintained in DMEM supplemented with 10% FCS and 5 ng/ml IL-3.

#### **2.1.5 UT7 cell line**

A human megakaryoblastic cell line was obtained from Dr P Simmons Division of Haematology, Hanson Centre for Cancer Research. The cells were maintained in RPMI supplemented with 10% FCS and 2 ng/ml IL-3.

#### **2.1.6 Ba/F-3 cells**

Ba/F-3 cells expressing GMR $\alpha$  and  $\beta_c$  were obtained from from S. Barry Division of Immunology, Hanson Centre for Cancer Research. The cells were maintained in DMEM supplemented with 10% FCS and 2 ng/ml GM-CSF.

### **2.1.7 TF-1.8 cells**

TF-1.8 cells ( a gift from Dr J Tavernier from Roche Research Ghent, Belgium) were maintained in RPMI-1640 medium supplemented with 10% FCS and 2ng/ml GM-CSF.

### **2.1.8 Chinese Hamster Ovary (CHO) cell Line**

A9/C7 CHO cell line expressing GM-CSFR $\alpha$ -chain and  $\beta_c$  was developed by Dr Jo Woodcock in this Laboratory as described previously(Hercus et al.1994b). The CHO cells were maintained in F12 medium supplemented with 10% FCS.

The CHO cell lines, s $\beta_c$  CHO and sGMR $\alpha$  CHO were developed as described previously for the GMR $\alpha$  CHO cell line, A9/C7 (Hercus et al.1994b). CHO lines expressing sGMR $\alpha$  or GMR $\alpha$  were subsequently co-transfected with either  $\beta_{cp}Rc/CMVpuro$  or s $\beta_{cp}Rc/CMVpuro$  by the same method and selected in 2.5 $\mu$ g/ml Puromycin (Calbiochem, La Jolla, CA). Cell surface expression of transfected receptors was confirmed by flow cytometry as described previously (Stomski et al.1996) and analysed on an EPICS Profile II Flow Cytometer (Coulter Electronics, Hialeah, FL).

### **2.1.9 Purification of human eosinophils and monocytes**

Eosinophils were purified from the peripheral blood of eosinophilic individuals by centrifugation on a hypertonic gradient of metrizamide as described previously (Vadas et al.1979). Monocytes were purified from the peripheral blood of normal

donors obtained from the Adelaide Red Cross Transfusion Service as described previously (Elliot et al.1989).

All factor dependent cell lines were routinely starved of growth factor overnight prior to cytokine treatment.

## **2.2 Antibodies**

### **2.2.1 Antibodies to cytokine receptors**

MAB against the GM-CSFR $\alpha$ , IL-3R $\alpha$ , IL-5R $\alpha$  and  $\beta_c$  chains were raised by immunizing mice with COS cells transiently transfected with GM-CSFR $\alpha$  or IL-3R $\alpha$  IL-5R $\alpha$  or  $\beta_c$  cDNA and selecting on CHO cell transfectants stably expressing each receptor chain. MAb 8G6 and 4H1 against the GM-CSFR $\alpha$ , MAb 9F5 and 6B6 against the IL-3R $\alpha$  chain, Mab A14 against the IL-5R $\alpha$  and MAb 4F3, 3D7 and 8E4 against  $\beta_c$  were selected for their ability to provide a strong signal in immunoprecipitations. MAb 8D10 against the GM-CSFR $\alpha$ , MAb 9F5 against the IL-3R $\alpha$  and MAb 1C1 against  $\beta_c$  and an anti-peptide polyclonal rabbit antibody against residues 131-241 of  $\beta_c$  were used for immunoblotting and were selected for giving a strong signal in Western blotting. MAb 7G3 is directed against the IL-3Ra chain and blocks IL-3 binding and IL-3-mediated functions (Sun et al.1996). Each MAb was produced as ascites fluid and purified by Protein G Sepharose. For immunoprecipitation experiments, MAb 9F5, 8G6, 4H1, 4F3 and 8E4 were directly coupled to Sepharose beads using CNBr-activated Sepharose 4B as previously described (Stomski et al.1992).

### **2.2.2 Anti-phosphotyrosine monoclonal antibodies**

MAB against phosphorylated tyrosine was either anti-phosphotyrosine-peroxidase conjugated 3-365-10(Boehringer Mannheim, Frankfurt, Germany) or PY20 antibody horse radish peroxidase conjugated (Sapphire Bioscience, Alexandria, NSW)

## **2.3 Cytokines**

### **2.3.1 Production of cytokines**

Recombinant human IL-3, GM-CSF, IL-5 and IL-3 mutant E22R were produced in *E. coli* and purified to homogeneity by reverse-phase HPLC(Barry et al.1994; Hercus et al.1994a). Cytokine purity and quantitation was determined by HPLC analysis. The unit activity of the cytokines based on the ED<sub>50</sub> values in a TF-1 proliferation assay (Kitamura et al.1989) was 0.03ng/ml GM-CSF, 0.1 ng/ml IL-3 and 0.3 ng/ml IL-5 equal to 1 unit respectively.

### **2.3.2 Radiolabelling cytokines**

Recombinant IL-3, and GM-CSF were radio-iodinated by the iodine monochloride method (Contreras et al.1983) to a specific activity of about 36 mCi/mg. Routinely 4 µg of protein was iodinated and separated from iodide ions on a Sephadex G-25 column (Pharmacia, North Ryde, NSW, Australia), eluted with PBS containing 0.02% v/v Tween 20, and the iodinated proteins stored at 4°C for up to 4 weeks. Prior to use, the radioligands were purified from Tween and non-protein-associated radioactivity by cation-exchange chromatography on a Carboxymethyl-Sephrose CL-6B column (Pharmacia, Uppsala, Sweden) which had been pre-equilibrated with

10 mM citrate-phosphate buffer (pH 2.6). The column was washed and the radioligand was eluted with binding medium consisting of RPMI 1640 supplemented with 10 mM HEPES, 0.5% BSA and 0.1% sodium azide.

#### **2.4 Saturation binding assays**

Binding assays were performed on CHO cells grown to confluency in 96 well plates over a concentration range of 10pM - 10nM <sup>125</sup>I-labelled GM-CSF in binding medium [RPMI containing 0.5% (w/v) BSA and 0.1% (w/v) Sodium Azide] with non-specific binding determined at each concentration with excess unlabelled GM-CSF. After incubation at room temperature for 2 hours radioligand was removed and the wells washed briefly twice in binding medium. Where stated, low affinity binding was then removed with five sequential 15 minute washes in binding medium. Specific counts were determined after lysis of the cell monolayer with subsequent transfer and counting on a  $\gamma$ -counter (Cobra Auto Gamma; Packard Instruments Co, Meriden, CT). Dissociation constants were calculated using the EBDA and LIGAND programs (Munson et al.1980) (Elsevier Biosoft, Cambridge, United Kingdom).

Binding assays were performed on soluble receptors in solution in a similar fashion to soluble receptor assays described previously (Nicola et al.1992). Aliquots of soluble receptor (100  $\mu$ l) were incubated with <sup>125</sup>I-labelled GM-CSF (10  $\mu$ l) over a concentration range of 0.5nM-20nM. An excess of unlabelled GM-CSF was added to assays to determine non-specific binding. Assays were incubated at room temperature for 1 hr. and then Con A-sepharose (10  $\mu$ l of 50% slurry in PBS) added

to each tube and allowed to bind over 1 hour. Sepharose (100 µl of 50% slurry in PBS) was then added to each assay to increase the amount of precipitable material and tubes centrifuged to pellet the beads. Pelleted beads were washed once in PBS and then the radioactivity determined by counting on a  $\gamma$ -counter.

## 2.5 Kinetic Binding Assays

Association kinetics were determined at 4°C with eosinophils and monocytes using radio-iodinated cytokines at 200 pM. Cells ( $2-4 \times 10^6$  per tube) were incubated in 0.15 ml of binding medium containing radioligand with or without 100 fold excess unlabelled cytokine in borosilicate tubes on a rotating table. Assays were harvested at time points after addition of radio-iodinated cytokine by overlaying onto 0.2 ml FCS and spinning for 30 s at maximum speed in a Beckman microfuge. The visible cell pellet was removed by cutting and the radioactivity in the pellet determined on the  $\gamma$ -counter. The apparent association rate ( $K_{Obs}$ ) was calculated using the KINETIC program (Elsevier Biosoft, Cambridge, United Kingdom) from the specific binding data.  $K_{Obs}$  is a composite function encompassing both on and off rates ( $K_{On}$  and  $K_{Off}$  respectively) from the receptor:

$$K_{Obs} = K_{On}[L] + K_{Off}$$

## 2.6 Proliferation Assay

GM-CSF dependent TF-1 cells or TF-1.8 cells were starved of GM-CSF for 24 to 48 hours before setting up proliferation assays. Briefly,  $1 \times 10^4$  cells were incubated in wells with preferred cytokines in the presence of a range of concentrations of ligand analogues for 48 hours at 37 °C. Wells were pulsed with 0.5 mCi/well  $^3\text{H}$ -thymidine

for 4 hours and then harvested onto a glass filter. Radioactivities of each well were determined by liquid scintillation. The results were expressed as disintegrations per minute (DPM).

## 2.7 Mutagenesis of human $\beta_c$ and expression plasmid constructs

Cysteine residues were substituted with alanines in the human  $\beta$  chain cDNA using oligonucleotide-directed mutagenesis (Altered-sites, Promega, Sydney, NSW, Australia) as described previously (Woodcock et al.1994). The mutations were confirmed by nucleotide sequencing and the mutant  $\beta_c$  cDNAs subcloned into the eukaryotic expression vector *pcDNA1* (Invitrogen, San Diego, CA). The IL-3R, GM-CSFR and IL-5R  $\alpha$ -chain cDNAs were cloned into the eukaryotic expression vector *pCDM8* (Invitrogen) for transfection (Woodcock et al.1994).

## 2.8 Plasmid construction

The cDNA for the human  $\beta_c$  was cloned by polymerase chain reaction (PCR) from cDNA prepared from the KMT-2 cell line (Barry et al.1994). A soluble form of the  $\beta_c$  ( $s\beta_c$ ) was created by PCR using the following synthetic oligonucleotides:

- 1) 5'-TGAATTCGCCTGTCCAGAGCTGACCAGGG-3' that starts 25 nucleotides 5' of the ATG and contains an engineered *HindIII* site and
- 2) 5'- ATACACTCTATATCACGACTCGGTGTCCCAGGAGCG -3' that contains an inframe termination codon immediately 5' of the transmembrane region and followed by an engineered *XbaI* site. The PCR product obtained from these primers was subcloned into the Neomycin resistance conferring expression vector pRc/CMV (Invitrogen Corporation, San Diego, CA) giving rise to  $s\beta_{cpRc/CMV}$ . A soluble

form of the human GMR $\alpha$  (sGMR $\alpha$ ) was made in a similar fashion using the following synthetic oligonucleotides:

- 1) 5'-ATACACAAGCTTAGCACCATGCTTCTCCTGGTG-3' that starts 18 nucleotides 5' of the ATG and contains an engineered *HindIII* site and
- 2) 5'-ATACACTCTAGATCACCCGTCGTCAGAACCAAATTC-3' that contains an inframe termination codon immediately 5' of the transmembrane region and followed by an engineered *XbaI* site. The PCR product obtained from this set of primers was subcloned into pRc/CMV to produce sGMR $\alpha$ pRc/CMV.

To allow for dual stable transfection of two receptors pRc/CMV was engineered such that the neomycin resistance gene (Neo<sup>R</sup>) was replaced with the puromycin resistance gene (*pac*) from pRuf puro (Jenkins et al.1995). Briefly, the 1.5kb *KpnI*-*BamHI* fragment from pRc/CMV containing Neo<sup>R</sup> and its flanking SV40 early promoter and poly-adenylation region was subcloned into pUC19. The Neo<sup>R</sup> gene was removed by *EcoRV*-*NaeI* digestion and *pac* introduced as a *Sall*-*ClaI* fragment from pRuf puro. The puromycin resistance gene plus flanking SV40 early promoter and poly-adenylation region was excised from pUC19 as a *KpnI*-*BamHI* fragment and subcloned into *KpnI*-partial *BamHI* digested s $\beta$ <sub>cp</sub>Rc/CMV resulting in s $\beta$ <sub>cp</sub>Rc/CMVpuro. Subsequently full length  $\beta$ <sub>c</sub> cDNA was introduced in on a *EcoRI*-*XbaI* fragment thereby generating  $\beta$ <sub>cp</sub>Rc/CMVpuro.

## 2.9 Purification of Recombinant Soluble Human $\beta$ <sub>c</sub> Receptor

Soluble  $\beta$ <sub>c</sub> protein was purified from conditioned medium from CHO cells stably expressing the protein using a 3D7 anti- $\beta$ <sub>c</sub> monoclonal antibody affinity column.

Bound soluble  $\beta_c$  was eluted with a linear gradient of 0-3M KSCN in 10mM Tris-HCl pH8.0 and subsequently buffer exchanged into phosphate buffered saline (PBS) containing 0.02% (vol/vol) Tween 20 [polyoxyethylene (20)- sorbitan monolaurate].

### **2.10 Deglycosylation conditions**

Deglycosylation of proteins was performed after immunoprecipitation with the protein still attached to the Protein-A sepharose beads. The immunoprecipitated protein was firstly incubated in 200mM sodium cacodylate pH 7.0, 0.1% SDS and then 0.75% NP40 with Neuraminidase, O-Glycanase (Genzyme) and N-Glycanase (New England Biolabs) for 18 hours at 37°C before separation by SDS-PAGE.

### **2.11 Analysis of receptor cell surface expression by flow cytometry**

Cell surface expression of transfected receptor subunits was confirmed by indirect immuno-fluorescence staining using anti-receptor  $\alpha$  and  $\beta$  chain specific monoclonal antibodies. Fifty microlitres of cells ( $10^5$  cells) were added to 50ml of optimally diluted antibody and incubated at 4°C for 30min. The cells were washed with PBS and then incubated with 1/50 fluoresceinated rabbit anti-mouse antibody (DDAF Silenus, Australia) for 30min at 4°C. The cells were then washed and resuspended in FACS FIX (phosphate-buffered saline with 2% glucose, 1% formaldehyde, and 0.02% sodium azide) and analysed on a Coulter Profile Flow Cytometer (Coulter Electronics, Hialeah, FL).

### **2.12 <sup>125</sup>I surface labelling of cells**

Cells were cell-surface labelled with <sup>125</sup>I by the lactoperoxidase method as described previously (Walsh et al.1977). Approximately 10<sup>8</sup> cells were washed twice in PBS and then labelled with 1mCi <sup>125</sup>I (NEN, AMRAD Pharmacia Biotech, Boronia, Vic, Australia) in PBS.

### **2.13 Immunoprecipitation of the cytokine receptors**

Either labelled or unlabelled cells were incubated with medium, IL-3, GM-CSF, IL-5 or IL-3 mutant E22R, for different times before lysing the cells. The cells were lysed in lysis buffer consisting of 137mM NaCl, 10mM Tris-HCl (pH 7.4), 10% glycerol, 1% Nonidet P-40 (NP40) with protease inhibitors (10µg/ml leupeptin, 2mM phenylmethsulfonyl fluoride, 10µg/ml aprotinin) and 2mM sodium vanadate for 30 mins at 4°C followed by centrifugation of the lysate for 15 mins at 12,000g 4°C . Following a 1 hour preclearance with Protein-A-sepharose (Pierce, Rockford, IL) at 4°C, the supernatant was incubated for 18 hours with anti-GM-CSFRα, anti-IL-3Rα, or anti-β<sub>c</sub> MAb coupled Sepharose beads for 2 hours at 4°C. After this, the beads were washed 6 times with lysis buffer before boiling for 5 min in SDS-PAGE sample buffer either in the presence or absence of 2-mercaptoethanol (ie. reducing or non-reducing) before separating immunoprecipitated proteins by SDS-PAGE. In some experiments, the alkylating agent iodoacetamide was added to the cells for 20 min at 4°C before or after the cells were incubated with IL-3.

## 2.14 SDS-PAGE

Immunoprecipitated proteins were analysed by one-dimensional (1D) or two-dimensional (2D) SDS-PAGE under reducing and non-reducing conditions. Proteins separated by 2D SDS-PAGE were subjected to separation under non-reducing conditions in tube gels followed by separation under reducing conditions in the second dimension (Phillips et al.1977). For 1D gels MW were estimated using commercially available MW markers either Biorad Broad Range Standards 161-0318 (Bio-rad Laboratories Pty. Ltd., Regents Park, NSW, Australia) or SeeBlue™ Pre-Stained Standards (Novex French's Forest, NSW, Australia). For 2D gels, the MW of the separated proteins was estimated by mixing the <sup>125</sup>I-labelled immunoprecipitated proteins with solubilized platelet proteins and using the known molecular weight of the major characterized platelet proteins visualized by Coomassie blue staining. The <sup>125</sup>I-labelled immunoprecipitated proteins were detected and quantified by means of a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Silver staining of gels was carried out as described previously (Morrissey,1981).

## 2.15 Immunoblot and ECL

Immunoprecipitated proteins separated by SDS-PAGE were transferred to nitrocellulose membrane by electroblotting. Routinely, nitrocellulose membranes were blocked in a solution of PBS/0.05% (vol/vol) Tween 20 (PBT) containing 1% (w/v) blocking reagent 1096 176 (Boehringer Mannheim, Frankfurt, Germany) and then probed with MAb's to anti-GM-CSFR $\alpha$  (8D10), anti-IL-3R $\alpha$  (9F5), IL-3R  $\beta_c$  (1C1 or rabbit polyclonal), peroxidase-conjugated anti-phosphotyrosine 3-365-10

(Boehringer Mannheim, Frankfurt, Germany) and horse radish peroxidase conjugated PY20 antibody (Sapphire Bioscience, Alexandria, NSW), followed where appropriate by either rabbit anti-mouse horseradish peroxidase (Dako) or goat anti-rabbit horseradish peroxidase (Dako). Immunoreactive proteins were detected by chemiluminescence using the the ECL kit (Amersham, Little Chalfont, U.K.) as per manufacturer's guidelines. Stripping of membranes was carried out by incubating nitrocellulose membrane for 30 minutes at 50 °C in 100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH 6.7 followed by two sequential washes in TNT. Membranes were reblocked for 1 hour before reprobing.

## *Chapter III*

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**HUMAN INTERLEUKIN-3 (IL-3) INDUCES  
DISULPHIDE-LINKED IL-3 RECEPTOR  $\alpha$  AND  $\beta$  CHAIN  
HETERODIMERIZATION WHICH IS REQUIRED FOR  
RECEPTOR ACTIVATION BUT NOT HIGH AFFINITY  
BINDING**

### 3.1 INTRODUCTION

Engagement of the human interleukin-3 receptor by IL-3 triggers a variety of cellular signals resulting in the preservation of cell viability, proliferation and differentiation of haemopoietic cells (Clark et al.1987; Metcalf,1989). Expression of the IL-3 receptor, whilst subject to regulation, is maintained during haemopoietic cell differentiation, and its activation on the mature cells leads to enhanced function of monocytes (Elliot et al.1989), eosinophils (Lopez et al.1987) basophils (Haak-Frendscho et al.1988; Lopez et al.1990) and neutrophils (Smith et al.1995). The IL-3 receptor has been shown to be expressed also on endothelial cells with activation by IL-3 stimulating cytokine release and the expression of adhesion molecules (Korpelainen et al.1995; Korpelainen et al.1993). The wide expression of the IL-3 receptor on haemopoietic cells and on cells of the vasculature suggests roles in haemopoiesis, allergy, atherosclerosis and chronic inflammation. However, the mechanism of IL-3 receptor activation remains unclear.

The human IL-3 receptor is a heterodimeric receptor consisting of an IL-3 specific  $\alpha$  chain (Kitamura et al.1991b), and a common  $\beta$  chain (Hayashida et al.1990) that is also a component of the receptors for GM-CSF and IL-5 (Lopez et al.1992a),(Nicola et al.1991). Both receptor chains belong to the cytokine receptor superfamily (Bazan,1990b), although it has been noted that the IL-3 receptor  $\alpha$  chain is more closely related to the GM-CSF and IL-5 receptor  $\alpha$  chains than to other cytokine receptors (Goodall et al.1993). Thus, these three  $\alpha$  chains can be recognized structurally, and possibly functionally, as a distinct subfamily.  $\beta_c$ , on the other hand, is structurally more closely related to gp130 and the IL-2 receptor  $\beta$  chain (Goodall et al.1993) and, analogous to these common receptor subunits, converts low affinity

ligand binding into high affinity binding, and acts as a signal transducer (Miyajima et al.1992).

The expression of both IL-3R $\alpha$  and  $\beta_c$  is necessary for triggering signalling and cellular proliferation in response to IL-3 (Kitamura et al.1992). Stimulation of cells with IL-3 leads to activation of JAK-2 (Quelle et al.1994; Silvennoinen et al.1993) and Lyn (Torigoe et al.1992) kinases, phosphatidylcholine hydrolysis and protein kinase C translocation (Rao et al.1995), activation of multiple isoforms of the signal transducer protein Stat 5 (Azam et al.1995; Mui et al.1995) and gene expression (Watanabe et al.1993). Although some form of receptor dimerization has been presumed to take place, the relative contribution of each receptor chain, the nature of their association and the implications for receptor activation are not known.

Receptor dimerization is recognized to be important for activation in many receptor systems. For example, receptor tyrosine kinases (Heldin,1995; Lev et al.1992; Ullrich et al.1990), as well as non tyrosine kinase receptors such as the G-CSF receptor (Hiraoka et al.1994), undergo homodimerization following ligand binding which leads to signalling. Homodimerization has also been observed in the EPO receptor (Miura et al.1993b) and in a mutant EPO receptor that is constitutively active (Watowich et al.1994). IL-6 receptor and CNTF receptor dimerization have also been shown to occur. These receptors are more analogous to the IL-3 receptor in that they comprise a binding subunit and signalling subunits (Davis et al.1993; Hibi et al.1990). In these cases ligand triggers subunit association but only dimerization of the signalling subunits, gp130 in the case of IL-6, and gp130 and LIFR $\beta$  in the case of CNTF, mediate signalling (Davis et al.1993; Murakami et

al.1993). In the IL-3/GM-CSF/IL-5 receptor system, however, the biochemical evidence of receptor dimerization has been missing. Furthermore, the requirements for the  $\alpha$  chain and  $\beta_c$  in receptor activation based on cell lines transfected with genetically manipulated receptors is controversial. On the one hand chimeric receptors comprising extracellular  $\alpha$  chains and intracellular  $\beta_c$  allow function in the presence of intact  $\beta_c$  and ligand, suggesting that  $\beta_c$  dimerization is sufficient for signalling (Eder et al.1994; Muto et al.1995; Takaki et al.1994). On the other hand, deletion of the cytoplasmic domain of the  $\alpha$  chains abolishes ligand-mediated stimulation suggesting that an intact  $\alpha$  chain may be required for receptor dimerization and signalling (Sakamaki K et al.1992; Weiss et al.1993).

Using primary human cells, cell lines and transfected cells the question of dimerization of IL-3R $\alpha$  and  $\beta_c$  upon stimulation with IL-3 is addressed. A comparison to IL-6 and the CNTF receptors is made, in particular does disulphide-linked dimerisation of the receptor occur.

## 3.2 RESULTS

### 3.2.1 IL-3 induces IL-3R $\alpha$ and $\beta_c$ association

In order to study the molecular events leading to IL-3 receptor activation in primary cells, MAb were developed specific for IL-3R $\alpha$  and for  $\beta_c$  which could give a strong signal in immunoprecipitation and Western blot analyses. From several MAb raised against COS cell transfectants overexpressing IL-3R $\alpha$  or  $\beta_c$ , three MAb were selected: MAb 9F5 specifically immunoprecipitated IL-3R $\alpha$  from stable CHO cell transfectants whilst MAb 4F3 immunoprecipitated  $\beta_c$  from CHO cells transfected with  $\beta_c$  cDNA. These MAb did not react with CHO cells expressing GM-CSFR $\alpha$ . For Western blot analyses, MAb 9F5 gave a strong signal on CHO cells expressing IL-3R $\alpha$  and MAb 1C1 gave the strongest signal on CHO cells expressing  $\beta_c$ . These MAb were used to screen several primary myeloid cells of which RG cells obtained from a patient with chronic myeloid leukaemia exhibited the largest number of receptor molecules as judged by flow cytometry, and were therefore used for subsequent studies.

In order to determine whether the IL-3R exists as a preformed complex or is ligand-induced, MAb 9F5 (anti-IL-3R $\alpha$ ) and MAb 4F3 (anti- $\beta_c$ ) were used in immunoprecipitation studies and the proteins separated on SDS-PAGE under reducing conditions. The results showed that that in the absence of IL-3, MAb 9F5 immunoprecipitated only IL-3R $\alpha$  and MAb 4F3 immunoprecipitated only  $\beta_c$  from <sup>125</sup>I surface-labelled RG cells (Fig. 3.1). However, the addition of IL-3 caused the co-immunoprecipitation of a 120,000 MW protein in the case of immunoprecipitation with MAb 9F5 (Fig. 3.1A), and of a 70,000 MW protein in the

case of immunoprecipitation with MAb 4F3 (Fig. 3.1B). The association of these proteins was rapid, and detectable within 30 sec. To determine the generality of this phenomenon further experiments were done using the human UT7 cell line which expresses IL-3 receptors, and COS cells transfected with the IL3 receptor  $\alpha$  and  $\beta$  chains. In both cases IL-3 induced the formation of the heterodimeric complex (Fig. 3.2A and 3.2B).

The MW of the co-immunoprecipitated proteins suggested that they corresponded to  $\beta_c$  when MAb 9F5 was used, and to IL-3R $\alpha$  when MAb 4F3 was used (Fig. 3.1). To test whether this was indeed the case, a Western blot analyses was performed using chemiluminescence as the detection method. In addition, and as a specificity control, the cells were alternatively incubated with GM-CSF (Fig. 3.3). It was found that MAb 9F5 immunoprecipitated IL-3R $\alpha$  together with a 120,000 MW protein in the presence of IL-3 but not in the presence of GM-CSF (Fig. 3.3A). Conversely MAb 4F3 immunoprecipitated  $\beta_c$  and a protein of 70,000 MW only in the presence of IL-3 (Fig. 3.3B). A consistent finding of a weak band of approximately 80,000 MW co-immunoprecipitating with  $\beta_c$  was shown to represent GM-CSFR $\alpha$ . To determine the identity of the co-immunoprecipitated bands I probed the proteins immunoprecipitated with MAb 9F5 (anti-IL-3R $\alpha$ ) by western blotting with MAb 1C1 (anti- $\beta_c$ ), and reciprocally, the proteins immunoprecipitated with MAb 4F3 (anti- $\beta_c$ ) with MAb 9F5 (anti-IL-3R $\alpha$ ). These data established that  $\beta_c$  co-immunoprecipitated with IL-3R $\alpha$  and IL-3R $\alpha$  co-immunoprecipitated with  $\beta_c$ , but only in the presence of IL-3 (Fig. 3.3B).

### **3.2.2 IL-3R complex formation is dependent on IL-3 contacting both IL-3R $\alpha$ and $\beta_c$**

To study whether IL-3 binding to both chains of the IL-3R was a prerequisite for IL-3R complex formation, co-immunoprecipitation studies were performed after preincubation with the MAb 7G3 which blocks IL-3 binding to IL-3R $\alpha$  (Sun et al.1996), and in the presence of the IL-3 mutant E22R which is defective in  $\beta_c$  interaction (Barry et al.1994). It was found that pre-incubation of cells with the blocking MAb 7G3 greatly reduced the ability of the  $\alpha$  chain to co-immunoprecipitate  $\beta_c$  (Fig. 3.4A) and of  $\beta_c$  to co-immunoprecipitate with IL-3R $\alpha$  in the presence of IL-3 (Fig. 3.4B). Experiments comparing the effects of the IL-3 mutant E22R with wild type IL-3 were performed over a range of ligand concentrations. Results showed that wild type IL-3 induced the co-immunoprecipitation of IL-3R $\alpha$  and  $\beta_c$  at a concentration of 0.1nM and maximally at 1.0nM (Fig. 3.5A and 3.5B). In contrast the IL-3 mutant E22R did not cause co-immunoprecipitation of IL-3R $\alpha$  and  $\beta_c$  at concentrations up to 100nM (Fig. 3.5C and 3.5D). At higher concentrations (4 $\mu$ M) of E22R some coimmunoprecipitation of IL-3R $\alpha$  and  $\beta_c$  was observed. Again, a protein of approximately 80,000 MW co-immunoprecipitated with  $\beta_c$  in the absence of wild type IL-3 (Fig. 3.5B) or in the presence of E22R (Fig. 3.5D) corresponding to GM-CSFR $\alpha$ . These experiments establish that IL-3 needs to contact both receptor chains in order to trigger receptor  $\alpha$  and  $\beta_c$  association.

### 3.2.3 IL-3 induces disulphide- and nondisulphide-linked receptor dimers.

To examine the possibility that IL-3 induces the covalent association of IL-3R $\alpha$  and  $\beta_c$  the next experiment was the immunoprecipitation of proteins under non-reducing conditions. The result showed that in the absence of IL-3 the MAb anti-IL-3R $\alpha$  immunoprecipitated only IL-3R $\alpha$  (Fig. 3.6A). The MAb anti- $\beta_c$ , on the other hand, immunoprecipitated mainly monomeric  $\beta_c$  but also consistently immunoprecipitated a faint band of 245,000 apparent MW (Fig. 3.6B). However, incubation with IL-3 not only led to the co-immunoprecipitation of IL-3R $\alpha$  and  $\beta_c$  as shown above but also induced the appearance of two high MW bands of approximately 215,000 MW and 245,000 apparent MW (Fig. 3.6). This was observed whether immunoprecipitations were performed with anti-IL-3R $\alpha$  MAb (Fig. 3.6A) or anti- $\beta_c$  MAb (Fig. 3.6B). The high MW complexes appeared to be disulphide-linked since treatment with the thiol-specific alkylating agent, iodoacetamide, prior to addition of IL-3, inhibited their appearance (Fig. 3.6A, track 4 and Fig. 3.6B, track 4). Despite the marked loss of the high MW complexes, iodoacetamide pre-treatment did not prevent IL-3R $\alpha$  and  $\beta_c$  co-immunoprecipitation (Fig. 3.6A, track 4 and Fig. 3.6B, track 4). Thus IL-3 induces IL-3R $\alpha$  and  $\beta_c$  association by covalent as well as non-covalent means. The controls used were iodoacetamide after IL-3 treatment in which case little or no reduction in the formation of the high MW complexes was observed (Fig. 3.6A, track 3 and Fig. 3.6B, track 3). Furthermore, the addition of IL-3 into the lysis buffer did not induce IL-3R $\alpha$  and  $\beta_c$  association (Fig. 3.6A, track 5 and Fig. 3.6B, track 5) indicating that  $\alpha$  and  $\beta_c$  association was not induced in solution. These controls demonstrate that the appearance of the high MW complexes requires free sulphhydryl groups and IL-3 acting on the intact cells.

Next was examined whether IL-3 itself was present in the immunoprecipitated complexes. The approach here was not to perform western blotting with anti-IL-3 antibodies since it was possible that IL-3 epitopes could be masked by bound receptor. Instead, the cells were stimulated with radiolabelled IL-3 in the absence or presence of a 100-fold excess of unlabelled IL-3. Results showed that  $^{125}\text{I}$ -IL-3 could be immunoprecipitated with MAb 9F5 (anti-IL-3R $\alpha$ ) or MAb 4F3 (anti- $\beta_c$ ) and this was inhibited by excess unlabelled IL-3 (Fig. 3.6C). However, IL-3 was not covalently bound to IL-3R $\alpha$  or  $\beta_c$  as demonstrated by its migration to the 15,000 MW region (Fig. 3.6C).

To identify the components of the disulphide-linked high MW bands, the next step was to perform two-dimensional SDS-PAGE and Western blot analysis. Two-dimensional gel electrophoresis of immunoprecipitates from  $^{125}\text{I}$ -labelled, IL-3 treated cells with MAb 9F5 (anti-IL-3R $\alpha$ ) and MAb 4F3 (anti- $\beta_c$ ) were performed in which the non-reduced samples were separated in the first dimension and then reduced before separation in the second dimension. The two major proteins seen on the diagonal were of 120,000 MW and of 70,000 MW corresponding to  $\beta_c$  and IL-3R $\alpha$  respectively (Fig. 3.7). The band of 215,000 MW in the non-reduced first dimension was resolved into two spots of 120,000 MW and 70,000 MW in the reduced dimension, consistent with  $\beta_c$  and IL-3R $\alpha$ , respectively. A broad, higher MW complex in the non-reduced dimension was also resolved into 120,000 MW and 70,000 MW components although this was less distinct than for the 215,000 MW complex. These experiments suggested that the two high MW bands seen under

non-reducing conditions contain IL-3R $\alpha$  and  $\beta_c$ . To examine this identity further an western blot of IL-3-stimulated cells immunoprecipitated with anti-IL-3R $\alpha$  and with anti- $\beta_c$  MAb. In the absence of IL-3 no high MW bands were immunoprecipitated with MAb anti-IL-3R $\alpha$  (Fig. 3.8A), whilst a band of 245,000 MW could be immunoprecipitated with MAb anti- $\beta_c$  (Fig. 3.8A) and also contained IL-3R $\alpha$  (Fig. 3.8B) as judged by Western blotting. After stimulation with IL-3, however, both MAb 9F5 (anti-IL-3R $\alpha$ ) and MAb 1C1 (anti- $\beta_c$ ) reacted with the 215,000 MW and 245,000 MW bands confirming that IL-3R $\alpha$  and  $\beta_c$  were both present in these disulphide-linked complexes (Fig. 3.8B and 3.8C).

#### **3.2.4 High affinity binding**

Significantly, prevention of disulphide-linked dimer formation by iodoacetamide did not affect high affinity binding of IL-3. Scatchard analysis of cells treated with iodoacetamide showed that high affinity binding was essentially the same as seen with untreated cells (Fig. 3.6D).

#### **3.2.5 Correlation between IL-3-induced disulphide-linked dimers and receptor activation**

In order to determine the functional consequences of IL-3 receptor dimerization, we examined the immunoprecipitated complexes for the presence of phosphorylated tyrosine. Immunoprecipitations were performed on  $^{125}\text{I}$  surface-labelled cells with anti-IL-3R $\alpha$  and anti  $\beta_c$  antibodies and probed the immunoprecipitates, separated under non-reducing or reducing conditions, with anti-phosphotyrosine antibodies by Western blotting. The results showed that the anti-phosphotyrosine MAb 3-365-10

reacted strongly with bands of 215,000 and 245,000 MW (>94% of phosphotyrosine) but little label (<6% of phosphotyrosine) was observed in the 120,000 MW region. This anti-phosphotyrosine staining was observed with immunoprecipitates using either 9F5 (anti-IL-3R $\alpha$ ) or 4F3 (anti- $\beta_c$ ) and disappeared when  $\alpha\beta$  covalent dimer formation was prevented by iodoacetamide (Fig. 3.9A). These results show that phosphorylation in response to IL-3 occurs primarily on the covalent  $\alpha\beta$  heterodimer, suggesting that  $\alpha\beta$  heterodimer formation is required for cellular activation. To ascertain whether both IL-3R $\alpha$  and  $\beta_c$  were being phosphorylated, the immunoprecipitates with anti-IL-3R $\alpha$  and anti- $\beta_c$  MAb were separated under reducing conditions before analysis by Western blotting with the anti-phosphotyrosine antibody 3-365-10. Under these conditions, only a phosphorylated band of about 120,000 MW was observed consistent with  $\beta_c$  but not IL-3R $\alpha$  being the phosphorylated protein present in the receptor dimer (Fig. 3.9A).

To further confirm the predominant phosphorylation of the covalent  $\alpha\beta$  heterodimer the human UT7 cell line was used (Fig. 3.9B). The onset of phosphorylation in the covalent  $\alpha\beta$  heterodimer was rapid and detectable at 1 minute (Fig. 3.9B). The proportion of tyrosine phosphorylation in these heterodimers at 1', 5', 15' was 91%, 87%, and 82% respectively. As with RG cells the formation of the covalent  $\alpha\beta$  heterodimer was prevented and tyrosine phosphorylation abolished by pretreatment of UT7 cells with iodoacetamide. To control for any possible toxic effects of iodoacetamide, UT7 cells were pretreated with this agent and then stimulated with PMA and calcium ionophore. Under the conditions used no inhibition of basal or

induced tyrosine phosphorylation was observed (Fig. 3.9C) indicating that iodoacetamide was not toxic to the cells.

### 3.3 Discussion

In this chapter it has been shown that human IL-3 binding to its receptor triggers the heterodimerization of its specific receptor binding subunit, IL-3R $\alpha$ , with  $\beta_c$  and that receptor heterodimerization is dependent on IL-3 contacting both receptor subunits. Both disulphide-linked as well as non disulphide-linked heterodimers were observed and, although IL-3 is required for their formation, IL-3 is not covalently attached to the dimers. Importantly, the disulphide-linked and not the noncovalently-linked heterodimer is shown to correlate with receptor activation but is not required for high affinity binding. These results are different from those in the IL-6, EPO and G-CSF receptor systems where receptor activation involves homodimerization of a single signalling subunit and may apply also to the related GM-CSF and IL-5 receptors.

Previous experiments have shown that IL-3 receptor activation leads to stimulation of JAK-2 (Silvennoinen et al.1993) and Lyn (Torigoe et al.1992) kinases, as well as the Ras-MAP kinase, PI3 kinase and PKC pathways (Sato et al.1994). The molecular basis of receptor activation has not been revealed although by analogy with other receptor systems it is believed to involve receptor dimerization. Receptor dimerization has been demonstrated in tyrosine kinase receptors (Heldin,1995; Ullrich et al.1990), as well as the G-CSF receptor (Hiraoka et al.1994), EPO receptor (Miura et al.1993b), IL-6 receptor (Murakami et al.1993) and CNTF receptor (Davis et al.1993). Dimerization of the IL-3/GM-CSF/IL-5 receptors has been proposed in ligand-induced as well as in ligand-independent receptor activation (D'Andrea et al.1994; Jenkins et al.1995) but no evidence has been forthcoming. The results presented in this chapter demonstrate both covalent and noncovalent dimerization of

the receptor, that it involves the ligand-binding subunit dimerizing with  $\beta_c$ , and that covalently-linkage of dimers is required for signalling. These results emphasize the importance of the  $\alpha$  chain not only as a ligand-binding subunit but also as a subunit required for signalling in primary cells. This is consistent with experiments where deletion of the cytoplasmic domain of  $\alpha$  chains abolishes signalling (Sakamaki K et al.1992; Weiss et al.1993). Other experiments have shown, however, that the cytoplasmic domain of  $\beta_c$  can substitute for the intracytoplasmic domain of the  $\alpha$  chain (Eder et al.1994; Muto et al.1995; Takaki et al.1994). The exact significance of these findings is not clear but may reflect the conserved nature of the proline-rich sequence present in the membrane proximal domain of both the  $\alpha$  chain and  $\beta_c$ , or that at least two molecules of  $\beta_c$  are required for signalling with the role of the  $\alpha$  chain being to recruit or facilitate the association of two molecules of  $\beta_c$ . These experiments demonstrate the presence of both IL-3R $\alpha$  and  $\beta_c$  in the complexes of 215,000 and 245,000 MW suggest that a heterodimer containing one  $\alpha$  chain and one  $\beta_c$  may be sufficient for signalling, although it cannot be ruled out the existence of higher MW oligomeric complexes. Definition of the stoichiometry of the active receptor complex would require direct measurements using purified receptor components as shown with the IL-6 receptor (Paonessa et al.1995; Ward et al.1994).

The presence of IL-3R $\alpha$  in the disulphide-linked IL-3 receptor dimers contrasts with the disulphide-linked dimerization of the IL-6 and CNTF receptors which involve only the signal transducer subunits. Thus, in the case of the IL-6 receptor, disulphide-linked dimerization involves only gp130 (Davis et al.1993), whilst in the case of the CNTF receptor gp130 and LIFR $\beta$  form disulphide-linked dimers (Davis

et al.1993), events which appear to initiate signal transduction. These findings suggest a fundamental difference in the functional contribution of IL-3R $\alpha$  compared with IL-6R $\alpha$  and CNTFR $\alpha$  in receptor activation, and suggest that IL-3R $\alpha$  is not only involved in the initial binding of ligand but also participates in signalling. These results are consistent with differences in the requirement for the cytoplasmic domain of the  $\alpha$  chains for signalling. Thus, whilst the cytoplasmic domain of the  $\alpha$  chains of the IL-3, GM-CSF and IL-5 sub-family of receptors are essential for signal transduction (Polotskaya et al.1993; Sakamaki K et al.1992), the cytoplasmic portion of IL-6R $\alpha$  is not (Yawata et al.1993). On the other hand the IL-3Ra behaves similarly to the LIF receptor which participates both in ligand binding and signalling.

Human IL-3 receptor heterodimerization upon addition of IL-3 was found to be very rapid (it is complete within 1 minute), and dose-dependent. An anti-IL3R $\alpha$  monoclonal antibody which inhibits IL-3 binding to this chain (Sun et al.1996) prevented IL-3-induced receptor heterodimerization. Similarly, the IL-3 mutant E22R which is selectively deficient at interacting with  $\beta_c$  (Barry et al.1994) failed to induce receptor dimerization in a concentration range in which wild type IL-3 did so. This indicates that IL-3 needs to contact both receptor chains to stabilize the complex and induce receptor dimerization.

Under reducing conditions of SDS-PAGE the IL-3-induced dimers were resolved into monomeric IL-3R $\alpha$  and  $\beta_c$ . Under non-reducing conditions, however, two high molecular weight complexes of 215,000 and 245,000 MW were also seen in immunoprecipitations performed with both anti-IL3R $\alpha$  and anti- $\beta_c$  MAb. These

were disulphide-linked dimers as judged by their disappearance in the presence of the thiol-specific alkylating agent iodoacetamide added before but not after incubation with IL-3. In the absence of IL-3 a faint band of about 245,000 apparent MW was consistently seen, but only in immunoprecipitations performed with MAb anti- $\beta_c$  (Fig. 3.6 and 3.8). Western blotting showed this band to contain  $\beta_c$  and IL-3R $\alpha$  (Fig. 3.8B), suggesting that a small proportion of receptors may exist as preformed, though inactive (see below) dimers. IL-3 was present in the IL-3-induced receptor dimers although it was not disulphide-linked to either receptor chain (Fig. 3.6).

The size of the two high MW complexes suggested that they might represent IL-3R $\alpha$  and  $\beta_c$  heterodimers or  $\beta_c$  homodimers. By performing two dimensional SDS-PAGE (Fig. 3.7) and Western blotting (Fig. 3.8) experiments, it was possible to show that both IL-3R $\alpha$  and  $\beta_c$  were present in the 215,000 and 245,000 MW bands. In addition, the two dimensional SDS-PAGE suggested that complexes of higher MW were also formed. The presence of both IL-3R $\alpha$  and  $\beta_c$  in these complexes may account for the 215,000 MW band, however, the 245,000 MW band may contain another protein in addition to IL-3R $\alpha$  and  $\beta_c$ . This accessory protein is not IL-3 as IL-3 is not disulphide-linked to either receptor chain. It is possible that a third, poorly-iodinated surface protein or a small cytoplasmic protein becomes covalently linked to the IL-3 receptor upon activation, however its presence and identity remain to be determined.

The presence of IL-3-induced disulphide-linked and non disulphide-linked heterodimers suggests two types of IL-3R $\alpha$  and  $\beta_c$  interaction, a non-covalent one

and one that is mediated by Cys-Cys bridging of the receptors. The reason for the presence of both types of complexes or the sequence of their appearance is not clear. It is possible that initially IL-3 binds to IL-3R $\alpha$  and the IL-3/IL-3R $\alpha$  complex binds  $\beta_c$  forming a high affinity complex. IL-3 high affinity binding then triggers a non-covalent association, with both events being mediated by the CRM 2 in  $\beta_c$  (Fig. 3.10). The main function of these events may be then to bring IL-3R $\alpha$  and  $\beta_c$  into close proximity thus facilitating a proportion of the dimers undergoing disulphide-linkage through the CRM1 of  $\beta_c$  (Fig. 3.10). These disulphide-linked dimers are therefore not required for high affinity binding but instead are then involved in recruiting tyrosine kinases which phosphorylate dimerized  $\beta_c$  and intracellular proteins. Alternatively, the association of IL-3R $\alpha$  and  $\beta_c$  may be primarily covalent but under SDS-PAGE, a proportion of this complex is disrupted by intramolecular disulphide bond rearrangement.

In this chapter it has been shown that by preventing disulphide-linked dimer formation it was possible to dissociate high affinity binding from receptor activation. Thus high affinity binding could take place in the absence of receptor activation (Fig. 3.6 and 3.9). It may therefore be possible to construct IL-3 antagonists that retain full high affinity binding but which are deficient at inducing disulphide-linked dimers. On the other hand, if high affinity binding inevitably leads to disulphide-linked dimer formation and cellular activation, antagonism of IL-3 may rely largely on compounds that prevent disulphide-linked dimerization.

Two lines of evidence showed that disulphide-linked receptor dimerization is involved in receptor activation. Firstly, the vast majority of the tyrosine phosphorylated bands immunoprecipitated by anti-IL-3R $\alpha$  or anti- $\beta_c$  MAb corresponded to the disulphide-linked heterodimers (Fig. 3.9). This is even more remarkable when it is considered that whilst <17% of the IL-3-dependent IL-3R $\alpha$  and  $\beta_c$  heterodimers are disulphide-linked, >94% of the tyrosine phosphorylation is associated with these dimers. Secondly, blocking of the thiol groups with iodoacetamide prevented receptor phosphorylation under conditions that did not affect cell viability and total cellular phosphorylation (Fig. 3.9C) or high affinity binding (Fig 3.6D). This is analogous to the IL-6 receptor where the presence of gp130 homodimers is accompanied by tyrosine phosphorylation of these proteins (Murakami et al.1993). The disulphide-linked heterodimerization of IL-3R $\alpha$  and  $\beta_c$  appears therefore essential for tyrosine phosphorylation which is known to involve the Janus family of tyrosine kinases (Silvennoinen et al.1993). Since both IL-3R $\alpha$  and  $\beta_c$  contain intracellular tyrosine residues, it was necessary to examine whether both receptor chains were being phosphorylated by reduction of the disulphide-linked dimers with DTT and probing with anti-phosphotyrosine antibodies. This revealed that  $\beta_c$  but not IL-3R $\alpha$  was the phosphorylated protein in the heterodimers (Fig. 3.9). This is consistent with activation of JAK-2 causing phosphorylation of  $\beta_c$  (Quelle et al.1994; Silvennoinen et al.1993). It should be noted that although disulphide-linked dimerization appears essential for receptor phosphorylation, it is possible that other functions are not affected and it would be of interest to examine functions such as proliferation under conditions that prevent disulphide-linked dimer formation. The

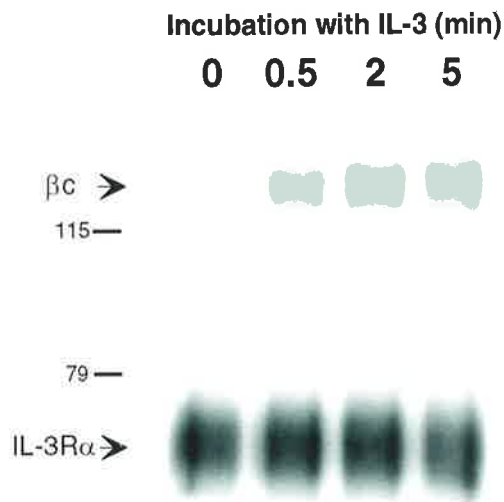
identification of the cysteines involved in disulphide formation and their mutation may reveal IL-3 activation pathways that occur independently of this process.

The IL-3-induced appearance of disulphide-linked IL-3R $\alpha$  and  $\beta_c$  dimers suggests the presence of free Cys in these molecules. Examination of the sequences of IL-3R $\alpha$  and  $\beta_c$  and modelling of the cytokine receptor modules (Elliott et al.1989), suggest that IL-3R $\alpha$  has two potentially unpaired Cys, one in the *N*-terminal domain at either positions 52, 68 or 76 in the *N*-terminal region, and one at position 195 in domain 1 of the cytokine receptor module (CRM) (Fig. 3.10). Similarly,  $\beta_c$  has two potentially free Cys, one at either positions 86, 91, 96 or 100 in domain 1, and one at position 234 in domain 2 of the CRM 1 (Fig. 3.10). It is interesting to note that, in contrast to the  $\alpha$  chains,  $\beta_c$  has two CRMs. Whilst the CRM2 has been implicated in binding IL-3, GM-CSF and IL-5 (Lock et al.1994; Woodcock et al.1996), the function of the CRM1 is unknown. This leads to a hypothesis that this is important for disulphide linkage and suggest that, analogous to the cytoplasmic domain of  $\beta_c$  (Sato et al.1994), the extracellular region can be viewed as having two functional domains, with CRM2 involved in ligand recognition and nondisulphide-linked heterodimerization (Fig. 3.10), and CRM1 involved in the final activation step which includes  $\alpha\beta$  disulphide formation. The possible stoichiometry of this complex is discussed in chapter 7.

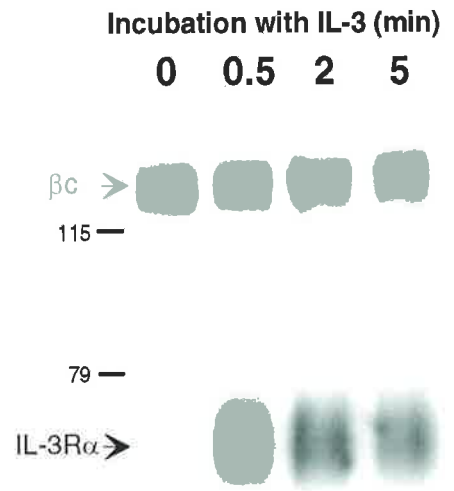
It is also worth noting that the *N*-terminal regions of IL-3R $\alpha$ , GM-CSFR $\alpha$  and IL-5R $\alpha$  are significantly conserved between each other and are present only in this family of receptors (Goodall et al.1993). Although the number of Cys in this region

varies between IL-3R $\alpha$  (3 Cys), GM-CSFR $\alpha$  (5 Cys) and IL-5R $\alpha$  (1 Cys), all three  $\alpha$  chains exhibit one unpaired Cys. This raises the possibility that these unpaired Cys are involved in disulphide-linked dimerization and suggests a functional reason for the conservation of this N-terminal region in the IL-3, GM-CSF, IL-5 receptor  $\alpha$  chain subfamily.

**Figure 3.1 IL-3 induces IL-3 receptor complex formation.** <sup>125</sup>I surface-labelled cells were incubated with 50nM IL-3 for different times at 4°C. After cell lysis proteins were immunoprecipitated with MAb 9F5 (anti-IL-3R $\alpha$ ) (A) or MAb 4F3 (anti- $\beta_c$ ) (B), separated on 7.5% SDS-PAGE under reducing conditions and visualized by phosphorimaging. The positions and MW (000's) of marker protein are indicated.

**A**

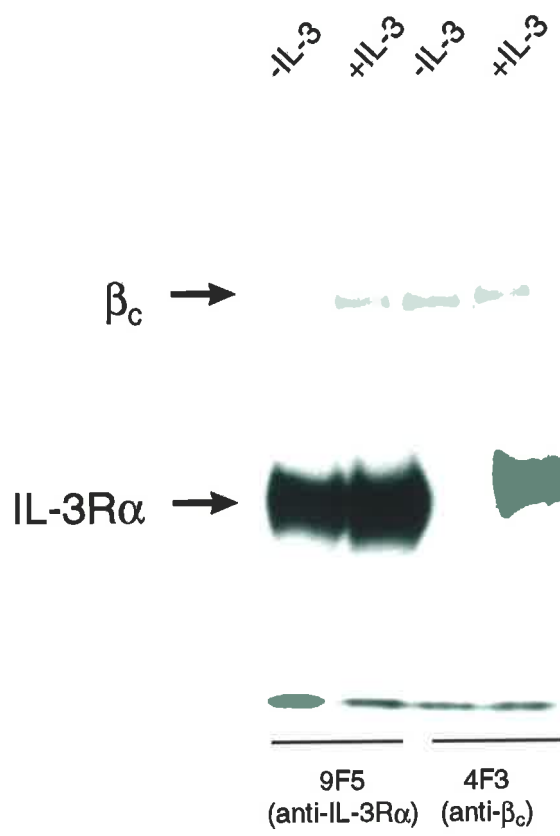
9F5 (anti-IL-3R $\alpha$ )

**B**

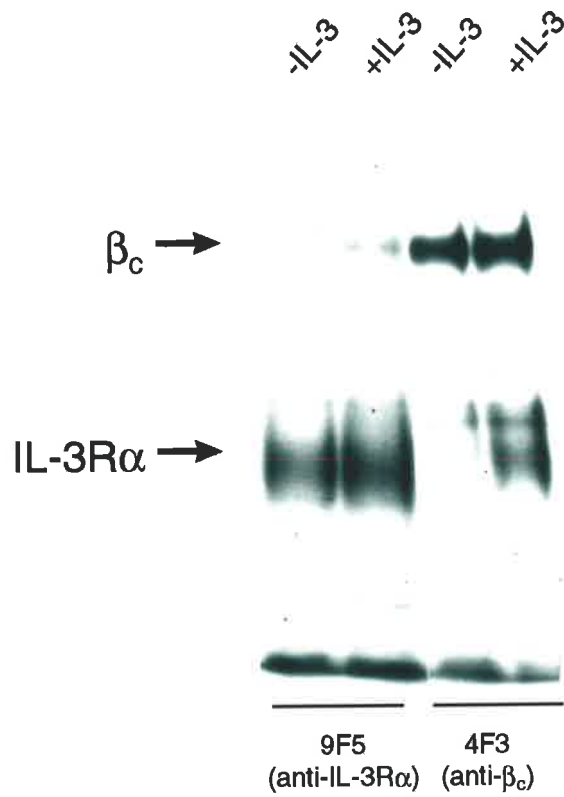
4F3 (anti- $\beta c$ )

**Figure 3.2 IL-3 induces IL-3 receptor complex formation in the human UT7 cell line and COS cells transfected with IL-3R $\alpha$  and  $\beta_c$ .** <sup>125</sup>I surface labelled cells UT7 cells (A), and COS cells transfected with IL-3R $\alpha$  and  $\beta_c$  (B) were incubated with 50nM IL-3 for 15' at 4°C. After cell lysis proteins were immunoprecipitated with MAb 9F5 (anti-IL-3R $\alpha$ ) or MAb 4F3 (anti- $\beta_c$ ), separated on 7.5% SDS-PAGE under reducing conditions and visualized by phosphorimaging. The positions and MW (000's) of marker protein are indicated.

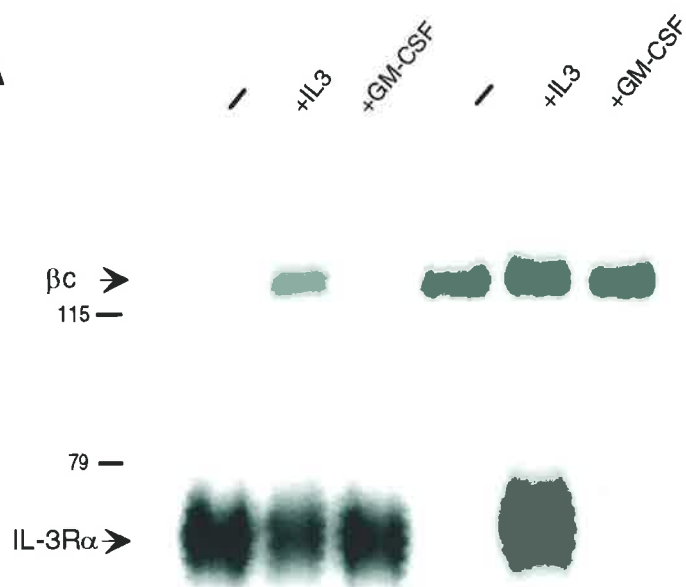
A



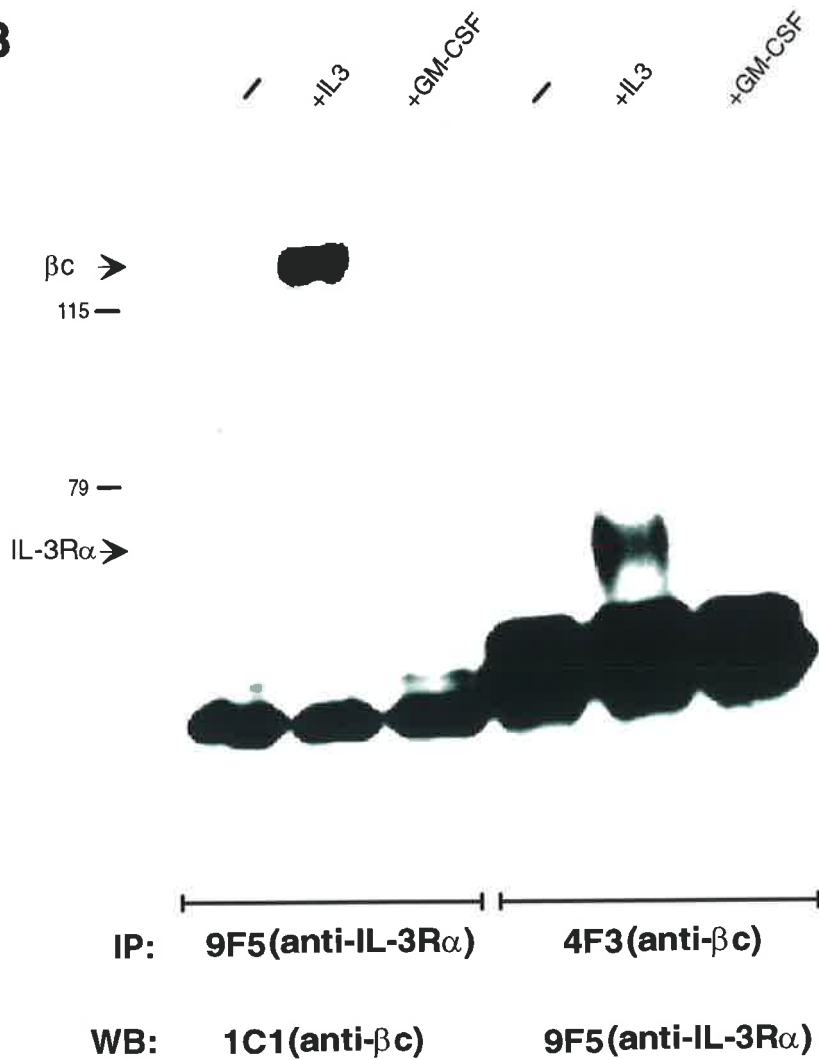
B



**Figure 3.3: Identification of co-immunoprecipitated bands as IL-3R $\alpha$  and  $\beta_c$  by Western blotting.** A) Immunoprecipitations using MAb 9F5 (anti IL-3R $\alpha$ ) or 4F3 (anti- $\beta_c$ ) and SDS-PAGE as described in Figure 1 from cells untreated (-) or treated with IL-3 (50nM) or GM-CSF (50nM) for 45 min at 4°C, and visualized by phosphorimaging. B) Western blot of A) using 1C1 (anti- $\beta_c$ ) or MAb 9F5 (anti IL-3R $\alpha$ ). Major band represents immunoreactivity of immunoprecipitating MAb.

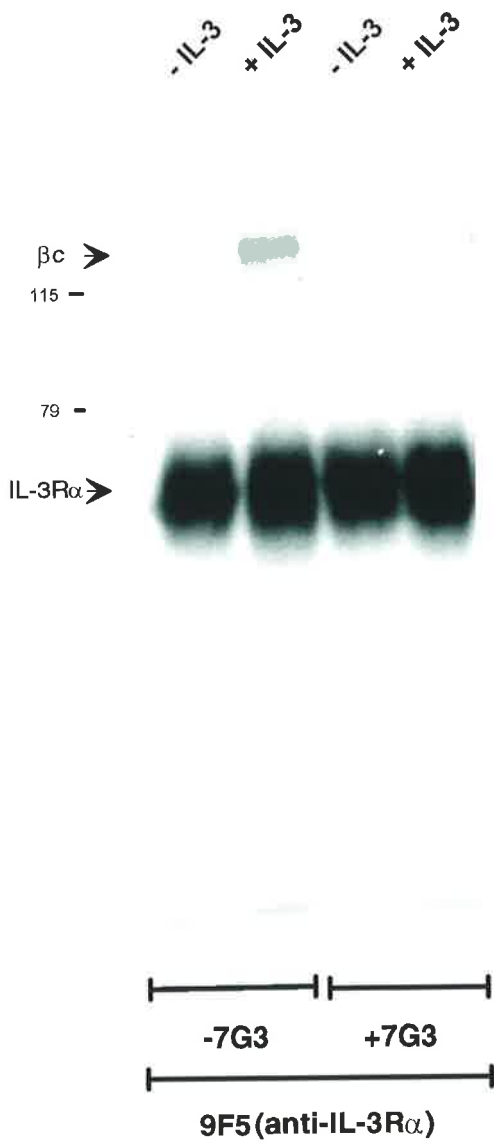
**A**

9F5(anti-IL-3R $\alpha$ ) 4F3(anti- $\beta c$ )

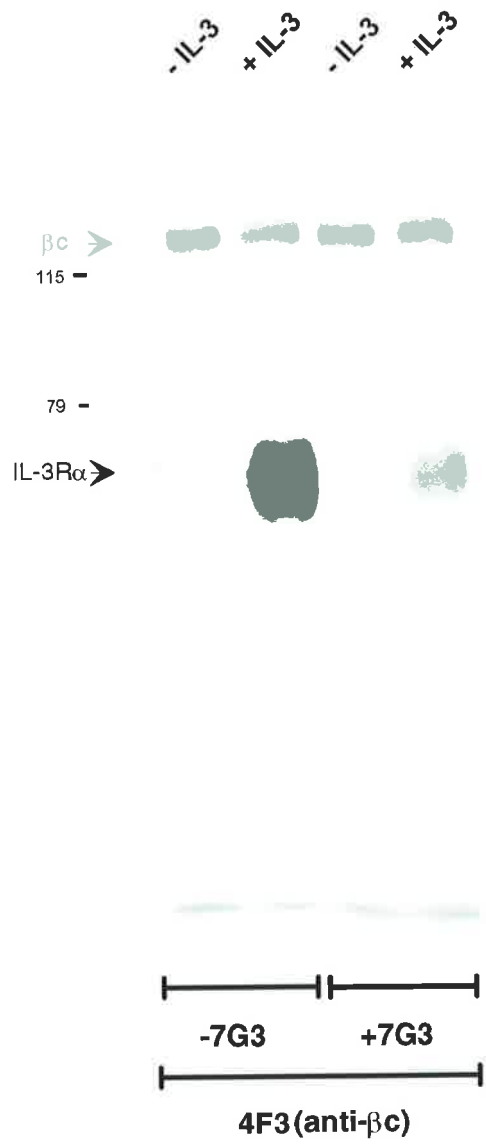
**B**

**Figure 3.4: The anti-IL-3R $\alpha$  blocking MAb 7G3 inhibits IL-3R $\alpha$  and  $\beta_c$  dimerization.** <sup>125</sup>I surface-labelled cells were preincubated with 1 $\mu$ g MAb 7G3 for 30 min at 4°C before treatment with or without IL-3 (50nM) for 45 min at 4°C. The cells were then lysed and the lysates subjected to immunoprecipitation with MAb 9F5 (anti-IL-3R $\alpha$ ) (A), or MAb 4F3 (anti- $\beta_c$ ) (B) as described in Figure 3.1.

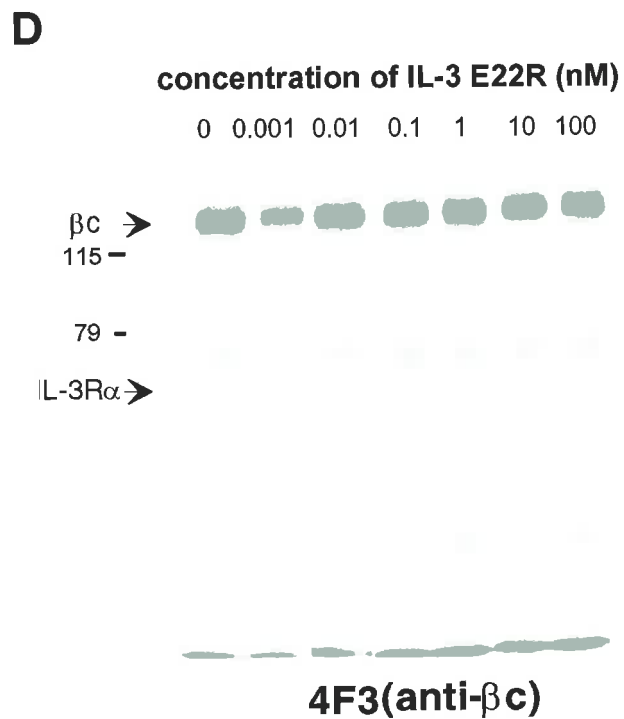
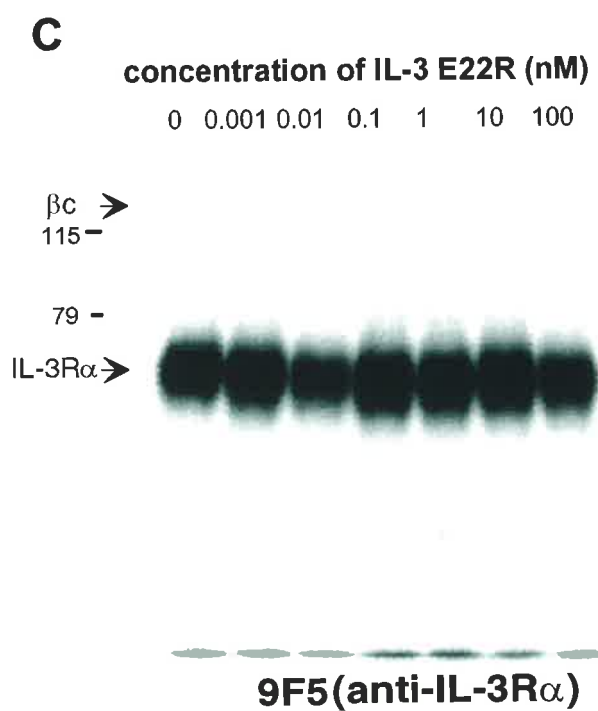
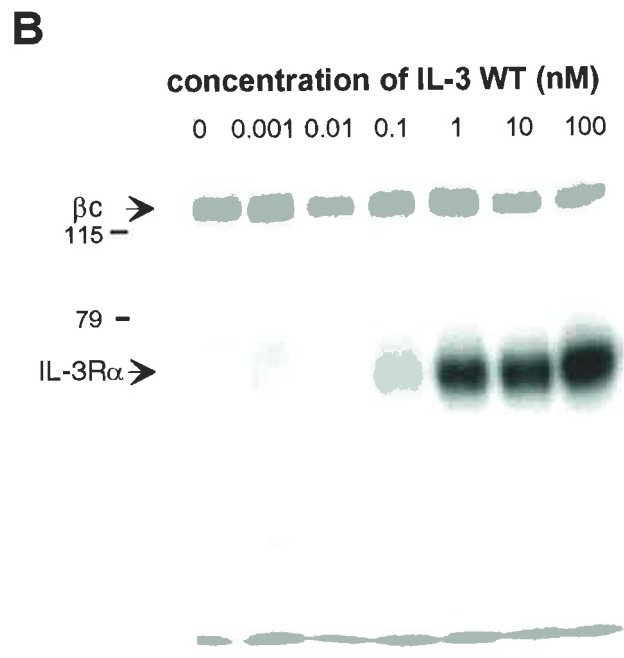
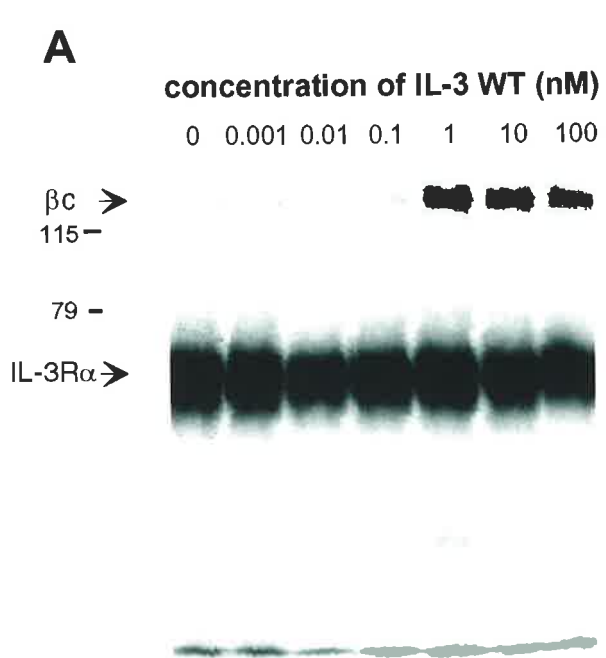
**A**



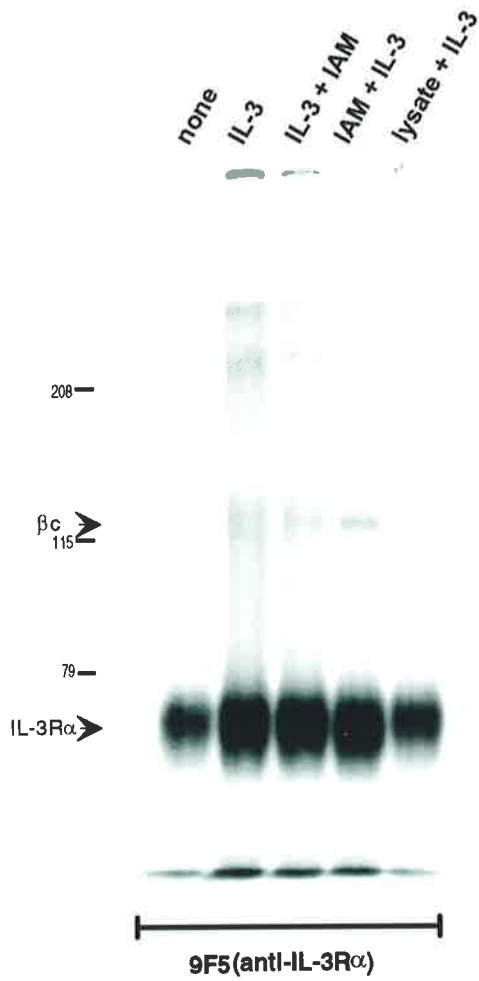
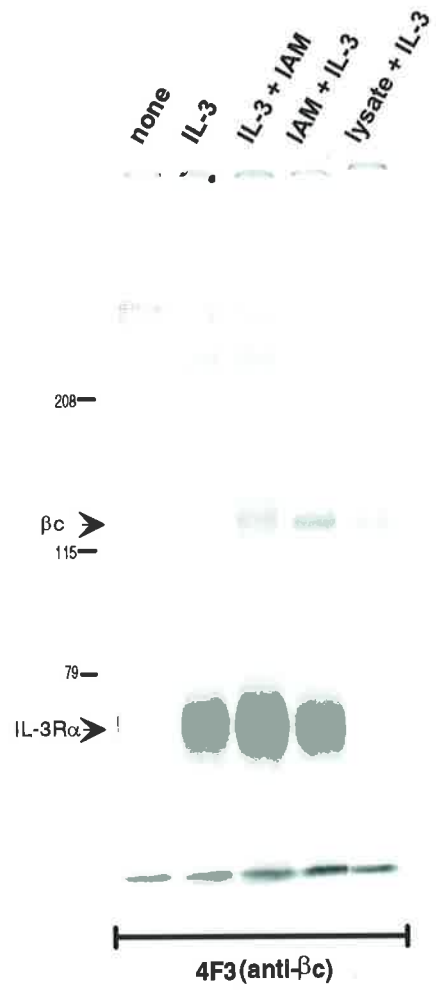
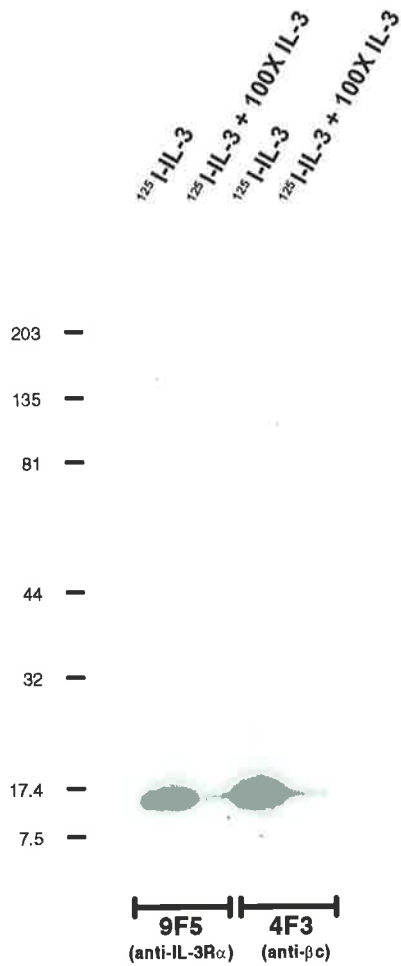
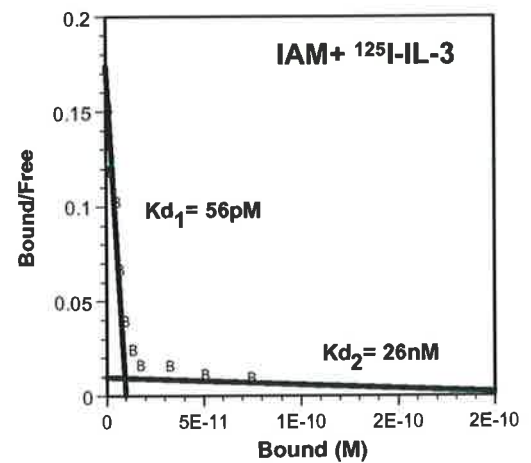
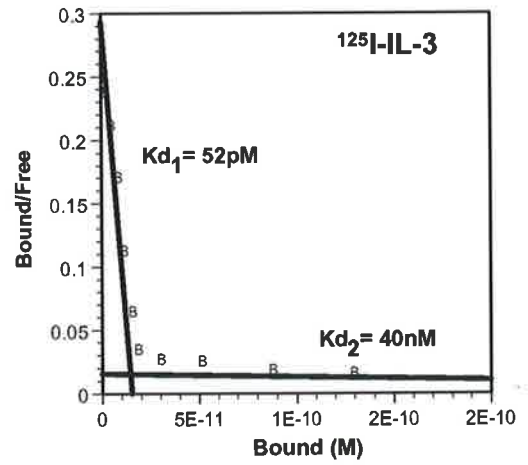
**B**



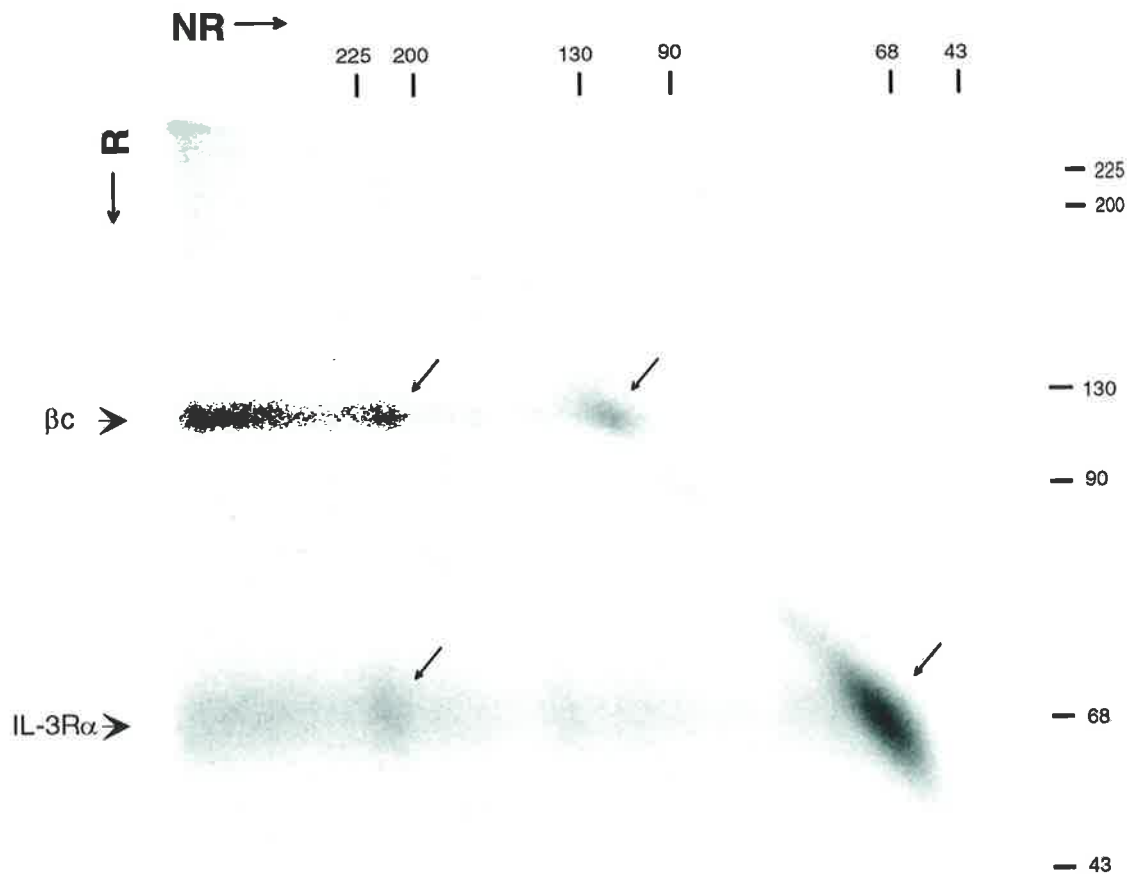
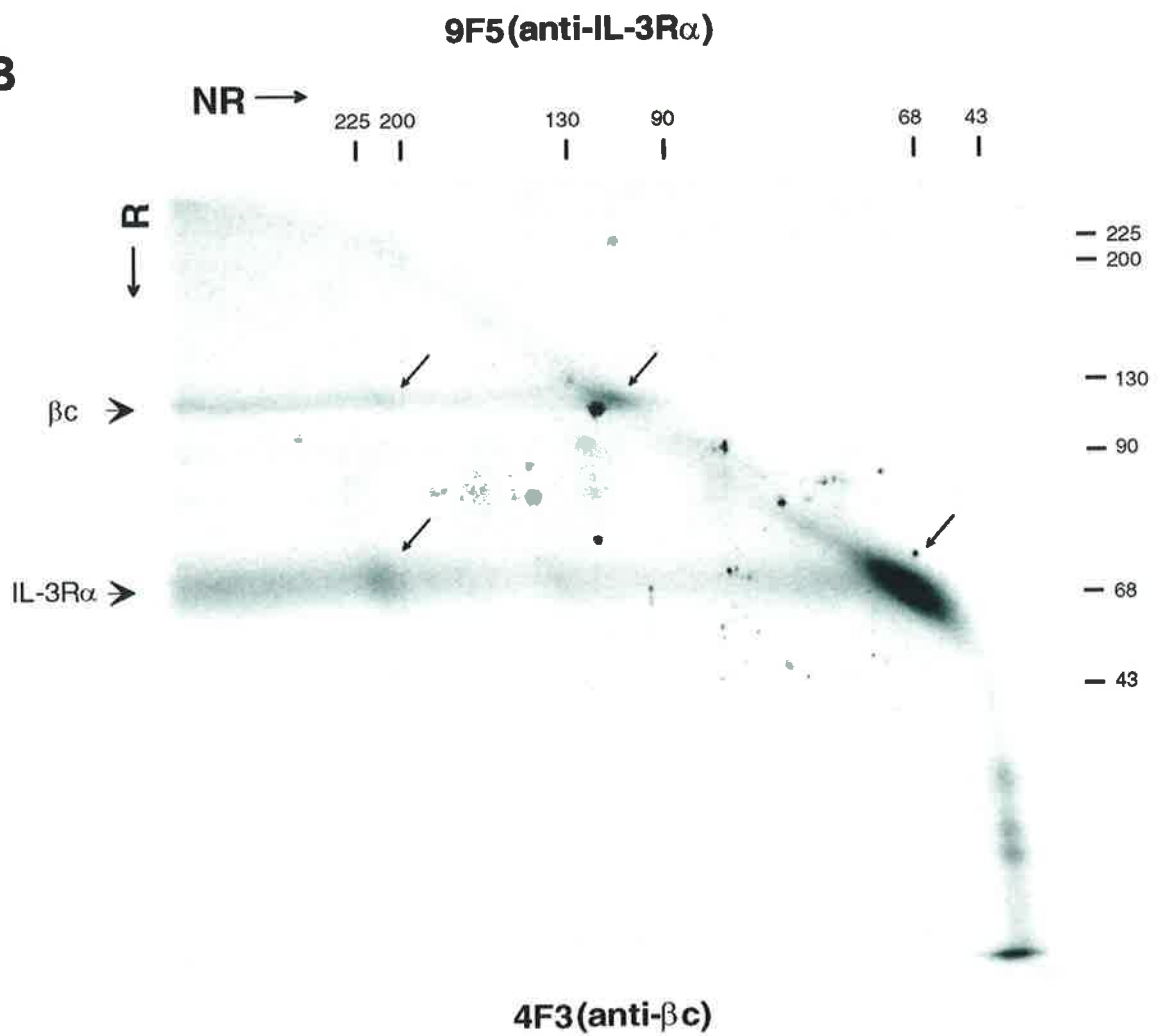
**Figure 3.5: The IL-3 analogue E22R is deficient at inducing IL-3R $\alpha$  and  $\beta_c$  dimerization.** <sup>125</sup>I surface-labelled cells were treated with different concentrations of wild type IL-3 (A and B) or with the IL-3 mutant E22R (C and D). The cells were then lysed and the lysates subjected to immunoprecipitation with MAb 9F5 (anti-IL-3R $\alpha$ ) (A and C), or MAb 4F3 (anti- $\beta_c$ ) (B and D) as described in Figure 3.1.



**Figure 3.6: IL-3 induces the formation of disulphide-linked receptor complexes.** A) and B) from left to right:  $^{125}\text{I}$  surface-labelled cells were incubated with either medium for 30 min at 37°C, IL-3 (50nM) for 30 min at 37°C, IL-3 (50nM) for 30 min at 37°C followed by iodoacetamide (IAM) (10mM) for 5 min at 21°C, iodoacetamide (10mM) for 5 min at 21°C followed by IL-3 (50nM) for 30 min at 37°C, or medium only and IL-3 (50nM) added to the cell lysate for 30 min at 4°C. Immunoprecipitations were carried out with MAb 9F5 (anti-IL-3R $\alpha$ ) in A) and with MAb 4F3 (anti- $\beta_c$ ) in B). C) Unlabelled cells were incubated with  $^{125}\text{I}$ -IL-3 (1nM) in the absence or presence of 100 fold excess unlabelled IL-3 before immunoprecipitation with MAb 9F5 (anti-IL-3R $\alpha$ ) and MAb 4F3 (anti- $\beta_c$ ). The immunoprecipitated proteins were separated under non-reducing conditions on a 6% SDS-PAGE. D) Scatchard transformation of  $^{125}\text{I}$ -IL-3 binding curves in untreated cells (top) or cells treated with iodoacetamide (10mM) for 20 min at 4°C (bottom).

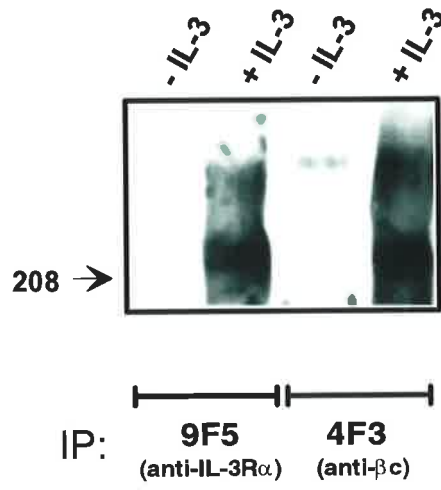
**A****B****C****D**

**Figure 3.7: Two-dimensional gel electrophoresis.** <sup>125</sup>I surface-labelled cells incubated with 50nM IL-3 for 30 min at 4°C and immunoprecipitated with MAb anti-IL-3R $\alpha$  (9F5) (A), and anti- $\beta_c$  (4F3) (B). The immunoprecipitates were separated under non-reducing conditions in the first dimension and under reducing conditions in the second dimension and visualized by phosphorimaging. The immunoprecipitates were mixed with unlabelled platelet extract and stained with Coomassie blue (not shown) to obtain more accurate MW estimates.

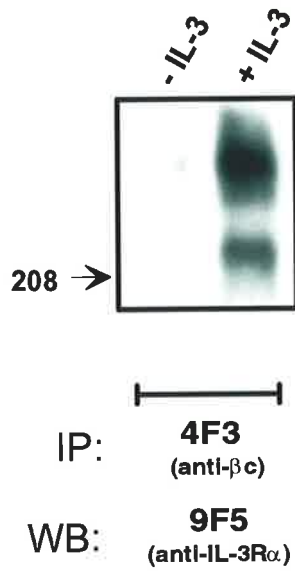
**A****B**

**Figure 3.8: IL-3-induced disulphide-linked complexes contain IL-3R $\alpha$  and  $\beta_c$ .** <sup>125</sup>I-surface-labelled cells were treated and immunoprecipitated as in Figure 3.6. A) Immunoprecipitation with MAb-9F5 (anti-IL-3R $\alpha$ ) and MAb 4F3 (anti- $\beta_c$ ). B) Immunoprecipitates with MAb 4F3 (anti- $\beta_c$ ) were analysed by Western blotting with Mab 9F5 (anti-IL-3R $\alpha$ ). C) Immunoprecipitates with MAb 9F5 (anti-IL3R $\alpha$ ) were analysed by Western blotting with MAb 1C1 (anti- $\beta_c$ ). Proteins were separated as described in Figure 3.6.

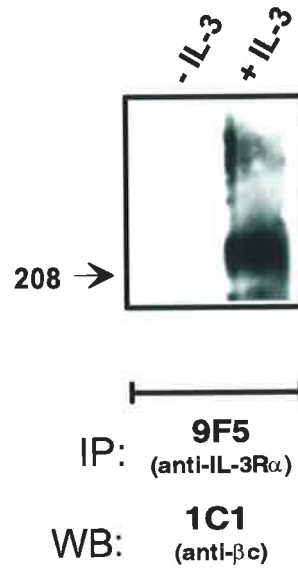
**A**



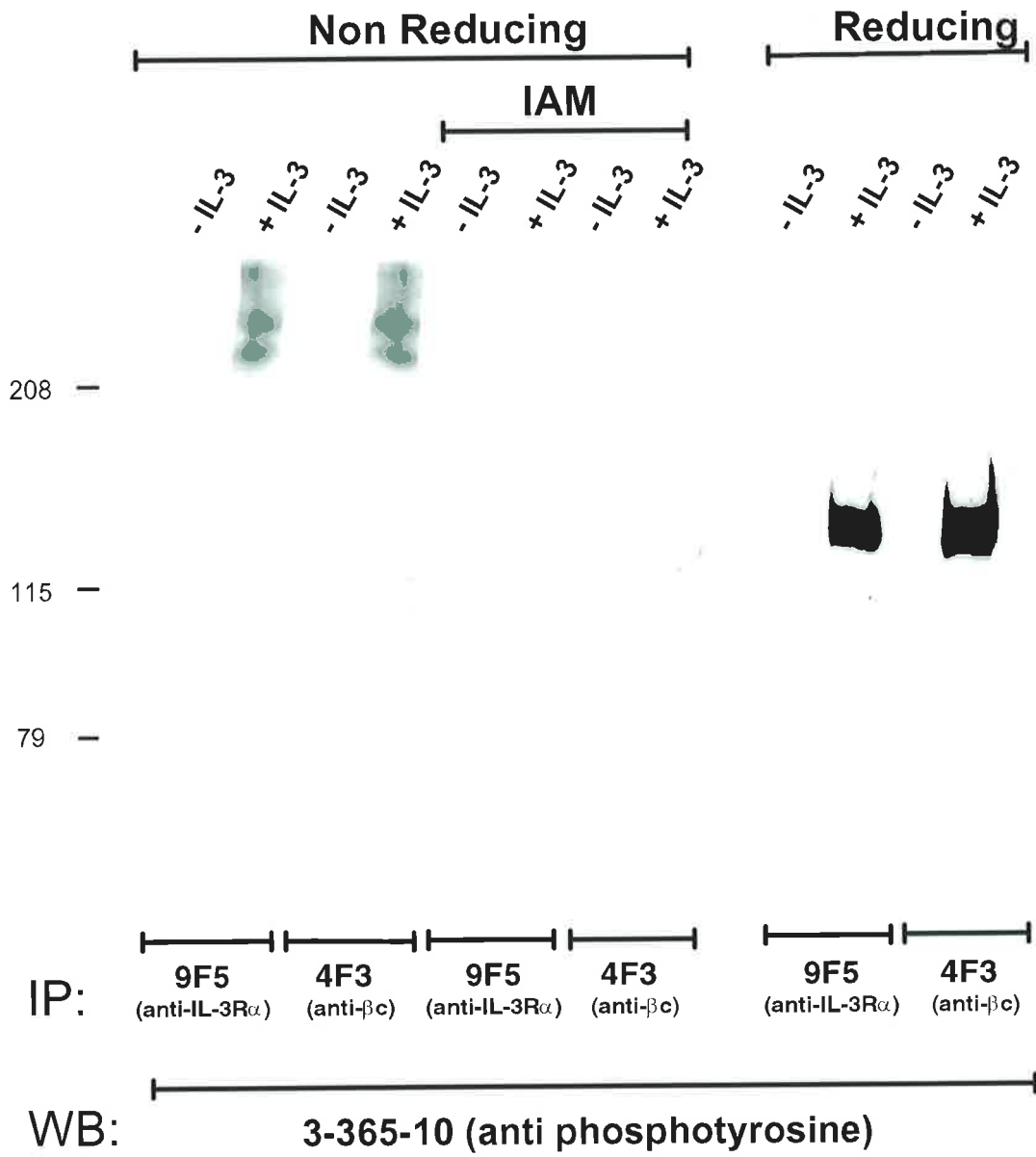
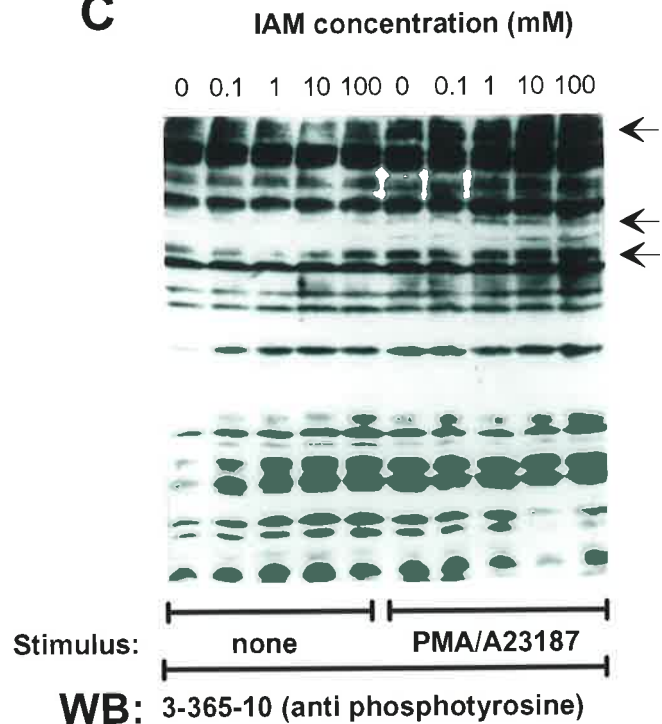
**B**



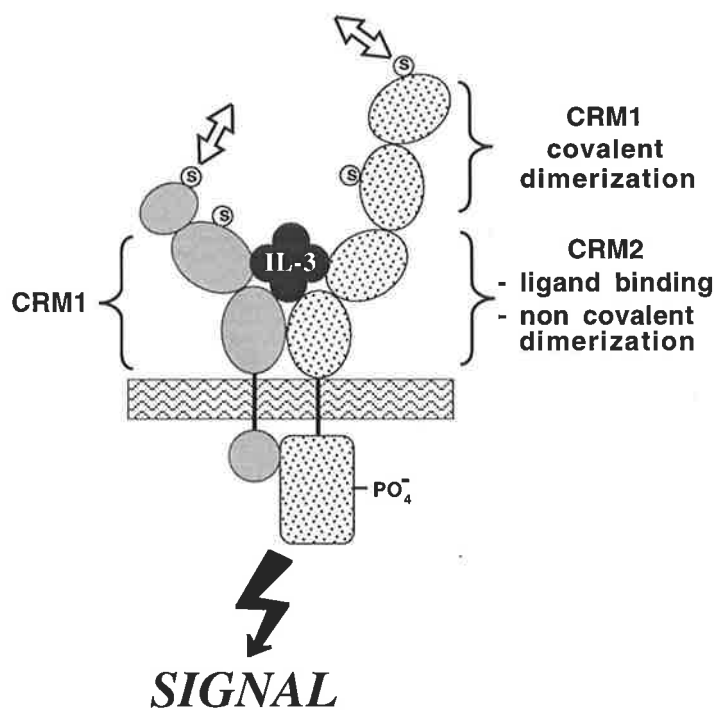
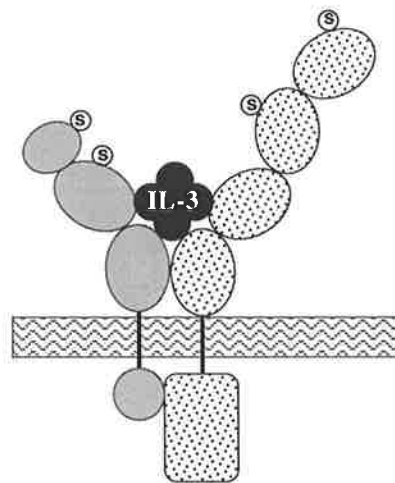
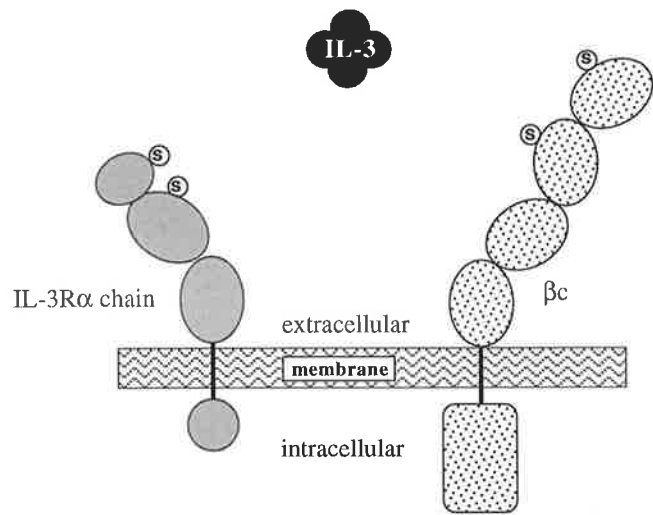
**C**



**Figure 3.9: Tyrosine phosphorylation of disulphide-linked receptor dimers.** A) Unlabelled RG cells were either untreated or incubated with IL-3 (50nM) for 30 min at 4°C with or without the prior addition of iodoacetamide (IAM) and immunoprecipitated with MAb 9F5 (anti-IL-3R $\alpha$ ) or MAb 4F3 (anti  $\beta_c$ ). The immunoprecipitates were separated under non reducing or reducing conditions on 6% SDS-PAGE and probed with the anti-phosphotyrosine antibody 3-365-10. B). Unlabelled UT7 cells were incubated with 50nM IL-3 for different times at 4°C. After cell lysis proteins were immunoprecipitated with MAb 9F5 (anti-IL-3R $\alpha$ ) and separated under non reducing conditions on 6% SDS-PAGE and probed with the anti-phosphotyrosine antibody 3-365-10. C). Human UT7 cells were untreated or treated with various concentrations of iodoacetamide for 20' at 4°C, and then either unstimulated or stimulated with PMA(50ng/ml)/calcium ionophore A23187(2uM) for 60' at 4°C. After cell lysis proteins were separated under reducing conditions on 12.5% SDS-PAGE and probed with the anti-phosphotyrosine antibody 3-365-10. The arrows indicate the position of the newly induced proteins.

**A****B****C**

**Figure 3.10: Model of human IL-3 receptor activation.** In the absence of IL-3, IL-3R $\alpha$  and  $\beta_c$  are not associated on the cell surface. The presence of IL-3 triggers IL-3 binding to IL-3R $\alpha$  initially and then  $\beta_c$ . IL-3 binding to  $\beta_c$  occurs through the cytokine receptor module CRM2 (Woodcock et al.1994; Woodcock et al.1996) and triggers dimerization. Two complementary mechanisms of receptor heterodimerization are proposed: a non covalent one probably involving the A-B loop in membrane proximal domains of the CRM2 of each chain analogous to the growth hormone receptor (De Vos et al. 1992), and a covalent association probably involving unpaired Cys in the *N*-terminal region and domain 1 of IL-3R $\alpha$  interacting with hitherto unpaired Cys in the CRM1 of  $\beta_c$  (see discussion). IL-3 is associated with the receptor dimer but is not covalently attached to it. Disulphide-linked receptor heterodimerization leads to phosphorylation of the dimerized  $\beta_c$ , but not of non-covalently associated monomeric  $\beta_c$  or IL-3R $\alpha$ , an event that leads to cellular activation.



## *Chapter IV*

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**THE HUMAN INTERLEUKIN-5 RECEPTOR UNDERGOES  
COVALENT AND NONCOVALENT DIMERIZATION AND  
PHOSPHORYLATION IN THE PRESENCE OF IL-5 BUT  
NOT IN THE PRESENCE OF THE IL-5 ANTAGONIST E13R**

#### 4.1 INTRODUCTION

The engagement of the human interleukin-5 receptor by IL-5 triggers a series of molecular events which lead to receptor activation. IL-5R expression is highly cell-lineage restricted compared to the IL-3R and the GM-CSFR. The IL-5R is expressed mainly on eosinophils and basophils. Stimulation of cells with IL-5 leads to increase in eosinophil production, function and survival (Sanderson,1992). Eosinophilic responses are usually seen in allergy, parasitic infections and in certain tumours. In allergy, eosinophilic responses are linked to asthma, atopic dermatitis and allergic rhinitis. (Lopez et al.1988; Drazen et al.1996).

It has been shown that IL-5R activation requires the IL-5R $\alpha$  and  $\beta_c$  to be in close proximity of each other. This has been shown through cross-linking studies with radio-iodinated IL-5 on cells expressing the IL-5R (Plaetinck et al.1990; Lopez et al.1991; Takaki S et al.1993; Tavernier et al.1991; Tavernier et al.1992). To identify the motifs required for interaction between IL-5 and IL-5 receptor, a number of residues have been targeted in both the receptor and the ligand. Mutants of the targeted residues in both IL-5 and IL-5R have been assessed. The residues targeted on the IL-5R  $\alpha$  chain include a region encompassing Asp<sup>55</sup>Asp<sup>56</sup>Tyr<sup>57</sup>Glu<sup>58</sup> which is located in the N-terminal FnIII domain. Structurally these residues are predicted to be in the C-D loop (Cornelis et al.1995). The region was initially identified through the use of chimeric IL-5R molecules and then the actual residues identified by alanine scanning mutagenesis (Cornelis et al.1995). Individual alanine substitution of these residues caused a significant loss in of IL-5 binding affinity (Cornelis et al.1995). Another alanine substitution mutant in the IL-5R $\alpha$ , in the E-F loop of

domain 1 of the CRM at position Arg<sup>188</sup>, also showed significant loss in binding to IL-5. This mutation was based on a structural comparison to the GH-GHR complex (Cornelis et al.1995). One more region in the IL-5R $\alpha$  a Cys residue in the N-terminal domain was identified through the use of isothiazolone derivatives. In the presence of isothiazolone derivatives IL-5 binding to IL-5R was completely blocked, an effect mediated by modification of sulphhydryl groups of cysteine residues. Loss of binding was due to the modification of a single Cys 86 residue in the IL-5R alpha chain, initially identified by protease cleavage and further confirmed by mutagenesis (Devos et al.1994).

The focus of identifying IL-5 interacting residues on the  $\beta_c$  has not been as intense as that in analysing the IL-5R $\alpha$ . The main reason has been that the affinity conversion of  $\beta_c$  is only 2-5 fold over that of IL-5R $\alpha$  alone compared to the GM-CSF and IL-3 receptors in which, the  $\beta$  chain confers a 20-50 fold and a 500-1000 fold increase respectively. This small change in affinity makes it difficult to use IL-5R as a model system for testing  $\beta_c$  mutations.

Nevertheless, the regions of  $\beta_c$  that were initially identified to be involved in binding to GM-CSF and IL-3, have now been shown to be important for IL-5. Initially these were modelled on the GHR. One contact area is a region of four residues Tyr<sup>365</sup>Glu<sup>366</sup>His<sup>367</sup>Ile<sup>368</sup> that is predicted to be between the B' and C' strand. Alanine substitution of residues in this region disrupted high affinity binding of GM-CSF and IL-5 although cross linking studies indicated that IL-5 still becomes associated with the  $\beta_c$  mutants(Woodcock et al.1996). A single substitution of Tyr<sup>365</sup> was sufficient

to completely abolish high affinity binding to GM-CSF and IL-5. (Woodcock et al.1996) A point mutation of Tyr<sup>421</sup> in the predicted F'-G' loop was also found to be critical for high affinity binding to GM-CSF, IL-3 and IL-5.

The IL-5R $\alpha$  chain requires a cytoplasmic domain for IL-5 cellular signalling to occur. It has been shown that truncation of the cytoplasmic region prevented IL-5 dependent growth signal (Takaki et al.1993). A region of well conserved proline residues is required for interaction with the  $\beta_c$  for growth and signal transduction. This conserved cytoplasmic region between Leu<sup>350</sup> and Pro<sup>355</sup> in mIL-5R is common to IL-5R, IL-3R and GM-CSFR (Takaki et al.1994). The truncated IL-5R $\alpha$  chain activity could be reconstituted with a chimeric receptor composed of IL-5R $\alpha$  extracellular and transmembrane domain with the  $\beta_c$  cytoplasmic domain. This suggests that interaction of the cytoplasmic domains of the  $\alpha\beta$  complex are required, to possibly either dimerize or induce a conformational change in the  $\beta_c$  for cell signalling to occur (Takaki et al.1994)

Airway hyperresponsiveness is a pathological condition associated with asthma (Drazen et al.1996). It has been shown in a murine model system that this inflammatory condition is dependent on IL-5. The IL-5 knockout mice fail to develop airway hyperresponsiveness after systemic allergen sensitisation followed by repeated aerosol challenge (Foster et al.1996). When these mice are reconstituted for IL-5 with the use of recombinant vaccinia viruses expressing a cDNA for this cytokine, the eosinophils return (Foster et al.1996). These findings show that IL-5 and eosinophils are mediators in the pathogenesis of allergic lung disease. Because

of this there has been a keen interest in developing non functional analogues of IL-5 that still bind the IL-5 receptor and can behave as specific IL-5 antagonists.

Human IL-5 exists as a disulphide linked homodimer and substitution of the Cys residue renders the protein inactive (Takatsu et al.1988; McKenzie et al.1991). Recently a monomeric form of IL-5 was engineered by lengthening the C-D loop which has almost native IL-5 activity (Dickason et al.1996). The short loop in native IL-5 prevents the presentation of helix D to the receptor, therefore the D helix is presented with the other A-C helices for a functional IL-5 molecule. This short loop is unique to IL-5.

Clusters of residues in the C-terminus of IL5 Glu<sup>89</sup>Glu<sup>90</sup>Arg<sup>91</sup> and Thr<sup>109</sup>Glu<sup>110</sup>Trp<sup>111</sup> as well as His<sup>38</sup>Lys<sup>39</sup>His<sup>41</sup> were identified as being important in binding the IL-5R $\alpha$  chain. The first evidence that the N-terminal region of IL-5 is involved in binding to the  $\beta_c$  was from IL-5/GM-CSF hybrid molecules. A hybrid was constructed by replacing amino acids 4-32 of hGM-CSF by amino acids 5-29 of mIL-5 (Shanafelt et al.1991). Structurally this is a region that was predicted to be the most likely to interact with the  $\beta_c$  receptor (Bazan,1990a; Parry et al.1991). This hybrid was able to elicit a full biological response indicating that a small discrete region in mGM-CSF is responsible for the interaction with the  $\beta_c$  (Shanafelt et al.1991). Sequence analysis of the predicted amino terminal helices within the cytokine receptor family predicted that a conserved negatively charged Glu residue in IL-5 may be the residue involved in the interaction with  $\beta_c$  (Shanafelt et al.1991; Lopez et al.1992b; Hercus et al.1994a; Barry et al.1994).

The same residue Glu<sup>13</sup> in the N-terminal region of IL-5 was identified through progressive replacement of clusters of charged residues (Graber et al.1995). The initial substitution of the Glu<sup>13</sup> with Ala gave a 10 fold reduction in a TF-1 proliferation assay (Graber et al.1995). Mutation of Glu<sup>13</sup> residue to either Gln, Lys or Arg residue produced IL-5 antagonists (Tavernier et al.1995; McKinnon et al.1997). These IL-5 analogues are able to promote high affinity binding but lack the ability to support proliferation. Therefore they are able to behave as IL-5 antagonists and compete for IL-5 activation as measured by proliferation. The E13K antagonist is interesting as it can still support eosinophil survival, thus suggesting that different cellular functions are initiated by IL-5R via different pathways (McKinnon et al.1997). Thus Glu<sup>13</sup> residue in IL-5 has been identified as the key residue for interactions with  $\beta_c$ .

The mechanism of IL-5 receptor activation was examined in this chapter. The issue of receptor dimerization was studied, in particular whether the IL-5R complex forms disulfide bonds as shown with the IL-3R complex (chapter 3). In addition the effect of the IL-5 analogue E13R on IL-5 induced receptor dimerization and activation was examined to understand the molecular basis of E13R antagonism.

## **4.2 RESULTS**

### **4.2.1 IL-5 induces IL-5R $\alpha$ and $\beta_c$ association**

In order to determine whether IL-5R exists as a preformed complex or is ligand induced. MAb A14 (anti-IL-5R $\alpha$ ) and MAb 8E4 (anti- $\beta_c$ ) were used in immunoprecipitation studies using TF1.8 cells which express IL-5R $\alpha$  and  $\beta_c$ . The immunoprecipitated proteins were separated on SDS-PAGE under reducing conditions. The results showed that in the absence of IL-5, MAb A14 did not co-immunoprecipitate  $\beta_c$  as visualised by western blotting with 1C1 (anti- $\beta_c$ ) (figure 4.1a). However, the addition of IL-5 caused the co-immunoprecipitation of a 120,000 MW protein in the case of immunoprecipitation with MAb A14 and western blotting with 1C1 (anti- $\beta_c$ ). The proteins associated rapidly to form an IL-5-induced heterodimeric complex (figure 4.1a). The band present in all tracks at approx 60,000 MW is non specific and is the immunoglobulin heavy chain.

### **4.2.2 IL-5 activates the IL-5R complex and induces a high order IL-5R $\alpha$ and $\beta_c$ dimerization**

In order to determine whether IL-5R $\alpha$  or  $\beta_c$  were phosphorylated either before or after ligand binding MAb A14 (anti-IL-5R $\alpha$ ) and MAb 8E4 (anti- $\beta_c$ ) were used in immunoprecipitation studies using TF1.8 cells. The immunoprecipitated proteins were separated on SDS-PAGE under reducing conditions. The results showed that in the absence of IL-5, immunoprecipitation with MAb A14 (anti-IL-5R $\alpha$ ) and MAb 8E4 (anti- $\beta_c$ ) did not immunoprecipitate any phosphorylated proteins either IL-5R $\alpha$  or  $\beta_c$  as visualised by western blotting with 3-356-10 (anti-phosphotyrosine) (figure 4.1b). However, the addition of IL-5 caused the immunoprecipitation of a 120,000

MW phosphorylated protein when either immunoprecipitated with MAb A14 (anti-IL-5R $\alpha$ ) or MAb 8E4 (anti- $\beta_c$ ) and western blotting with 3-356-10 (anti-phosphotyrosine) (figure 4.1b). Reprobing of the nitrocellulose filter with MAb 1C1 (anti- $\beta_c$ ) identified the phosphorylated proteins as  $\beta_c$  (figure 4.1 a and b).

I have previously established that stimulation of cells with IL-3 leads to the formation of disulfide-linked heterodimers of IL-3 receptor  $\alpha$  and  $\beta_c$  chain (chapter 3; (Stomski et al.1996)). To examine the possibility that IL-5 activation of the IL-5R is linked to the covalent association of IL-5R $\alpha$  and  $\beta_c$  TF1.8 cells were stimulated with either IL-5, IL-3 or medium prior to lysing and immunoprecipitated with 8E4 (anti $\beta_c$ ). The immunoprecipitated proteins were separated on SDS-PAGE under non-reducing conditions and western blotted with 3-356-10 (anti-phosphotyrosine). The nitrocellulose filters were reprobed with MAb 1C1 (anti- $\beta_c$ ) to verify the presence of  $\beta_c$ . IL-3 stimulated cells were used as a positive control for disulphide-linked dimerization. The results showed that cells stimulated with IL-3 showed the formation of a disulphide-linked high order complex and that this was activated (Fig. 4.2a). With IL-5 the results show that the IL-5 receptor also forms disulfide-linked complexes which are similarly accompanied by  $\beta_c$  phosphorylation (Fig. 4.2b). The relative proportion of phosphorylated  $\beta_c$  in the disulfide-linked heterodimer and in monomeric  $\beta_c$  varied between IL-3 and IL-5 receptors (Fig. 4a&4b). This may be due to kinetic differences in receptor assembly or in the stability of each receptor heterodimer.

#### **4.2.3 IL-5 analogue E13R inhibits IL-5 induced proliferation**

TF-1.8 cells were stimulated with either IL-5 or E13R in a range of concentrations varying from 0.0001 to 1000ng/ml of ligand. The wild type IL-5 induced the proliferation of the TF-1.8 cells in a concentration dependent fashion ( $ED_{50} = 3.0\text{ng/ml}$ ), while E13R was unable to stimulate proliferation within this range of concentrations. Since E13R was unable to elicit a proliferative response in TF1.8 cells, this mutant was then tested for its ability to antagonise the effects of IL-5 in a proliferation assay. As seen in Fig 4.3, E13R antagonised the effect of IL-5 in a concentration dependent manner. Proliferation of TF1.8 cells, in the presence of 3ng/ml wild type IL-5 was totally inhibited by 1000ng/ml of E13R.

#### **4.2.4 IL-5 analogue E13R inhibits IL-5 receptor activation**

To examine the molecular basis of E13R-mediated antagonism, TF-1.8 cells were stimulated with either IL-5 (100ng/ml), E13R (5ug/ml) or media for 10 minutes at 37° C. The cells were lysed and immunoprecipitated with 8E4 (anti $\beta_c$ ). The immunoprecipitated proteins were separated on SDS-PAGE, either under non reducing or reducing conditions and western blotted with 3-356-10 (anti-phosphotyrosine). The nitrocellulose filters were reprobed with MAb 1C1 (anti- $\beta_c$ ) to verify the presence of  $\beta_c$ . The results showed that E13R does not activate the IL-5R as measured by phosphorylation of  $\beta_c$  even at a 50 fold excess of E13R compared to wild type IL-5 (Fig. 4.4 a and b). The presence of  $\beta_c$  was confirmed by reprobing the filters with MAb 1C1 (anti- $\beta_c$ ) (Fig. 4.4c).

In a second set of experiments TF-1.8 cells were pre-incubated with increasing concentrations of E13R and then stimulated with IL-5 (5ng/ml) for 10 minutes. E13R inhibited IL-5 induced phosphorylation of  $\beta_c$  when present in at least a 500 fold excess compared to the IL-5 stimulating dose (Fig. 4.4d)

### 4.3 DISCUSSION

In this chapter it has been shown that human IL-5 binding to its receptor triggers the heterodimerization of its specific receptor binding subunit, IL-5R $\alpha$ , with  $\beta_c$  and that receptor heterodimerization is dependent on IL-5. Both disulphide-linked and non disulphide-linked heterodimers were observed. In previous experiments it has been shown that IL-5R receptor heterodimerizes in the presence of ligand, using cross linking reagents (Tavernier et al.1991). This is the first report of co-association of the IL-5R $\alpha$  and  $\beta_c$  as immunoprecipitated with a MAb. In particular this is the first report of covalent association of the IL-5R.

The activated  $\beta_c$  is shown to be present, both as a monomer and in the covalently linked  $\alpha\beta$  complex as detected by one of earliest signalling events, phosphorylation. The presence of the IL-5R $\alpha$  chain in the activated covalently associated complex supports the findings that the IL-5R  $\alpha$  chain N-terminal cysteine must be available for an active receptor complex (Devos et al.1994). The covalent association of  $\alpha$  and  $\beta$  chains for an active receptor complex has now been shown for both IL-3R and IL-5R. This emphasises the importance of the  $\alpha$  chain not only as a ligand binding subunit but also as a subunit required for cell signalling.

The presence of the IL-5R $\alpha$  in the disulphide-linked IL-5 receptor dimer is consistent with an emerging theme in the IL-3/IL-5/GM-CSF receptor sub family, that of  $\alpha$  chains being involved in receptor activation. This contrasts with the disulphide-linked dimerization of the IL-6 and CNTF receptors which only involve the signal transducer units in the disulphide linked dimers (Davis et al.1993), but on the other

hand similar to the LIF receptor which participates in both ligand binding and signalling (Stahl et al.1993).

In this receptor sub family for signalling to occur, the need for ligand to contact both  $\alpha$  and  $\beta$  chains appears to be essential . A number of ligand mutants have been developed through mutational analysis that alter the  $\beta_c$  binding sites. These molecules are either devoid of agonist activity or can act as agonists with significantly reduced activity. The IL-3 mutant E22R retains agonist activity for TF-1 proliferation at 20,000-fold reduced potency relative to wild type IL-3 (Barry et al.1994). The GM-CSF mutant E21R lacks agonist activity, is a specific GM-CSF antagonist and also induces a apoptotic signal rather than survival (Hercus et al.1994a; Iversen et al.1997a). For IL-5 a number of mutants of residue 13 have been described. E13A exhibits partial agonist activity in a TF-1 proliferation assay (Graber et al.1995), E13Q is completely inactive and exhibited antagonists properties (Tavernier et al.1995) and E13K which lacks agonist activity and is an antagonist in both a TF-1 proliferation assay and an eosinophil activation assay, while it exhibits agonist activity in promoting eosinophil survival (McKinnon et al.1997).

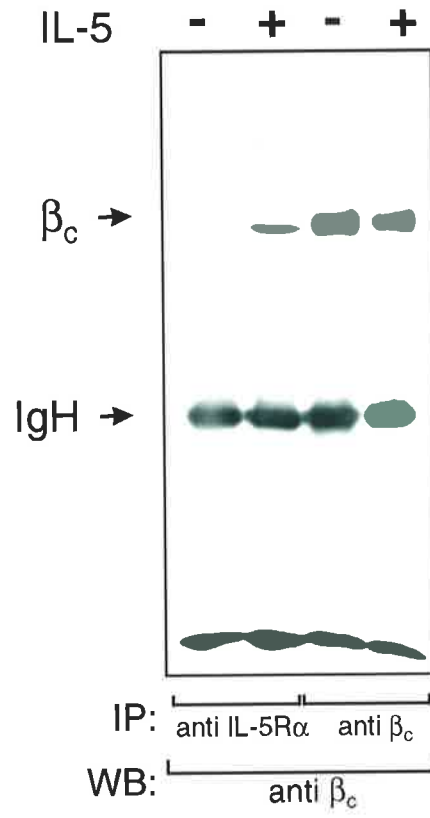
The IL-5 mutant E13R used in this study behaved in a similar fashion to E13Q and E13K. It was completely inactive as an agonist and exhibited antagonists properties in both TF-1.8 proliferation assay, and in activation of  $\beta_c$  as measured by phosphorylation.

Significant differences are emerging between each of the specific ligand analogues. Each of the ligand mutants is targeting the same contact site of the  $\beta_c$  but the output signal is quite different. IL-3 (E22R), is an agonist with reduced potency; GM-CSF (E21R) is a full antagonist and induces apoptosis; IL-5 (E13K/R) is an antagonist of proliferation but E13K supports eosinophil survival.

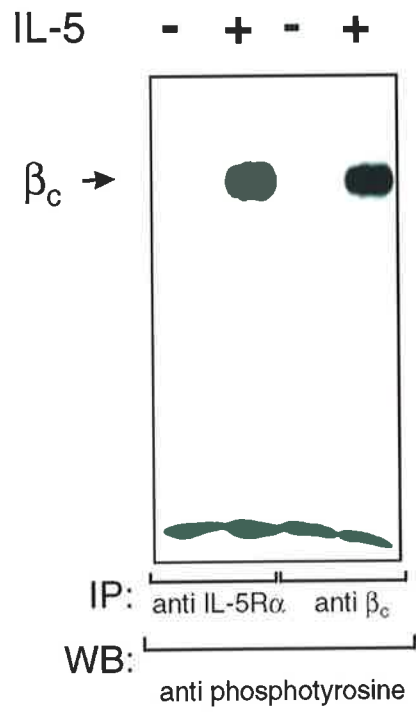
A common theme for this receptor subfamily is emerging, namely, that disulphide-linked receptor dimerization is associated with receptor activation. The IL-5 analogue E13R may fail to induce disulphide-linked dimerization and therefore prevent receptor signalling as measured by phosphorylation and proliferation. The lack of disulphide-linked hetero-dimerization is likely to be the reason for the lack of activation, since it has been shown by a number of groups through Scatchard analysis that all of the IL-5 analogues tested are able to bind to the receptors with high affinity (Tavernier et al.1995; McKinnon et al.1997). Similarly, IL-3 is able to bind IL-3R with high affinity, but is not able to activate the receptor in the presence of thiol-specific alkylating reagents that prevent formation of high order complexes. However, at present there is no direct evidence that disulphide-linked dimerization of the receptors is required for receptor phosphorylation. To prove that disulphide-linked dimers are required for phosphorylation, mutating the appropriate Cys residues that are involved in disulphide-dimerization would provide definitive proof. This is addressed in chapter 6.

**Figure 4.1 IL-5 induces IL-5R $\alpha$  and  $\beta_c$  association and activates the  $\beta_c$ .** After TF1.8 cells were untreated (-) or treated with IL-5 (3.8nM) for 5 min at 37°C cells were lysed and the proteins immunoprecipitated with MAb A14 (anti IL-5Ra) or MAb 8E4 (anti- $\beta_c$ ), separated on 7.5% SDS-PAGE under reducing conditions. Identification of co-immunoprecipitated bands was identified by western blotting with (A) MAb1C1 (anti- $\beta_c$ ) and (B) anti-phosphotyrosine antibody 3-365-10. A major band at 60 kD represents immunoreactivity of immunoprecipitating MAb heavy chain.

(A)

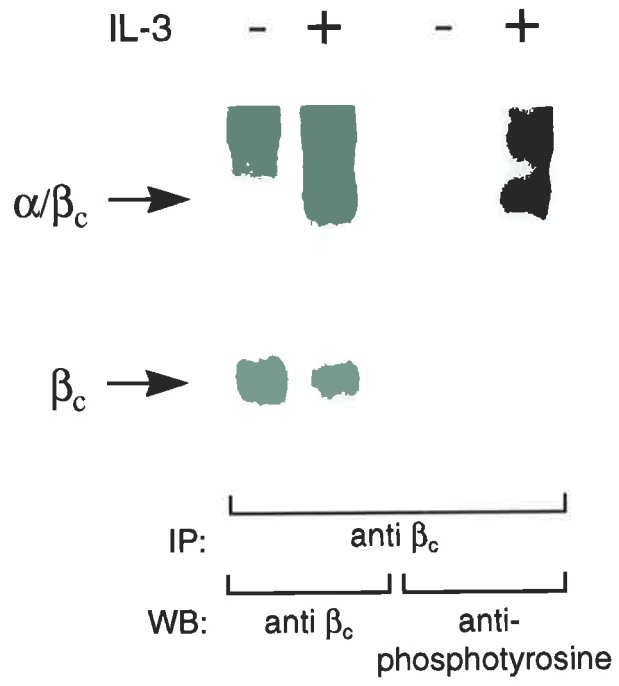


(B)

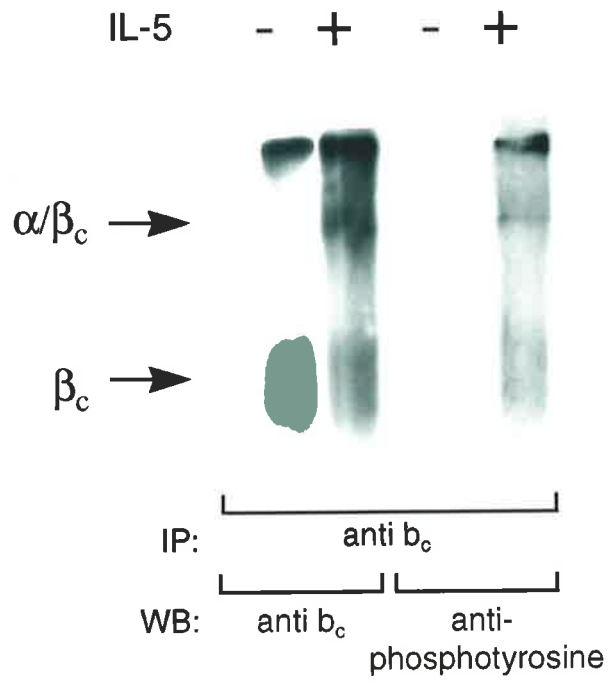


**Figure 4.2 Ligand-induced disulfide-linked IL-3, and IL-5 receptor dimerization results in phosphorylation of  $\beta_c$ .** TF1.8 cells were either incubated with medium alone (-) or stimulated with medium containing 6.5 nM IL-3 (A), 3.8 nM IL-5 (B) for 5 minutes at 37°C. After cell lysis, proteins were immunoprecipitated with anti- $\beta_c$  MAb 8E4 and the immunoprecipitates were separated under non reducing conditions on an SDS-7.5% polyacrylamide gel and transferred onto nitrocellulose filters. The filters were then probed either with MAb 1C1 anti- $\beta_c$ , or anti-phosphotyrosine antibody 3-365-10.

**(A)**

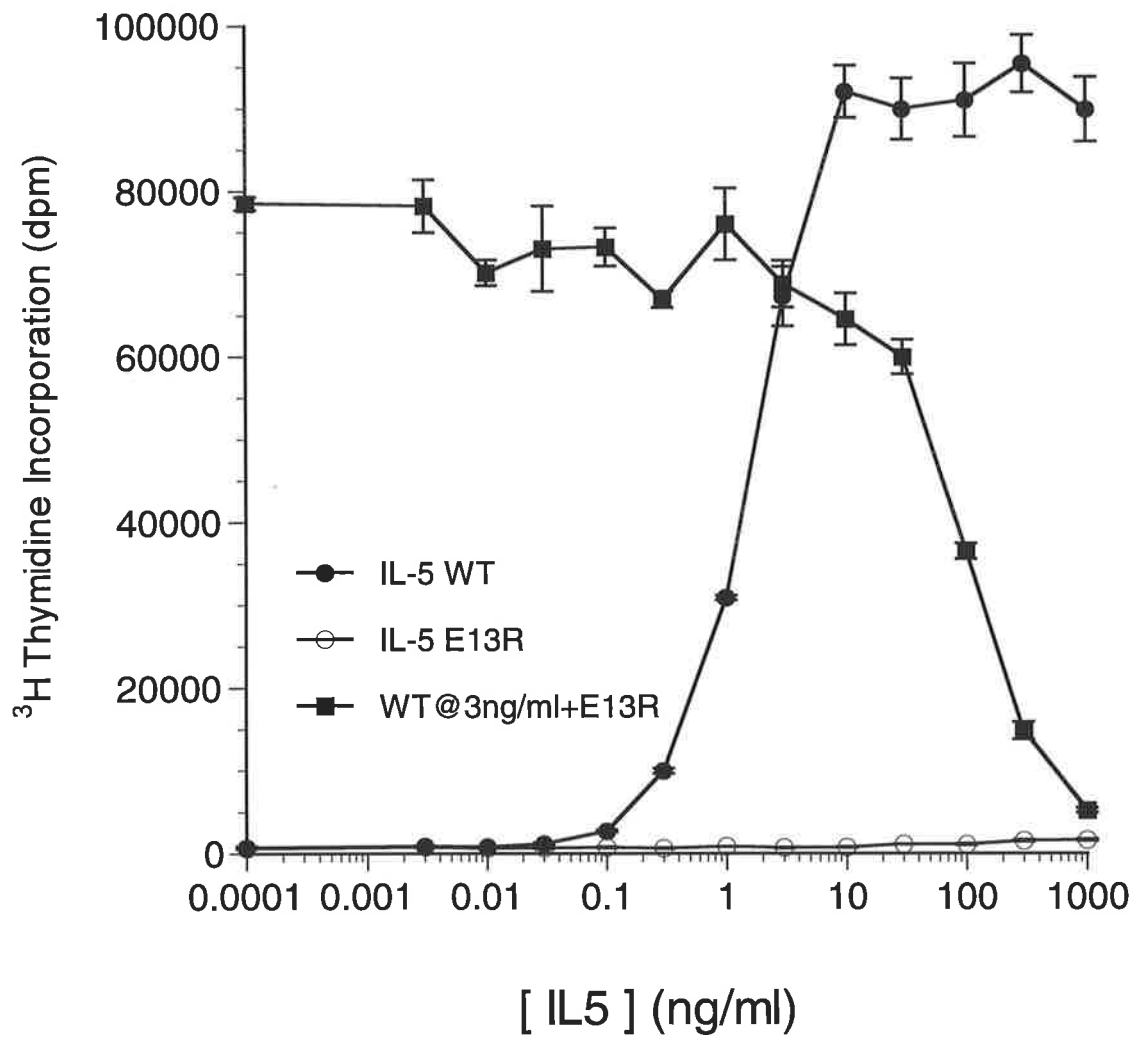


**(B)**

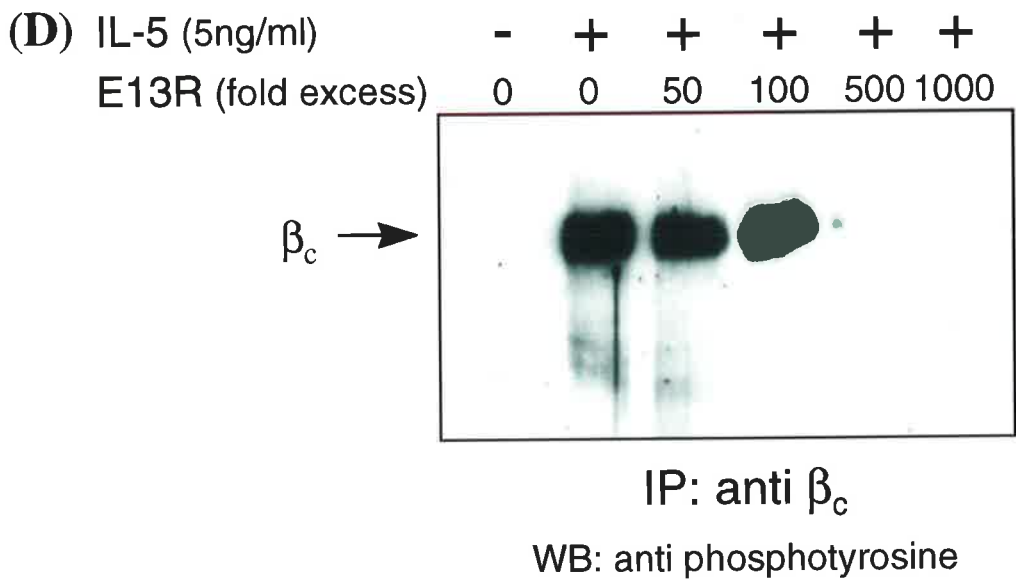
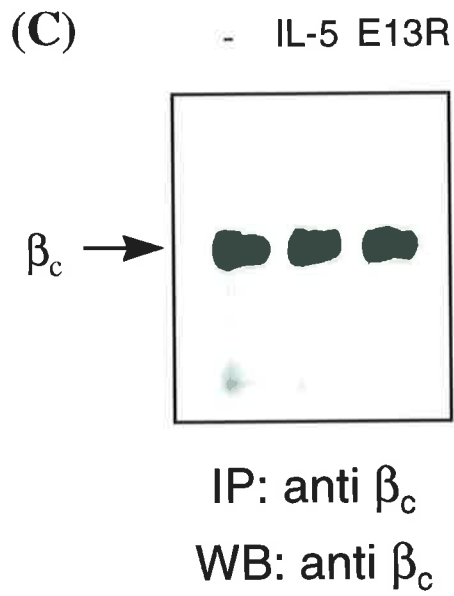
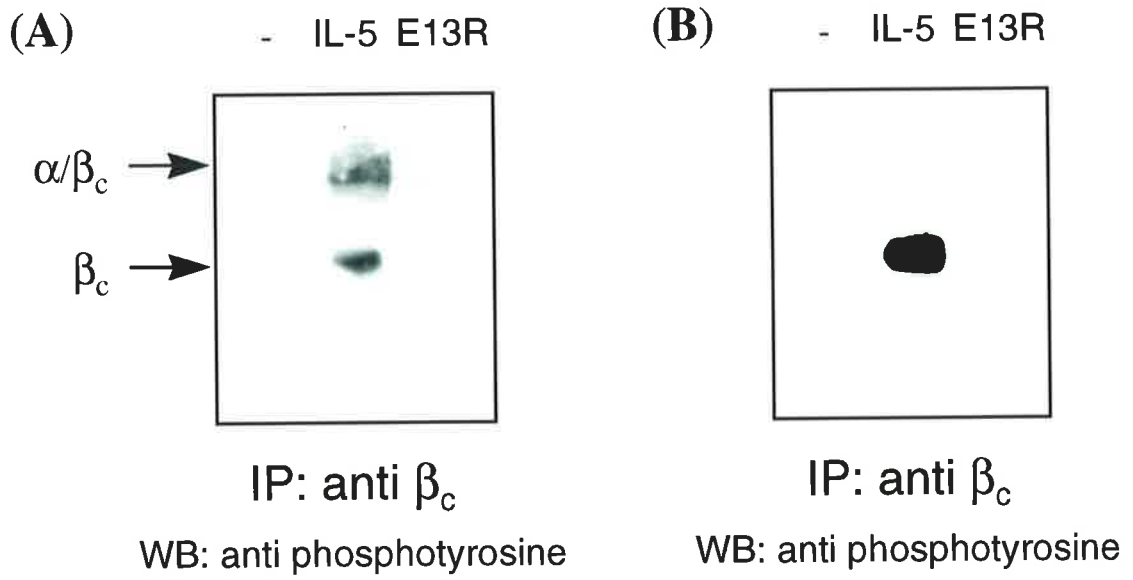


**Figure 4.3 Inhibition of IL-5 mediated proliferation by IL-5 analogue E13R.**

Titration of IL-5 WT (●) and IL-5 E13R (○) mediated proliferation are shown. In the antagonist experiment E13R (■) was titrated against IL-5 at 3 ng/ml. Each value represents the mean of triplicate determinations, and error bars represent the SEM.



**Figure 4.4** An IL-5 antagonist, E13R, does not induce disulphide-linked receptor dimerization or phosphorylation of  $\beta_c$  and inhibits IL-5 induced phosphorylation. TF1.8 cells were either incubated with medium alone (-) or treated with medium containing 3.8 nM IL-5 (IL-5) or 200 nM IL-5 analogue (E13R) for 5 minutes at 37 °C (A, B, C). In (D) TF1.8 cells were preincubated with various concentrations of E13R (0-5000 ng/ml) for 10 min. and then IL-5 was added at a final concentration of 5ng/ml and stimulated for 5 min.. After cell lysis, proteins were immunoprecipitated with MAb 8E4 (anti- $\beta_c$ ) and the immunoprecipitates were resolved either under nonreducing (A) or reducing (B, C, D) conditions on an SDS-7.5% polyacrylamide gel and transferred to nitrocellulose filters. The filters were then probed either with anti-phosphotyrosine antibody 3-365-10 (A,B) or anti- $\beta_c$  MAb 1C1 (C).



## *Chapter V*

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**THE HUMAN GM-CSF RECEPTOR EXISTS AS A  
PREFORMED RECEPTOR COMPLEX THAT CAN  
BE ACTIVATED BY GM-CSF, IL-3 OR IL-5.**

## 5.1 INTRODUCTION

GM-CSF is a pleiotropic cytokine that exhibits effects on most cell types in the haemopoietic compartment (Metcalf,1986; Clark et al.1987). GM-CSF exhibits overlapping biological activities with IL-3 on several haemopoietic cells as it shares a similar pattern of receptor expression and to the sharing a common signal transducing receptor subunit that is also shared with IL-5 (Lopez et al.1992a). Indeed, on eosinophils that express GM-CSF, IL-3 and IL-5 receptors, these three cytokines stimulate the same functions with very similar potency (95). The functional receptors for GM-CSF, IL-3 and IL-5 are closely related and are composed two subunits: a ligand specific  $\alpha$  chain and the communal  $\beta_c$  (Hayashida et al.1990; Kitamura et al.1991b; Tavernier et al.1991). The receptor  $\alpha$  chains bind their cognate cytokine ligands with low affinity but generally are unable to mediate signalling alone, although some reports have suggested a role for GMR $\alpha$  chain in glucose transport (Ding et al.1994). The communal  $\beta$  chain,  $\beta_c$ , is unable to bind any cytokine alone, but confers high affinity binding on a ligand  $\alpha$  chain complex ( $K_d \sim 100$  pM) and is required for receptor signalling (Hayashida et al.1990; Kitamura et al.1991b; Tavernier et al.1991). Functional high affinity receptors for GM-CSF, IL-3 or IL-5 can be reconstituted on cells that do not normally express these receptors by co-expressing cytokine specific  $\alpha$  chains and  $\beta_c$  (Kitamura et al.1991a; Kitamura et al.1992; Takaki et al.1993), however, the relationship and assembly of these subunits on the cell surface are unknown.

The mechanism of activation of the GM-CSF receptor is likely to involve receptor dimerization, although the molecular basis of this phenomenon is poorly understood.

Ligand-induced receptor dimerization is a common theme amongst the cytokine receptor superfamily and is usually a prerequisite for receptor activation. For example, IL-6 induces IL-6R $\alpha$  and gp130 dimerization (Taga et al.1989) with homodimerization of gp 130 causing receptor phosphorylation (Murakami et al.1993). Similarly, ciliary neurotrophic factor induces receptor dimerization and subsequent receptor activation (Davis et al.1993). In the case of the IL-3 receptor which is closely related to the GM-CSF receptor (Goodall et al.1993), IL-3 induces receptor  $\alpha$ : $\beta_C$  heterodimerization followed by covalent disulphide bridging between receptor  $\alpha$  chain and  $\beta_C$  ( chapter 3, ; Stomski et al.1996). The structural similarities and functional overlap between the GM-CSF and IL-3 receptor systems has suggested that activation of the GM-CSF receptor may follow a similar pattern of events. Indeed GM-CSF has been shown to induce co-association of GMR $\alpha$  with  $\beta_C$  (Eder et al.1994) and a general mechanism has been noted that involves disulphide bridging between receptor  $\alpha$  chain and a cysteine motif in  $\beta_C$  that is essential for activation of GM-CSF, IL-3 and IL-5 receptors ( chapter 3, ; chapter 4, ; Stomski et al.1996; Bagley et al.1997). Despite exhibiting some common features of activation with other receptors, the GM-CSF receptors also appear to exhibit some unusual features. For example, mutant forms of GMR $\alpha$  that are deficient in GM-CSF binding when expressed alone on cells, are able to support binding when co-expressed with  $\beta_C$  (Ronco et al.1994; Rajotte et al.1997), suggesting that  $\beta_C$  can compensate for losses in binding affinity. Conversely, a mutation in GM-CSF abolishes the ability of the molecule to compete for low affinity binding but retains the ability to compete for high affinity binding (Hercus et al.1994b). Lastly, a recent report showed that a naturally occurring soluble form of GMR $\alpha$  is retained at the cell

surface when co-expressed with  $\beta_C$ , although co-immunoprecipitation of the two subunits could only be demonstrated in the presence of GM-CSF (Murray et al.1996).

We report here that the human GM-CSF receptor exists as both an inducible complex and, unlike other cytokine receptors, as a preformed receptor complex. Using monoclonal antibodies specific for the GM-CSF receptor  $\alpha$  and  $\beta_C$  we found that both subunits could be co-immunoprecipitated in the absence of GM-CSF whether they were surface expressed, or expressed as soluble forms by the same cells. Consistent with there being two types of GM-CSF receptor complex we demonstrate in kinetic experiments on eosinophils that GM-CSF exhibits unique association kinetics with two types of binding site; one type exhibits association kinetics very similar to those of IL-3 and IL-5, whereas the other type shows virtually instantaneous association. Significantly, stimulation of cells not only with GM-CSF but also with IL-3 and IL-5 induces the phosphorylation of  $\beta_C$  associated with GMR $\alpha$ . A model is proposed in which IL-3 and IL-5 recruit the preformed GM-CSF receptor into a high order complex raising the possibility that some of the biological activities of IL-3 and IL-5 are mediated indirectly through activation of the preformed GM-CSF receptor complex.

## 5.2 RESULTS

### 5.2.1 GMR $\alpha$ and $\beta_C$ are pre-associated on the cell surface

In chapter 3 in studies on IL-3 receptor complex formation an observation was made that co-immunoprecipitation of an 80,000 MW protein with  $\beta_C$  from  $^{125}\text{I}$ -surface-labelled primary CML cells (RG cells) in the absence of exogenous stimuli (Stomski et al.1996). The size of this protein suggested it could be the GM-CSF receptor  $\alpha$  chain (GMR $\alpha$ ). To examine this possibility, immunoprecipitation of  $^{125}\text{I}$ -surface-labelled CML cells either in the presence or absence of GM-CSF or IL-3 with anti-GMR $\alpha$ , anti-IL-3R $\alpha$  or anti- $\beta_C$  antibodies were performed. Immunoprecipitation of unstimulated cells with anti-GMR $\alpha$  MAb 8G6 immunoprecipitated a protein of 80,000 MW consistent with the size of GMR $\alpha$  (Fig. 5.1A). A second protein of 120,000 MW, corresponding in size to  $\beta_C$  co-immunoprecipitated with GMR $\alpha$  in the absence of GM-CSF and its level did not significantly increase with the addition of GM-CSF (Fig. 5.1A). Reciprocally, immunoprecipitation with anti- $\beta_C$  MAb 4F3 immunoprecipitated both the 120,000 MW  $\beta_C$  protein and the 80,000 MW GMR $\alpha$  protein in the presence or absence of GM-CSF (Fig. 5.1A). Co-immunoprecipitation of GMR $\alpha$  with  $\beta_C$  with either anti-GMR $\alpha$  or anti- $\beta_C$  antibodies could be the result of these antibodies recognising similar epitopes on both receptor chains. However, in previous studies it has been shown that these antibodies are absolutely specific for their respective receptor chains and show no cross-reactivity ( chapter 3, ; Woodcock et al.1994; Stomski et al.1996).

In contrast to the co-immunoprecipitation seen with GMR $\alpha$  and  $\beta_C$ , co-immunoprecipitation of IL-3R $\alpha$  and  $\beta_C$  by either anti-IL-3R $\alpha$  or anti- $\beta_C$  antibodies

was only seen in the presence of IL-3 (Fig. 5.1B) as shown previously. ( chapter 3, ; Stomski et al.1996). The phosphorimage signal for the IL-3 receptor (Fig. 5.1B) is strong relative to the signal obtained for GM-CSF receptor (Fig. 5.1A) owing to the high level of IL-3 receptor expression relative to GM-CSF receptor on these cells (Lopez et al.1990). As stated previously, a protein of 80,000 MW, consistent in size with GMR $\alpha$  co-immunoprecipitated with  $\beta_c$  in either the presence or absence of IL-3 although at much weaker intensity than either  $\beta_c$  or IL-3R $\alpha$  (Στομσκι ετ αλ.1996) and is hence not visible at the exposure shown (Fig. 5.1B).

To confirm the identity of the 120,000 MW protein co-immunoprecipitated by anti-GMR $\alpha$  MAb 8G6, immunoprecipitations carried out with unlabelled cells before and after treatment with GM-CSF using anti-GMR $\alpha$  MAb 8G6, anti- $\beta_c$  MAb 4F3 and anti-IL-3R $\alpha$  MAb 9F5. After western transfer an immunoblot with anti- $\beta_c$  antibody was carried out. An 120,000 MW protein was clearly detected in the presence or absence of GM-CSF in both GMR $\alpha$  and  $\beta_c$  immunoprecipitates but not in the IL-3R $\alpha$  immunoprecipitate (Fig. 5.1C). This indicates that  $\beta_c$  is associated with GMR $\alpha$  but not with IL-3R $\alpha$  in the absence of added cytokine on these primary CML cells.

One possible explanation for the pre-association of GMR $\alpha$  with  $\beta_c$  was the autocrine production of GM-CSF by the CML cells. However GM-CSF protein was not detected with either enzyme-linked immunoabsorbant assay or GM-CSF mRNA by northern analysis or reverse transcription-PCR. Nevertheless, to confirm the GM-CSF-independent association between GMR $\alpha$  and  $\beta_c$  and to determine the

generality of this observation, immunoprecipitations were carried out on a human GM-CSF dependent cell line (Mo7e) and on a mouse cell line (Ba/F-3) transfected with the human GM-CSF receptor. Mo7e cells maintained in IL-3, and murine Ba/F-3 cells expressing human GMR $\alpha$  and  $\beta_C$  maintained in GM-CSF were starved overnight prior to GM-CSF stimulation. Cells were  $^{125}\text{I}$ -surface labelled and proteins were immunoprecipitated with anti-GMR $\alpha$  MAb 8G6 or anti- $\beta_C$  MAb 8E4 before and after treatment with GM-CSF. The results showed the co-immunoprecipitation of the 120,000 MW  $\beta_C$  protein and the 80,000 MW GMR $\alpha$  protein with either antibody in the presence or absence of GM-CSF (Fig. 5.2A and 5.2B) although the signal observed on Mo7e cells was weak relative to the CML and Ba/F-3 cells, presumably due to low receptor expression. However, the relative intensity of the two proteins immunoprecipitated from Mo7e cells was similar regardless of whether GM-CSF was present or not whereas with the GM-CSF receptor expressing Ba/F-3 cells, GM-CSF stimulation enhanced the association of  $\beta_C$  with GMR $\alpha$  (Fig. 5.2A and 5.2B) indicating that only a proportion of GM-CSF receptors are preformed in these cells. In order to confirm that the 120,000 MW protein co-immunoprecipitated from Ba/F-3 cells with GMR $\alpha$  was human  $\beta_C$  and not a mouse  $\beta$  chain protein, an immunoblot was carried out on the immunoprecipitates with anti- $\beta_C$  antibody. The anti- $\beta_C$  antibody was highly immunoreactive against the 120,000 MW protein immunoprecipitated by anti-GMR $\alpha$  antibody in either the presence or absence of GM-CSF confirming the 120,000 MW protein as human  $\beta_C$  (Fig. 5.2C). Re-probing the immunoblot with anti-phosphotyrosine antibody PY20, showed that the  $\beta_C$  was phosphorylated only after treatment of the Ba/F-3 cells with GM-CSF (Fig. 5.2C) indicating that the preformed GMR $\alpha$ : $\beta_C$  complex is not activated and that

this complex was not the result of residual cytokine on the cells after overnight factor depletion. These findings strongly suggest that  $\text{GMR}\alpha$  and  $\beta_{\text{C}}$  are associated at the cell surface in the absence of GM-CSF as a preformed complex. In some experiments IL-3 is able to modulate the degree of preformed complex present.

### **5.2.2 A soluble form of $\beta_{\text{C}}$ interacts with cell surface expressed $\text{GMR}\alpha$ .**

To determine whether the extracellular portions of  $\text{GMR}\alpha$  and  $\beta_{\text{C}}$  are sufficient for ligand independent  $\text{GMR}\alpha:\beta_{\text{C}}$  interaction we made a construct encoding a soluble form of  $\beta_{\text{C}}$  ( $s\beta_{\text{C}}$ ), comprising the entire extracellular domain but lacking the transmembrane and cytoplasmic regions, and examined its ability to associate with  $\text{GMR}\alpha$ . Initial characterisation of  $s\beta_{\text{C}}$  was carried out by transfection into CHO cells and affinity purification of conditioned medium on an anti- $\beta_{\text{C}}$  antibody 3D7 coupled to CNBr-activated sepharose column. Two proteins of 55,000 and 65,000 MW were specifically eluted from the affinity column and visualised on a reducing SDS-PAGE gel by silver staining (Fig. 5.3A). These two proteins were also detected after western transfer by immunoblotting with anti- $\beta_{\text{C}}$  antibody (1C1) (Fig. 5.3B) implying that they represent two forms of  $s\beta_{\text{C}}$  protein. Intriguingly when the eluted  $s\beta_{\text{C}}$  fractions were run on SDS-PAGE under non-reducing conditions proteins of 120,000 MW and higher were seen by silver staining (Fig. 5.3A) and also by anti- $\beta_{\text{C}}$  immunoblotting (Fig. 5.3B) suggesting that the  $s\beta_{\text{C}}$  forms disulphide-linked dimers and higher order complexes. A similar phenomenon was observed with a soluble form of the mouse IL-3 specific  $\beta$  chain, sAIC2A (Ogorochi et al.1992) and may relate to the ability of  $\beta_{\text{C}}$  to spontaneously form dimers as previously noted (Stomski et al.1996; Muto et al.1996; Bagley et al.1997).

The association of  $s\beta_C$  with  $GMR\alpha$  was studied by transfecting the  $s\beta_C$  construct into CHO cells expressing  $GMR\alpha$  and monitoring  $s\beta_C$  retention at the cell surface with anti- $\beta_C$  MAb. Initial flow cytometric analysis demonstrated specific binding of anti- $\beta_C$  MAb on the surface of CHO cells co-expressing  $s\beta_C$  and  $GMR\alpha$  but not on CHO cells expressing  $s\beta_C$  alone. Importantly, the specific association of  $s\beta_C$  with  $GMR\alpha$  on the surface of CHO cells could be also demonstrated by co-immunoprecipitation experiments. In these experiments it also sought to establish that the retained  $\beta_C$  reactivity detected on the  $GMR\alpha$  expressing CHO cells was indeed  $s\beta_C$  and not another protein with a common epitope or a fusion protein produced by an anomalous transfection event. In order to examine surface expressed  $\beta_C$  specifically and avoid involvement of  $\beta_C$  from intracellular compartments, CHO cells expressing either full length or soluble  $\beta_C$  with or without  $GMR\alpha$  were surface labelled with  $^{125}I$  and  $\beta_C$  protein immunoprecipitated using an anti- $\beta_C$  antibody (8E4). A single  $^{125}I$ -labelled protein of 120,000 MW was immunoprecipitated from CHO cells expressing full length  $\beta_C$  (Fig. 5.4A). Two  $^{125}I$ -labelled proteins of 55,000 and 65,000 MW were immunoprecipitated from CHO cells expressing  $GMR\alpha$  and  $s\beta_C$  (Fig. 5.4A) that corresponded in size to the  $s\beta_C$  proteins detected in cell supernatants (Fig. 5.3). No labelled protein was immunoprecipitated from CHO cells expressing  $s\beta_C$  alone indicating that the  $s\beta_C$  retained on the surface of  $GMR\alpha$  expressing cells does not represent  $s\beta_C$  protein in the process of secretion but is specifically retained by  $GMR\alpha$ .

To investigate the nature of the  $s\beta_C$  protein doublet detected on  $GMR\alpha$  expressing CHO cells, *in vitro* deglycosylation was carried out on the immunoprecipitated protein prior to SDS-PAGE. The two  $^{125}I$ -labelled  $s\beta_C$  proteins were both rendered to a 50,000 MW protein (Fig. 5.4A). Similarly the two 55,000 and 65,000 MW forms of  $s\beta_C$  immunoprecipitated from conditioned medium were converted to a 50,000 MW protein after *in vitro* deglycosylation as seen by immunoblot using anti- $\beta_C$  antibody (3D7) (Fig. 5.4B). This demonstrates that the 55,000 and 65,000 MW proteins represent differentially glycosylated forms of  $s\beta_C$  as has previously been observed with the full length  $\beta_C$  (Ogorochi et al.1992) and that both forms are retained on  $GMR\alpha$  expressing cells.

In order to determine whether the  $GMR\alpha:s\beta_C$  complex is able bind GM-CSF with high affinity, saturation binding assays were carried out on  $GMR\alpha$  CHO cells co-expressing a similar amount of either  $s\beta_C$  or full length  $\beta_C$ . Due to the very high level of  $GMR\alpha$  chain expression on these transfectants ( $5 \times 10^5$  sites per cell as determined by Scatchard analysis) no high affinity sites could be detected directly from either transfectant (Fig. 5.5A and 5.5B). In order to reduce this interference, dissociation of weakly bound radioligand was carried out after binding, thereby removing ligand interacting with low affinity receptors. Using this approach high affinity binding sites ( $K_d$  236 pM) were detectable on  $GMR\alpha$  cells co-expressing full length  $\beta_C$  (Fig. 5.5A) but not on those co-expressing  $s\beta_C$  ( $K_d$  5.3 nM) (Fig. 5.5B). This implies that the  $s\beta_C$  protein is unable to confer full high affinity binding on the GM-CSF: $GMR\alpha$  complex, a function that may require a conformational change facilitated by the transmembrane and cytoplasmic regions of  $\beta_C$ .

### 5.2.3 Soluble GMR $\alpha$ and $\beta_C$ can exist as a complex and bind GM-CSF

Based on the demonstration of a preformed complex between GMR $\alpha$  and  $\beta_C$  on the cell surface and also retention of  $s\beta_C$  by cells expressing GMR $\alpha$  chain it suspected that it may be possible to observe co-association of a soluble form of GMR $\alpha$  and  $s\beta_C$  in solution. To test this idea a soluble carboxy-truncated form of GMR $\alpha$  was constructed that comprised of only the extracellular portion of the receptor, termed sGMR $\alpha$ . By immunoprecipitation and immunoblotting using a GMR $\alpha$  chain specific antibody (8G6) a 65,000 MW protein was detected in the medium of CHO cells transfected with this construct indicating that the soluble GMR $\alpha$  protein was expressed and was able to bind GM-CSF specifically with low affinity ( $K_d$  13.7 nM).

The sGMR $\alpha$  construct was then co-transfected together with the  $s\beta_C$  encoding cDNA into CHO cells. Both soluble proteins were detectable by immunoprecipitation and western blotting with appropriate antibodies in the cell medium of co-transfected cells. Significantly,  $s\beta_C$  protein was detected by immunoblot when immunoprecipitated not only with anti- $\beta_C$  (4F3) but also anti-GMR $\alpha$  antibody (8G6) but not an irrelevant antibody (9F5) (Fig.5.6A). This suggests that some but not all sGMR $\alpha$  is associated with  $s\beta_C$  in solution. Immunoprecipitation of a mixture of conditioned medium from cells expressing sGMR $\alpha$  and  $s\beta_C$  separately did not result in co-immunoprecipitation of the two chains. This is consistent with the retention of  $s\beta_C$  on GMR $\alpha$  expressing CHO cells in that it appears that co-expression of the two soluble receptor chains is required for the association to occur.

To determine whether the sGMR $\alpha$ :s $\beta_c$  complex is capable of binding ligand, conditioned medium from cells expressing sGMR $\alpha$  and s $\beta_c$  was incubated with GM-CSF and subsequently immunoprecipitation carried out with anti-GM-CSF antibody. Immunoblotting of the precipitated material revealed that s $\beta_c$  was associated with the anti-GM-CSF immunoprecipitated complex when conditioned medium from cells co-expressing the two receptor proteins was used, but not when conditioned medium from cells expressing the two chains separately was mixed (Fig. 5.6B). This implies that the association of s $\beta_c$  with GM-CSF is dependent on its interaction with sGMR $\alpha$  in conditioned medium from cells co-expressing sGMR $\alpha$  and s $\beta_c$ .

#### **5.2.4 GM-CSF exhibits rapid receptor association compared with IL-3 and IL-5.**

In order to examine whether a preformed GM-CSF receptor complex may influence the kinetics of GM-CSF binding, the kinetics of association of  $^{125}\text{I}$ -GM-CSF to primary human eosinophils and monocytes were examined. These cells were used as they express IL-3 receptors and in the case of eosinophils, IL-5 receptors as well as GM-CSF receptors thus allowing comparison between different receptor systems.

The association of GM-CSF was compared with IL-3 and IL-5 on human eosinophils in binding studies carried out at 4 °C with 200 pM  $^{125}\text{I}$ -labelled cytokine in which specific binding was determined over a 24 hour time-course (Fig. 5.7A). The results showed that GM-CSF binding approached equilibrium faster than IL-3 and IL-5 and binding was detected at very early time points. Curve fitting analysis revealed that a

significantly improved fit was obtained for GM-CSF association when binding was resolved into two classes of binding site (Table 5.1): one site exhibiting a rapid approach to equilibrium about 1000 fold faster than IL-3 or IL-5 and the other exhibiting similar apparent association kinetics to IL-3 and IL-5 (Table 5.1). Only a small proportion of the GM-CSF binding sites exhibit rapid binding kinetics, with the majority behaving like IL-3 and IL-5 receptors with a slower apparent association (Table 5.1). In previous studies it was shown that eosinophils exhibit only high

**Table 1** Kinetic parameters<sup>a</sup> for <sup>125</sup>I-CSF interaction with eosinophils

<b><sup>125</sup>I-CSF</b>	<b>K<sub>obs</sub><sup>b</sup> (min<sup>-1</sup>)</b>	<b>No. sites<sup>c</sup></b>
GM-CSF <sup>d</sup>	2.5 ± 1.2	15
	0.0061 ± 0.0015	105
IL-3	0.0071 ± 0.0024	90
IL-5	0.005 ± 0.002	160

<sup>a</sup>Determined as described in materials and methods.

<sup>b</sup>Apparent association rate.

<sup>c</sup>Number of binding sites exhibiting K<sub>obs</sub>.

<sup>d</sup>Statistical fit of 1 vs 2 sites P=0.005

affinity binding sites for GM-CSF, IL-3 (Lopez et al.1989) and IL-5 (Lopez et al.1991). From these studies it appears that the GM-CSF receptors exists in two pools that exhibit different kinetic properties.

On monocytes, as on eosinophils, the kinetics of GM-CSF binding was rapid and approached equilibrium faster than IL-3 binding (Fig. 5.7B). It has previously been shown that the approach to equilibrium by GM-CSF is approximately ten times faster than IL-3 (Elliott et al.1992). The rate of approach to equilibrium of IL-3 on monocytes is comparable to that seen for IL-3 and IL-5 and the slower binding site for GM-CSF on eosinophils suggesting that association at these sites may involve similar mechanisms whereas GM-CSF binding to the rapidly associating sites on eosinophils and monocytes is different.

### **5.2.5 The preformed GMR $\alpha$ : $\beta_C$ can be phosphorylated in response to IL-3 and IL-5.**

The functional significance of the preformed GMR $\alpha$ : $\beta_C$  complex was examined by means of receptor activation studies. It is known that in the course of activation of the GM-CSF, IL-3 and IL-5 receptors,  $\beta_C$  becomes phosphorylated in response to ligand binding (Kitamura et al.1991b; Duronio et al.1992), a process that requires the ligand specific  $\alpha$  chain. We have examined the phosphorylation of  $\beta_C$  induced by cytokines in Mo7e and TF-1.8 cells and have found that phosphorylated  $\beta_C$  can be detected by anti-phosphotyrosine (PY20) immunoblot after treatment with GM-CSF and immunoprecipitation with either anti- $\beta_C$  (8E4) or anti-GMR $\alpha$  (8G6) antibody (Fig. 5.8A and 5.8B). Similarly, treating Mo7e cells with IL-3 also resulted in  $\beta_C$  phosphorylation that was immunoprecipitable by either anti- $\beta_C$  (8E4) or anti-IL-3R $\alpha$  antibody (9F5) (Fig. 5.8A). Strikingly however, we found that anti-GMR $\alpha$  antibody also immunoprecipitated phosphorylated  $\beta_C$  in cells treated with IL-3, indicating that GMR $\alpha$  is associated with the IL-3-induced receptor complex (Fig. 5.8A). Similar

results were obtained in TF-1.8 cells with the addition that anti-GMR $\alpha$  antibody also immunoprecipitated  $\beta_c$  phosphorylated in response to IL-5 (Fig. 5.8B). Treatment of TF-1.8 cells with erythropoietin however did not result in  $\beta_c$  phosphorylation, indicating that  $\beta_c$  phosphorylation is specific to GM-CSF, IL-3 and IL-5 and not a general activation event. The involvement of GMR $\alpha$  in the IL-3- and IL-5- induced receptor complexes is specific to GMR $\alpha$  and may be mediated by the preformed GMR $\alpha$ : $\beta_c$  complex. Thus these findings raise the possibility that the preformed GMR $\alpha$ : $\beta_c$  complex can be recruited into an active receptor complex induced not only by GM-CSF but also by IL-3 or IL-5.

#### **5.2.6 GM-CSF induces high order GM-CSFR $\alpha$ and $\beta_c$ dimerization and activates the GM-CSFR complex**

With the IL-3R and IL-5R disulphide-linked dimerization has been shown between each of the binding  $\alpha$  subunits and  $\beta_c$  leading to phosphorylation ( chapter 3, ; chapter 4, ). Stimulation of GM-CSFR with GM-CSF using starved Mo7e cells leads to covalent and non covalent heterodimerization of binding  $\alpha$  subunits and  $\beta_c$ . In contrast to the IL-3R, where the majority of the phosphotyrosine reactivity is associated with the disulphide\_linked complex, the GMR $\alpha$  shows phosphorylation of both the disulphide and non disulphide-linked  $\beta_c$  (Fig. 5.9).

### 5.3 DISCUSSION

The existence of a  $\text{GMR}\alpha:\beta_{\text{C}}$  complex that is formed in the absence of GM-CSF has been demonstrated in this chapter. Also it has been observed that this ligand-independent association between  $\text{GMR}\alpha$  and  $\beta_{\text{C}}$  with both cell surface expressed receptors in several cell lines and also with carboxy-truncated soluble forms of the receptor subunits. The number of preformed  $\text{GMR}\alpha:\beta_{\text{C}}$  complexes observed on cells varied from cell to cell. In some cases all of the  $\text{GMR}\alpha$  and  $\beta_{\text{C}}$  chains were apparently co-associated and no further association was induced by GM-CSF treatment, whereas on other cells only a component of  $\text{GMR}\alpha$ s and  $\beta_{\text{C}}$ s were pre-associated and further association was induced by GM-CSF treatment. This suggests that two pools of GM-CSF receptors exist; preformed complexes and ligand induced complexes.

The notion of two GM-CSF receptor pools is consistent with previous experiments demonstrating that GM-CSF induces  $\text{GMR}\alpha$  and  $\beta_{\text{C}}$  association (Eder et al.1994) and reconciles this observation with that of Ronco et al (Ronco et al.1994), who suggested that the GM-CSF receptor may exist as a preformed complex. This possibility was raised by the inability of a mutant  $\text{GMR}\alpha$  to bind GM-CSF unless it was co-expressed with  $\beta_{\text{C}}$ . This was interpreted as  $\beta_{\text{C}}$  pre-associated with  $\text{GMR}\alpha$  compensating for the loss of GM-CSF binding on the mutant  $\text{GMR}\alpha$ . In an analogous manner a GM-CSF helix D mutant showed no detectable binding to  $\text{GMR}\alpha$  alone, yet could bind to cells expressing both  $\text{GMR}\alpha$  and  $\beta_{\text{C}}$  (Hercus et al.1994b), possibly reflecting the effect of a  $\text{GMR}\alpha:\beta_{\text{C}}$  preformed complex.

By using soluble receptor constructs it was possible to show the formation of sGMR $\alpha$ :s $\beta_C$  complexes in solution, indicating that the extracellular domains of the two proteins are sufficient to mediate the interaction. This in turn is dependent on the two soluble receptor chains being expressed by the same cell, because neither addition of s $\beta_C$  to GMR $\alpha$  expressing cells nor combining separately expressed sGMR $\alpha$  and s $\beta_C$  resulted in complex formation. This suggests that the association between the two proteins occurs as the proteins reach the cell surface, possibly before or during transport to the cell surface. However, interestingly, the retention of s $\beta_C$  by cells expressing GMR $\alpha$  did not result in a detectable increase in affinity for GM-CSF, in contrast to full length  $\beta_C$  that confers high affinity binding on the GM-CSF:GMR $\alpha$  complex. Under the dissociation conditions used it is possible that binding of intermediate affinity was lost and so we can only conclude that s $\beta_C$  is unable to confer full high affinity binding on GMR $\alpha$  expressing cells. This deficiency in binding with s $\beta_C$  may be due to the  $\beta_C$  lacking transmembrane and extracellular portions. These findings are consistent with recent studies where a naturally occurring soluble form of GMR $\alpha$  was found to be retained on the cell surface when co-expressed with full length  $\beta_C$  on BHK cells (Murray et al.1996). The soluble GMR $\alpha$  conferred GM-CSF binding on the cells albeit with intermediate affinity indicating some deficit in the interaction with  $\beta_C$ . These observations suggest that the transmembrane and cytoplasmic regions of these receptor subunits may be required for conformational changes and optimal high affinity binding. Alternatively these associations observed with soluble forms of the receptor may not represent normal receptor interactions.



The precise regions in the extracellular domains of  $GMR\alpha$  and  $\beta_C$  that mediate their spontaneous association in the cell membrane and in solution are not known. From modelling studies and comparison with the growth hormone crystal structure (De Vos et al.1992) the A-B loop and the E strand in the fourth domain of  $\beta_C$  appear to be good candidates for interaction with the second domain of the cytokine receptor module of  $GMR\alpha$ . It is worth noting that insertions, deletions and point mutations in this domain of  $\beta_C$  lead to factor independent activation (Gonda et al.1997). It is possible that various perturbations of an already preformed complex may result in receptor activation. There was no evidence of receptor activation in my experiments, as measured by anti-phosphotyrosine reactivity of the preformed complex (Fig. 5.2C). However, it would be interesting to examine this possibility with  $\beta_C$  mutants and indeed in human leukaemias.

In seeking to determine the functional significance of the preformed  $GMR\alpha:\beta_C$  complex, kinetic analysis for GM-CSF association was performed. Using normal cells expressing GM-CSF receptor it was found that the association of GM-CSF to both eosinophils and monocytes is more rapid relative to IL-3 and IL-5 and, in the case of eosinophils is bimodal. In previous studies it has been shown that eosinophils exhibit only high affinity binding sites for GM-CSF, IL-3 (Lopez et al.1989) and IL-5 (Lopez et al.1991). This suggests that there are sufficient  $\beta_C$ s to support full affinity conversion of GM-CSF receptors and that the receptors exist in two forms; one form approaches equilibrium very rapidly and a second form binds with similar kinetics to IL-3 and IL-5. This is consistent with the presence of two pools of receptor for GM-CSF; a small number of receptors that bind GM-CSF

rapidly, possibly representing preformed complexes as described here, and a larger pool, possibly comprised of free  $\text{GMR}\alpha$ s and  $\beta_{\text{C}}$ s that exhibit slower association on GM-CSF binding akin to IL-3 and IL-5 binding. We have previously reported that GM-CSF binds more rapidly to monocytes (Elliott et al.1992) and induces their adhesion faster than IL-3 (Elliott et al.1990). The presence of preformed  $\text{GMR}\alpha:\beta_{\text{C}}$  complexes may also account for these kinetic difference on monocytes by providing a pool of preformed receptors that rapidly associate with GM-CSF.

The binding cross competition exhibited between GM-CSF, IL-3 and IL-5 has previously been described on normal (Elliott et al.1989; Lopez et al.1989; Park et al.1989a) and leukemia cells (Gesner et al.1988; Park et al.1989b). The molecular basis of this phenomenon is the competition between GM-CSF: $\text{GMR}\alpha$ , IL-3:IL-3 $\text{R}\alpha$  and IL-5:IL-5 $\text{R}\alpha$  for  $\beta_{\text{C}}$  interaction. The proposed preformed  $\text{GMR}\alpha:\beta_{\text{C}}$  complex might be expected to have an effect on this phenomenon, sequestering  $\beta_{\text{C}}$  for the exclusive binding of GM-CSF. However, cross competition experiments carried out previously on eosinophils (Lopez et al.1989) and CML cells (Lopez et al.1990) show that IL-3 is able to compete for  $^{125}\text{I}$ -GM-CSF binding effectively with up to 90% competition. This suggests that the  $\beta_{\text{C}}$  associated with  $\text{GMR}\alpha$  in the preformed complex is in equilibrium with free  $\beta_{\text{C}}$  and is therefore competable by IL-3. This may also explain the relative numbers of preformed complexes observed on cells in that the level of preformed complex would be dependent on the relative level of expression of  $\beta_{\text{C}}$ . Thus cells that express excess  $\beta_{\text{C}}$  and thus exhibit high affinity binding sites only may have relatively more preformed sites compared to cells that express limiting amounts of  $\beta_{\text{C}}$ .

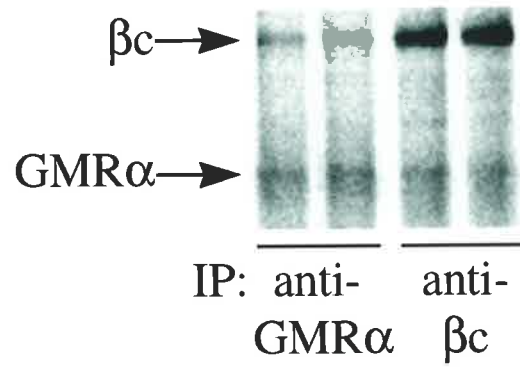
The stoichiometry of the active GM-CSF receptor is not known and may involve a  $\text{GMR}\alpha:\beta_{\text{C}}$  ratio of 1:1 or a 2:2 complex. Because of the disulphide-linked  $\text{GMR}\alpha:\beta_{\text{C}}$  heterodimer and molecular modelling of the extracellular region of  $\beta_{\text{C}}$  the second possibility is favoured (Bagley et al.1997). This is also consistent with the observations that at least two molecules of  $\text{GMR}\alpha$  are required for receptor activation (Lia et al.1996) and that phosphorylation of  $\beta_{\text{C}}$  dimers (Ogorochi et al.1992) and disulphide-linked  $\beta_{\text{C}}$  containing complexes (Fig 5.9) occurs in response to GM-CSF. In this model, binding of ligand may render cysteine residues in  $\text{GMR}\alpha$  and  $\beta_{\text{C}}$  reactive leading to disulphide bond formation across two receptors in a hexameric configuration, thus bringing together two  $\beta_{\text{C}}$  associated JAK-2 molecules thereby inducing receptor activation (Fig.5.10A). Given the observation of the preformed  $\text{GMR}\alpha:\beta_{\text{C}}$  it is possible to speculate that it could be recruited into an IL-3 or IL-5 receptor complex (Fig. 5.10B). Consistent with this possibility it was found that anti-GMR $\alpha$  antibodies immunoprecipitated phosphorylated  $\beta_{\text{C}}$  when cells were stimulated not only with GM-CSF but also with IL-3 and IL-5. In contrast, GM-CSF did not induce phosphorylation of  $\beta_{\text{C}}$  associated with IL-3R $\alpha$  (Fig. 5.8A) consistent with IL-3 receptor existing only as a fully inducible receptor.

The unidirectional activation of the GM-CSF receptor by IL-3 is reminiscent of trans-down modulation experiments in the mouse where IL-3 was found to trans-down modulate GM-CSF, M-CSF and G-CSF receptors but GM-CSF or G-CSF were unable to trans-down modulate the mouse IL-3 receptor (Walker et al.1985; Nicola,1987). The transphosphorylation of  $\text{GMR}\alpha$  associated  $\beta_{\text{C}}$  which was shown

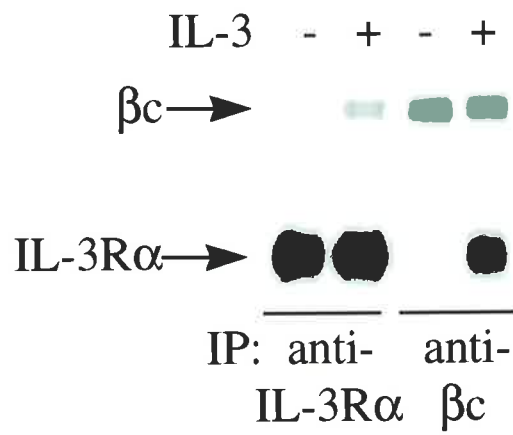
in these experiments appears to be limited to the GM-CSF/IL-3/IL-5 receptor system in that erythropoietin is ineffective and is probably a reflection of the unique mode of assembly of this heterodimeric receptor family. GM-CSF receptors are expressed by many cells of the haemopoietic lineage and intriguingly most cells that express either IL-3 or IL-5 receptors also express GM-CSF receptors. The data presented here suggests that IL-3 and IL-5 are able to activate preformed GM-CSF receptors thus raising the possibility that the biological functions of IL-3 and IL-5 are mediated in part by signalling through the GM-CSF receptor. A further possibility is that the GM-CSF preformed complex may act to potentiate the effects of IL-3, IL-5 and GM-CSF by reducing the need for multiple ligand-induced heterodimerization events. A single receptor oligomerization event (ie. hexameric complex formation) in the absence of preformed complexes would require the formation of two ligand induced receptor heterodimers. The presence of preformed complexes however, may theoretically reduce the number of ligand- induced receptor heterodimers required to produce a functional signal.

**Figure 5.1 Co-immunoprecipitation of GMR $\alpha$  and  $\beta_c$  from primary CML cells.** **A & B** CML cells were  $^{125}\text{I}$ -surface-labelled, treated with (+) or without (-) GM-CSF or IL-3 for 5 mins. at 4 $^{\circ}\text{C}$  and immunoprecipitation carried out either with anti-GMR $\alpha$  (8G6) anti-IL-3R $\alpha$  (9F5) or anti- $\beta_c$  (4F3) MoAb. Immunoprecipitated proteins were separated on 7.5% SDS-PAGE and visualised by phosphorimager and are presented at exposure levels appropriate for the specific signal obtained. **C** Proteins immunoprecipitated from CML cells with different anti-receptor antibodies either in the presence (+) or absence (-) of GM-CSF were subjected to western transfer and immunoblotted using a polyclonal anti- $\beta_c$  antibody.

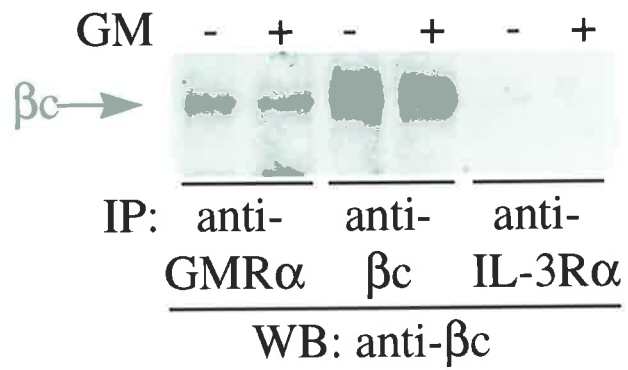
**A**



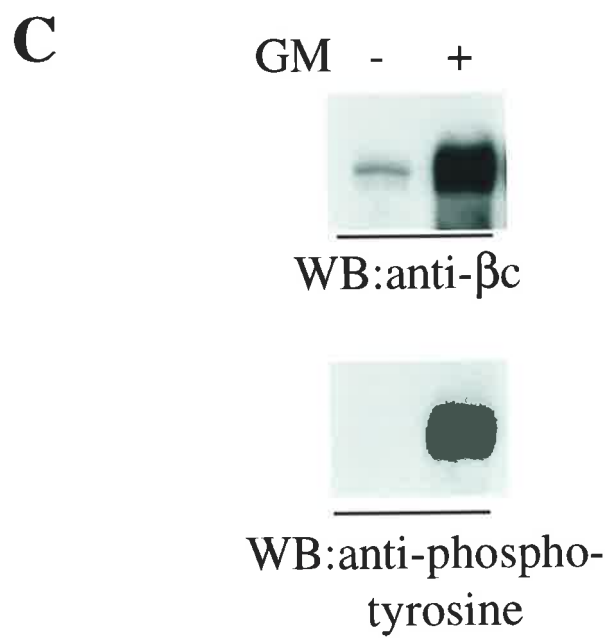
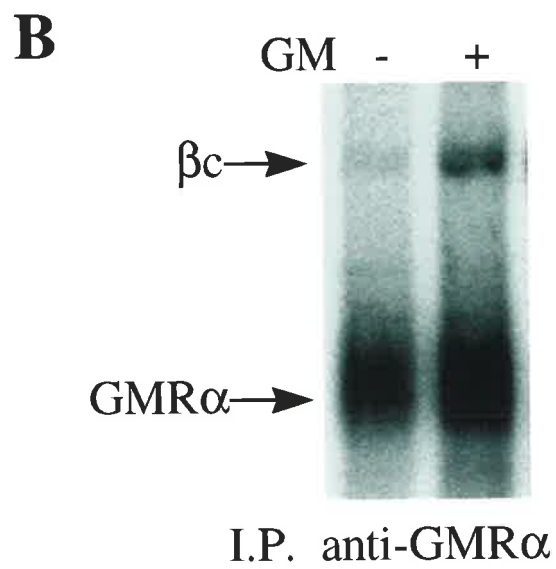
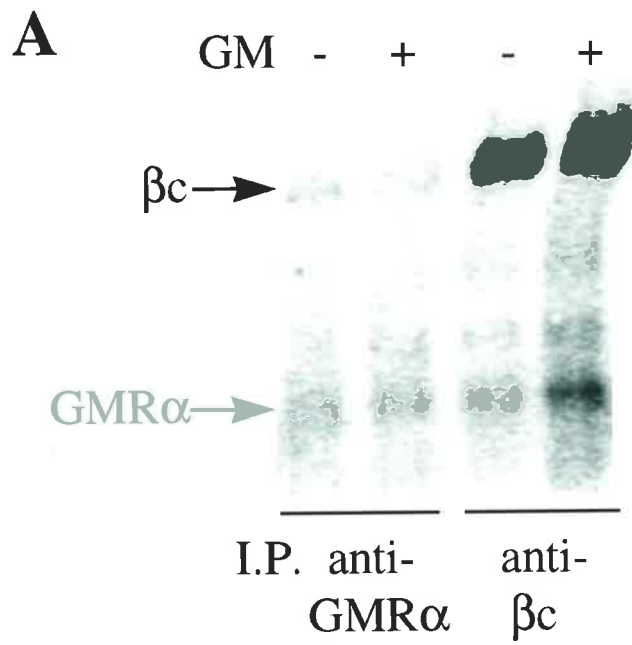
**B**



**C**

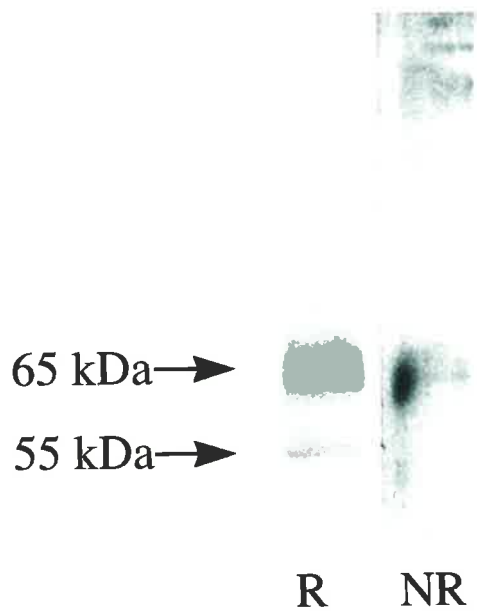


**Figure 5.2 Co-immunoprecipitation of GMR $\alpha$  and  $\beta_c$  from Mo7e and hGMR $\alpha$ /h $\beta_c$  expressing Ba/F-3 cells.** **A** Mo7e cells were starved overnight and  $^{125}\text{I}$ -surface-labelled, treated with (+) or without (-) GM-CSF for 5 minutes and immunoprecipitation carried out either with anti-GMR $\alpha$  MoAb (8G6) or anti- $\beta_c$  MoAb (4F3). Immunoprecipitated proteins were separated on 7.5% SDS-PAGE under reducing conditions and the gel exposed to phosphorimager. **B** hGMR $\alpha$ /h $\beta_c$  expressing Ba/F-3 cells were starved overnight and  $^{125}\text{I}$ -surface-labelled, treated with (+) or without (-) GM-CSF for 5 minutes and immunoprecipitation carried out either with anti-GMR $\alpha$  MoAb (8G6). Immunoprecipitated proteins were separated on 7.5 % SDS-PAGE under reducing conditions and the gel exposed to phosphorimager. **C** Proteins immunoprecipitated from hGMR $\alpha$ /h $\beta_c$  expressing Ba/F-3 cells with anti-GMR $\alpha$  MoAb (8G6) either in the presence (+) or absence (-) of GM-CSF were subjected to western transfer and immunoblotted using anti- $\beta_c$  antibody 1C1 (upper panel) or anti-phosphotyrosine antibody PY20 (lower panel).

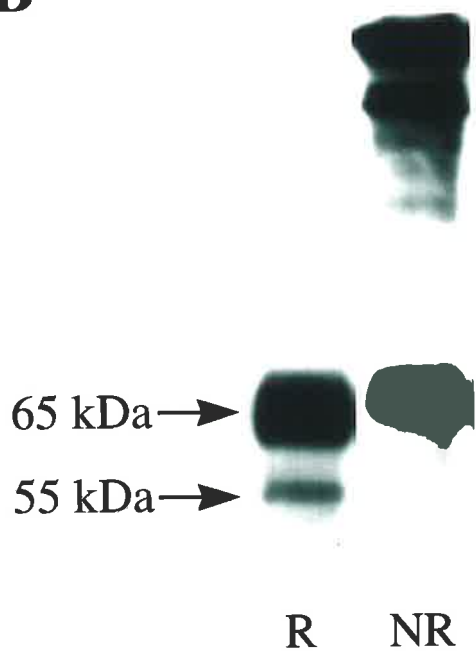


**Figure 5.3 Soluble  $\beta_C$  protein was purified from conditioned medium from CHO transfectants.** Soluble purified  $\beta_C$  protein was run under reducing (R) or non-reducing (NR) conditions on 10% SDS-PAGE and either **A** silver stained or **B** subjected to western transfer and immunoblotted with anti- $\beta_C$  antibody (1C1).

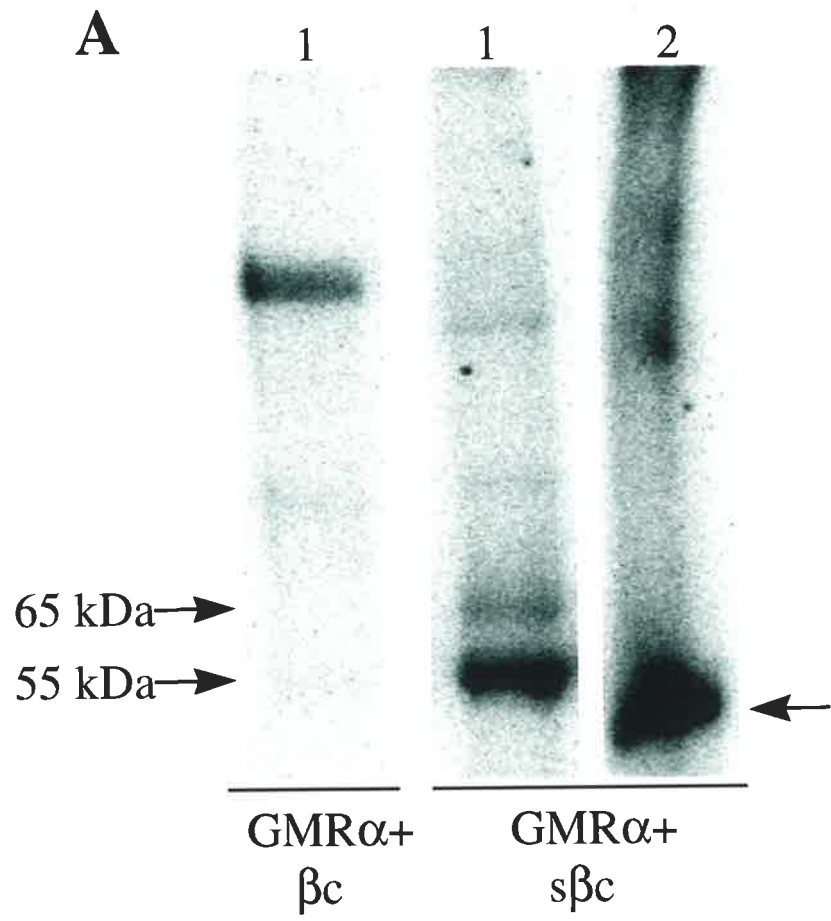
**A**



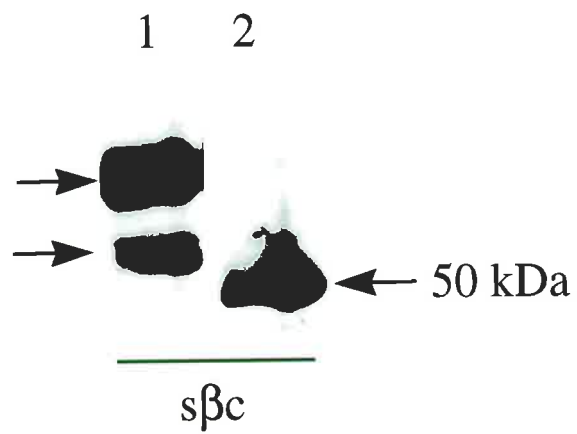
**B**



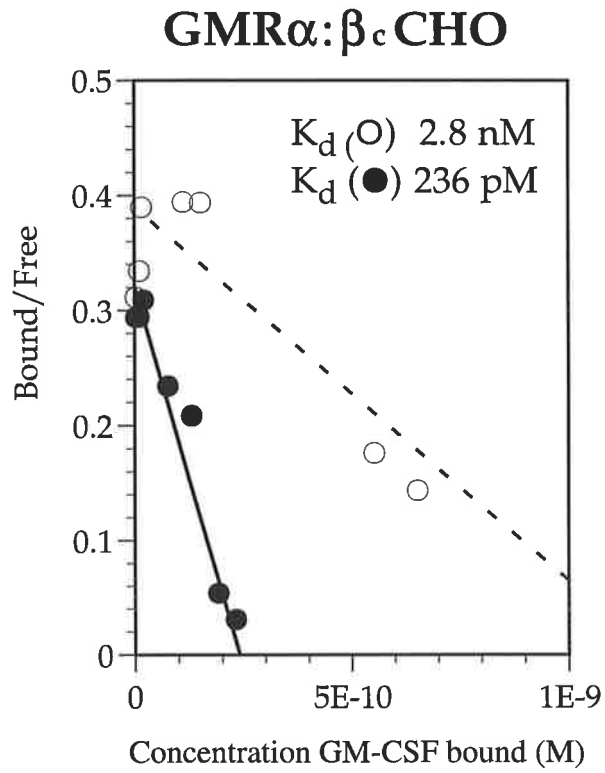
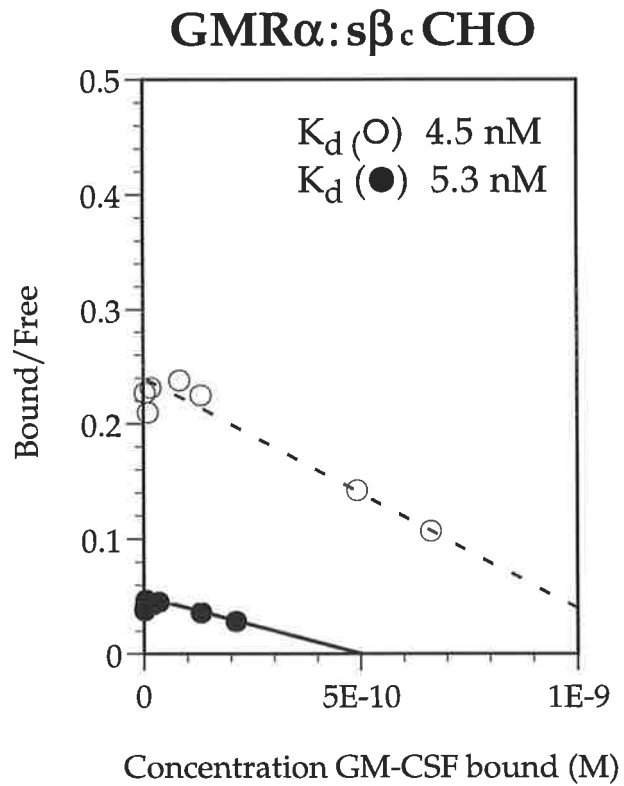
**Figure 5.4 Soluble  $\beta_C$  is retained on the surface of GMR $\alpha$  expressing CHO cells.** **A** CHO cells expressing GMR $\alpha$  and either full-length  $\beta_C$  ( $\beta_C$ ) or soluble  $\beta_C$  ( $s\beta_C$ ) were  $^{125}\text{I}$ -surface-labelled and immunoprecipitation carried out with anti- $\beta_C$  MoAb (8E4). The immunoprecipitated proteins were then either incubated with (2) or without (1) deglycosylating enzymes and subsequently separated on 7.5% SDS-PAGE under reducing conditions and visualised by phosphorimager. **B** Soluble  $\beta_C$  was immunoprecipitated from the medium of CHO cells co-expressing GMR $\alpha$  and soluble  $\beta_C$  ( $s\beta_C$ ) and the immunoprecipitated proteins either subjected to enzymatic deglycosylation (2) or not (1) and subsequently separated on 7.5% SDS-PAGE. Western transfer was then carried out and immunoblotting with anti- $\beta_C$  antibody 1C1.



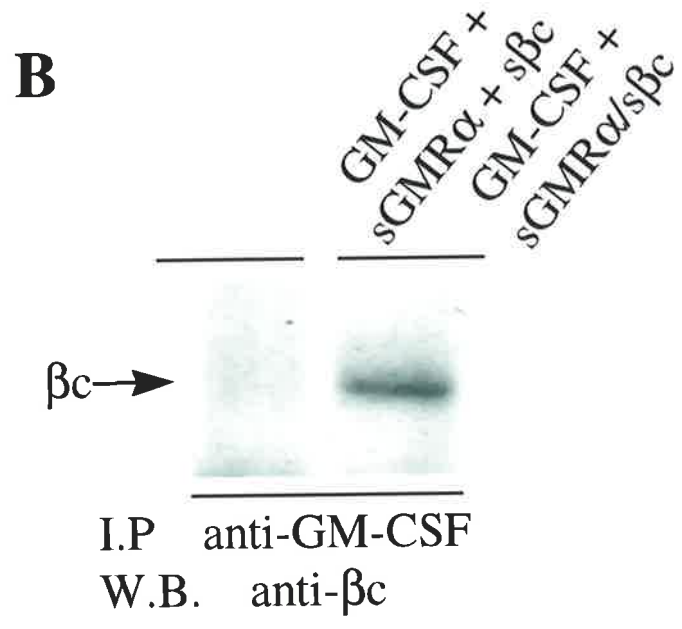
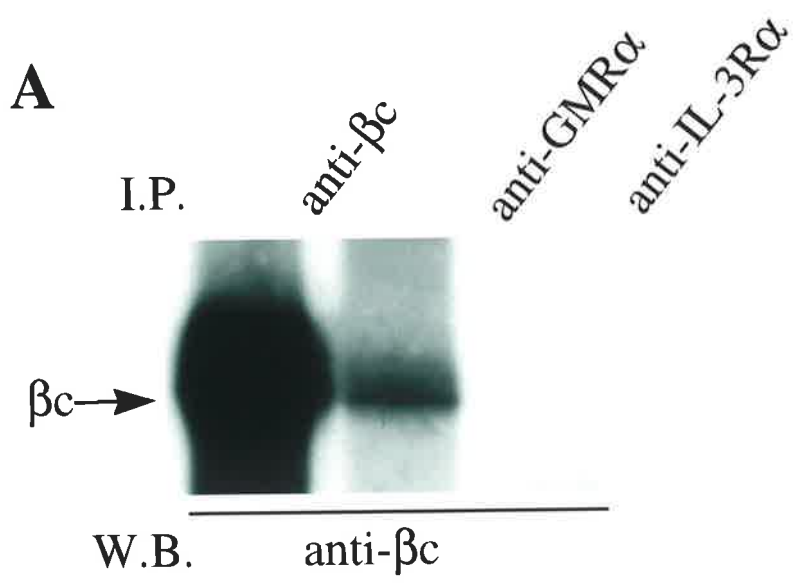
**B**



**Figure 5.5** Scatchard transformation of saturation binding studies carried out on CHO cells co-expressing: **A** GMR $\alpha$  and full length  $\beta_C$ , **B** GMR $\alpha$  and soluble  $\beta_C$  (s $\beta_C$ ). Binding assays were carried out with  $^{125}\text{I}$ -labelled GM-CSF over a concentration range of 10 pM- 10 nM. After binding had reached equilibrium the cells were washed briefly and either lysed directly (O ) or subjected to four 15 minute washes to remove ligand bound with low affinity (●). The broken line indicates the best fit for non-dissociated binding and the solid line the best fit for dissociated binding.

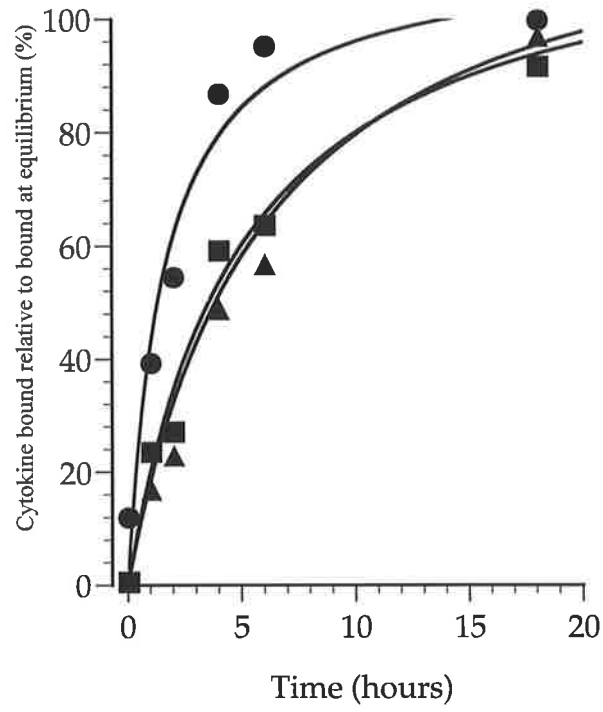
**A****B**

**Figure 5.6 Soluble forms of GMR $\alpha$  and  $\beta_C$  spontaneously associate when co-expressed and bind GM-CSF.** **A** Conditioned medium from CHO cells co-expressing soluble GMR $\alpha$  and soluble  $\beta_C$  was immunoprecipitated using either anti- $\beta_C$  (4F3), anti-GMR $\alpha$  (8G6) or a control antibody (9F5), the proteins separated on 10% SDS-PAGE, western transferred and immunoblotted with anti- $\beta_C$  antibody 1C1. **B** Conditioned medium from CHO cells either co-expressing soluble GMR $\alpha$  and soluble  $\beta_C$  (sGMR $\alpha$ /s $\beta_C$ ) or a mixture of conditioned medium from CHO cells expressing the soluble proteins separately (sGMR $\alpha$ + $\beta_C$ ) were incubated with GM-CSF and immunoprecipitation carried out with anti-GM-CSF antibody. Proteins were separated on 10% SDS-PAGE, western transferred and then immunoblotted with anti- $\beta_C$  antibody 1C1.

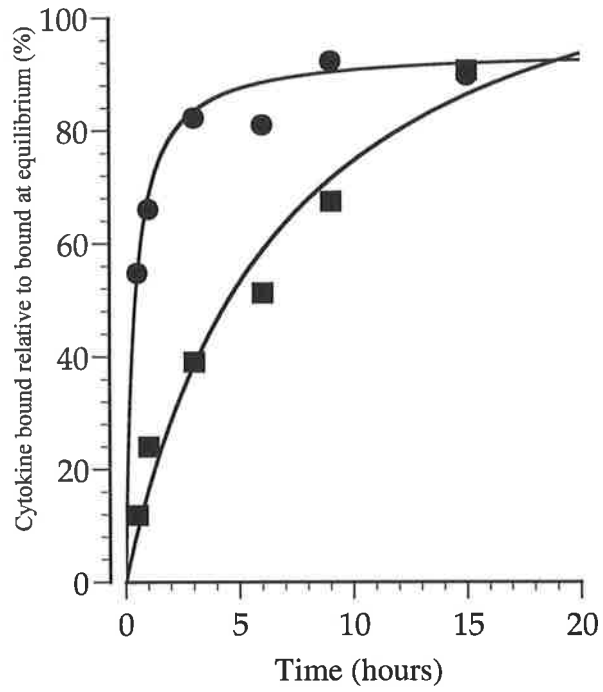


**Figure 5.7 Association kinetics.** Association kinetics of  $^{125}\text{I}$ -labelled cytokines binding to **A** eosinophils, and **B & C** monocytes at 4\_C with 150 pM  $^{125}\text{I}$ -CSF: ● GM-CSF, ■ IL-3, ▲ IL-5.

**A**

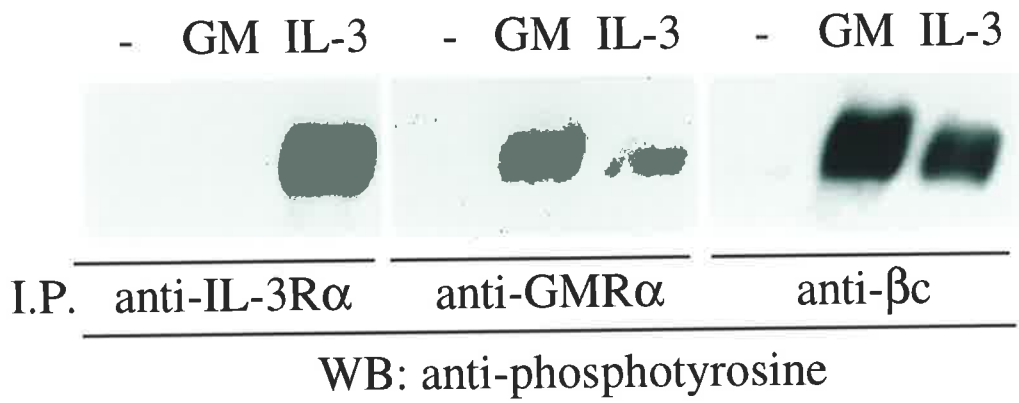


**B**

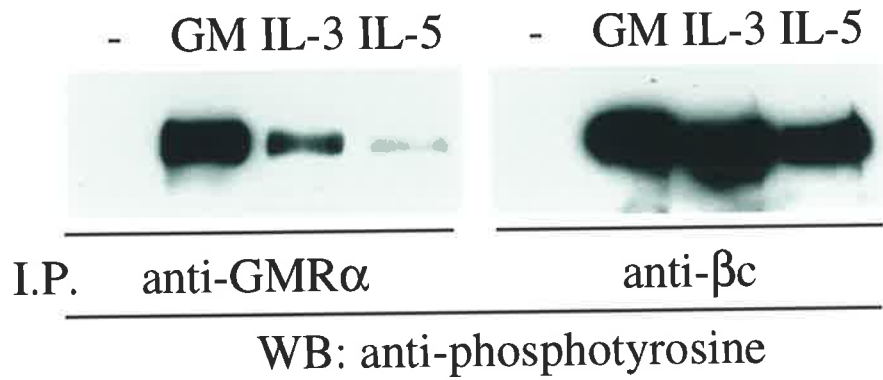


**Figure 5.8**  $\beta_c$  phosphorylated in response to GM-CSF, IL-3 or IL-5 is immunoprecipitable by anti-GMR $\alpha$  antibody. **A** Mo7e cells were starved overnight and then treated with either 100 pM GM-CSF, IL-3 or medium for 5 minutes at 4\_C and then immunoprecipitated with either anti-IL-3R $\alpha$  (9F5), anti-GMR $\alpha$  (8G6) or anti- $\beta_c$  (8E4) antibody. **B** TF-1.8 cells were starved overnight and then treated with either 100 pM GM-CSF, IL-3 or IL-5 or medium for 5 minutes at 4\_C and then immunoprecipitated with either anti-GMR $\alpha$  (8G6) or anti- $\beta_c$  (8E4) antibody. Immunoprecipitated proteins were separated on 7.5% SDS-PAGE, western transferred and then immunoblotted with anti-phosphotyrosine antibody (PY20).

**A**



**B**



**Figure 5.9 Disulphide-linked receptor complexes.** Immunoprecipitation of MO7e cells with anti- $\beta_c$  monoclonal antibody and immunoblotting with anti-phosphotyrosine antibody. The cells were either untreated or treated with GM-CSF or IL-3. Proteins were separated on a 7.5% SDS-PAGE gel under non reducing or reducing (4% 2-ME) conditions.

A

$\alpha/\beta_c \rightarrow$

$\beta_c \rightarrow$



- +GM-CSF +IL-3

B

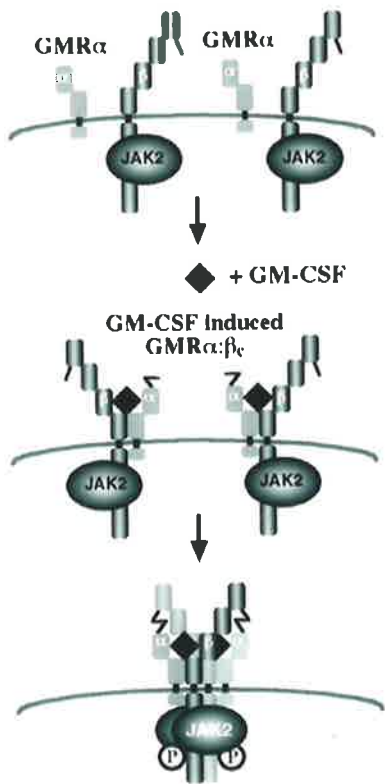
$\beta_c \rightarrow$



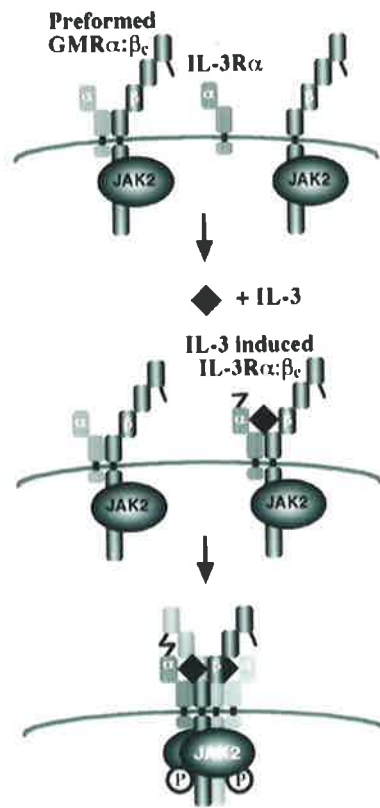
- +GM-CSF +IL-3

**Figure 5.10 Proposed models for assembly of (A) GM-CSF-, IL-3- and IL-5-induced receptor complexes, and (B) preformed GM-CSF receptor complexes into activated receptors.** In A)  $GMR\alpha$ ,  $IL-3R\alpha$  or  $IL-5R\alpha$  are in close proximity to (though not associated with)  $\beta_C$  on the cell surface. Ligand binding to the appropriate  $\alpha$  chain induces  $\alpha:\beta_C$  heterodimerization and a conformational change in  $\alpha$  chain that allows its disulphide linkage to  $\beta_C$ . Modelling of  $\beta_C$  suggests that this bridging would only be possible if the unpaired cysteines in the  $\alpha$  chain of receptor 1 formed a disulphide bridging with cysteine of  $\beta_C$  in receptor 2 (Bagley et al.1997). The bringing together of two  $\beta_C$  with their associated JAK-2 molecules would then lead to receptor activation. In B) it is postulated that the binding of IL-3 or IL-5 to their specific  $\alpha$  chain and  $\beta_C$  triggers a conformational change in the  $\alpha$  subunit analogous to model A. In this case however, a disulphide bridge can be formed between the free cysteine in the  $IL-3R\alpha$  or  $IL-5R\alpha$  and a cysteine in  $\beta_C$  that is already non-covalently associated with  $GMR\alpha$  chain in a preformed complex. This interaction may be sufficient to bring together two  $\beta_C$  and two JAK-2 kinases leading to receptor activation. This model raises the possibility that some of the functions mediated by IL-3 and IL-5 are mediated inducibly through the activation of a preformed  $GMR\alpha:\beta_C$  complex.

**A. Induced GM-CSF/IL-3/IL-5 receptor complexes**



**B. Preformed GMR $\alpha$ : $\beta_c$  recruited into IL-3/IL-5 receptor complexes**



## *Chapter VI*

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**IDENTIFICATION OF A CYS MOTIF IN THE COMMON  $\beta$   
CHAIN OF THE IL-3, GM-CSF AND IL-5 RECEPTORS  
ESSENTIAL FOR DISULFIDE-LINKED RECEPTOR  
HETERODIMERIZATION AND ACTIVATION OF ALL THREE  
RECEPTORS**

## 6.1 INTRODUCTION

Cytokine receptor dimerization is a common theme in receptor activation (Heldin,1995). Following the binding of the cognate ligand to cytokine receptors a sequential process takes place whereby receptor subunits associate and recruit cytoplasmic signalling molecules leading to receptor activation and cellular signalling (Ihle,1995). The general process of receptor dimerization exhibits variations amongst the cytokine receptor superfamily and may involve homodimerization or heterodimerization events depending on receptor subunit composition (Ihle et al.1995; Bagley et al.1997). In the case of the growth hormone receptor, growth hormone binds initially to one receptor subunit and induces its homodimerization with a second identical subunit (Cunningham et al.1991). A similar process probably takes place with erythropoietin and G-CSF leading in both cases to receptor homodimerization and activation (Miura et al.1993b; Hiraoka et al.1994).

With cytokine receptors that comprise multiple subunits, receptor activation is accompanied by homodimerization or heterodimerization of the signalling subunits. For example, in the IL-6 receptor system IL-6 induces dimerization of IL-6R $\alpha$  with gp130 (Hibi et al.1990), homodimerization of gp130 and receptor activation (Murakami et al.1993). On the other hand the binding of CNTF to CNTFR $\alpha$  induces its association with gp130 and the LIF receptor (LIFR) and the heterodimerization of gp130 and the LIFR is accompanied by receptor activation (Davis et al.1993). Similarly, heterodimerization of IL-2R $\beta$  and  $\gamma$  subunits is necessary for IL-2 receptor activation (Nakamura et al.1994; Nelson et al.1994). Interestingly, in these cases

each receptor  $\alpha$  chain constitutes the major binding subunit but does not seem to form part of the signalling receptor complex .

The mechanism of activation of the GM-CSF/IL-3/IL-5 receptor system exhibits similar features to the mechanism employed by the above receptors although some unique features are becoming evident. One of the most important differences is the contribution that each receptor  $\alpha$  chain makes to signalling. This is manifested in two ways: firstly, unlike IL-6R $\alpha$ , CNTFR $\alpha$  and the IL-2R $\alpha$  chains, the cytoplasmic domains of GM-CSFR $\alpha$ , IL-3R $\alpha$  and IL-5R $\alpha$  are all required for full receptor activation and signalling (Sakamaki K et al.1992; Rapoport et al.1996; Takaki et al.1994; Weiss et al.1993). Secondly, IL-3R $\alpha$  and GM-CSFR $\alpha$  form disulfide-linked dimers with the common  $\beta$  chain ( $\beta_c$ ) of their receptor as shown for IL-3R in chapter 3 (Stomski et al.1996; Bagley et al.1997).

Disulfide-linked dimerization of receptor subunits has been demonstrated for several cytokine receptors and is associated with receptor activation. Two gp130 molecules form disulfide-linked homodimers and become tyrosine phosphorylated in the presence of IL-6 (Murakami et al.1993). Disulfide-linked dimers of gp130 and LIFR are formed and both subunits become tyrosine phosphorylated in cells also expressing CNTFR $\alpha$  and stimulated with CNTF (Davis et al.1993). Similarly, disulfide-mediated dimerization of IL-3R $\alpha$  with  $\beta_c$  and of GM-CSFR $\alpha$  with  $\beta_c$  is accompanied by tyrosine phosphorylation of  $\beta_c$  (Bagley et al.1997). In all these cases, however, tyrosine phosphorylation is observed in the disulfide-linked dimers as well as in the monomeric molecules, and hence it is not clear which is the critical

species for receptor activation. Furthermore, the location of the cysteines involved in disulfide-linkage is not known, nor is it apparent whether they constitute a functionally conserved motif in the cytokine receptor superfamily.

The extracellular region of  $\beta_c$  comprises four fibronectin type III-like domains grouped into two CRM. To further characterize which cysteine residues are involved, single alanine substitutions of candidate cysteine residues were performed in the N-terminal CRM of the IL-3, GM-CSF and IL-5 receptor  $\beta_c$  and examined for their contribution to disulfide-linked receptor dimerization, high affinity ligand binding and receptor activation. In this chapter it is shown that Cys 86 and Cys 91 of  $\beta_c$  are critical for disulfide-linked receptor dimerization but not for noncovalent receptor dimerization or for high affinity binding of ligand. Importantly, Cys 86 and Cys 91 are shown to be essential for the activation of IL-3, GM-CSF and IL-5 receptors. The fact that these cysteines can be aligned with cysteines at similar positions in mouse  $\beta_c$  and  $\beta_{IL-3}$  but not in other receptor subunits suggests that they represent a functional motif restricted to the IL-3, GM-CSF and IL-5 subfamily of cytokine receptors.

## 6.2 RESULTS

### 6.2.1 Rationale for mutagenesis of N-terminal Cysteine residues of $\beta_C$

In order to study the molecular events involved in the activation of the IL-3, GM-CSF and IL-5 receptors, several extracellular cysteine residues of  $\beta_C$  were replaced by alanine residues. So as to target Cys available for intermolecular interactions, it was sought to avoid Cys involved in structurally-important intramolecular disulfide bonds. By homology with other cytokine receptors, domains one and three are expected to possess two disulfide bonds each. This is clearly the case with domain three which contains only four Cys residues. However, domain one  $\beta_C$  possesses seven Cys of which only Cys 34, Cys 45 and Cys 75 could be aligned readily with equivalent Cys in other receptors (Fig 6.1). Of the remaining Cys residues, Cys 86, Cys 91 and Cys 96 are conserved in murine  $\beta_C$ . Of these, Cys 96 is followed by an Ile at position 98 which aligns with conserved hydrophobic residues in other cytokine receptors suggesting that is this Cys that is part of the second conserved disulfide bond. Cys 96, Cys 91 are proposed to lie in an extended loop between the D and E beta strands of the first domain. Although Cys 86 and Cys 91 were favoured candidates for intermolecular disulfide bond formation, It was chosen to also mutate the nearby Cys 96 and Cys 100 and the single Cys residue in domain 2 at position 234.

### 6.2.2 Mutation of Cys 86 and Cys 91 selectively disrupt ligand-induced, disulfide-linked heterodimer formation

Expression plasmids encoding IL-3R  $\alpha$  and wild type (wt) or C86A, C91A, C96A, C100A, and C234A mutant  $\beta_C$  were co-transfected into COS cells. After 48h the

cells were  $^{125}\text{I}$  surface-labelled and either left unstimulated or stimulated with IL-3. IL-3R $\alpha$  and  $\beta_c$  were then immunoprecipitated with specific monoclonal antibodies (MAb) 9F5 or 8E4 respectively, and the proteins resolved by 6% SDS-PAGE under either nonreducing or reducing conditions. It was found that the  $\beta_c$  mutants C100A and C234A behaved very similarly to wt  $\beta_c$ . Both mutants allowed the formation of two high molecular weight complexes in response to IL-3 (Fig. 6.2A), which, as with wt  $\beta_c$ , contain IL-3R $\alpha$  and  $\beta_c$  ((Stomski et al.1996), and data not shown). These two complexes were immunoprecipitated by both anti-IL-3R $\alpha$  MAb 9F5 and anti- $\beta_c$  MAb 8E4 (Fig. 6.2A). In the absence of IL-3, the anti-IL-3R $\alpha$  MAb 9F5, immunoprecipitated only monomeric IL-3R $\alpha$  whereas the anti- $\beta_c$  MAb 8E4 immunoprecipitated monomeric  $\beta_c$  as well as the high molecular weight complex corresponding to disulfide-linked  $\beta_c$  homodimers (Stomski et al.1996; Bagley et al.1997) (Fig. 6.2A). As with the disulfide-linked dimers, the noncovalent IL-3R $\alpha$  and  $\beta_c$  heterodimers were not affected by mutating Cys 100 or 234 as both the anti-IL-3R $\alpha$  MAb and the anti- $\beta_c$  MAb co-immunoprecipitated both IL-3R $\alpha$  and  $\beta_c$  and only in the presence of IL-3 (Fig. 6.2B).

In contrast, the mutants C86A, C91A and C96A had a profound effect on disulfide-linked receptor dimerization. In the presence of IL-3, anti-IL-3R $\alpha$  MAb 9F5 and anti- $\beta_c$  MAb 8E4 did not immunoprecipitate the high molecular weight complexes corresponding to IL-3R $\alpha$  and  $\beta_c$  heterodimers (Fig. 6.2A). In fact, under non-reducing conditions very little or no monomeric  $\beta_c$  was immunoprecipitated by either MAb with most of the label being observed in the high molecular weight region probably representing aggregated  $\beta_c$ . With the anti-IL-3R $\alpha$  MAb 9F5 a non specific

band migrating slightly faster than  $\beta_c$  was seen (Fig. 6.2A). Under reducing conditions, however, monomeric  $\beta_c$  could be detected (Fig. 6.2B). An important difference was noted between the C86A and C91A mutants on one hand and the C96A mutant on the other hand. Anti-IL-3R $\alpha$  MAb 9F5 co-immunoprecipitated C86A and C91A in the presence of IL-3 but did not co-immunoprecipitate C96A (Fig. 6.2). Reciprocally, in the presence of IL-3, the anti- $\beta_c$  MAb co-immunoprecipitated IL-3R $\alpha$  with C86A and C91A but not with C96A (Fig. 6.2). This was more clearly seen under reducing conditions (Fig. 6.2B) than non reducing conditions (Fig. 6.2A) where an overall lower signal was observed.

To verify that the surface expression levels of the individual  $\beta_c$  mutants was similar to  $\beta_c$  wt, COS cells transfected with the various constructs were analysed by flow cytometry (FACS). FACS analysis indicated that the surface expression of wt  $\beta_c$  and the Cys $\rightarrow$ Ala  $\beta_c$  mutants was very similar both in terms of percentage of transfected cells expressing the different receptor subunits and in absolute levels (Fig. 6.3) suggesting that the mutations did not affect subunit transport and expression at the cell surface.

### **6.2.3 The $\beta_c$ mutants C86A and C91A do not disrupt IL-3 and GM-CSF high affinity binding**

Next was examined the ability of Cys mutants of  $\beta_c$  to support high affinity IL-3 or GM-CSF binding. COS cells were transfected with IL-3R $\alpha$  and GM-CSFR $\alpha$  and either wt  $\beta_c$  or the Cys $\rightarrow$ Ala  $\beta_c$  mutants and subjected to saturation binding studies with  $^{125}$ I-IL3 and  $^{125}$ I-GM-CSF. Scatchard transformation of the saturation binding

curves were performed and the  $K_d$  and receptor numbers determined using the Ligand program. It was found that  $\beta_c$  bearing Cys→Ala substitutions at positions 86, 91, 100 and 234 were able to form high affinity binding sites (Fig 6.4 and Table 6.1). The range of affinities for IL-3 high affinity binding of these Cys→Ala  $\beta_c$  substitution mutants varied from 31 to 280 pM compared with 330 pM for wt  $\beta_c$ , while GM-CSF high affinity binding ranged from 27 to 230 pM compared with 120 pM for wild type  $\beta_c$ . In contrast, COS cell transfectants expressing the C96A  $\beta_c$  showed no detectable high affinity binding (Fig. 6.4 and Table 6.1).

Although  $\beta_c$  mutants C86A and C91A were able to support high affinity binding of IL-3 and GM-CSF, a reduction in the number of high affinity receptors was observed compared with wild type  $\beta_c$  and the C100A and C234A analogues (Fig. 6.4 and Table 6.1). This is probably a reflection of the tendency of C86A and C91A  $\beta_c$  analogues to oligomerize as observed in the immunoprecipitations under non-reducing conditions (Fig. 6.2A), thereby reducing the amount of free  $\beta_c$  available for interaction with  $\alpha$  chain.

#### **6.2.4 C86A and C91A abolish IL-3-, GM-CSF- and IL-5-dependent tyrosine-phosphorylation of $\beta_c$**

It has been previously established that stimulation of cells with IL-3 leads to the formation of disulfide-linked heterodimers of IL-3 receptor  $\alpha$  and  $\beta_c$  chain, which is associated with phosphorylation of  $\beta_c$  (Stomski et al.1996). Similarly, in the case of the GM-CSF receptor, receptor heterodimerization occurs upon stimulation with GM-CSF and this is accompanied by  $\beta_c$  phosphorylation (Bagley et al.1997) It is

shown that GM-CSF, IL-3 and IL-5 receptors form disulfide-linked complexes which are similarly accompanied by  $\beta_c$  phosphorylation. It was possible to take advantage of the inability of  $\beta_c$  mutants to form disulfide-linked heterodimers to determine whether the formation of these is a necessary for receptor activation or whether noncovalent dimerization is sufficient for activation as measured by  $\beta_c$  phosphorylation. Transfections of wt  $\beta_c$  and the different  $\beta_c$  mutants in HEK293T cells together with JAK-2 and either IL-3R $\alpha$ , GM-CSFR $\alpha$  or IL-5R $\alpha$  chain cDNA. After 48h the cells were either not treated or treated with IL-3, GM-CSF or IL-5, lysed and immunoprecipitated with MAb 8E4 anti- $\beta_c$ . The immunoprecipitates were separated on SDS-PAGE gels under reducing conditions and western blotted with anti phosphotyrosine antibody. Mutants C100A and C234A and wt  $\beta_c$ , which heterodimerize with the receptor  $\alpha$  chain in a disulfide-linked manner in response to ligand, showed phosphorylation of  $\beta_c$ . In contrast, mutants C86A, C91A and C96A which have lost the ability to heterodimerize in a disulfide-linked manner in response to ligand have lost the potential to be phosphorylated in response to IL-3, GM-CSF or IL-5 (Fig. 6.6).

The expression of all mutants compared with wt was monitored by flow cytometry and by western blot analysis with antibodies to  $\beta_c$  and indicated that the levels were very similar between all the mutants.(Fig. 6.3). Since the number of high affinity sites for mutants C86A and C91A was decreased, the lack of phosphorylation with these two mutants could have been the result of a decrease in sensitivity due to less mutant  $\beta_c$  being heterodimerized compared with the total amount of mutant  $\beta_c$  expressed. To address this possibility the receptor  $\beta_c$  only heterodimerized to the IL-

3R  $\alpha$  chain by immunoprecipitating with IL-3R  $\alpha$  chain antibody and western blotting with anti phosphotyrosine antibody. The results were identical to those seen when the  $\beta_c$  was directly immunoprecipitated, indicating that cysteines in position 86 and 91 are essential for receptor tyrosine phosphorylation (Fig. 6.7)

**Table 6.1 Effect of alanine substitutions of Cys 86, 91, 96, 100 and 234 of  $\beta_c$  on IL-3 and GM-CSF high affinity binding**

		IL-3		GM-CSF	
		Kd1 (pM)	Number/cell	Kd1	Number/cell
Wt $\beta_c$	Exp 1	330 $\pm$ 25.3	7230 $\pm$ 409	119 $\pm$ 44.5	4700 $\pm$ 1570
	Exp2	261 $\pm$ 29.6	7110 $\pm$ 662	40.9 $\pm$ 6.0	2770 $\pm$ 265
C86A	Exp 1	41.9 $\pm$ 15.5	289 $\pm$ 60	63 $\pm$ 18	723 $\pm$ 169
	Exp 2	45.6 $\pm$ 3.5	1325 $\pm$ 71	28.5 $\pm$ 11.0	1108 $\pm$ 313
C91A	Exp 1	31.0 $\pm$ 8.9	530 $\pm$ 79	27.4 $\pm$ 3.9	421 $\pm$ 42
	Exp 2	35.8 $\pm$ 5.1	1040 $\pm$ 94	44.6 $\pm$ 123.1	1340 $\pm$ 1610
C96A	Exp 1	-	0	-	0
	Exp 2	-	0	-	0
C100A	Exp 1	277 $\pm$ 15.7	7830 $\pm$ 241	230 $\pm$ 43.3	8790 $\pm$ 1450
	Exp 2	270 $\pm$ 36.0	13500 $\pm$ 1337	25.6 $\pm$ 5.4	3310 $\pm$ 374
C234A	Exp 1	279.2 $\pm$ 22.0	10100 $\pm$ 566	164 $\pm$ 30.5	9270 $\pm$ 1450
	Exp 2	193 $\pm$ 19.3	11800 $\pm$ 710	90 $\pm$ 8.1	2650 $\pm$ 156

COS cells were transfected with IL-3R $\alpha$ , GM-CSFR $\alpha$  and either wild type  $\beta_c$  or mutated  $\beta_c$  carrying alanine substitutions of Cys at positions 86, 91, 96, 100 and 234 and subjected to saturation binding studies with  $^{125}$ I-IL-3 and  $^{125}$ I-GM-CSF. The radioiodinated ligand concentration for both IL-3 and GM-CSF ranged from 10 pM to 10  $\mu$ M. Non specific binding was determined in the presence of 1  $\mu$ M unlabelled ligand. Scatchard transformation of the saturation binding curves were performed and the Kd and receptor numbers determined using the LIGAND program. In the case of IL-3 due to the extreme low affinity of IL-3R $\alpha$ , the affinity was estimated to be 50 nM based on previous studies (Vadas et al. 1979). In the case of GM-CSF binding a two site fit was statistically preferred ( $p < 0.05$ ) with all the  $\beta_c$  constructs except for C96A in which no high affinity sites were detected. Values from two representative experiments are shown. Experiment 2 is the same as the experiment shown in Fig 6.4.

### 6.3 DISCUSSION

In this chapter it has been shown that disulphide-linked heterodimerization of the GM-CSF, IL-3 and IL-5 receptors is essential for receptor activation by the cognate ligand. Furthermore it has been identified that Cys 86 and Cys 91 in the N-terminal domain of  $\beta_c$  are the key Cys involved in heterodimerization with the  $\alpha$  chain of each receptor. These Cys constitute a conserved motif present only in human  $\beta_c$  and mouse  $\beta_c$  and  $\beta_{IL-3}$  suggesting that it subserves a specialized function restricted to the GM-CSF, IL-3 and IL-5 receptor family.

It has been previously shown that the human IL-3 and GM-CSF receptors undergo both non covalent and disulfide-linked dimerization upon ligand binding (Bagley et al.1997). These observations now been extended to the IL-5 receptor demonstrating that disulfide-linked dimerization is a common theme in this receptor subfamily. To identify the Cys residues in  $\beta_c$  responsible for disulfide linkage with the IL-3, GM-CSF and IL-5 receptor  $\alpha$  chains mutagenesis of five of the eight Cys residues in the N-terminal CRM of  $\beta_c$  which, from alignment with other cytokine receptors, included the best candidates for intermolecular interactions. Results indicated a range of sensitivities to mutation of these Cys residues that could be correlated with their interspecies and interreceptor conservation.

The first class of Cys residue is exemplified by Cys 100 and Cys 234. These residues are not conserved with even the closely-related mouse  $\beta$ -chains and results indicated no phenotype on replacing them with alanine residues. The second class is represented by Cys 96 which is apparently a conserved residue in both the mouse  $\beta$ -

chains and the cytokine receptor family at large (Bazan,1990b) and is inferred to be involved in a structurally-conserved disulfide bond. This residue is apparently required for the structural integrity of the first domain of h $\beta_c$  if not the entire extracellular portion of the molecule. Although the C96A mutation permitted cell-surface expression of h $\beta_c$ , it did not support high affinity binding of GM-CSF or IL-3 despite the substitution being well removed in sequence and presumably spatially distant, in sequence from the fourth domain of the receptor that encompasses the majority of the ligand-recognition determinants (Woodcock et al.1994; Woodcock et al.1996). The exact molecular basis for this observation is uncertain but may be related to sequestration of h $\beta_c$  into very large aggregates (Fig 6.2A) that obscure the ligand-contact site.

The third and most interesting class of cysteine mutation is that of C86A and C91A, analogues that have lost their ability to form disulfide-linked heterodimers but still retain the ability to associate noncovalently with the  $\alpha$  subunit upon stimulation with ligand (Fig 6.2). Although these mutants exhibited some propensity to aggregate in the absence of stimulation, unlike C96A, they retain the ability to interact with ligand as judged by their ability to support high affinity binding. Importantly, these analogues are deficient in receptor activation as measured by phosphorylation of tyrosine residues in the cytoplasmic tail of the receptor.

The observation of identical phenotypes with either mutation C86A or C91A suggests that these residues may cooperate functionally in the native receptor such as via formation of an additional intramolecular disulfide. This is consistent with the

molecular modelling of  $\beta_c$  which suggests that Cys 86 and Cys 91 are sufficiently close to allow the formation of a strained disulfide bond. In the presence of ligand this strained disulfide may undergo disulfide exchange with a free sulfhydryl group from the  $\alpha$ -chain that is brought into proximity via ligand-dependent noncovalent association. In the absence of their normal partners, these residues may become prone to adventitious disulfide formation leading to the observed formation of aggregates. This is further borne out by the binding data which shows an appreciable reduction in number of high affinity sites with Cys mutants C86A and C91A despite good surface expression. Similar disulfide mediated aggregation has been observed with artificially truncated soluble forms of human  $\beta_c$  (Woodcock et al.1997) and with soluble forms of mouse  $\beta_{IL3}$  (Cunningham et al.1990).

Previous experiments have noted a correlation between disulfide-linked receptor dimerization and receptor activation. IL-6 induces covalent dimerization of two molecules of gp130 (Murakami et al.1993), and CNTF induces covalent dimerization of gp130 with the LIF receptor (Davis et al.1993). Similarly, IL-3, GM-CSF, and IL-5 induce covalent dimerization of  $\beta_c$  with the corresponding  $\alpha$  chain. In all these cases concomitant phosphorylation of the receptor has been observed, however, a causal relationship has not been established. The use of the C86A and C91A mutants gave the opportunity to demonstrate that non-covalent receptor associations are not sufficient for receptor activation and that this requires disulfide-linkage of receptor subunits. Results in this chapter illustrate the need of disulfide linkage for ligand-dependent receptor activation and identifies the responsible Cys residues. The role of covalent dimerization seems to be to ensure concomitant dimerization of the

cytoplasmic portions of  $\beta_c$  facilitating transphosphorylation of associated kinases of the JAK family. Indeed, experiments using chimeric receptors that cause artificial dimerization of the cytoplasmic portions of  $\beta_c$  lead to their activation (Muto et al.1995; Shikama et al.1996) as do chimeras that induce direct dimerization of JAK (Nakamura et al.1996). Although they are essential for normal activation (Takaki et al.1994; Weiss et al.1993; Rapoport et al.1996), the role of the cytoplasmic domains of the receptor  $\alpha$ -chains remains unclear although they may serve to orientate to  $\beta$ -chains so as to juxtapose correctly the JAK molecules or to participate directly in certain functions (Spielholz et al.1995; Patel et al.1996).

Cys 86 and Cys 91 in the  $\beta_c$  of the IL-3, GM-CSF, IL-5 receptor represent a functional motif that appears to be restricted to this receptor family. The two crucial Cys residues are found in human and mouse  $\beta$ -chains but do not align with Cys residues in other receptors. In gp130, which contains 13 Cys in its extracellular domain, no good Cys candidates are found in the equivalent region. In other receptors such as IL-2R $\beta$  and the second CRM of the thrombopoietin receptor (TPOR) extra pairs of Cys residues in both the human and mouse proteins can be found, however, they may be involved in supernumerary intramolecular disulfides.

The  $\beta$ -chain forms intermolecular disulfides specifically with the  $\alpha$ -chains of the GM-CSF, IL-3 or IL-5 receptors. A common feature of these  $\alpha$  chains is the presence of an N-terminal FnIII-like domains of a type restricted to this subfamily of cytokine receptors. In the case of IL-5 receptor  $\alpha$  chain there is only one unpaired cysteine residue and this is in the N-terminal domain. Similarly GM-CSFR and IL-

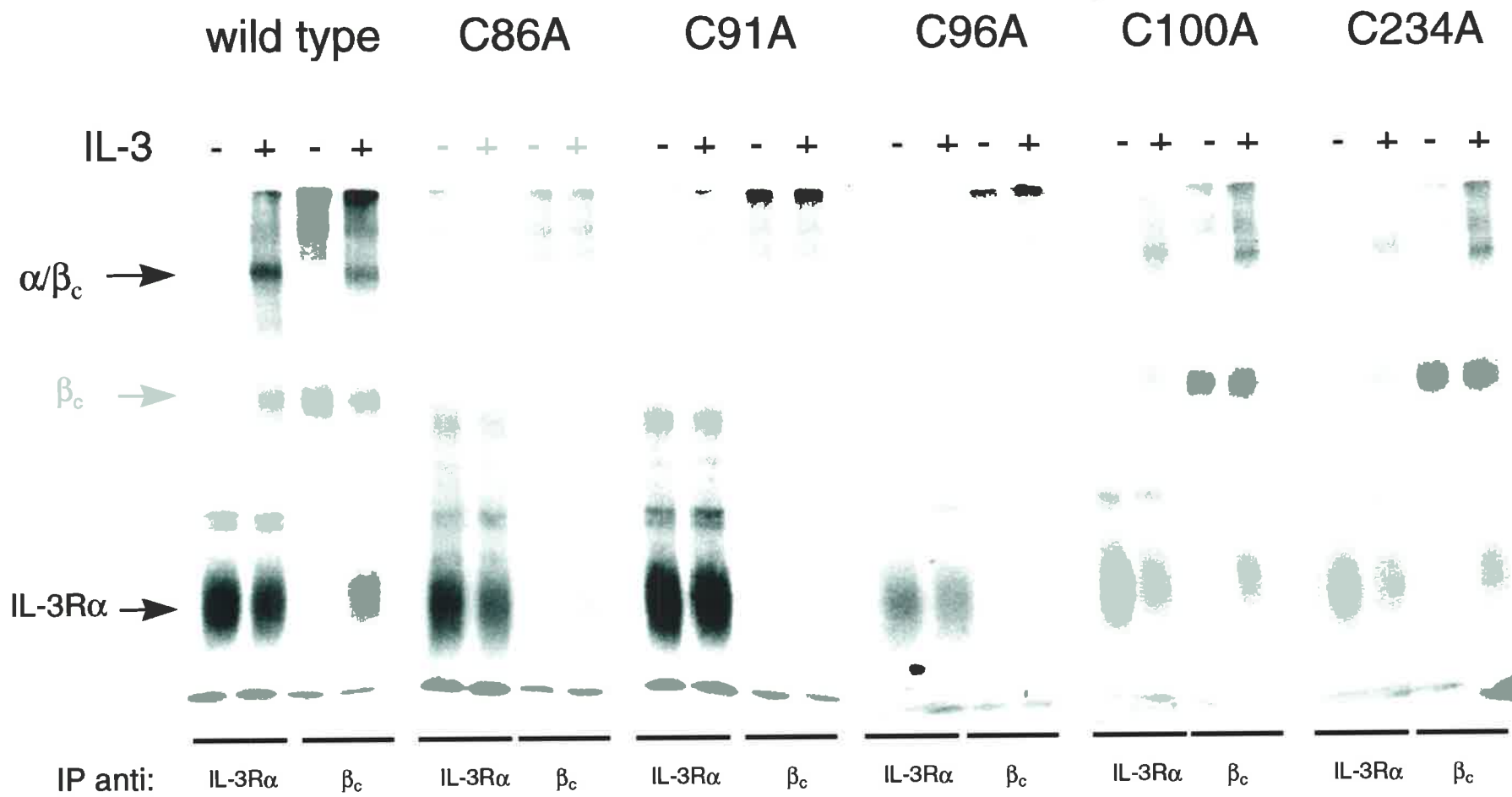
3R  $\alpha$  chains have unpaired cysteine residues in their N-terminal domains. The CRM domains of IL-5R and GM-CSFR  $\alpha$  chains do not contain any unpaired cysteine residues. Only in the case of IL-3R  $\alpha$  chain there an unpaired cysteine present in the CRM region. Analysis of the cysteine residues in the IL-5R  $\alpha$  chain has shown that mutation of either Cys 249 or Cys 296 in domain 2 of the CRM results in the complete loss of binding to IL-5 (Devos et al.1994). These residues are apparently required for structural integrity of the CRM region, therefore are partners in a intramolecular disulfide bond. Since all three receptor  $\alpha$  chains possess an uneven number of Cys residues in the N-terminal domain, the Cys residues in this domain are the most likely candidates to act as partners for  $\beta_c$ .

**Figure 6.1 Alignment of domain 1 of the cytokine receptor module (CRM) present in the common  $\beta$  chain of the GM-CSF, IL-3 and IL-5 receptors and other signalling subunits of the cytokine receptor superfamily.** Four conserved Cys form the basis of the alignment, with the second Cys followed by a conserved Trp, and the fourth Cys followed by a hydrophobic residue at the  $i+2$  position. The sequences of human as well as mouse  $\beta_c$  and  $\beta_{IL-3}$  are shown with the numbering corresponding to the human sequence with residue 1 being the initiation Met. Human and mouse  $\beta$  subunits are aligned with human gp130, the IL-2 receptor (R)  $\beta$  and  $\gamma$  chains, the erythropoietin (EPO)R, and the growth hormone (GH)R. The dashes represent spaces introduced to optimize the alignment.

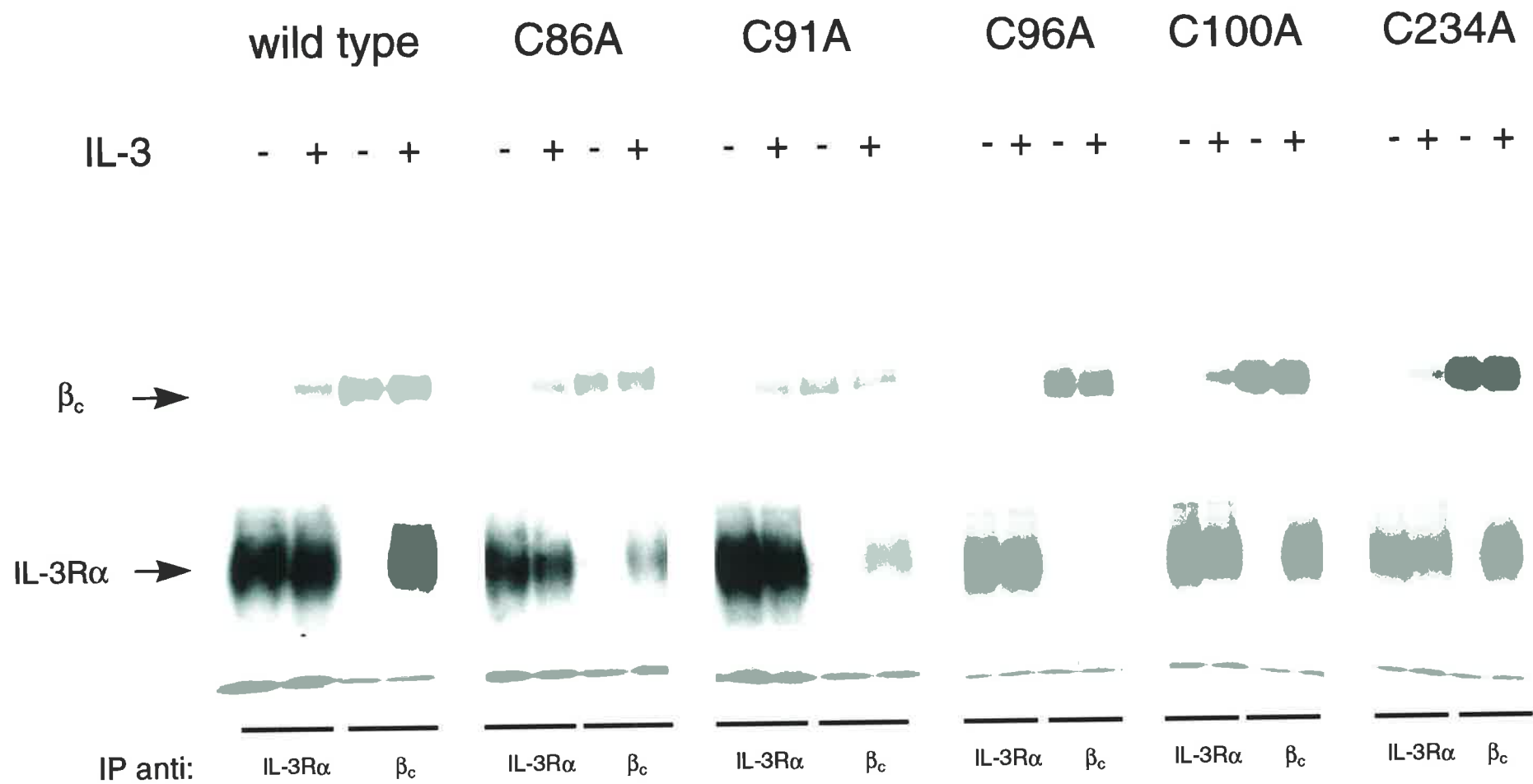
## CRM domain1

		86	91	96	100
h $\beta$ c CRM1	T I P L Q T L R C Y N D Y T S H I T C R W A D T Q D A Q R L - - V N V T L I R R V N E D - - - L L E P V S C D L S D D M P W S A C P - H P R C V P R R C V I P - - - - - - - - - - C Q S F V V T D V D Y F S F Q P D R P L G T R L T V T L T Q H V -				
m $\beta$ c CRM1	T V P L K T L C C Y N D Y T N H I I C S W A D T E D A Q G L - - I N M T L - Y H Q L E K - - - K Q P V S C E L S E K L M W S E C P S S H R C V P R R C V I P - - - - - - - - - - Y T R F S I T N E D Y Y S F R P D S D L G I Q L M V P L A Q N V -				
m $\beta$ IL-3 CRM1	T V P L K T L E C Y N D Y T N R I I C S W A D T E D A Q G L - - I N M T L L Y H Q L D K - - - I Q S V S C E L S E K L M W S E C P S S H R C V P R R C V I P - - - - - - - - - - Y T R F S N G D N D Y Y S F O P D R D L G I Q L M V P L A Q H V -				
h gp130	P E K P K N L S C I V N E G K K M R C E W D G G R E T H L E T N F T L K S E W A T - - - - - H K F A D C K A - - - - - K R D T P T S C T V D Y - - - - - S T V Y F V N I E V W V E A E N A L G K V T S D H I N F D P V Y K V K -				
h IL-2R $\beta$	V N G T S Q F T C F Y N S R A N I S C V W S Q D G A - - - - - L Q D T S C Q V H A W P D - - - - - R R R W N Q T C E L L P V S Q A S W A C N L I L G A P D S Q K L T T V - - - D I V T L R V L C R E G V R W R V				
h IL-2R $\gamma$	T L P L P E V C C F V F N V E Y M N C T W N S S S E P Q P T N L - T L H Y W Y K N S D N - - - D K V Q K C S H Y L F S - - - - - E E I T S G C Q L Q K K E I - - H L Y Q T F V V Q L Q D P R E P R R Q A T Q M L K L Q N L V I P W A P E N L				
h EPOR	A R G P E E L L C F T E R L E D L V C F W E E A A S A G V G P - G N Y S F S Y Q L E D - - - - - E P W K L C R L H Q A P T - - - - - A R G A V R F W C S L P T A D T - - S S F V P L E L R V T A A S - - - - - G A P R Y H R V I H I N E V V - - -				
h GHR	S K E P K F T K C R S P E R E T F S C H W T D E V H H G T K N L G P I Q L F Y T R R N T Q E W T Q E W K E C P D Y V - - - - - S A G E N S C Y F N S S F T - - S I W I P Y C I K L T S N G - - - - - G T V D E K C F S V D E I V - - -				

**Figure 6.2A Substitutions of Cys 86 and Cys 91 in  $\beta_c$  abolish disulfide-linked IL-3R $\alpha$  and  $\beta_c$  heterodimerization without affecting their noncovalent association.** COS cells transfected with IL-3R $\alpha$  and either wild type or mutant  $\beta_c$  C86A, C91A, C96A, C100A and C234A were  $^{125}$ I-surface labelled and incubated in medium without (-IL-3) or with 6.5 nM IL-3 (+IL-3) for 5 min at 4°C. After cell lysis, proteins were immunoprecipitated with MAb 9F5 (anti IL-3R $\alpha$ ) or MAb 8E4 (anti  $\beta_c$ ). The immunoprecipitated proteins were separated under non reducing conditions on an SDS-6% polyacrylamide gel and visualised by PhosphorImaging.

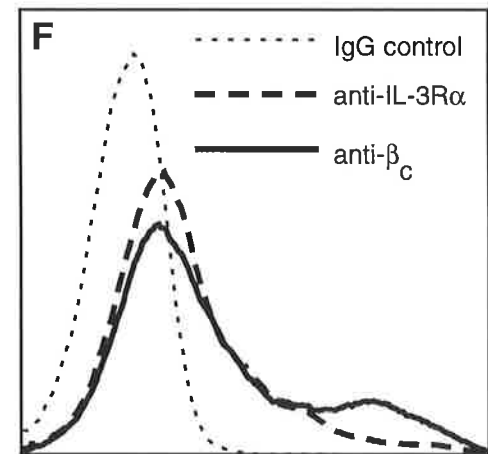
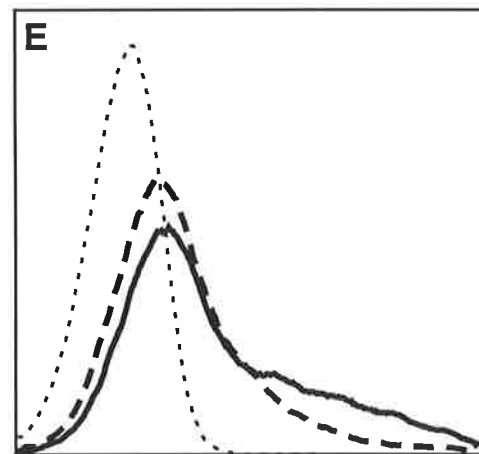
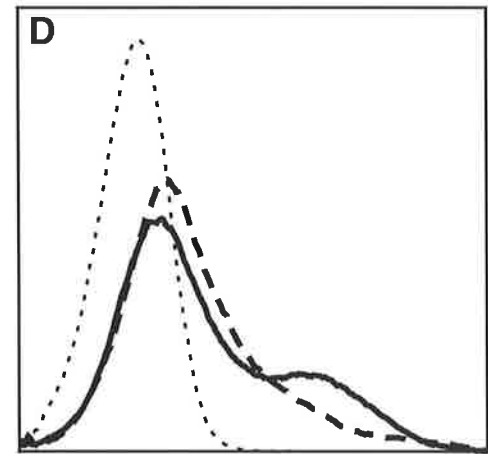
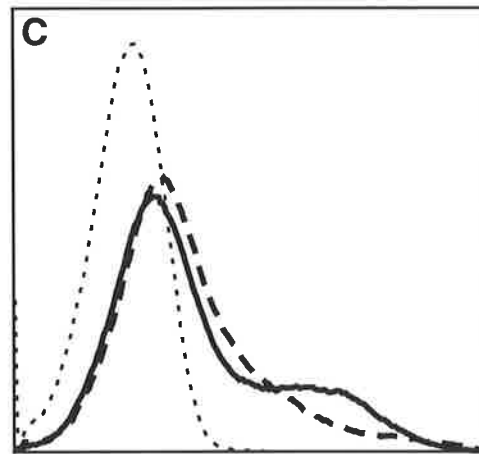
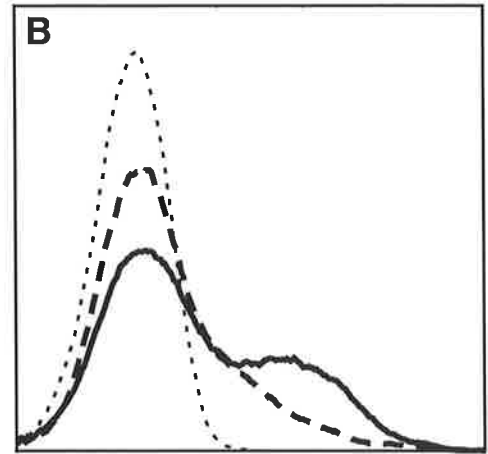
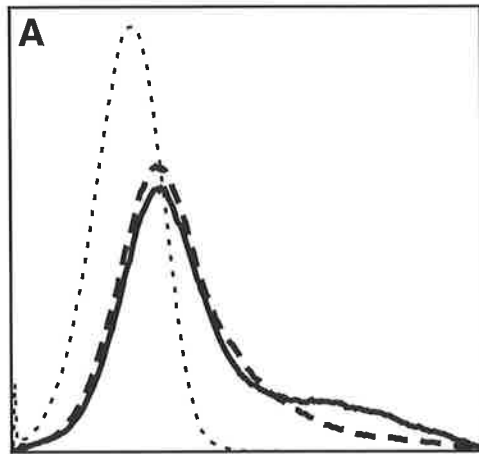


**Figure 6.2B Substitutions of Cys 86 and Cys 91 in  $\beta_c$  abolish disulfide-linked IL-3R $\alpha$  and  $\beta_c$  heterodimerization without affecting their noncovalent association.** COS cells transfected with IL-3R $\alpha$  and either wild type or mutant  $\beta_c$  C86A, C91A, C96A, C100A and C234A were  $^{125}$ I-surface labelled and incubated in medium without (-IL-3) or with 6.5 nM IL-3 (+IL-3) for 5 min at 4°C. After cell lysis, proteins were immunoprecipitated with MAb 9F5 (anti IL-3R $\alpha$ ) or MAb 8E4 (anti  $\beta_c$ ). The immunoprecipitated proteins were separated under reducing conditions on an SDS-7.5% polyacrylamide gel and visualised by PhosphorImaging.



**Figure 6.3** Surface expression of IL-3R $\alpha$ , GM-CSFR $\alpha$  and wild type or mutant  $\beta_c$  transfected into COS cells as measured by flow cytometry. COS cells expressing both IL-3R $\alpha$  and GM-CSFR $\alpha$  chains together with wild type or mutant  $\beta_c$  C86A, C91A, C96A, C100A and C234A were stained with negative control antibody mouse IgG1 (control), MAb 9F5 (anti IL-3R $\alpha$ ), MAb 4H1 (anti GM-CSFR $\alpha$ ) and MAb 8E4 (anti- $\beta_c$ ).

number of cells



relative fluorescence intensity

**Figure 6.4** High affinity  $^{125}\text{I}$ -IL-3 and  $^{125}\text{I}$ -GM-CSF binding using Cys→Ala  $\beta_c$  mutants. COS cells expressing both IL-3R $\alpha$  and GM-CSFR $\alpha$  chains together with wild type  $\beta_c$  (open circles) or  $\beta_c$  mutants (filled circles) containing different Cys→Ala substitutions were subjected to Scatchard transformation of saturation binding curves. The derived values are shown in Table 6.1.

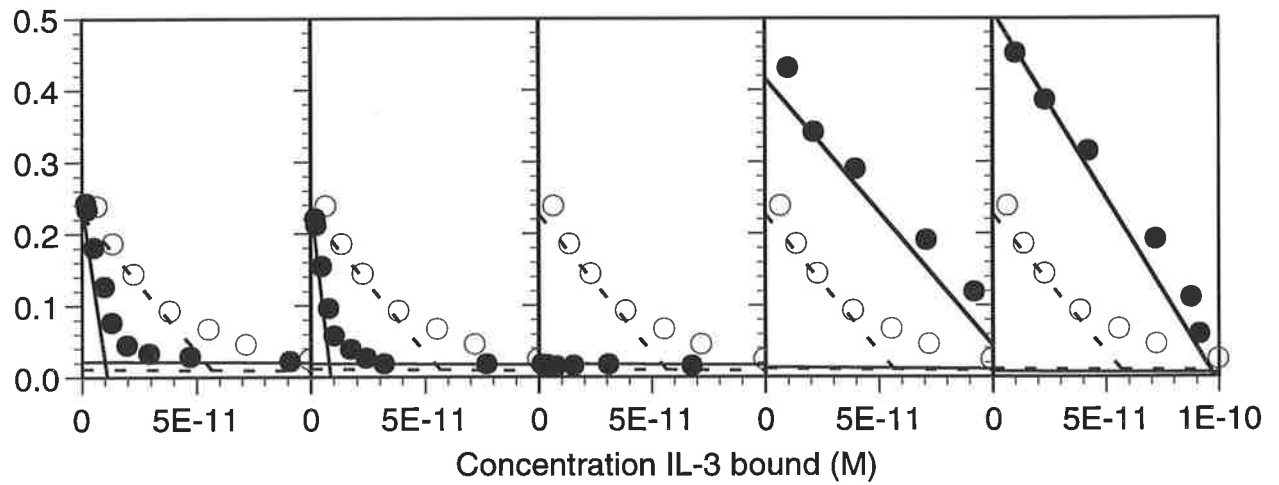
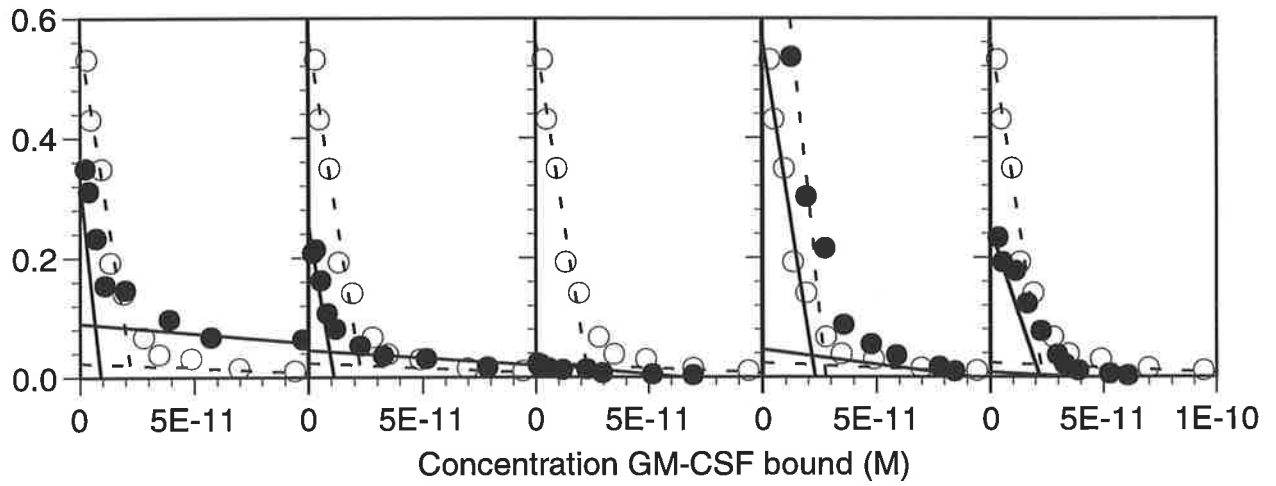
**C86A**

**C91A**

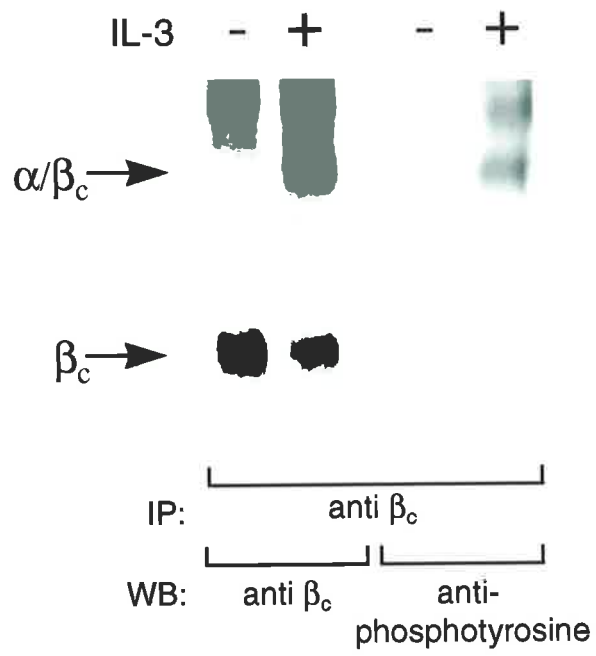
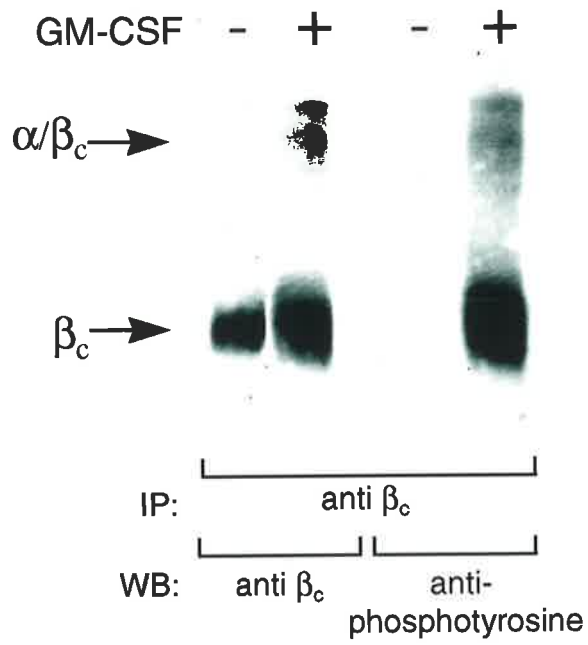
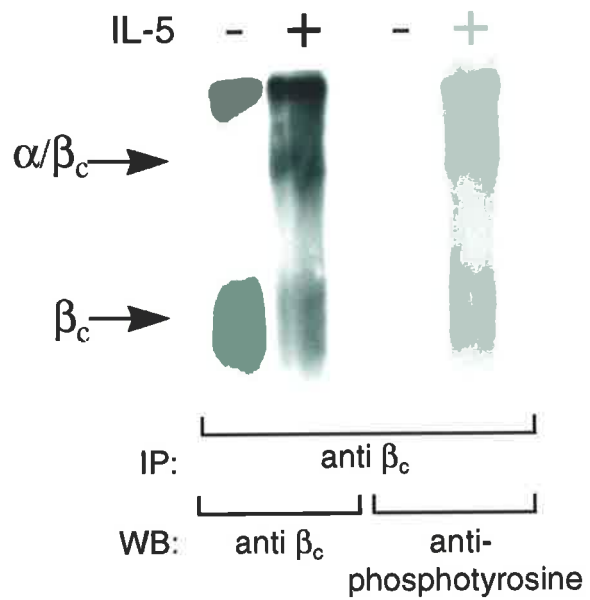
**C96A**

**C100A**

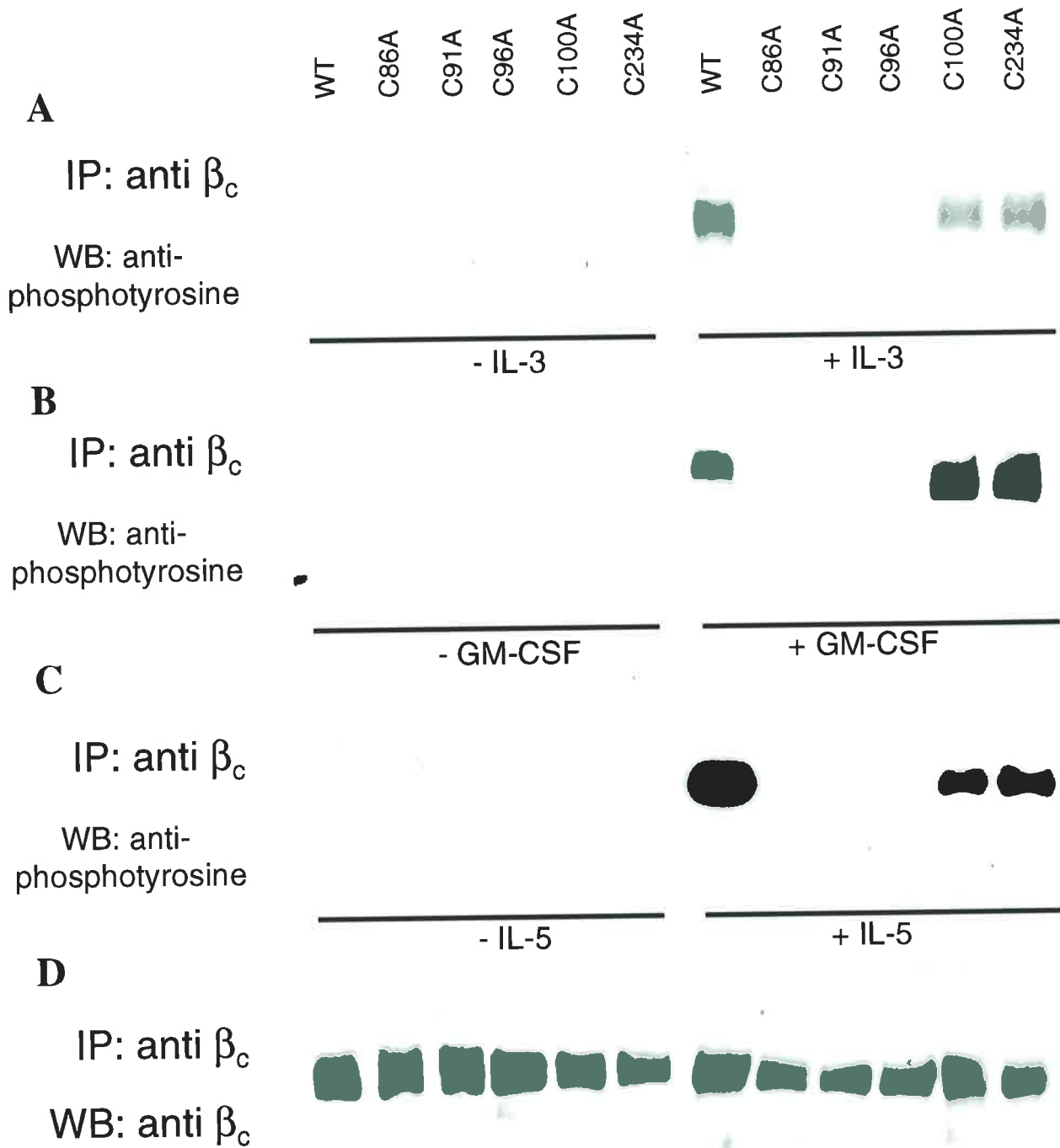
**C234A**



**Figure 6.5 Ligand-induced disulfide-linked IL-3, GM-CSF and IL-5 receptor dimerization results in phosphorylation of  $\beta_c$ .** TF1.8 cells were either incubated with medium alone (-) or stimulated with medium containing 6.5 nM IL-3 (A), 6.5 nM GM-CSF (B) or 6.5 nM IL-5 (C) for 5 minutes at 37°C. After cell lysis, proteins were immunoprecipitated with anti- $\beta_c$  MAb 8E4 and the immunoprecipitates were separated under non reducing conditions on an SDS-7.5% polyacrylamide gel and transferred onto nitrocellulose filters. The filters were then probed either with MAb 1C1 anti- $\beta_c$ , or anti-phosphotyrosine antibody 3-365-10.

**A****B****C**

**Figure 6.6 Alanine substitutions Cys 91, Cys 96 and Cys 100 of  $\beta_c$  abolish IL-3, GM-CSF and IL-5 induced tyrosine phosphorylation of  $\beta_c$ .** HEK293T cells transfected with either IL-3R $\alpha$  (A), GM-CSFR $\alpha$  (B), or IL-5R $\alpha$  (C) together with wild type or mutant  $\beta_c$  were incubated with either medium alone (-) or with medium containing 6.5 nM IL-3 (+IL-3), 6.5 nM GM-CSF (+GM-CSF) or 6.5 nM IL-5 (+IL-5) for 5 min at 4°C. After cell lysis, proteins were immunoprecipitated with anti  $\beta_c$  MAb 8E4 and the immunoprecipitates were separated under reducing conditions on an SDS-7.5% polyacrylamide gel transferred to nitrocellulose and probed with anti-phosphotyrosine antibody 3-365-10 (A, B and C). To control for the amount of  $\beta_c$  present in the filters were also probed with anti  $\beta_c$ , MAb and 1C1 (D).



**Figure 6.7** Ligand-induced heterodimers of IL-3R $\alpha$  and  $\beta_c$  with C86A and C91A substitution lack tyrosine phosphorylation of  $\beta_c$ . HEK293T cells transfected with IL-3R $\alpha$  together with either wild type or mutant  $\beta_c$  were incubated with medium alone (-) or with medium containing 6.5 nM IL-3 (+IL-3) for 5 min at 4°C. After cell lysis, proteins were immunoprecipitated with MAb 9F5 (IL-3R $\alpha$ ) and the immunoprecipitates were separated under reducing conditions on a SDS-7.5% polyacrylamide gel, transferred onto nitrocellulose and probed with anti phosphotyrosine antibody 3-365-10 (A). To control for similar amounts of immunoprecipitated  $\beta_c$  the filters were also probed with MAb 1C1 anti  $\beta_c$  (B).



## *Chapter VII*

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### **GENERAL DISCUSSION**

## 7.1 DISCUSSION

In this thesis it has been shown that human GM-CSF, IL-3 and IL-5 binding to GM-CSFR $\alpha$ , IL-3R $\alpha$  and IL-5R $\alpha$  respectively triggers the heterodimerization of its specific receptor binding  $\alpha$  subunit with  $\beta_c$  leading to receptor activation. The existence of a GMR $\alpha$ : $\beta_c$  complex that is formed in the absence of GM-CSF has also been demonstrated. Ligand-induced receptor heterodimerization is dependent on GM-CSF, IL-3 or IL-5 contacting both receptor subunits. Both disulphide-linked as well as non disulphide-linked heterodimers were observed and, although ligand is necessary for their formation, ligand is not covalently attached to the dimers. Importantly, the disulphide-linked and not the noncovalently-linked heterodimer is shown to be required for receptor activation but not high affinity binding. These results are different from those in the IL-6, EPO and G-CSF receptor systems where receptor activation involves homodimerization of a single signalling subunit.

Earlier experiments have shown that GM-CSF, IL-3 and IL-5 receptor activation leads to stimulation of JAK-2 (Quelle et al.1994; Silvennoinen et al.1993) and Lyn (Torigoe et al.1992) kinases, as well as the Ras-MAP kinase, PI3 kinase and PKC pathways (Sato et al.1994). Receptor dimerization has been demonstrated in tyrosine kinase receptors (Heldin,1995; Ullrich et al.1990), as well as the G-CSF receptor (Hiraoka et al.1994), EPO receptor (Miura et al.1993b), IL-6 receptor (Murakami et al.1993) and CNTF receptor (Davis et al.1993). Dimerization of the GM-CSF, IL-3 and IL-5 receptors has been proposed in ligand-induced as well as in ligand-independent receptor activation (D'Andrea et al.1994; Jenkins et al.1995). The results presented in this thesis illustrate both covalent and noncovalent dimerization

of the receptor, that it involves the ligand-binding subunit dimerizing with  $\beta_c$ , and that covalent-linkage of dimers is required for signalling. These results stress the importance of the  $\alpha$  chain not only as a ligand-binding subunit but also as a subunit required for signalling in cells. This is in concordance with experiments where deletion of the cytoplasmic domain of  $\alpha$  chains annuls signalling (Sakamaki K et al.1992; Weiss et al.1993). Other experiments have shown, however, that the cytoplasmic domain of  $\beta_c$  can substitute for the intracytoplasmic domain of the  $\alpha$  chain (Eder et al.1994; Muto et al.1995; Takaki et al.1994). The exact importance of these findings is not clear but may relate to the conserved nature of the proline-rich sequence present in the membrane proximal domain of both the  $\alpha$  chain and  $\beta_c$ , or that at least two molecules of  $\beta_c$  are required for signalling with the role of the  $\alpha$  chain being to recruit or facilitate the association of two molecules of  $\beta_c$ . The experiments demonstrated the presence of both  $\alpha$  and  $\beta_c$  in the high order complexes, suggesting that heterodimers containing  $\alpha$  chain and  $\beta_c$  are necessary for signalling.

The presence of  $\alpha$  subunits in the disulphide-linked receptor dimers differs from the disulphide-linked dimerization of the IL-6 and CNTF receptors which involve only the signal transducer subunits. Therefore, in the case of the IL-6 receptor, disulphide-linked dimerization involves only gp130 (Murakami et al.1993), whilst in the case of the CNTF receptor gp130 and LIFR $\beta$  form disulphide-linked dimers (Davis et al.1993) The outcome of this dimerization is to initiate signal transduction. This suggests that there is a basic difference in the functional contribution of GM-CSFR $\alpha$ , IL-3R $\alpha$  and IL-5R $\alpha$  compared with IL-6R $\alpha$  and CNTFR $\alpha$  in receptor

activation, and implies that  $\alpha$  subunits are not only involved in the initial binding of ligand but also participate in signalling. The findings are agreeable with differences in the need for the cytoplasmic domain of the  $\alpha$  chains for signalling. Thus, the cytoplasmic domain of the  $\alpha$  chains of the IL-3, GM-CSF and IL-5 sub-family of receptors is essential for signal transduction (Polotskaya et al.1993; Sakamaki K et al.1992) whilst, the cytoplasmic portion of IL-6R $\alpha$  is not (Yawata et al.1993).

Interestingly the existence of a GMR $\alpha$ : $\beta_C$  complex that is formed in the absence of GM-CSF has been demonstrated. It has been observed that this ligand-independent association between GMR $\alpha$  and  $\beta_C$  occurs in several cell lines and in all cases the preformed complex is not activated in the absence of ligand. The abundance of preformed GMR $\alpha$ : $\beta_C$  complexes observed on cells varied from cell to cell. In certain cases all of the GMR $\alpha$  and  $\beta_C$  chains were co-associated and no further association was induced by GM-CSF treatment, whereas on other cells only a fraction of GMR $\alpha$ s and  $\beta_C$ s were pre-associated and further association was induced by GM-CSF treatment. From these results it can be proposed that two pools of GM-CSF receptors exist: preformed complexes and ligand-induced complexes.

These findings may explain previous apparently contradictory data. One group has previously demonstrated that GM-CSF induces GMR $\alpha$  and  $\beta_C$  association (Eder et al.1994) and the second that that the GM-CSF receptor may exist as a preformed complex (Ronco et al.1994). This was proposed to be due to the inability of a mutant GMR $\alpha$  to bind GM-CSF unless it was co-expressed with  $\beta_C$ . From this observation it was interpreted that  $\beta_C$  pre-associated with GMR $\alpha$  compensated for

the loss of GM-CSF binding on the mutant GMR $\alpha$ . Similarly GM-CSF helix D mutants showed no detectable binding to GMR $\alpha$  alone, however it was able to bind cells expressing both GMR $\alpha$  and  $\beta_C$  possibly reflecting the effect of a GMR $\alpha$ : $\beta_C$  preformed complex (Hercus et al.1994b). The findings presented in chapter 5 in this thesis show for the first time biochemical evidence of the existence of a preformed GMR complex and shows that  $\alpha$ - $\beta$  association is mediated by the extracellular regions.

The exact regions in the extracellular domains of GMR $\alpha$  and  $\beta_C$  that mediate spontaneous association are not known. From modelling studies and relating to the growth hormone crystal structure (De Vos et al.1992) the A-B loop and the E strand in the fourth domain of  $\beta_C$  appear to be worthy possibilities for interaction with the second domain of the cytokine receptor module of GMR $\alpha$ . Insertions, deletions and point mutations in this domain of  $\beta_C$  lead to factor independent activation (Gonda et al.1997). It would be important to perform alanine scanning mutagenesis in these loops to define the residues involved in  $\alpha$ - $\beta$  preassociations.

This observation demonstrates for the first time that the GM-CSFR is unlike the other cytokine receptors that require ligand to induce receptor subunit association. The presence of a preformed GM-CSFR complex may explain the different ligand binding kinetics . As GM-CSF binding to monocytes and eosinophils is much more rapid than IL-3 and IL-5 (chapter 5) this may be due to the difference between a preformed “ready to go” receptor complex, compared to a ligand-induced complex. The exact significance of this unique receptor arrangement is unclear but it may

explain the transphosphorylation of the GM-CSF receptor observed when cells are stimulated with IL-3 or IL-5. This is similar to the trans-down modulation experiments in the mouse where IL-3 was found to trans-down modulate GM-CSF, M-CSF and G-CSF receptors but GM-CSF or G-CSF were unable to trans-down modulate the mouse IL-3 receptor (Walker et al.1985; Nicola,1987). The transphosphorylation phenomena of GMR $\alpha$  associated  $\beta_c$  which was observed in these studies appears to be specific to the GM-CSF/IL-3/IL-5 receptor system. The expression of the GM-CSF receptors has been shown on many cells of the haemopoietic lineage and it is fascinating that most cells that express either IL-3 or IL-5 receptors also express GM-CSF receptors. Since IL-3 and IL-5 are able to activate preformed GM-CSF receptors, this leads to the speculation that the biological functions of IL-3 and IL-5 are partially governed by signalling through the GM-CSF receptor. This suggests that the GM-CSF preformed complex may act to potentiate the effects of IL-3 and IL-5 or indeed mediate same specific functions.

In this receptor sub family for signalling to occur, the need for ligand to contact both  $\alpha$  and  $\beta$  chains appears to be essential . A number of ligand mutants have been developed through mutational analysis that alter the  $\beta_c$  binding sites. These molecules are either devoid of agonist activity or can act as agonists with significantly reduced activity. The need for ligand contact to both subunits for receptor activation has also been addressed through the use of MAbs that compete for ligand binding sites. An IL-3 analogue IL-3 E22R has markedly reduced ability to induce proliferation, a 20,000 fold decrease in potency (Barry et al.1994). This mutant has the Glu<sup>22</sup> residue in helix A of IL-3 substituted with an Arg residue

(Barry et al.1994). IL-3 E22R is still a weak agonist and ligand induced subunit dimerization is just detectable at a concentration of 4ug/ml which is a vast quantity of mutant to show minimal dimerization (chapter 3). The decrease in bindability of IL-3 E22R directly corresponds to the decrease in proliferation and in turn ability to form  $\alpha\beta$  dimers. This argument is further supported by the use of blocking MAbs 7G3 and QP1. MAb 7G3 is an anti-IL-3 receptor  $\alpha$  chain antibody capable of blocking IL-3 binding to IL-3R $\alpha$  and MAb QP-1 is an antibody raised to the  $\beta_c$  that blocks IL-3, IL-5 and GM-CSF binding to  $\beta_c$  (chapter 3 (Sun et al.1996; Sun et al. manuscript in preparation)). Both of these antibodies are capable of blocking  $\alpha\beta$  dimerization of receptors and this in turn blocks phosphorylation of  $\beta_c$ , and cellular proliferation (chapter 3 (Sun et al.1996; Sun et al. manuscript in preparation)).

A number of IL-5 analogues have been produced that act as antagonists. These analogues are directly analogous to the IL-3 E22R analogue with either a charge reversal substitution or a non charged residue at the conserved  $\beta_c$  interacting glutamate residue, Glu<sup>13</sup> in helix A of IL-5. The residues substituted for Glu<sup>13</sup> are Lys, Arg and Gln. All three mutants have been shown to lack the ability to induce proliferation in TF1.8 cells and in addition antagonize IL-5 proliferation response (chapter 4, (Tavernier et al.1995; McKinnon et al.1997)). I have shown that IL-5 E13R does not phosphorylate the  $\beta_c$  and is able to antagonise IL-5 inducible phosphorylation (chapter 4). The unique feature of E13Q and E13K mutants is that they are reported to bind with high affinity to the IL-5R and in addition E13K is able to support eosinophil survival but not proliferation. (Tavernier et al.1995; McKinnon et al.1997).

The third receptor mutant in this subfamily is the GM-CSF analogue GM-CSF E21R. This analogue is directly analogous to the IL-3 E22R analogue with a charge reversal substitution at the conserved  $\beta_c$  interacting glutamate residue, Glu<sup>21</sup> in helix A of GM-CSF (Hercus et al.1994a). This mutant is similar to the IL-5 mutants, in that is not an agonist and instead is able to antagonise GM-CSF induced proliferation in TF1.8 cells, (Hercus et al.1994a). The two differences are that GM-CSF is unable to bind with high affinity to GM-CSFR and the second one is that it actively induces apoptosis of several cell types (Iversen et al.1997b). The apoptotic activity of E21R is dependent on both GM-CSFR $\alpha$  and  $\beta_c$  expression (Iversen et al.1997a) which is interesting given that GM-CSF E21R interacts only with the GM-CSFR $\alpha$  chain. The existence of a preformed GM-CSF receptor complex may explain the molecular basis of the apoptotic activity of GM-CSF E21R. The GM-CSF E21R mutant does induce selective internalization of GM-CSFR $\alpha$  (Iversen, unpublished data) whereas wild type GM-CSF induces internalization of both GM-CSFR $\alpha$  and  $\beta_c$  (Iversen, unpublished data). Therefore GM-CSF E21R depletes the cell surface of GM-CSFR $\alpha$  chains which presumably disrupts the GM-CSF preformed complex. This implies that the preformed complex may have a role in cell survival and when disrupted by GM-CSF E21R causes the cells to apoptose.

A common theme for this receptor subfamily is developing, namely, that disulphide-linked receptor dimerisation is a prerequisite for receptor activation. The IL-5 analogue E13R must be competent at preventing disulphide-linked dimerization and therefore obstruct receptor signalling as measured by phosphorylation and

proliferation. The prevention of disulphide-linked hetero-dimerisation is the key to lack of activation, whereas a number of groups have exemplified through Scatchard analysis, that all of the IL-5 analogues tested are able bind to the receptors with high affinity (Tavernier et al.1995; McKinnon et al.1997). In addition, the IL-3R pretreated with thiol-specific alkylating reagents retain induction of high affinity sites, but the receptor could not be activated nor high order complexes could be induced.

The disulphide-linked high order complexes were seen in immunoprecipitations performed with both anti- $\alpha$ chain MAb and anti- $\beta_c$  MAb. Initially, these disulphide-linked dimers, disappeared in the presence of the thiol-specific alkylating agent iodoacetamide added before but not after incubation with ligand. By performing two dimensional non reducing-reducing SDS-PAGE the second dimension resolved the high order complexes into  $\alpha$  subunit and  $\beta_c$ . The presence of ligand-induced disulphide-linked and non disulphide-linked heterodimers suggests two types of  $\alpha$  subunit and  $\beta_c$  interaction, a non-covalent one and one that is mediated by Cys-Cys bridging of the receptors. It has been shown that by preventing disulphide-linked dimer formation it was possible to dissociate high affinity binding from receptor activation.

To determine which of the Cys residues in  $\beta_c$  was responsible for disulfide linkage with the GM-CSF, IL-3 and IL-5 receptor  $\alpha$  chains mutagenesis of five of the eight Cys residues in the N-terminal CRM of  $\beta_c$  were mutated. The five mutated were the best possibilities for intermolecular interactions. The findings from mutation of

these Cys residues show that the results relate to interspecies and inter-receptor conservation. The two notable mutations were Cys 86 and Cys 91 in the N-terminal domain of  $\beta_c$  as these are the key Cys residues involved in heterodimerization with the  $\alpha$  chain of each receptor. These Cys residues form a conserved motif which is unique to human  $\beta_c$  and mouse  $\beta_c$  and  $\beta_{IL-3}$  suggesting that it subserves a specialised function restricted to the GM-CSF, IL-3 and IL-5 receptor family.

Cysteine mutations of C86A and C91A results in analogues that lack the ability to form disulfide-linked heterodimers but are competent to associate noncovalently with the  $\alpha$  subunit upon stimulation with ligand. Although these mutants were inclined to homodimerize in the absence of stimulation, they have kept the ability to interact with ligand as judged by their ability to support high affinity binding. Importantly, these analogues are deficient in phosphorylation of tyrosine residues in  $\beta_c$ , however receptor-mediated functions and downstream signalling remains to be ascertained.

The phenotypes displayed by each of the mutations C86A or C91A suggests that these residues may functionally collaborate in the native receptor such as via formation of an additional intramolecular disulfide. This observation is compatible with molecular modelling of  $\beta_c$  which suggests that Cys 86 and Cys 91 are sufficiently close to allow the formation of such a disulfide bond. Upon ligand binding the receptor, this disulphide bond is proposed to undergo disulfide exchange with a free sulfhydryl group from the  $\alpha$ -chain that is brought into proximity via ligand-dependent noncovalent association.

The development of the C86A and C91A mutants has permitted the demonstration of non-covalent receptor associations as not sufficient for receptor tyrosine phosphorylation and that this requires disulfide-linkage of receptor subunits. The role of covalent dimerization appears to be to secure concomitant dimerization of the cytoplasmic portions of  $\beta_c$  facilitating transphosphorylation of associated kinases of the JAK family. This has been shown in experiments using chimeric receptors that cause artificial dimerization of the cytoplasmic portions of  $\beta_c$  lead to their activation (Muto et al.1995; Shikama et al.1996) and direct dimerization of JAK has been shown to transduce a growth signal (Nakamura et al.1996). Even though they are essential for normal activation (Takaki et al.1994; Weiss et al.1993; Rapoport et al.1996), the role of the cytoplasmic domains of the receptor  $\alpha$ -chains remains ambiguous, however, the prediction is that they may serve to orientate the  $\beta$ -chains so as to juxtapose correctly the JAK molecules or otherwise be involved directly in certain functions (Spielholz et al.1995; Patel et al.1996).

The  $\beta$ -chain forms intermolecular disulfides specifically with the  $\alpha$ -chains of the GM-CSF, IL-3 or IL-5 receptors. A common characteristic of these  $\alpha$  chains is the presence of an N-terminal FnIII-like domains but of a unique type which only occur in this subfamily of cytokine receptors. Within this N-terminal domain, all three receptor  $\alpha$  chains possess an uneven number of Cys residues in the N-terminal domain suggesting that these Cys residues are the most likely candidates to act as partners for  $\beta_c$ . The IL-5R $\alpha$  has only a single Cys residue in the N-terminal domain,

at position 86, and this residue has been shown to be important for IL-5 binding to the receptor (Devos et al.1994).

The stoichiometry of the IL-3, GM-CSF, IL-5 receptor complexes is not known. The formation of an intermolecular disulfide bond between Cys 86 or Cys 91 of  $\beta_C$  and a Cys in the N-terminal domain of a receptor  $\alpha$ -chain could occur potentially with either the  $\alpha$ -chain with which it shares ligand or a second  $\alpha$ -chain recruited as part of a hexameric complex as seen with the IL-6 receptor (Paonessa et al.1995). Since the individual FnIII-like domains of  $\beta_C$  are likely to be fairly rigid units of length 3.5 to 4.5 nm, the ability of  $\beta_C$  to contact  $\alpha$ -chain will depend on the interdomain angles that it can adopt. In this, it is possible to be guided by the empirical structures of other proteins bearing pairs of FnIII-like domains. In fibronectin itself, the angles of tilt between domains range from 12° to 52° (Leahy et al.1996) while the interdomain angles in the class 2 cytokine receptors, IFN $\gamma$  receptor and tissue factor are 45° (Walter et al.1995) and 60° (Harlos et al.1994) respectively.  $\beta_C$  is a class 1 cytokine receptor and the angles observed in the known structures of this family, GHR (Cunningham et al.1991) and EPOR (Livnah et al.1996) are approximately 90°. It is therefore reasonable to infer that the angles between domains 1&2 and 3&4 of  $\beta_C$  will also be approximately 90°.

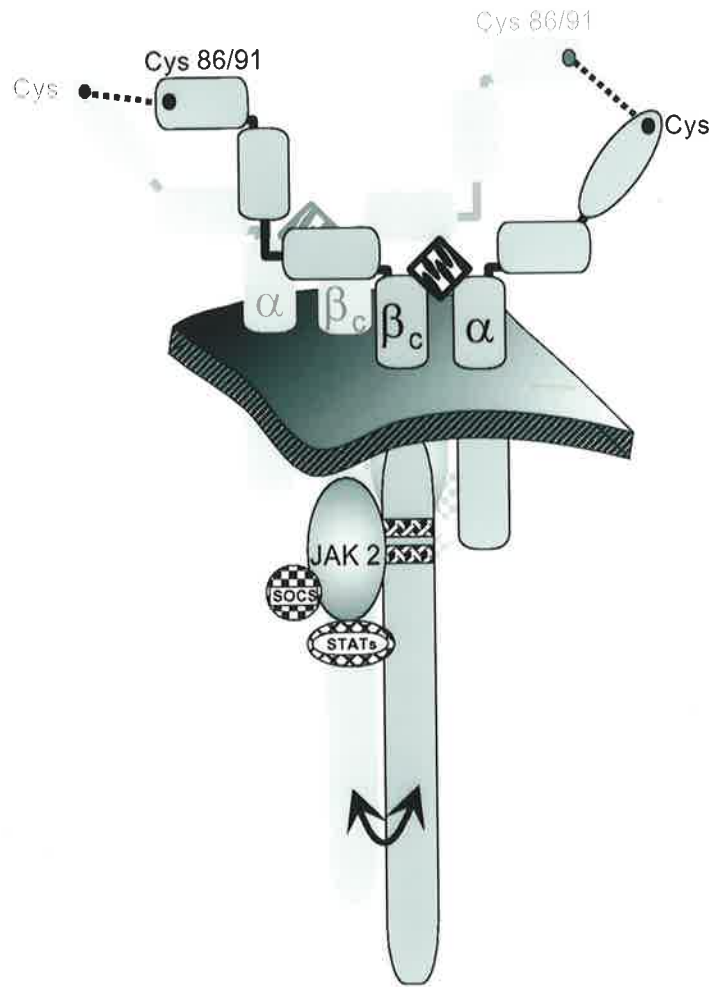
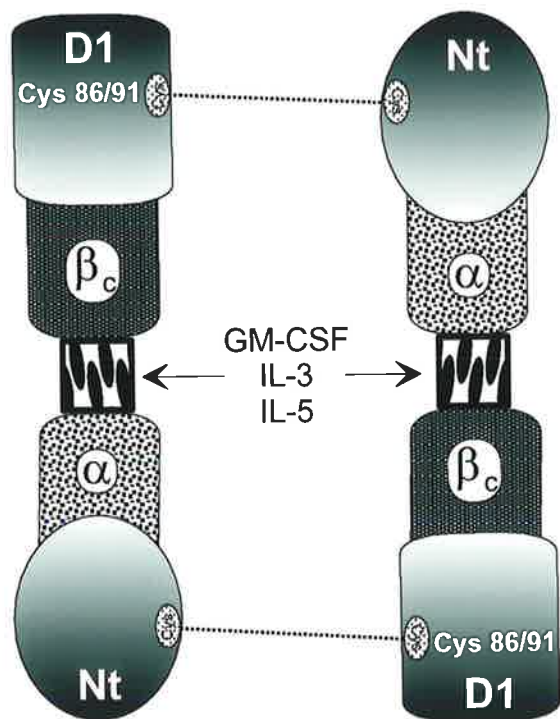
The conformation of the linker peptide between domains 2 and 3 of  $\beta_C$  cannot be gauged by reference to homologous structures but the extent of the peptide between the C-terminus of the G strand of domain 2 and the A strand of domain 3 together with the occurrence of a conserved Pro-Gly-Asp motif that is also present between

domains 2 and 3 of TPOR suggests that this may form a specific hinge region. Similarly, the  $\alpha$ -chains for GM-CSF, IL-3 and IL-5 have Gly residues at the border of the N-terminal domain and the first domain of the CRM and this lies approximately 10nm from the  $\beta_c$  hinge. The reactive Cys residues in  $\beta_c$  are predicted to lie in the D/E loop of domain 1, well-removed from the ligand interaction site and only 6nm from the  $\beta_c$  hinge. Thus, it appears that these residues could not interact readily with a Cys residue in the N-terminal domain of the  $\alpha$ -chain within a receptor heterodimer even if the hinges were highly kinked. It is proposed that the intermolecular disulfide bond forms between  $\beta_c$  and an  $\alpha$ -chain from a second receptor heterodimer (Fig 6.1) as this can be accommodated readily with respect to both the sizes of the domains and their interdomain angles.

Based on the likely orientation of their N-terminal domains with respect to the CRM of the  $\alpha$ -chains, it is favoured that a receptor complex arranged clockwise when viewed from outside the cell in the order  $\alpha$ -chain<sub>1</sub>/ligand/ $\beta$ -chain<sub>1</sub>S-S $\alpha$ -chain<sub>2</sub>ligand/ $\beta$ -chain<sub>2</sub>. The disulfide-linkage of  $\beta_c$  in one receptor heterodimer to an  $\alpha$  chain in a second receptor heterodimer would facilitate juxtaposing two  $\beta_c$  molecules with their associated JAK kinases and induce receptor phosphorylation. This initial association may also facilitate the formation of a second disulfide between  $\beta_c$  in receptor 2 and an  $\alpha$  chain in receptor 1 (Fig 6.1A and B). The formation of a 2:2:2 complex is also consistent with the requirement of two  $\alpha$  chains in an active ligand-receptor complex (Lia et al.1996). On the other hand a 1:1:1 stoichiometry has been suggested from experiments using chimeras of  $\alpha$  and  $\beta_c$  with fos and jun leucine zippers although the formation of higher order

complexes was not excluded (Patel et al.1996). The direct measurement of  $\alpha$ - $\beta_c$  interactions in solution may ultimately resolve this question. Furthermore the effect of these mutants on functional phenotype needs to be analysed. The stable expression of these mutations in CTLL cells would allow to measure there effect on proliferation. On the other hand cell lines like M1 cells expressing these mutants would permit to detect their effect on cell differentiation. Through these experiments it will be possilble to define the effect that disulpide-linked heterodimerisation has on cellular functions.

**Figure 7.1 Proposed model for assembly of GM-CSF-, IL-3- and IL-5-induced receptor complexes.** The binding of GM-CSF, IL-3 and IL-5 to GMR $\alpha$ -, IL-3R $\alpha$ - or IL-5R $\alpha$ -chain respectively induces  $\alpha$ : $\beta_C$  heterodimerization and a conformational change in  $\alpha$  chain that allows its disulphide linkage to  $\beta_C$ . Modelling of  $\beta_C$  suggests that this bridging would only be possible if the unpaired cysteines in the  $\alpha$  chain N terminal (Nt) of receptor 1 formed a disulphide bridging with cysteine at position 86 or 91 in domain 1 (D1) of  $\beta_C$  in receptor 2. The bringing together of two  $\beta_C$  with their associated JAK-2 molecules would then lead to receptor activation. A cartoon model representing the side view is shown in (A) and a top view in (B)

**A****B**

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## Human Interleukin-3 (IL-3) Induces Disulfide-Linked IL-3 Receptor $\alpha$ - and $\beta$ -Chain Heterodimerization, Which Is Required for Receptor Activation but Not High-Affinity Binding

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The human interleukin-3 receptor (IL-3R) is a heterodimer that comprises an IL-3-specific  $\alpha$  chain (IL-3R $\alpha$ ) and a common  $\beta$  chain ( $\beta_c$ ) that is shared with the receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5. These receptors belong to the cytokine receptor superfamily, but they are structurally and functionally more related to each other and thus make up a distinct subfamily. Although activation of the normal receptor occurs only in the presence of ligand, the underlying mechanisms are not known. We show here that human IL-3 induces heterodimerization of IL-3R $\alpha$  and  $\beta_c$  and that disulfide linkage of these chains is involved in receptor activation but not high-affinity binding. Monoclonal antibodies (MAb) to IL-3R $\alpha$  and  $\beta_c$  were developed which immunoprecipitated, in the absence of IL-3, the respective chains from cells labelled with <sup>125</sup>I on the cell surface. However, in the presence of IL-3, each MAb immunoprecipitated both IL-3R $\alpha$  and  $\beta_c$ . IL-3-induced receptor dimers were disulfide and nondisulfide linked and were dependent on IL-3 interacting with both IL-3R $\alpha$  and  $\beta_c$ . In the presence of IL-3 and under nonreducing conditions, MAb to either IL-3R $\alpha$  or  $\beta_c$  immunoprecipitated complexes with apparent molecular weights of 215,000 and 245,000 and IL-3R $\alpha$  and  $\beta_c$  monomers. Preincubation with iodoacetamide prevented the formation of the two high-molecular-weight complexes without affecting noncovalent dimer formation or high-affinity IL-3 binding. Two-dimensional gel electrophoresis and Western blotting (immunoblotting) demonstrated the presence of both IL-3R $\alpha$  and  $\beta_c$  in the disulfide-linked complexes. IL-3 could also be coimmunoprecipitated with anti-IL-3R $\alpha$  or anti- $\beta_c$  MAb, but it was not covalently attached to the receptor. Following IL-3 stimulation, only the disulfide-linked heterodimers exhibited reactivity with antiphosphotyrosine antibodies, with  $\beta_c$  but not IL-3R $\alpha$  being the phosphorylated species. A model of IL-3R activation is proposed which may be also applicable to the related GM-CSF and IL-5 receptors.

Engagement of the human interleukin-3 receptor (IL-3R) by IL-3 triggers a variety of cellular signals resulting in the preservation of cell viability, proliferation, and differentiation of hemopoietic cells (4, 27). Expression of the IL-3R, while subject to regulation, is maintained during hemopoietic cell differentiation, and its activation on the mature cells leads to enhanced function of monocytes (10), eosinophils (26), basophils (12, 24), and neutrophils (44). The IL-3R has been shown to be expressed also on endothelial cells with activation by IL-3 stimulating cytokine release and the expression of adhesion molecules (20, 21). The wide expression of the IL-3R on hemopoietic cells and on cells of the blood system suggests roles in hemopoiesis, allergy, atherosclerosis, and chronic inflammation. However, the mechanism of IL-3R activation remains unclear.

The human IL-3R is a heterodimeric receptor consisting of an IL-3-specific  $\alpha$  chain (IL-3R $\alpha$ ) (19) and a common  $\beta$  chain ( $\beta_c$ ) (13) that is also a component of the receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 (reviewed in references 25 and 34). Both receptor chains belong to the cytokine receptor superfamily (3), although it has been noted that the IL-3R $\alpha$  chain is more closely related to the

GM-CSF receptor  $\alpha$  chain (GM-CSFR $\alpha$ ) and IL-5R $\alpha$  chains than to other cytokine receptors (11). Thus, these three  $\alpha$  chains can be recognized structurally and possibly functionally as a distinct subfamily.  $\beta_c$ , on the other hand, is structurally more closely related to gp130 and the IL-2R $\beta$  chain (11) and, analogous to these common receptor subunits, it converts low-affinity ligand binding to high-affinity ligand binding and acts as a signal transducer (29).

The expression of both IL-3R $\alpha$  and  $\beta_c$  is necessary for triggering signalling and cellular proliferation in response to IL-3 (18). Stimulation of cells with IL-3 leads to activation of JAK-2 (38, 43) and Lyn (48) kinases, phosphatidylcholine hydrolysis and protein kinase C translocation (39), activation of multiple isoforms of the signal transducer protein Stat 5 (1, 30), and gene expression (52). Although some form of receptor dimerization has been presumed to take place, the relative contribution of each receptor chain, the nature of their association, and the implications for receptor activation are not known.

Receptor dimerization is recognized to be important for activation in many receptor systems. For example, receptor tyrosine kinases (14, 22, 49), as well as nontyrosine kinase receptors such as the G-CSFR (16), undergo homodimerization following ligand binding, which leads to signalling. Homodimerization has also been observed in the erythropoietin (EPO) receptor (28) and in a mutant EPO receptor that is constitutively active (53). IL-6R and ciliary neurotrophic factor receptor (CNTFR) dimerization have also been shown to occur. These receptors are more analogous to the IL-3R in that

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they consist of a binding subunit and signalling subunits (7, 15). In these cases, ligand triggers subunit association but only dimerization of the signalling subunits; the signalling subunits, gp130 in the case of IL-6, and gp130 and the leukemia inhibitory factor-binding protein (LIFR) in the case of CNTF, mediate signalling (7, 32). In the IL-3R, GM-CSFR, and IL-5R system, however, the biochemical evidence of receptor dimerization has been missing. Furthermore, the requirements for the  $\alpha$  chain and  $\beta_c$  in receptor activation based on cell lines transfected with genetically manipulated receptors is controversial. On the one hand, chimeric receptors consisting of extracellular  $\alpha$  chains and intracellular  $\beta_c$  allow function in the presence of intact  $\beta_c$  and ligand, suggesting that  $\beta_c$  dimerization is sufficient for signalling (9, 33, 47). On the other hand, deletion of the cytoplasmic domain of the  $\alpha$  chains abolishes ligand-mediated stimulation, suggesting that an intact  $\alpha$  chain may be required for receptor dimerization and signalling (40, 54). Using primary human cells, we show here that IL-3R $\alpha$  and  $\beta_c$  undergo dimerization following stimulation with IL-3, and that, unlike the IL-6R and CNTFR, the  $\alpha$  chain of the IL-3R is part of the disulfide- and the non-disulfide-linked dimers. Furthermore, the disulfide-linked IL-3R $\alpha$ - $\beta_c$  heterodimer is shown to be required for receptor activation and phosphorylation of  $\beta_c$  but not for affinity conversion. Given the conservation of IL-3R $\alpha$  with the GM-CSFR and IL-5R $\alpha$  chains and the common nature of the  $\beta$  chain, the results presented here may also be applicable to the GM-CSFR and IL-5R.

#### MATERIALS AND METHODS

**Cytokines and cells.** Human IL-3, GM-CSF, and the IL-3 mutant E22R were produced in *Escherichia coli* and purified to homogeneity by reverse-phase high-pressure liquid chromatography (2). Quantitation was performed by integration of peak absorbance of protein stained with Coomassie blue R-250 after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). IL-3 was radiolabelled with  $^{125}\text{I}$  by the ICI method as previously described (5). The cells used, unless otherwise indicated, were primary human leukemic cells obtained from the blood of a patient with chronic myeloid leukemia (CML cells) after separation on Ficoll-Paque (Pharmacia). CML cells were T- and B-cell antigen negative, CD33 and CD34 positive, and IL-3R $\alpha$ , GM-CSFR $\alpha$ , and  $\beta_c$  positive, as judged by flow cytometry with specific monoclonal antibodies (MAB) and then with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Ig). The other two types of cells used were the human UT7 cell line and COS cells transfected with IL-3R $\alpha$  and  $\beta_c$  cDNAs. The  $\beta_c$  DNA construct was cloned in pSG5 (gift from S. Barry and R. D'Andrea).

**Anti-IL-3R MAB.** MAB against the IL-3R $\alpha$  and  $\beta$  chains were raised by immunizing mice with COS cells transiently transfected with the IL-3R $\alpha$  or  $\beta_c$  cDNA and selecting on CHO cell transfectants stably expressing each receptor chain. MAB 9F5 against the IL-3R $\alpha$  chain and MAB 4F3 against  $\beta_c$  were selected for their ability to provide a strong signal in immunoprecipitation analysis, and MAB 1C1 against  $\beta_c$  and MAB 9F5 were selected for giving strong signals on Western blot (immunoblot) analysis. MAB 7G3 is directed against the IL-3R $\alpha$  chain and blocks IL-3 binding and IL-3-mediated functions (46). Each MAB was produced as ascitic fluid and purified by protein G Sepharose. For immunoprecipitation experiments, MAB 9F5 and 4F3 were directly coupled to Sepharose beads by using CNBr-activated Sepharose 4B as previously described (45).

**Antiphosphotyrosine MAB.** The MAB against phosphorylated tyrosine was the peroxidase-conjugated antiphosphotyrosine 3-365-10 (Boehringer Mannheim, Frankfurt, Germany).

**Fluorescence-activated cell sorting staining.** Fifty microliters of cells ( $10^5$  cells) was added to 50  $\mu\text{l}$  of optimally diluted antibody and incubated at 4°C for 30 min. The cells were washed with phosphate-buffered saline (PBS) and then incubated with 1/50 fluoresceinated rabbit anti-mouse antibody (DDAF; Silenus, Melbourne, Australia) for 30 min at 4°C. The cells were then washed and resuspended in FACS FIX (PBS with 2% glucose, 1% formaldehyde, and 0.02% sodium azide) and analyzed with a Coulter Profile Flow Cytometer (Coulter Electronics, Hialeah, Fla.).

**Immunoprecipitation of the IL-3R.** CML cells were surface labelled with  $^{125}\text{I}$ . Usually  $10^8$  cells in 1 ml of PBS were mixed with 1 mCi of  $^{125}\text{I}$  (NEN) and radiolabelled by the lactoperoxidase method as described previously (50). Following labelling, the cells were incubated with medium, IL-3, GM-CSF, or IL-3 mutant E22R, for different times before being lysed with lysis buffer composed of 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 10% glycerol, 1% Nonidet P-40, and protease and phosphatase inhibitors (10  $\mu\text{g}$  of aprotinin per ml, 10  $\mu\text{g}$  of leupeptin per ml, 2 mM phenylmethylsulfonyl fluoride, and 2 mM sodium vana-

date). After 30 min at 4°C, the lysate was centrifuged at  $10,000 \times g$  for 15 min, and the supernatant was removed, precleared with mouse Ig-coupled Sepharose beads for 18 h at 4°C, and incubated with anti-IL-3R $\alpha$  MAB, anti- $\beta_c$  MAB, or anti-phosphotyrosine-coupled Sepharose beads for 2 h at 4°C. The beads were then washed 6 times with lysis buffer before being boiled for 5 min in SDS-PAGE sample buffer and the immunoprecipitated proteins were separated by SDS-PAGE. In some experiments, the alkylating agent iodoacetamide was added to the cells for 20 min at 4°C before or after the cells were incubated with IL-3.

**SDS-PAGE.** Immunoprecipitated proteins were analyzed by one-dimensional (1D) or two-dimensional (2D) SDS-PAGE under reducing and nonreducing conditions. Separation of proteins by 1D SDS-PAGE and reducing conditions utilized 7.5% polyacrylamide linear gels and boiling in SDS sample buffer containing 4% 2-mercaptoethanol. Proteins were separated under nonreducing conditions on SDS-6% polyacrylamide linear gels and the samples were boiled in SDS buffer without 2-mercaptoethanol. Proteins separated by 2D SDS-PAGE were subjected to separation under nonreducing conditions in tube gels followed by separation under reducing conditions in the second dimension (36). For 1D gels, molecular weights (MW) were estimated with commercially available MW markers (Bio-Rad Broad Range Standards 161-0318). For 2D gels, the MW of the separated proteins were estimated by mixing the  $^{125}\text{I}$ -labelled immunoprecipitated proteins with solubilized platelet proteins and by using the known MW of the major characterized platelet proteins visualized by Coomassie blue staining. The  $^{125}\text{I}$ -labelled immunoprecipitated proteins were detected and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

**Western blotting (immunoblotting).** Immunoprecipitated proteins separated by SDS-PAGE were transferred onto nitrocellulose filters by electroblotting. The filters were blocked with a solution consisting of 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.85% Tween 20 containing 5% bovine serum albumin and then probed with either anti-IL-3R $\alpha$  MAB (9F5), anti-IL-3R $\beta_c$  (1C1), or anti-phosphotyrosine (3-365-10) MAB followed where appropriate by goat anti-mouse Ig coupled to horseradish peroxidase. In order to detect radiolabelled immunoprecipitated proteins and Western blot reactivity on the same filters, the chemiluminescence method with an ECL kit (Amersham, Little Chalfont, United Kingdom) was used per the manufacturer's guidelines.

**Scatchard analysis.** Binding assays were performed with  $0.5 \times 10^5$  to  $1 \times 10^5$  cells in 150  $\mu\text{l}$  of binding medium. Cells were incubated in the presence of a concentration range of 10 pM to 10 nM  $^{125}\text{I}$ -labelled IL-3. Nonspecific binding was determined from samples containing 1  $\mu\text{M}$  unlabelled IL-3. The nonspecific binding component for datum points obtained at lower radioligand concentrations was obtained by interpolation. After incubation at 21°C for 2 h with shaking, cell-associated radioligand was separated from free radioligand by overlaying the cell suspension on a 0.2-ml cushion of fetal calf serum and centrifuging for 10 s at maximum speed in a microcentrifuge. The visible cell pellet was removed by cutting, and radioactivity was determined with a Cobra 5010  $\gamma$ -counter (Packard, Meriden, Conn.). Dissociation constants were calculated by using the EBDA and LIGAND programs (31) (Biosoft, Cambridge, United Kingdom).

#### RESULTS

**IL-3 induces IL-3R $\alpha$  and  $\beta_c$  association.** In order to study the molecular events leading to IL-3R activation in primary cells, we developed MAB specific for IL-3R $\alpha$  and for  $\beta_c$  which could give strong signals in immunoprecipitation and Western blot analyses. From several MAB raised against COS cell transfectants overexpressing IL-3R $\alpha$  or  $\beta_c$ , three MAB were selected: MAB 9F5 specifically immunoprecipitated IL-3R $\alpha$  from stable CHO cell transfectants, while MAB 4F3 immunoprecipitated  $\beta_c$  from CHO cells transfected with  $\beta_c$  cDNA. These MAB did not react with CHO cells expressing GM-CSFR $\alpha$  (data not shown). For Western blot analyses, MAB 9F5 gave a strong signal on CHO cells expressing IL-3R $\alpha$  and MAB 1C1 gave the strongest signal on CHO cells expressing  $\beta_c$ . These MAB were used to screen several primary myeloid cells; of these cell lines, CML cells obtained from a patient with chronic myeloid leukemia exhibited the largest number of receptor molecules, as judged by flow cytometry, and were therefore used for subsequent experiments (data not shown).

In order to determine whether the IL-3R exists as a pre-formed complex or is induced by ligand, MAB 9F5 (anti-IL-3R $\alpha$ ) and MAB 4F3 (anti- $\beta_c$ ) were used in immunoprecipitation experiments and the proteins were separated on SDS-polyacrylamide gels under reducing conditions. We found that in the absence of IL-3, MAB 9F5 immunoprecipitated only IL-3R $\alpha$  and MAB 4F3 immunoprecipitated only  $\beta_c$  from  $^{125}\text{I}$ -

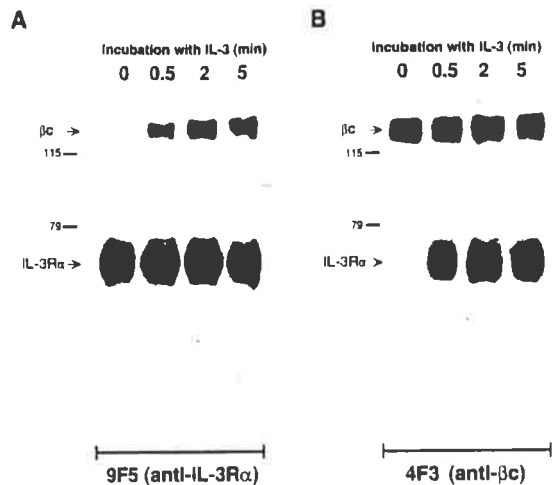


FIG. 1. IL-3 induces IL-3R complex formation. Cells labelled with  $^{125}\text{I}$  on the cell surface were incubated with 50 nM IL-3 for different times at  $4^{\circ}\text{C}$ . After cell lysis, proteins were immunoprecipitated with MAb 9F5 (anti-IL-3R $\alpha$ ) (A) or MAb 4F3 (anti- $\beta_c$ ) (B), separated on SDS-7.5% polyacrylamide gels under reducing conditions, and visualized by PhosphorImaging. The positions and MW (in thousands) of marker proteins are shown to the left of the gels.

surface-labelled CML cells (Fig. 1). However, the addition of IL-3 caused the coimmunoprecipitation of a 120,000-MW protein in the case of immunoprecipitation with MAb 9F5 (Fig. 1A) and of a 70,000-MW protein in the case of immunoprecipitation with MAb 4F3 (Fig. 1B). The association of these proteins was rapid and detectable within 30 s. To determine the generality of this phenomenon, we performed further experiments using the human UT7 cell line which expresses IL-3R and COS cells transfected with the IL-3R $\alpha$  and  $\beta_c$  chains. In both cases, IL-3 induced the formation of the heterodimeric complex (Fig. 2A and B).

The MW of the coimmunoprecipitated proteins suggested that they corresponded to  $\beta_c$  when MAb 9F5 was used and to IL-3R $\alpha$  when MAb 4F3 was used (Fig. 1). To test whether this was indeed the case, we performed Western blot analyses using chemiluminescence as the detection method. In addition, and as a specificity control, the cells were incubated with GM-CSF (Fig. 3). We found that MAb 9F5 immunoprecipitated IL-3R $\alpha$  together with a 120,000-MW protein in the presence of IL-3 but not in the presence of GM-CSF (Fig. 3A). Conversely, MAb 4F3 immunoprecipitated  $\beta_c$  and a 70,000-MW protein only in the presence of IL-3 (Fig. 3B). We consistently found a weak band with an MW of approximately 80,000 coimmunoprecipitating with  $\beta_c$ , and other experiments showed that this band represents GM-CSFR $\alpha$  (data not shown). To determine the identities of the coimmunoprecipitated bands, we probed the proteins immunoprecipitated with MAb 9F5 (anti-IL-3R $\alpha$ ) by Western blotting with MAb 1C1 (anti- $\beta_c$ ), and reciprocally, the proteins immunoprecipitated with MAb 4F3 (anti- $\beta_c$ ) with MAb 9F5 (anti-IL-3R $\alpha$ ). These data established that  $\beta_c$  coimmunoprecipitated with IL-3R $\alpha$  and IL-3R $\alpha$  coimmunoprecipitated with  $\beta_c$ , but only in the presence of IL-3 (Fig. 3B).

**IL-3R complex formation is dependent on IL-3 contacting both IL-3R $\alpha$  and  $\beta_c$ .** To study whether IL-3 binding to both chains of the IL-3R was a prerequisite for IL-3R complex formation, we performed coimmunoprecipitation experiments after preincubation of the cells with MAb 7G3, which blocks IL-3 binding to IL-3R $\alpha$  (46), and in the presence of the IL-3

mutant E22R, which has defective  $\beta_c$  interaction (2). We found that preincubation of cells with the blocking MAb (7G3) greatly reduced the abilities of the  $\alpha$  chain to coimmunoprecipitate  $\beta_c$  (Fig. 4A) and of  $\beta_c$  to coimmunoprecipitate with IL-3R $\alpha$  in the presence of IL-3 (Fig. 4B). Experiments comparing the effects of the IL-3 mutant E22R with wild-type IL-3 were performed over a range of ligand concentrations. We found that wild-type IL-3 induced the coimmunoprecipitation of IL-3R $\alpha$  and  $\beta_c$  at a concentration of 0.1 nM and maximally at 1.0 nM (Fig. 5A and B). In contrast, the IL-3 mutant E22R did not cause coimmunoprecipitation of IL-3R $\alpha$  and  $\beta_c$  at concentrations up to 100 nM (Fig. 5C and D). At higher concentrations (4  $\mu\text{M}$ ) of E22R, some coimmunoprecipitation of IL-3R $\alpha$  and  $\beta_c$  was observed (data not shown). Again, a protein with an MW of approximately 80,000 coimmunoprecipitated with  $\beta_c$  in the absence of wild-type IL-3 (Fig. 5B) or in the presence of E22R (Fig. 5D) corresponding to GM-CSFR $\alpha$ . These experiments establish that IL-3 needs to contact both receptor chains in order to trigger receptor  $\alpha$  and  $\beta_c$  association.

**IL-3 induces disulfide- and nondisulfide-linked receptor dimers.** To examine the possibility that IL-3 induces the covalent association of IL-3R $\alpha$  and  $\beta_c$ , we next analyzed the immunoprecipitated proteins under nonreducing conditions. We found that in the absence of IL-3, the anti-IL-3R $\alpha$  MAb immunoprecipitated only IL-3R $\alpha$  (Fig. 6A). The anti- $\beta_c$  MAb, on the other hand, immunoprecipitated mainly monomeric  $\beta_c$  but also consistently immunoprecipitated a faint band with an apparent MW of 245,000 (Fig. 6B). However, incubation with IL-3 not only led to the coimmunoprecipitation of IL-3R $\alpha$  and  $\beta_c$  as shown above but also induced the appearance of two high-MW bands with apparent MW of approximately 215,000 and 245,000 (Fig. 6). This was observed whether immunoprecipitations were performed with anti-IL-3R $\alpha$  MAb (Fig. 6A) or anti- $\beta_c$  MAb (Fig. 6B). The high-MW complexes appeared to be disulfide linked, since treatment with the thiol-specific al-

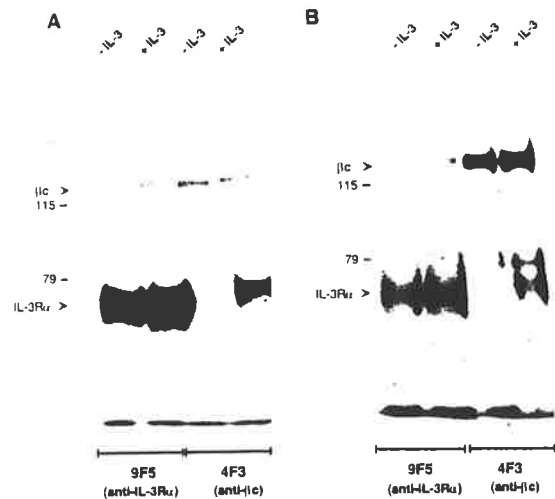


FIG. 2. IL-3 induces IL-3R complex formation in the human UT7 cell line and COS cells transfected with IL-3R $\alpha$  and  $\beta_c$ .  $^{125}\text{I}$ -surface-labelled cells UT7 (A) and COS cells transfected with IL-3R $\alpha$  and  $\beta_c$  (B) were incubated with 50 nM IL-3 (+IL-3) for 15 min at  $4^{\circ}\text{C}$ . After cell lysis, proteins were immunoprecipitated with MAb 9F5 (anti-IL-3R $\alpha$ ) or MAb 4F3 (anti- $\beta_c$ ), separated on SDS-7.5% polyacrylamide gels under reducing conditions, and visualized by PhosphorImaging. The positions and MW (in thousands) of marker proteins are shown to the left of the gels.

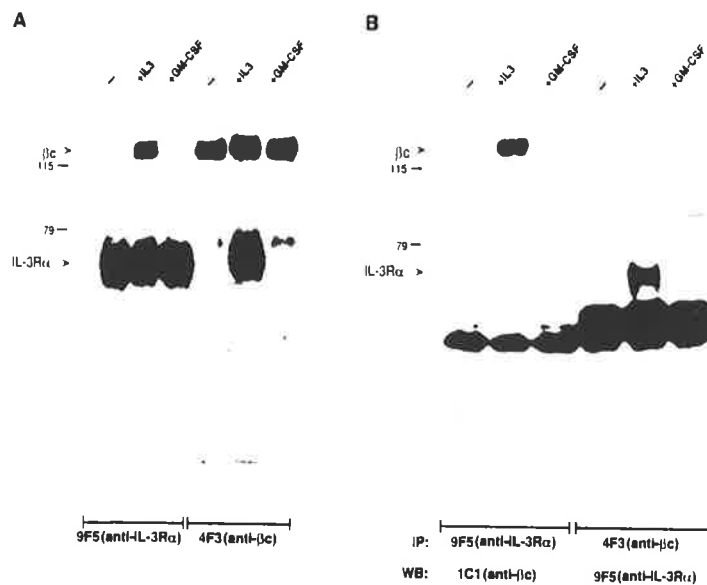


FIG. 3. Identification of coimmunoprecipitated bands as IL-3R $\alpha$  and  $\beta_c$  by Western blotting. (A) Immunoprecipitations using MAb 9F5 (anti-IL-3R $\alpha$ ) or 4F3 (anti- $\beta_c$ ) and SDS-PAGE as described in the legend to Fig. 1 from cells not treated (-) or treated (+) with IL-3 (50 nM) or GM-CSF (50 nM) for 45 min at 4°C and visualized by PhosphorImaging. (B) Western blot of panel A using MAb 1C1 (anti- $\beta_c$ ) or MAb 9F5 (anti-IL-3R $\alpha$ ). The major band shows the immunoreactivity of the immunoprecipitating MAb. The positions and MW (in thousands) of marker proteins are shown to the left of the gels. IP, immunoprecipitation; WB, Western blotting.

kylation agent iodoacetamide prior to the addition of IL-3 inhibited their appearance (Fig. 6A and B, lanes IAM+IL-3). Despite the marked loss of the high-MW complexes, iodoacetamide pretreatment did not prevent IL-3R $\alpha$  and  $\beta_c$  coimmu-

noprecipitation (Fig. 6A and B, lanes IAM+IL-3). Thus, IL-3 induces IL-3R $\alpha$  and  $\beta_c$  association by covalent and noncovalent means. For controls, we used iodoacetamide after IL-3 treatment in which case little or no reduction in the formation of the high-MW complexes was observed (Fig. 6A and B, lanes IL-3+IAM). Furthermore, the addition of IL-3 to the lysis buffer did not induce IL-3R $\alpha$  and  $\beta_c$  association (Fig. 6A and B, lanes lysate+IL-3), indicating that  $\alpha$  and  $\beta_c$  association was not induced in solution. These controls demonstrate that the appearance of the high-MW complexes requires free sulfhydryl groups and IL-3 acting on the intact cells.

We next examined whether IL-3 itself was present in the immunoprecipitated complexes. We did not perform Western blotting with anti-IL-3 antibodies, since it was possible that IL-3 epitopes could be masked by bound receptor. Instead, we stimulated the cells with radiolabelled IL-3 in the absence or presence of a 100-fold excess of unlabelled IL-3. We found that  $^{125}$ I-labelled IL-3 could be immunoprecipitated with MAb 9F5 (anti-IL-3R $\alpha$ ) or MAb 4F3 (anti- $\beta_c$ ) and this immunoprecipitation was inhibited by excess unlabelled IL-3 (Fig. 6C). However, IL-3 was not covalently bound to IL-3R $\alpha$  or  $\beta_c$ , as demonstrated by its migration to the 15,000-MW region (Fig. 6C).

Significantly, prevention of disulfide-linked dimer formation by iodoacetamide did not affect high-affinity binding of IL-3. Scatchard analysis of cells treated with iodoacetamide showed that high-affinity binding was essentially the same as that seen with untreated cells (Fig. 6D).

To identify the components of the disulfide-linked high-MW bands, we next performed 2D SDS-PAGE and Western blot analysis. 2D gel electrophoresis of immunoprecipitates from  $^{125}$ I-labelled, IL-3-treated cells with MAb 9F5 (anti-IL-3R $\alpha$ ) and MAb 4F3 (anti- $\beta_c$ ), in which the nonreduced samples were separated in the first dimension and then reduced before separation in the second dimension, were performed. The two major proteins seen on the diagonal had MW of 120,000 and 70,000, corresponding to the MW of  $\beta_c$  and IL-3R $\alpha$ , respec-

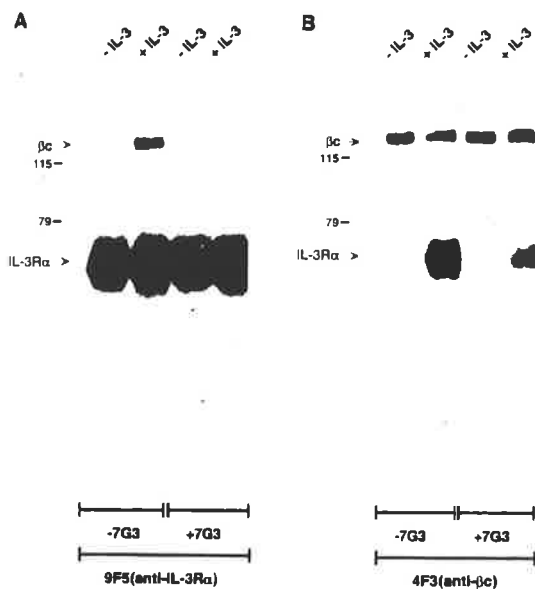


FIG. 4. The anti-IL-3R $\alpha$  blocking MAb 7G3 inhibits IL-3R $\alpha$  and  $\beta_c$  dimerization.  $^{125}$ I-surface-labelled cells were preincubated with (+) 1  $\mu$ g of MAb 7G3 for 30 min at 4°C before treatment with (+) or without (-) IL-3 (50 nM) for 45 min at 4°C. The cells were then lysed, and the lysates were subjected to immunoprecipitation with MAb 9F5 (anti-IL-3R $\alpha$ ) (A) or MAb 4F3 (anti- $\beta_c$ ) (B) as described in the legend to Fig. 1. The positions and MW (in thousands) are shown to the left of the gels.

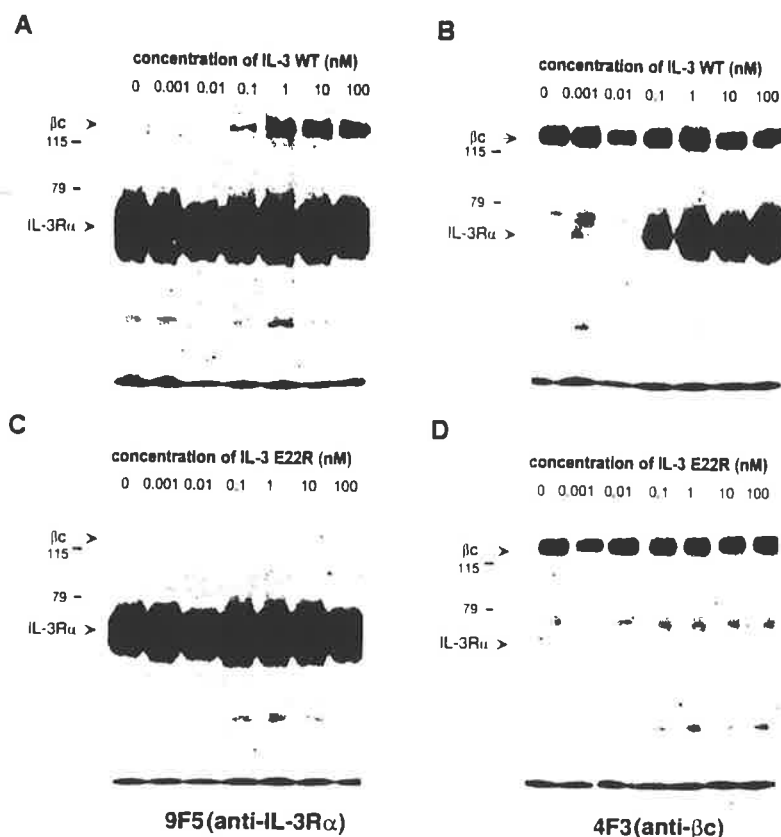


FIG. 5. The IL-3 analog E22R exhibits deficient induction of IL-3R $\alpha$  and  $\beta_c$  dimerization.  $^{125}$ I-surface-labelled cells were treated with different concentrations of wild-type (WT) IL-3 or with the IL-3 mutant E22R. The cells were then lysed, and the lysates were subjected to immunoprecipitation with MAb 9F5 (anti-IL-3R $\alpha$ ) (A and C) or MAb 4F3 (anti- $\beta_c$ ) (B and D) as described in the legend to Fig. 1. The positions and MW (in thousands) of marker proteins are shown to the left of the gels.

tively (Fig. 7). The 215,000-MW band in the nonreduced first dimension was resolved into two spots with MW of 120,000 and 70,000 in the reduced dimension, consistent with the MW of  $\beta_c$  and IL-3R $\alpha$ , respectively. A broad, higher-MW complex in the nonreduced dimension was also resolved into 120,000- and 70,000-MW components, although this was less distinct than for the 215,000-MW complex. These experiments suggested that the two high-MW bands seen under nonreducing conditions contain IL-3R $\alpha$  and  $\beta_c$ . To examine this identity further, we performed Western blotting of IL-3-stimulated cells immunoprecipitated with anti-IL-3R $\alpha$  and anti- $\beta_c$  MAb. In the absence of IL-3, no high-MW bands were immunoprecipitated with anti-IL-3R $\alpha$  MAb (Fig. 8A), while a 245,000-MW band could be immunoprecipitated with anti- $\beta_c$  MAb (Fig. 8A) and also contained IL-3R $\alpha$  (Fig. 8B), as judged by Western blotting. After stimulation with IL-3, however, both MAb 9F5 (anti-IL-3R $\alpha$ ) and MAb 1C1 (anti- $\beta_c$ ) reacted with the 215,000- and 245,000-MW bands, confirming that IL-3R $\alpha$  and  $\beta_c$  were both present in these disulfide-linked complexes (Fig. 8B and C).

**IL-3-induced disulfide-linked dimers are required for receptor activation.** In order to determine the functional consequences of IL-3R dimerization, we examined the immunoprecipitated complexes for the presence of phosphorylated tyrosine. We performed immunoprecipitations on  $^{125}$ I-surface-

labelled cells with anti-IL-3R $\alpha$  and anti- $\beta_c$  antibodies and probed the immunoprecipitates, separated under nonreducing or reducing conditions, with antiphosphotyrosine antibodies by Western blotting. The results showed that the antiphosphotyrosine MAb 3-365-10 reacted strongly with 215,000- and 245,000-MW bands (>94% of phosphotyrosine), but little label (<6% of phosphotyrosine) was observed in the 120,000-MW region. This antiphosphotyrosine staining was observed with immunoprecipitates by using either MAb 9F5 (anti-IL-3R $\alpha$ ) or 4F3 (anti- $\beta_c$ ) and disappeared when  $\alpha\beta$  covalent dimer formation was prevented by iodoacetamide (Fig. 9A). These results show that phosphorylation in response to IL-3 occurs primarily on the covalent  $\alpha\beta$  heterodimer, suggesting that  $\alpha\beta$  heterodimer formation is required for cellular activation. To ascertain whether both IL-3R $\alpha$  and  $\beta_c$  were being phosphorylated, the immunoprecipitates with anti-IL-3R $\alpha$  and anti- $\beta_c$  MAb were separated under reducing conditions before analysis by Western blotting with the antiphosphotyrosine antibody 3-365-10. Under these conditions, only a phosphorylated band with an MW of about 120,000 was observed, consistent with  $\beta_c$  but not IL-3R $\alpha$  being the phosphorylated protein present in the receptor dimer (Fig. 9A).

We further confirmed the predominant phosphorylation of the covalent  $\alpha\beta$  heterodimer by using the human UT7 cell line (Fig. 9B). The onset of phosphorylation in the covalent  $\alpha\beta$

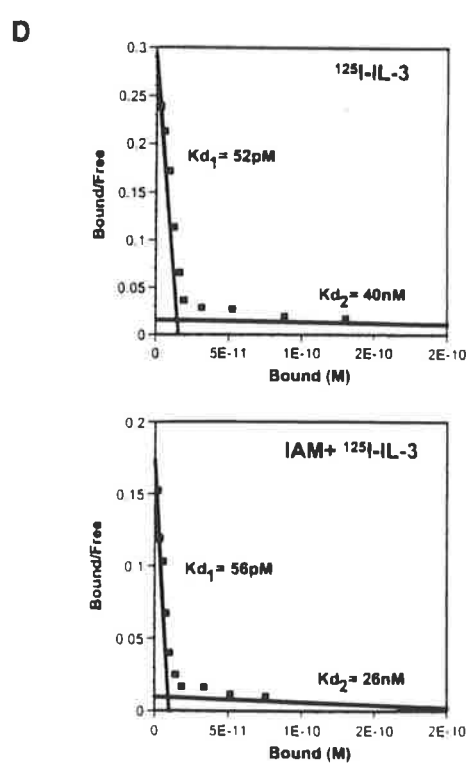
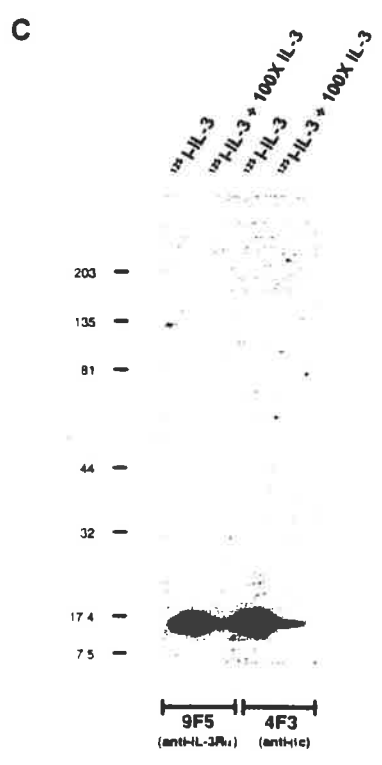
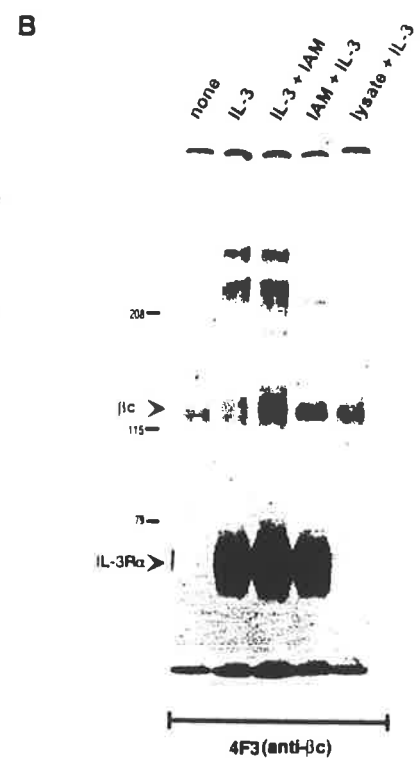
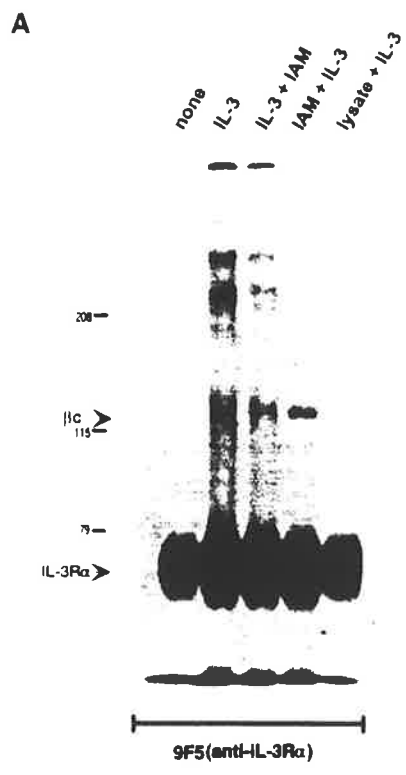


FIG. 6. IL-3 induces the formation of disulfide-linked receptor complexes. (A and B) From left to right, the lanes contain  $^{125}\text{I}$ -surface-labelled cells incubated with either medium for 30 min at 37°C, IL-3 (50 nM) for 30 min at 37°C, IL-3 (50 nM) for 30 min at 37°C and then with iodoacetamide (IAM) (10 mM) for 5 min at 21°C, iodoacetamide (10 mM) for 5 min at 21°C and then with IL-3 (50 nM) for 30 min at 37°C, or medium only and IL-3 (50 nM) added to the cell lysate for 30 min at 4°C. Immunoprecipitations were carried out with MAb 9F5 (anti-IL-3R $\alpha$ ) (A) and with MAb 4F3 (anti- $\beta_c$ ) (B). (C) Unlabelled cells were incubated with  $^{125}\text{I}$ -labelled IL-3 (1 nM) in the absence or presence of 100-fold-excess (100 $\times$ ) unlabelled IL-3 before immunoprecipitation with MAb 9F5 (anti-IL-3R $\alpha$ ) and MAb 4F3 (anti- $\beta_c$ ). The immunoprecipitated proteins were separated under nonreducing conditions on an SDS-6% polyacrylamide gel. (D) Scatchard transformation of  $^{125}\text{I}$ -labelled IL-3 binding curves in untreated cells (top) or cells treated with iodoacetamide (10 mM) for 20 min at 4°C (bottom).

heterodimer was rapid and detectable at 1 min (Fig. 9B). The proportions of tyrosine phosphorylation in these heterodimers at 1, 5, and 15 min were 91, 87, and 82%, respectively. As with CML cells, the formation of the covalent  $\alpha\beta$  heterodimer was prevented and tyrosine phosphorylation was abolished by pretreatment of UT7 cells with iodoacetamide (data not shown). To control for any possible toxic effects of iodoacetamide, UT7 cells were pretreated with this agent and then stimulated with phorbol myristate acetate and calcium ionophore. Under the

conditions used, no inhibition of basal or induced tyrosine phosphorylation was observed (Fig. 9C), indicating that iodoacetamide was not toxic to the cells.

## DISCUSSION

We show here that human IL-3 binding to its receptor triggers the heterodimerization of its specific receptor binding subunit, IL-3R $\alpha$ , with  $\beta_c$  and that receptor heterodimerization is dependent on IL-3 contacting both receptor subunits. Both disulfide-linked and non-disulfide-linked heterodimers were observed and, although IL-3 is required for their formation, IL-3 is not covalently attached to the dimers. Importantly, the disulfide-linked heterodimer and not the noncovalently linked heterodimer is shown to be required for receptor activation but not high-affinity binding. These results are different from those in the IL-6, EPO, and G-CSF receptor systems where receptor activation involves homodimerization of a single signalling subunit and may apply also to the related GM-CSF and IL-5 receptors.

Previous experiments have shown that IL-3R activation leads to stimulation of JAK-2 (43) and Lyn (48) kinases, as well as the Ras-mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase C pathways (41). The molecular basis of receptor activation has not been revealed, although by analogy with other receptor systems, it is believed to involve receptor dimerization. Receptor dimerization has been demonstrated in tyrosine kinase receptors (14, 49), as well as the G-CSFR (16), EPO receptor (28), IL-6R (32), and CNTFR (7). Dimerization of the IL-3R, GM-CSFR, or IL-5R has been proposed in ligand-induced and ligand-independent receptor activation (6, 17), but no evidence has been forthcoming. The results presented here demonstrate both covalent and noncovalent dimerization of the receptor, that it involves the ligand-binding subunit dimerizing with  $\beta_c$ , and that covalent linkage of dimers is required for signalling. These results emphasize the importance of the  $\alpha$  chain not only as a ligand-binding subunit but also as a subunit required for signalling in primary cells. This is consistent with experiments where deletion of the cytoplasmic domain of  $\alpha$  chains abolishes signalling (40, 54). Other experiments have shown, however, that the cytoplasmic domain of  $\beta_c$  can substitute for the intracytoplasmic domain of the  $\alpha$  chain (9, 33, 47). The exact significance of these findings is not clear but these findings may reflect the conserved nature of the proline-rich sequence present in the membrane-proximal domain of both the  $\alpha$  chain and  $\beta_c$  or the fact that at least two molecules of  $\beta_c$  are required for signalling, with the role of the  $\alpha$  chain being to recruit or facilitate the association of two molecules of  $\beta_c$ . Our experiments demonstrating the presence of both IL-3R $\alpha$  and  $\beta_c$  in the 215,000- and 245,000-MW complexes suggest that a heterodimer containing one  $\alpha$  chain and one  $\beta_c$  may be sufficient for signalling, although we cannot rule out the existence of higher-MW oligomeric complexes. Definition of the stoichiometry of the active receptor complex would require direct measurements using purified receptor components as shown with the IL-6R (35, 51).

The presence of IL-3R $\alpha$  in the disulfide-linked IL-3R dimers

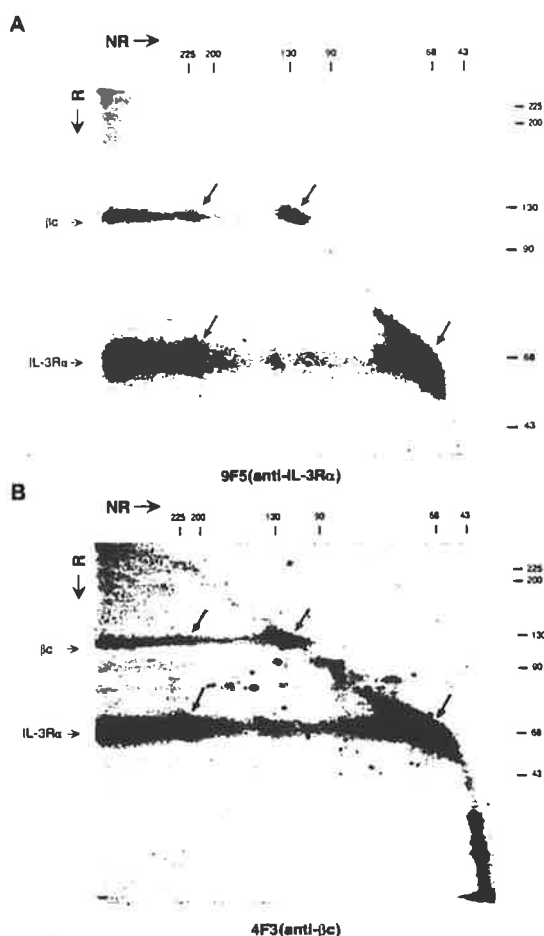


FIG. 7. 2D gel electrophoresis of  $^{125}\text{I}$ -surface-labelled cells incubated with 50 nM IL-3 for 30 min at 4°C and immunoprecipitated with anti-IL-3R $\alpha$  MAb (9F5) (A) or anti- $\beta_c$  MAb (4F3) (B). The immunoprecipitates were separated under nonreducing (NR) conditions in the first dimension and under reducing (R) conditions in the second dimension and visualized by PhosphorImaging. The immunoprecipitates were mixed with unlabelled platelet extract and stained with Coomassie blue (not shown) to obtain more accurate MW estimates. The positions and MW (in thousands) of proteins are shown at the sides of the gels.

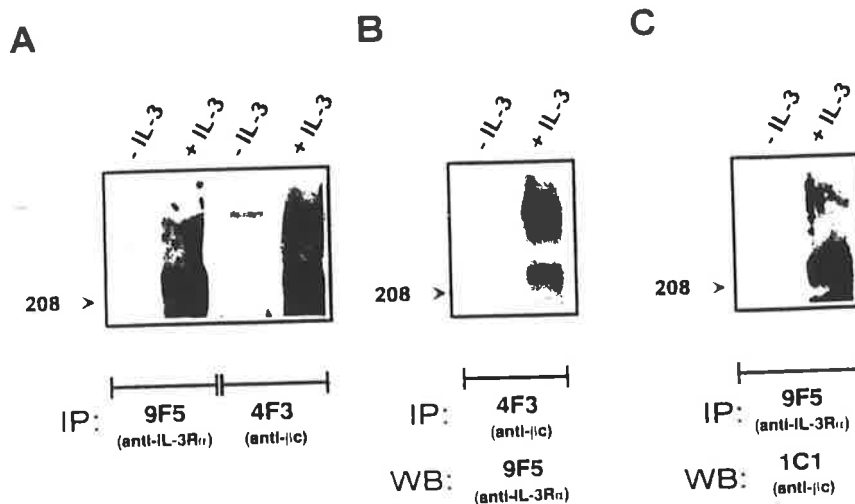


FIG. 8. IL-3-induced disulfide-linked complexes contain IL-3R $\alpha$  and  $\beta_c$ .  $^{125}$ I-surface-labelled cells were treated and immunoprecipitated as described in the legend to Fig. 6. (A) Immunoprecipitation (IP) with MAb 9F5 (anti-IL-3R $\alpha$ ) and MAb 4F3 (anti- $\beta_c$ ). (B) Immunoprecipitates with MAb 4F3 (anti- $\beta_c$ ) were analyzed by Western blotting (WB) with MAb 9F5 (anti-IL-3R $\alpha$ ). (C) Immunoprecipitates with MAb 9F5 (anti-IL-3R $\alpha$ ) were analyzed by Western blotting with MAb 1C1 (anti- $\beta_c$ ). Proteins were separated as described in the legend to Fig. 6. The position and MW (in thousands) of marker protein are shown to the left of the gels.

contrasts with the disulfide-linked dimerization of the IL-6 and CNTF receptors which involve only the signal transducer subunits. Thus, in the case of the IL-6R, disulfide-linked dimerization involves only gp130 (7), while in the case of the CNTFR, gp130 and LIFR form disulfide-linked dimers (7), events which appear to initiate signal transduction. These findings suggest a fundamental difference in the functional contribution of IL-3R $\alpha$  compared with IL-6R $\alpha$  and CNTFR $\alpha$  in receptor activation and suggest that IL-3R $\alpha$  is not only involved in the initial binding of ligand but also participates in signalling. These results are consistent with differences in the requirement for the cytoplasmic domain of the  $\alpha$  chains for signalling. Thus, while the cytoplasmic domain of the  $\alpha$  chains of the IL-3, GM-CSF, and IL-5 subfamily of receptors are essential for signal transduction (37, 40), the cytoplasmic portion of IL-6R $\alpha$  is not (57). On the other hand, IL-3R $\alpha$  behaves like the LIFR, which participates both in ligand binding and signalling.

Human IL-3R heterodimerization upon addition of IL-3 was found to be very rapid (it is complete within 1 min) and dose dependent. An anti-IL-3R $\alpha$  monoclonal antibody which inhibits IL-3 binding to this chain (46) prevented IL-3-induced receptor heterodimerization. Similarly, the IL-3 mutant E22R which is selectively deficient at interacting with  $\beta_c$  (2) failed to induce receptor dimerization in a concentration range in which wild-type IL-3 did so. This indicates that IL-3 needs to contact both receptor chains to stabilize the complex and induce receptor dimerization.

Under reducing conditions of SDS-PAGE, the IL-3-induced dimers were resolved into monomeric IL-3R $\alpha$  and  $\beta_c$ . Under nonreducing conditions, however, two high-MW complexes with MW of 215,000 and 245,000 were also seen in immunoprecipitations performed with both anti-IL-3R $\alpha$  and anti- $\beta_c$  MAb. These were disulfide-linked dimers, as judged by their disappearance in the presence of the thiol-specific alkylating agent iodoacetamide added before but not after incubation with IL-3. In the absence of IL-3, a faint band with an apparent MW of about 245,000 was consistently seen, but only in immunoprecipitations performed with MAb anti- $\beta_c$  (Fig. 6 and 8). Western blotting showed that this band contains  $\beta_c$  (data

not shown) and IL-3R $\alpha$  (Fig. 8B), suggesting that a small proportion of receptors may exist as preformed, though inactive (see below) dimers. IL-3 was present in the IL-3-induced receptor dimers, although it was not disulfide linked to either receptor chain (Fig. 6).

The sizes of the two high-MW complexes suggested that they might represent IL-3R $\alpha$  and  $\beta_c$  heterodimers or  $\beta_c$  homodimers. By performing 2D SDS-PAGE (Fig. 7) and Western blotting (Fig. 8) experiments, we could show that both IL-3R $\alpha$  and  $\beta_c$  were present in the 215,000- and 245,000-MW bands. In addition, the 2D SDS-PAGE suggested that higher-MW complexes were also formed. The presence of both IL-3R $\alpha$  and  $\beta_c$  in these complexes may account for the 215,000-MW band, but the 245,000-MW band may contain another protein in addition to IL-3R $\alpha$  and  $\beta_c$ . This accessory protein is not IL-3, as IL-3 is not disulfide linked to either receptor chain (see above). It is possible that a third, poorly iodinated surface protein or a small cytoplasmic protein becomes covalently linked to the IL-3R upon activation, but its presence and identity remain to be determined.

The presence of IL-3-induced disulfide-linked and non-disulfide-linked heterodimers suggests two types of IL-3R $\alpha$  and  $\beta_c$  interaction, a noncovalent one and one that is mediated by Cys-Cys bridging of the receptors. The reason for the presence of both types of complexes or the sequence of their appearance is not clear. It is possible that initially IL-3 binds to IL-3R $\alpha$  and the IL-3-IL-3R $\alpha$  complex binds  $\beta_c$ , forming a high-affinity complex. IL-3 high-affinity binding then triggers a noncovalent association, with both events being mediated by cytokine receptor module 2 (CRM2) in  $\beta_c$  (Fig. 10). The main function of these events may be then to bring IL-3R $\alpha$  and  $\beta_c$  into close proximity, thus facilitating a proportion of the dimers undergoing disulfide linkage through CRM1 of  $\beta_c$  (Fig. 10). These disulfide-linked dimers are therefore not required for high-affinity binding but instead are then involved in recruiting tyrosine kinases which phosphorylate dimerized  $\beta_c$  and intracellular proteins. Alternatively, the association of IL-3R $\alpha$  and  $\beta_c$  may be primarily covalent, but after SDS-PAGE, a propor-

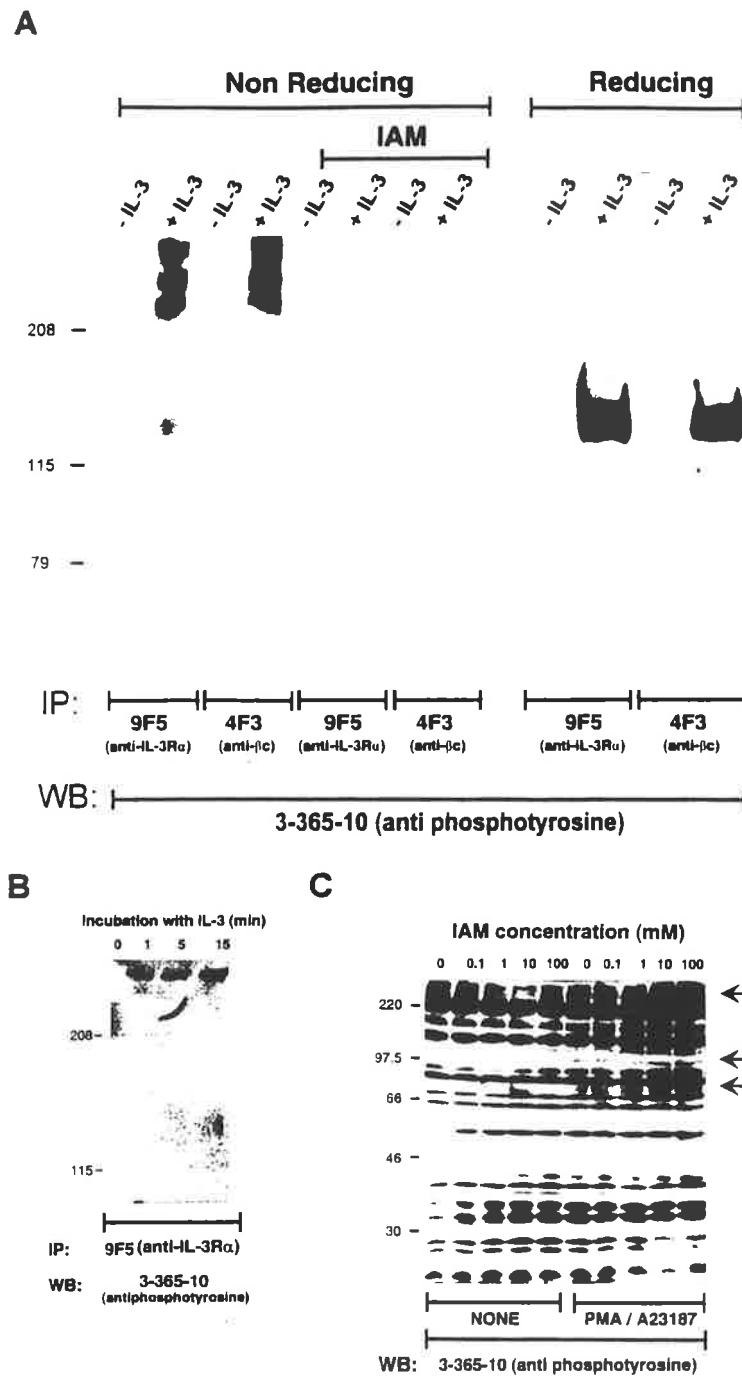


FIG. 9. Tyrosine phosphorylation of disulfide-linked receptor dimers. (A) Unlabelled CML cells were either not treated ( $-IL-3$ ) or incubated with  $IL-3$  ( $50$  nM) ( $+IL-3$ ) for  $30$  min at  $4^\circ C$  with or without the prior addition of iodoacetamide (IAM) and immunoprecipitated with MAb 9F5 (anti- $IL-3R_\alpha$ ) or MAb 4F3 (anti- $\beta_c$ ). The immunoprecipitates were separated under nonreducing or reducing conditions on SDS-6% polyacrylamide gels and probed with the antiphosphotyrosine antibody 3-365-10. (B) Unlabelled UT7 cells were incubated with  $50$  nM  $IL-3$  for different times at  $4^\circ C$ . After cell lysis, proteins were immunoprecipitated with MAb 9F5 (anti- $IL-3R_\alpha$ ) and separated under nonreducing conditions on an SDS-6% polyacrylamide gel and probed with the antiphosphotyrosine antibody 3-365-10. (C) Human UT7 cells were not treated or treated with various concentrations of iodoacetamide for  $20$  min at  $4^\circ C$ , and then either not stimulated or stimulated with phorbol myristate acetate ( $50$  ng/ml) and calcium ionophore A23187 ( $2$   $\mu M$ ) (PMA/A23187) for  $60$  min at  $4^\circ C$ . After cell lysis, proteins were separated under reducing conditions on SDS-12.5% polyacrylamide gels and probed with the antiphosphotyrosine antibody 3-365-10. The arrows indicate the positions of the newly induced proteins. MW (in thousands) of marker proteins are shown to the left of the gels. IP, immunoprecipitation; WB, Western blotting.

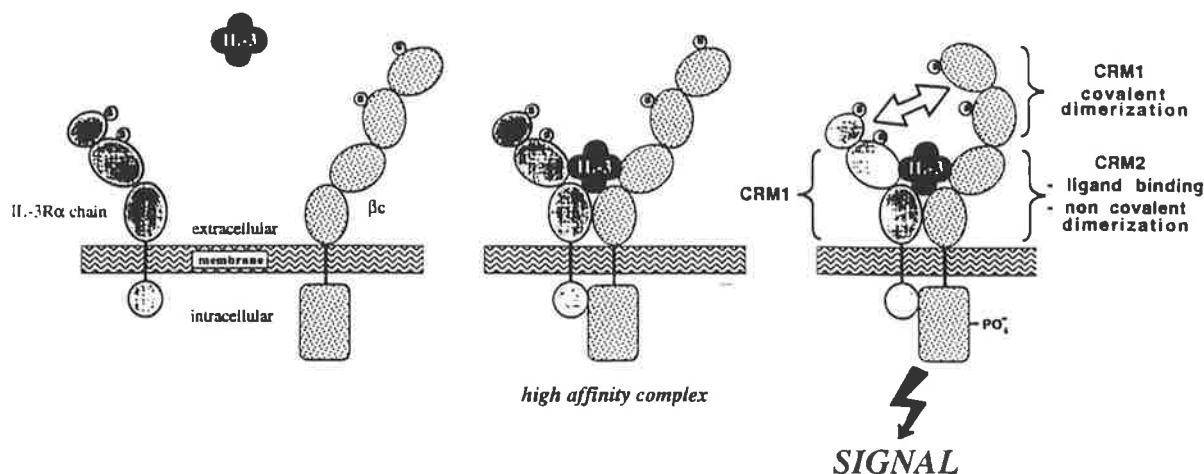


FIG. 10. Model of human IL-3R activation. In the absence of IL-3, IL-3R $\alpha$  and  $\beta_c$  are not associated on the cell surface. The presence of IL-3 triggers IL-3 binding to IL-3R $\alpha$  initially and then to  $\beta_c$ . IL-3 binding to  $\beta_c$  occurs through the cytokine receptor module CRM2 (55, 56) and triggers dimerization. Two complementary mechanisms of receptor heterodimerization are proposed: a noncovalent one probably involving the A-B loop in membrane-proximal domains of the CRM2 of each chain analogous to the growth hormone receptor (8) and a covalent association probably involving unpaired Cys residues in the N-terminal region and domain 1 of IL-3R $\alpha$  interacting with hitherto unpaired Cys residues in the CRM1 of  $\beta_c$  (see Discussion). IL-3 is associated with the receptor dimer but is not covalently attached to it. Disulfide-linked receptor heterodimerization leads to phosphorylation of the dimerized  $\beta_c$ , but not to phosphorylation of noncovalently associated  $\beta_c$  or IL-3R $\alpha$ , an event that leads to cellular activation.

tion of this complex is disrupted by intramolecular disulfide bond rearrangement.

We found that by preventing disulfide-linked dimer formation, it was possible to dissociate high-affinity binding from receptor activation. Thus, high-affinity binding could take place in the absence of receptor activation (Fig. 6 and 9). It may therefore be possible to construct IL-3 antagonists that retain full high-affinity binding but which are deficient at inducing disulfide-linked dimers. On the other hand, if high-affinity binding inevitably leads to disulfide-linked dimer formation and cellular activation, antagonism of IL-3 may rely largely on compounds that prevent disulfide-linked dimerization.

Two lines of evidence showed that disulfide-linked receptor dimerization is involved in receptor activation. First, the vast majority of the tyrosine-phosphorylated bands immunoprecipitated by anti-IL-3R $\alpha$  or anti- $\beta_c$  MAb corresponded to the disulfide-linked heterodimers (Fig. 9). This is even more remarkable when it is considered that while <17% of the IL-3-dependent IL-3R $\alpha$  and  $\beta_c$  heterodimers are disulfide linked, >94% of the tyrosine phosphorylation is associated with these dimers. Second, blocking of the thiol groups with iodoacetamide prevented receptor phosphorylation under conditions that did not affect cell viability and total cellular phosphorylation (Fig. 9C) or high-affinity binding (Fig. 6D), although we cannot rule out inactivation of JAK-2. This is analogous to the IL-6 receptor, where the presence of gp130 homodimers is accompanied by tyrosine phosphorylation of these proteins (32). Therefore, the disulfide-linked heterodimerization of IL-3R $\alpha$  and  $\beta_c$  appears to be essential for tyrosine phosphorylation, which is known to involve the Janus family of tyrosine kinases (43). Since both IL-3R $\alpha$  and  $\beta_c$  contain intracellular tyrosine residues, we examined whether both receptor chains were being phosphorylated by reduction of the disulfide-linked dimers with dithiothreitol and probing with antiphosphotyrosine antibodies. This revealed that  $\beta_c$  but not IL-3R $\alpha$  was the phosphorylated protein in the heterodimers (Fig. 9). This is consistent with preassociated JAK-2 causing phosphorylation of  $\beta_c$  (38, 43). It should be noted that although disulfide-linked

dimerization appears essential for receptor phosphorylation, it is possible that other functions are not affected, and it would be of interest to examine functions, such as proliferation, under conditions that prevent disulfide-linked dimer formation. The identification of the cysteines involved in disulfide formation and their mutation may reveal IL-3 activation pathways that occur independently of this process.

The IL-3-induced appearance of disulfide-linked IL-3R $\alpha$  and  $\beta_c$  dimers suggests the presence of free Cys in these molecules. Examination of the sequences of IL-3R $\alpha$  and  $\beta_c$  and modelling of the cytokine receptor modules (11) suggest that IL-3R $\alpha$  has two potentially unpaired Cys residues, one in the N-terminal domain at position 52, 68, or 76 and one at position 195 in domain 1 of the CRM (Fig. 10). Similarly,  $\beta_c$  has two potentially free Cys residues, one at position 91, 96, or 100 in domain 1 and one at position 234 in domain 2 of CRM1 (Fig. 10). It is interesting to note that, in contrast to the  $\alpha$  chains,  $\beta_c$  has two CRMs. While CRM2 has been implicated in binding IL-3, GM-CSF, and IL-5 (23, 56), the function of CRM1 is unknown. We hypothesize that this is important for disulfide linkage and suggest that, analogous to the cytoplasmic domain of  $\beta_c$  (42), the extracellular region can be viewed as having two functional domains, with CRM2 involved in ligand recognition and nondisulfide-linked heterodimerization (Fig. 10) and CRM1 involved in the final activation step which includes  $\alpha\beta$  disulfide formation.

It is also worth noting that the N-terminal regions of IL-3R $\alpha$ , GM-CSFR $\alpha$ , and IL-5R $\alpha$  are significantly conserved between each other and are present only in this family of receptors (11). Although the number of Cys residues in this region varies in IL-3R $\alpha$  (3 Cys), GM-CSFR $\alpha$  (5 Cys), and IL-5R $\alpha$  (1 Cys), all three  $\alpha$  chains exhibit one unpaired Cys. This raises the possibility that these unpaired Cys residues are involved in disulfide-linked dimerization and suggests a functional reason for the conservation of this N-terminal region in the IL-3R $\alpha$ , GM-CSFR $\alpha$ , and IL-5R $\alpha$  family.

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**Identification of a Cys motif in the common  $\beta$  chain of the IL-3, GM-CSF and IL-5 receptors  
essential for disulfide-linked receptor heterodimerization and activation of all three receptors \***

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## ABSTRACT

The human IL-3 and GM-CSF receptors undergo covalent dimerization of the respective specific  $\alpha$  chains with the common  $\beta_c$  subunit ( $\beta_c$ ) in the presence of the cognate ligand. We have now performed alanine substitutions of individual Cys residues in  $\beta_c$  to identify the Cys residues involved and their contribution to activation of the IL-3, GM-CSF and IL-5 receptors. We found that substitution of Cys 86, Cys 91 and Cys 96 in  $\beta_c$  but not of Cys 100 or Cys 234 abrogated disulfide-linked IL-3 receptor dimerization. However, whilst Cys 86 and Cys 91  $\beta_c$  mutants retained their ability to form non-disulfide-linked dimers with IL-3R $\alpha$ , substitution of Cys 96 eliminated this interaction. Binding studies demonstrated that all  $\beta_c$  mutants with the exception of C96A supported high affinity binding of IL-3 and GM-CSF. In receptor activation experiments we found that  $\beta_c$  mutants C86A, C91A and C96A but not C100A or C234A abolished phosphorylation of  $\beta_c$  in response to IL-3, GM-CSF or IL-5. These data show that whilst Cys 96 is important for the structural integrity of  $\beta_c$ , Cys 86 and Cys 91 participate in disulfide-linked receptor heterodimerization and that this linkage is essential for tyrosine phosphorylation of  $\beta_c$ . Sequence alignment of  $\beta_c$  with other cytokine receptor signalling subunits in the light of these data shows that Cys 86 and Cys 91 represent a motif restricted to human and mouse  $\beta$  chains suggesting a unique mechanism of activation utilized by the IL-3, GM-CSF and IL-5 receptors.

## INTRODUCTION

Cytokine receptor dimerization is a common theme in receptor activation (1). Following the binding of the cognate ligand to cytokine receptors a sequential process takes place whereby receptor subunits associate and recruit cytoplasmic signalling molecules leading to receptor activation and cellular signalling (2). The general process of receptor dimerization exhibits variations amongst the cytokine receptor superfamily and may involve homodimerization or heterodimerization events depending on receptor subunit composition (3,4). In the case of the growth hormone receptor, growth hormone binds initially to one receptor subunit and induces its homodimerization with a second identical subunit (5). A similar process probably takes place with erythropoietin and G-CSF leading in both cases to receptor homodimerization and activation (6,7).

With cytokine receptors that comprise multiple subunits, receptor activation is accompanied by homodimerization or heterodimerization of the signalling subunits. For example, in the IL-6 receptor system IL-6 induces dimerization of IL-6R $\alpha$  with gp130 (8), homodimerization of gp130 and receptor activation (9). On the other hand the binding of CNTF to CNTFR $\alpha$  induces its association with gp130 and the LIF receptor (LIFR) and the heterodimerization of gp130 and the LIFR is accompanied by receptor activation (10). Similarly, heterodimerization of IL-2R $\beta$  and  $\gamma$  subunits is necessary for IL-2 receptor activation (11,12). Interestingly, in these cases each receptor  $\alpha$  chain constitutes the major binding subunit but does not seem to form part of the signalling receptor complex.

The mechanism of activation of the GM-CSF/IL-3/IL-5 receptor system exhibits similar features to the mechanism employed by the above receptors although some unique features are becoming evident. One of the most important differences is the contribution that each receptor  $\alpha$  chain makes to signalling. This is manifested in two ways: firstly, unlike IL-6R $\alpha$ , CNTFR $\alpha$  and the IL-2R $\alpha$ , the cytoplasmic domains of GM-CSFR $\alpha$ , IL-3R $\alpha$  and IL-5R $\alpha$  are all required for full receptor activation and signalling (13-16). Secondly, IL-3R $\alpha$  and GM-CSFR $\alpha$  form disulfide-linked dimers with the common  $\beta$  chain ( $\beta_c$ ) of their receptor (4,17). The disulfide-mediated dimerization of IL-3R $\alpha$  with  $\beta_c$  and of GM-CSFR $\alpha$  with  $\beta_c$  is accompanied by tyrosine phosphorylation of  $\beta_c$  (4). In all these cases, however, tyrosine phosphorylation is observed in the disulfide-linked dimers as well as in the monomeric molecules, and hence it is not clear which is the critical species for receptor activation. Furthermore, the location of the cysteines involved in disulfide-linkage is not known, nor is it apparent whether they constitute a functionally conserved motif in the cytokine receptor superfamily. We have now performed single alanine substitutions of candidate cysteine residues in the N-terminal CRM of the IL-3, GM-CSF and IL-5 receptor  $\beta_c$  and examined their contribution to disulfide-linked receptor dimerization, high affinity ligand binding and receptor activation.

## MATERIALS AND METHODS

### Mutagenesis of human $\beta_C$ and expression plasmid constructs

Cysteine residues were substituted with alanines in the human  $\beta$  chain cDNA using oligonucleotide-directed mutagenesis (Altered-sites, Promega, Sydney, NSW, Australia) as described previously (18). The mutations were confirmed by nucleotide sequencing and the mutant  $\beta_C$  cDNAs subcloned into the eukaryotic expression vector *pcDNA1* (Invitrogen, San Diego, CA). The IL-3R, GM-CSFR and IL-5R  $\alpha$ -chain cDNAs were cloned into the eukaryotic expression vector *pCDM8* (Invitrogen) for transfection (18).

### Cell culture and DNA transfection

COS cells were maintained in RPMI-1640 supplemented with 10 % v/v fetal calf serum (FCS) and transfected by electroporation. Routinely  $2 \times 10^7$  COS cells were co-transfected in 0.8 ml PBS at 0°C with 25  $\mu$ g of wild type or mutated  $\beta_C$  cDNA together with 10  $\mu$ g of GM-CSFR and 10  $\mu$ g IL-3R  $\alpha$ -chain cDNA at 500  $\mu$ F with 300 V. After electroporation cells were centrifuged through a 1 ml cushion of FCS and cells were plated in either 25 ml of medium per 150 cm<sup>2</sup> flask or 24 well plates for binding analysis. Transfectants were incubated for 2 days prior to cytokine treatment (18).

The HEK293T cell line derived from the adenovirus type transformed human embryonic kidney 293 cell line, containing the simian virus 40 large tumour antigen (19) were maintained in RPMI-1640 medium supplemented with 10% v/v FCS. On the day before transfection,  $1.4 \times 10^6$  cells were plated into 6 cm tissue culture dishes to adhere overnight. Four hours after a medium change, 6  $\mu$ g of wild type or mutated  $\beta_C$  cDNA together with 4  $\mu$ g of GM-CSFR $\alpha$  or 4  $\mu$ g IL-3R $\alpha$  or 4  $\mu$ g IL-5R $\alpha$  cDNA and 0.5  $\mu$ g of JAK-2 cDNA were added to cells in the form of a calcium phosphate precipitate (20), and the cells were placed in an incubator for 4 h to permit the uptake of the DNA-calcium phosphate precipitate. The cells were then washed, replated in 4 plates/150 cm<sup>2</sup> and placed in the incubator for 48h prior to cytokine treatment.

### GM-CSF, IL-3 and IL-5

Recombinant human IL-3 and GM-CSF and IL-5 were produced in *E.coli* essentially as described before (21,22). Cytokine purity and quantitation was determined by HPLC analysis. The unit activity of the cytokines based on the ED<sub>50</sub> values in a TF-1 proliferation assay (23) was 0.03ng/ml GM-CSF, 0.1 ng/ml IL-3 and 0.3 ng/ml IL-5 equal to 1 unit respectively.

### Radiolabelling cytokines

Recombinant IL-3, and GM-CSF were radio-iodinated by the iodine monochloride method (24) to a specific activity of about 36 mCi/mg. Routinely 4  $\mu$ g of protein was iodinated and separated from iodide ions on a Sephadex G-25 column (Pharmacia, North Ryde, NSW, Australia), eluted with PBS containing 0.02% v/v Tween 20, and the iodinated proteins stored at 4°C for up to 4 weeks.

### Saturation binding assays

Binding assays were performed on confluent monolayers in 24 well plates over a concentration range of 10pM - 10nM <sup>125</sup>I-labelled GM-CSF or IL-3 in binding medium [RPMI containing 0.5% (w/v) BSA/0.1% (w/v) sodium azide] essentially as described previously (25). After incubation at room temperature for 2 hours, radioligand was removed and the cells briefly washed twice in binding medium. Specific counts were determined after lysis of the cell monolayer with subsequent transfer and counting on a  $\gamma$ -counter (Cobra Auto Gamma; Packard Instruments Co, Meriden, CT). Dissociation constants were calculated using the EBDA and LIGAND programs (26) (Biosoft, Cambridge, U.K.).

### Monoclonal antibodies to the IL-3, GM-CSF and IL-5 receptors

Monoclonal antibodies directed against GM-CSFR $\alpha$ , IL-3R $\alpha$ , IL-5R $\alpha$  or  $\beta_C$  were generated as previously described (27) and purified and characterized as detailed elsewhere (17,18,27). The monoclonal antibodies 8E4, 4H1, 9F5 and A14 were selected for their ability to specifically immunoprecipitate  $\beta_C$ , GMR $\alpha$  and IL-3R $\alpha$  and IL-5R $\alpha$  respectively. The monoclonal antibody 1C1 conjugated to biotin was used for immunoblotting  $\beta_C$  and 8E4, 4H1, 9F5 and A14 were used for cell

surface expression staining for  $\beta_C$ , GMR $\alpha$ , IL3R $\alpha$  and IL5R $\alpha$  respectively. The monoclonal antibodies were purified from ascites as described (27). The MAb against phosphorylated tyrosine was the peroxidase conjugated anti-phosphotyrosine 3-365-10 (Boehringer Mannheim, Rose Park, S. Australia).

#### **Analysis of receptor cell surface expression by flow cytometry**

Cell surface expression of transfected receptor subunits was confirmed by indirect immunofluorescence staining using anti-receptor  $\alpha$  and  $\beta$  chain specific monoclonal antibodies. Staining was performed as described previously (17) and analysed with a EPICS Profile II Flow Cytometer (Coulter Electronics, Hialeah, FL).

#### **Surface labelling of cells and immunoprecipitation.**

COS cells were cell-surface labelled with  $^{125}\text{I}$  by the lactoperoxidase method as described previously (28). Approximately  $10^8$  cells were washed twice in PBS and then labelled with 1mCi  $^{125}\text{I}$  (NEN, AMRAD Pharmacia Biotech, Boronia, Vic, Australia) in PBS. Cells were lysed in lysis buffer consisting of 137mM NaCl, 10mM Tris-HCl (pH 7.4), 10% glycerol, 1% Nonidet P-40 (NP40) with protease inhibitors (10 $\mu\text{g}/\text{ml}$  leupeptin, 2mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$  aprotinin) and 2mM sodium vanadate for 30 mins at 4°C followed by centrifugation of the lysate for 15 mins at 12,000g 4°C. Following a 1 hour pre-clearance with Protein-A-sepharose (Pierce, Rockford, IL) at 4°C, the supernatant was incubated for 18 hours with 5 $\mu\text{g}/\text{ml}$  antibody. Protein-immunoglobulin complexes were captured by incubation for 1 hour with Protein-A-sepharose followed by 6 subsequent washes in lysis buffer. Samples were boiled for 10 mins in SDS sample load buffer either in the presence or absence of 2-mercaptoethanol (ie. reducing or non-reducing) before separating immunoprecipitated proteins by SDS-PAGE. Immunoprecipitation from HEK293T cells was carried out similarly except the cells were not surface labelled.

#### **SDS-Polyacrylamide Gel Electrophoresis**

Immunoprecipitated proteins were analysed by SDS-PAGE on polyacrylamide gels. Samples were boiled in SDS loading buffer for 5 minutes prior to loading. Molecular weights (MW) were estimated using SeeBlue<sup>TM</sup> Pre-Stained Standards (Novex French's Forest, NSW, Australia). Radiolabelled proteins were visualised using an ImageQuant Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

#### **Immunoblot and ECL**

Immunoprecipitated proteins separated by SDS-PAGE were transferred to nitrocellulose membrane by electroblotting. Routinely, nitrocellulose membranes were blocked in a solution of PBS/0.05% (v/v) Tween 20 (PBT) containing 1% (w/v) blocking reagent 1096 176 (Boehringer Mannheim) and probed with either peroxidase-conjugated anti-phosphotyrosine 3-365-10 (Boehringer Mannheim) or anti- $\beta_C$  (1C1-biotin) followed by Streptavidin-POD (Boehringer Mannheim). Immunoreactive proteins were detected by chemiluminescence using the ECL kit (Amersham, Little Chalfont, U.K.) following the manufacturer's instructions.

## **Results**

### **Rationale for mutagenesis of N-terminal Cysteine residues of $\beta_C$**

In order to study the molecular events involved in the activation of the IL-3, GM-CSF and IL-5 receptors, we replaced several extracellular cysteine residues of  $\beta_C$  by alanine residues. So as to target Cys available for intermolecular interactions, we sought to avoid Cys involved in structurally-important intramolecular disulfide bonds. By homology with other cytokine receptors, domains one and three are expected to possess two disulfide bonds each. This is clearly the case with domain three which contains only four Cys residues. However, domain one  $\beta_C$  possesses seven Cys of which only Cys 34, Cys 45 and Cys 75 could be aligned readily with equivalent Cys in other receptors (Fig 1). Of the remaining Cys residues, Cys 86, Cys 91 and Cys 96 are conserved in murine  $\beta_C$ . Of these, Cys 96 is followed by an Ile at position 98 which aligns with conserved hydrophobic residues in other cytokine receptors suggesting that is this Cys that is part of the second conserved disulfide bond. Cys 96, Cys 91 are proposed to lie in an extended loop between the D and E beta strands of the first domain. Although

Cys 86 and Cys 91 were favoured candidates for intermolecular disulfide bond formation, we chose to also mutate the nearby Cys 96 and Cys 100 and the single Cys residue in domain 2 at position 234.

### **Mutation of Cys 86 and Cys 91 selectively disrupt ligand-induced, disulfide-linked heterodimer formation**

Expression plasmids encoding IL-3R  $\alpha$  and wild type (wt) or C86A, C91A, C96A, C100A, and C234A mutant  $\beta_c$  were co-transfected into COS cells. After 48h the cells were  $^{125}\text{I}$  surface-labelled and either left unstimulated or stimulated with IL-3. IL-3R $\alpha$  and  $\beta_c$  were then immunoprecipitated with specific monoclonal antibodies (MAb) 9F5 or 8E4 respectively, and the proteins resolved by 6% SDS-PAGE under either nonreducing or reducing conditions. We found that the  $\beta_c$  mutants C100A and C234A behaved very similarly to wt  $\beta_c$ . Both mutants allowed the formation of two high molecular weight complexes in response to IL-3 (Fig. 2A), which, as with wt  $\beta_c$ , contain IL-3R $\alpha$  and  $\beta_c$  (17), and data not shown). These two complexes were immunoprecipitated by both anti-IL-3R $\alpha$  MAb 9F5 and anti- $\beta_c$  MAb 8E4 (Fig. 2A). In the absence of IL-3, the anti-IL-3R $\alpha$  MAb 9F5, immunoprecipitated only monomeric IL-3R $\alpha$  whereas the anti- $\beta_c$  MAb 8E4 immunoprecipitated monomeric  $\beta_c$  as well as the high molecular weight complex corresponding to disulfide-linked  $\beta_c$  homodimers (4,17) (Fig. 2A). As with the disulfide-linked dimers, the noncovalent IL-3R $\alpha$  and  $\beta_c$  heterodimers were not affected by mutating Cys 100 or 234 as both the anti-IL-3R $\alpha$  MAb and the anti- $\beta_c$  MAb co-immunoprecipitated both IL-3R $\alpha$  and  $\beta_c$  and only in the presence of IL-3 (Fig. 2B).

In contrast, the mutants C86A, C91A and C96A had a profound effect on disulfide-linked receptor dimerization. In the presence of IL-3, anti-IL-3R $\alpha$  MAb 9F5 and anti- $\beta_c$  MAb 8E4 did not immunoprecipitate the high molecular weight complexes corresponding to IL-3R $\alpha$  and  $\beta_c$  heterodimers (Fig. 2A). In fact, under non-reducing conditions very little or no monomeric  $\beta_c$  was immunoprecipitated by either MAb with most of the label being observed in the high molecular weight region probably representing aggregated  $\beta_c$ . With the anti-IL-3R $\alpha$  MAb 9F5 a non specific band migrating slightly faster than  $\beta_c$  was seen (Fig. 2A). Under reducing conditions, however, monomeric  $\beta_c$  could be detected (Fig. 2B). An important difference was noted between the C86A and C91A mutants on one hand and the C96A mutant on the other hand. Anti-IL-3R $\alpha$  MAb 9F5 co-immunoprecipitated C86A and C91A in the presence of IL-3 but did not co-immunoprecipitate C96A (Fig. 2). Reciprocally, in the presence of IL-3, the anti- $\beta_c$  MAb co-immunoprecipitated IL-3R $\alpha$  with C86A and C91A but not with C96A (Fig. 2). This was more clearly seen under reducing conditions (Fig. 2B) than non reducing conditions (Fig. 2A) where an overall lower signal was observed.

To verify that the surface expression levels of the individual  $\beta_c$  mutants was similar to  $\beta_c$  wt, COS cells transfected with the various constructs were analysed by flow cytometry (FACS). FACS analysis indicated that the surface expression of wt  $\beta_c$  and the Cys $\rightarrow$ Ala  $\beta_c$  mutants was very similar both in terms of percentage of transfected cells expressing the different receptor subunits and in absolute levels (Fig. 3) suggesting that the mutations did not affect subunit transport and expression at the cell surface.

### **The $\beta_c$ mutants C86A and C91A do not disrupt IL-3 and GM-CSF high affinity binding**

We next examined the ability of Cys mutants of  $\beta_c$  to support high affinity IL-3 or GM-CSF binding. COS cells were transfected with IL-3R $\alpha$  and GM-CSFR $\alpha$  and either wt  $\beta_c$  or the Cys $\rightarrow$ Ala  $\beta_c$  mutants and subjected to saturation binding studies with  $^{125}\text{I}$ -IL3 and  $^{125}\text{I}$ -GM-CSF. Scatchard transformation of the saturation binding curves were performed and the  $K_d$  and receptor numbers determined using the Ligand program. We found that  $\beta_c$  bearing Cys $\rightarrow$ Ala substitutions at positions 86, 91, 100 and 234 were able to form high affinity binding sites (Fig 4 and Table 1). The range of affinities for IL-3 high affinity binding of these Cys $\rightarrow$ Ala  $\beta_c$  substitution mutants varied from 31 to 280 pM compared with 330 pM for wt  $\beta_c$ , while GM-CSF high affinity binding ranged from 27 to 230 pM compared with 120 pM for wild type  $\beta_c$ . In contrast, COS cell transfectants expressing the C96A  $\beta_c$  showed no detectable high affinity binding (Fig. 4 and Table 1).

Although  $\beta_c$  mutants C86A and C91A were able to support high affinity binding of IL-3 and GM-CSF, a reduction in the number of high affinity receptors was observed compared with wild type  $\beta_c$  and the C100A and C234A analogues (Fig. 4 and Table 1). This is probably a reflection of the tendency of C86A and C91A  $\beta_c$  analogues to oligomerize as observed in the immunoprecipitations under

non-reducing conditions (Fig. 2A), thereby reducing the amount of free  $\beta_c$  available for interaction with  $\alpha$  chain.

### **C86A and C91A abolish IL-3-, GM-CSF- and IL-5-dependent tyrosine-phosphorylation of $\beta_c$**

It has been previously established that stimulation of cells with IL-3 leads to the formation of disulfide-linked heterodimers of IL-3 receptor  $\alpha$  and  $\beta_c$  chain, which is associated with phosphorylation of  $\beta_c$  (17). Similarly, in the case of the GM-CSF receptor, receptor heterodimerization occurs upon stimulation with GM-CSF and this is accompanied by  $\beta_c$  phosphorylation (4). Here we show that as well as the IL-3 and GM-CSF receptors, the IL-5 receptor also forms disulfide-linked complexes which are similarly accompanied by  $\beta_c$  phosphorylation (Fig. 5). The relative proportion of phosphorylated  $\beta_c$  in the disulfide-linked heterodimer and in monomeric  $\beta_c$  varied between the three receptors. This may be due to kinetic differences in receptor assembly or in the stability of each receptor heterodimer. We have now taken advantage of the inability of  $\beta_c$  mutants to form disulfide-linked heterodimers to determine whether the formation of these is a necessary for receptor activation or whether noncovalent dimerization is sufficient for activation as measured by  $\beta_c$  phosphorylation. We transfected wt  $\beta_c$  and the different  $\beta_c$  mutants in HEK293T cells together with JAK-2 and either IL-3R $\alpha$ , GM-CSFR $\alpha$  or IL-5R $\alpha$  chain cDNA. After 48h the cells were either not treated or treated with IL-3, GM-CSF or IL-5, lysed and immunoprecipitated with MAb 8E4 anti- $\beta_c$ . The immunoprecipitates were separated on SDS-PAGE gels under reducing conditions and western blotted with antiphosphotyrosine antibody. Mutants C100A and C234A and wt  $\beta_c$ , which heterodimerize with the receptor  $\alpha$  chain in a disulfide-linked manner in response to ligand, showed phosphorylation of  $\beta_c$ . In contrast, mutants C86A, C91A and C96A which have lost the ability to heterodimerize in a disulfide-linked manner in response to ligand have lost the potential to be phosphorylated in response to IL-3, GM-CSF or IL-5 (Fig. 6).

The expression of all mutants compared with wt was monitored by flow cytometry and by western blot analysis with antibodies to  $\beta_c$  and indicated that the levels were very similar between all the mutants (Fig. 3 and Fig. 5D). Since the number of high affinity sites for mutants C86A and C91A was decreased, the lack of phosphorylation with these two mutants could have been the result of a decrease in sensitivity due to less mutant  $\beta_c$  being heterodimerized compared with the total amount of mutant  $\beta_c$  expressed. To address this possibility we have examined only  $\beta_c$  heterodimerized to the IL-3R $\alpha$  by immunoprecipitating with IL-3R $\alpha$  antibody and western blotting with antiphosphotyrosine antibody. The results were identical to those seen when the  $\beta_c$  was directly immunoprecipitated, indicating that cysteines in position 86 and 91 are essential for receptor tyrosine phosphorylation (Fig. 7)

## **DISCUSSION**

We show here that disulfide-linked heterodimerization of the GM-CSF, IL-3 and IL-5 receptors is essential for receptor activation by the cognate ligand. Furthermore we have identified Cys 86 and Cys 91 in the N-terminal domain of  $\beta_c$  as the key Cys involved in heterodimerization with the  $\alpha$  chain of each receptor. Comparison with other cytokine receptors indicates that these Cys constitute a conserved motif present only in human and mouse  $\beta_c$  and in  $\beta_{IL-3}$  suggesting that it subserves a specialized function restricted to the GM-CSF, IL-3 and IL-5 receptor family.

We have previously shown that the human IL-3 and GM-CSF receptors undergo both non covalent and disulfide-linked dimerization upon ligand binding (4). We have now extended these observations to the IL-5 receptor demonstrating that disulfide-linked dimerization is a common theme in this receptor subfamily. To identify the Cys residues in  $\beta_c$  responsible for disulfide linkage with the IL-3, GM-CSF and IL-5 receptor  $\alpha$  chains we mutagenized five of the eight Cys residues in the N-terminal CRM of  $\beta_c$  which, from alignment with other cytokine receptors, represented the best candidates for intermolecular interactions. We found a range of sensitivities to mutation of these Cys residues that could be correlated with their interspecies and interreceptor conservation.

The first class of Cys residue is exemplified by Cys 100 and Cys 234. These residues are not conserved with even the closely-related mouse  $\beta$ -chains and we find no phenotype on replacing them with alanine residues. The second class is represented by Cys 96 which is apparently a conserved residue in both the mouse  $\beta$ -chains and the cytokine receptor family at large (29) and is inferred to be involved in a structurally-conserved disulfide bond. This residue is apparently required for the

structural integrity of the first domain of  $h\beta_c$  if not the entire extracellular portion of the molecule. Although the C96A mutation permitted cell-surface expression of  $h\beta_c$ , it did not support high affinity binding of GM-CSF or IL-3 despite the substitution being well removed in sequence, and presumably spatially distant, from the fourth domain of the receptor that encompasses the majority of the ligand-recognition determinants (18,25). The exact molecular basis for this observation is uncertain but may be related to sequestration of  $h\beta_c$  into very large aggregates (Fig 2A) that obscure the ligand-contact site.

The third and most interesting class of cysteine mutation is that of C86A and C91A, analogues that have lost their ability to form disulfide-linked heterodimers but still retain the ability to associate noncovalently with the  $\alpha$  subunit upon stimulation with ligand (Fig 2). Although these mutants exhibited some propensity to aggregate in the absence of stimulation, they retain the ability to interact with ligand as judged by their ability to support high affinity binding. Importantly, these analogues are deficient in phosphorylation of tyrosine residues in  $\beta_c$ , however receptor-mediated functions and downstream signalling remains to be ascertained.

The observation of identical phenotypes with either mutation C86A or C91A suggests that these residues may cooperate functionally in the native receptor such as via formation of an additional intramolecular disulfide. This is consistent with our molecular modelling of  $\beta_c$  which suggests that Cys 86 and Cys 91 are sufficiently close to allow the formation of such a disulfide bond (unpublished observations). In the presence of ligand this bond is proposed to undergo disulfide exchange with a free sulfhydryl group from the  $\alpha$ -chain that is brought into proximity via ligand-dependent noncovalent association.

Previous experiments have noted a correlation between disulfide-linked receptor dimerization and receptor activation. IL-6 induces covalent dimerization of two molecules of gp130 (9), and CNTF induces covalent dimerization of gp130 with the LIF receptor (10). Similarly, IL-3 (Fig 5a), GM-CSF (Fig 5b), and IL-5 (Fig 5c) induce covalent dimerization of  $\beta_c$  with the corresponding  $\alpha$  chain. In all these cases concomitant phosphorylation of the receptor has been observed, however, a causal relationship has not been established. The use of the C86A and C91A mutants allowed us to demonstrate that non-covalent receptor associations are not sufficient for receptor tyrosine phosphorylation and that this requires disulfide-linkage of receptor subunits. The role of covalent dimerization seems to be to ensure concomitant dimerization of the cytoplasmic portions of  $\beta_c$  facilitating transphosphorylation of associated kinases of the JAK family. Indeed, experiments using chimeric receptors that cause artificial dimerization of the cytoplasmic portions of  $\beta_c$  lead to their activation (30,31) and direct dimerization of JAK has been shown to transduce a growth signal (32). Although they are essential for normal activation (14-16), the role of the cytoplasmic domains of the receptor  $\alpha$ -chains remains unclear although they may serve to orientate to  $\beta$ -chains so as to juxtapose correctly the JAK molecules or to participate directly in certain functions (33,34).

The  $\beta$ -chain forms intermolecular disulfides specifically with the  $\alpha$ -chains of the GM-CSF, IL-3 or IL-5 receptors. A common feature of these  $\alpha$  chains is the presence of an N-terminal FnIII-like domains of a type restricted to this subfamily of cytokine receptors. Within this N-terminal domain, all three receptor  $\alpha$  chains possess an uneven number of Cys residues in the N-terminal domain suggesting that these Cys residues are the most likely candidates to act as partners for  $\beta_c$ . The IL-5R $\alpha$  has only a single Cys residue in the N-terminal domain, at position 86, and this residue has been shown to be important for IL-5 binding to the receptor (35).

The stoichiometry of the IL-3, GM-CSF, IL-5 receptor complexes is not known. The formation of an intermolecular disulfide bond between Cys 86 or Cys 91 of  $\beta_c$  and a Cys in the N-terminal domain of a receptor  $\alpha$ -chain could occur potentially with either the  $\alpha$ -chain with which it shares ligand or a second  $\alpha$ -chain recruited as part of a hexameric complex as seen with the IL-6 receptor (36). Since the individual FnIII-like domains of the receptor  $\alpha$ -chains and  $\beta_c$  are likely to be fairly rigid units of length 3.5 to 4.5 nm, the ability of  $\beta_c$  to contact  $\alpha$ -chain will depend on the interdomain angles that they can adopt. The receptor  $\alpha$ - and  $\beta_c$  chains are class 1 cytokine receptors and the angles observed between the two domains of the CRM in the known structures of this family, GHR (5) and EPOR (37) are approximately 90°. It is therefore reasonable to infer that the angles between domains 1&2 and 3&4 of

$\beta_c$  and between domains 2&3 of the receptor  $\alpha$ -chains will also be approximately 90°. The conformation of the linker peptides between domains 2 and 3 of  $\beta_c$  or between domains 1 and 2 of the receptor  $\alpha$ -chains cannot be gauged by reference to homologous structures. Even if the linker peptides permitted the membrane-distal portions to fold back over the cytokine-binding portions of the receptors, this would be unlikely to facilitate a sufficiently close approach of Cys residues to allow formation of the observed intermolecular disulfide bonds. Rather, we propose that the intermolecular disulfide bond forms between  $\beta_c$  and an  $\alpha$ -chain from a second receptor heterodimer (Fig 8) as this can be accommodated readily with respect to both the sizes of the domains and their interdomain angles.

Based on the likely orientation of their N-terminal domains with respect to the CRM of the  $\alpha$ -chains, we favour a receptor complex arranged clockwise when viewed from outside the cell in the order  $\alpha$ -chain<sub>1</sub>/ligand<sub>1</sub>/ $\beta$ -chain<sub>1</sub>S-S $\alpha$ -chain<sub>2</sub>/ligand<sub>2</sub>/ $\beta$ -chain<sub>2</sub>. The disulfide-linkage of  $\beta_c$  in one receptor heterodimer to an  $\alpha$  chain in a second receptor heterodimer would facilitate juxtaposing two  $\beta_c$  molecules with their associated JAK kinases and induce receptor phosphorylation. This initial association may also facilitate the formation of a second disulfide between  $\beta_c$  in receptor 2 and an  $\alpha$  chain in receptor 1 (Fig 8A and B). The formation of a 2:2:2 complex is also consistent with the requirement of two  $\alpha$  chains in an active ligand-receptor complex (38). On the other hand a 1:1:1 stoichiometry has been suggested from experiments using chimeras of  $\alpha$  and  $\beta_c$  with fos and jun leucine zippers although the formation of higher order complexes was not excluded (34). The direct measurement of  $\alpha$ - $\beta_c$  interactions in solution may ultimately resolve this question.

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## FIGURE LEGENDS

**Figure 1 Alignment of domain 1 of the cytokine receptor module (CRM) present in the common  $\beta$  chain of the GM-CSF, IL-3 and IL-5 receptors and other signalling subunits of the cytokine receptor superfamily.** Four conserved Cys form the basis of the alignment, with the second Cys followed by a conserved Trp, and the fourth Cys followed by a hydrophobic residue at the i+2 position. The sequences of human as well as mouse  $\beta_c$  and  $\beta_{IL-3}$  are shown with the numbering corresponding to the human sequence with residue 1 being the initiation Met. Human and mouse  $\beta$  subunits are aligned with human gp130, the IL-2 receptor (R)  $\beta$  and  $\gamma$  chains, the erythropoietin (EPO)R, and the growth hormone (GH)R. The dashes represent spaces introduced to optimize the alignment.

**Figure 2 Substitutions of Cys 86 and Cys 91 in  $\beta_c$  abolish disulfide-linked IL-3R $\alpha$  and  $\beta_c$  heterodimerization without affecting their noncovalent association.** COS cells transfected with IL-3R $\alpha$  and either wild type or mutant  $\beta_c$  C86A, C91A, C96A, C100A and C234A were  $^{125}$ I-surface labelled and incubated in medium without (-IL-3) or with 6.5 nM IL-3 (+IL-3) for 5 min at 4°C. After cell lysis, proteins were immunoprecipitated with MAb 9F5 (anti IL-3R $\alpha$ ) or MAb 8E4 (anti  $\beta_c$ ). The immunoprecipitated proteins were separated either under non reducing conditions on an SDS-6% polyacrylamide gel (A), or under reducing conditions on an SDS-7.5% polyacrylamide gel (B) and visualised by PhosphorImaging.

**Figure 3 Surface expression of IL-3R $\alpha$ , GM-CSFR $\alpha$  and wild type or mutant  $\beta_c$  transfected into COS cells as measured by flow cytometry.** COS cells expressing both IL-3R $\alpha$  and GM-CSFR $\alpha$  chains together with wild type or mutant  $\beta_c$  C86A, C91A, C96A, C100A and C234A were stained with negative control antibody mouse IgG1 (control), MAb 9F5 (anti IL-3R $\alpha$ ), MAb 4H1 (anti GM-CSFR $\alpha$ ) and MAb 8E4 (anti- $\beta_c$ ).

**Figure 4 High affinity  $^{125}$ I-IL-3 and  $^{125}$ I-GM-CSF binding using Cys $\rightarrow$ Ala  $\beta_c$  mutants.** COS cells expressing both IL-3R $\alpha$  and GM-CSFR $\alpha$  chains together with wild type  $\beta_c$  (open circles) or  $\beta_c$  mutants (filled circles) containing different Cys $\rightarrow$ Ala substitutions were subjected to Scatchard transformation of saturation binding curves. The derived values are shown in Table 1.

**Figure 5 Ligand-induced disulfide-linked IL-3, GM-CSF and IL-5 receptor dimerization results in phosphorylation of  $\beta_c$ .** TF1.8 cells were either incubated with medium alone (-) or stimulated with medium containing 6.5 nM IL-3 (A), 6.5 nM GM-CSF (B) or 6.5 nM IL-5 (C) for 5 minutes at 37°C. After cell lysis, proteins were immunoprecipitated with anti- $\beta_c$  MAb 8E4 and the immunoprecipitates were separated under non reducing conditions on an SDS-7.5% polyacrylamide gel and transferred onto nitrocellulose filters. The filters were then probed either with MAb 1C1 anti- $\beta_c$ , or anti-phosphotyrosine antibody 3-365-10.

**Figure 6 Alanine substitutions Cys 91, Cys 96 and Cys 100 of  $\beta_c$  abolish IL-3, GM-CSF and IL-5 induced tyrosine phosphorylation of  $\beta_c$ .** HEK293T cells transfected with either IL-3R $\alpha$  (A), GM-CSFR $\alpha$  (B), or IL-5R $\alpha$  (C) together with wild type or mutant  $\beta_c$  were incubated with either medium alone (-) or with medium containing 6.5 nM IL-3 (+IL-3), 6.5 nM GM-CSF (+GM-CSF) or 6.5 nM IL-5 (+IL-5) for 5 min at 4°C. After cell lysis, proteins were immunoprecipitated with anti  $\beta_c$  MAb 8E4 and the immunoprecipitates were separated under reducing conditions on an SDS-7.5% polyacrylamide

gel transferred to nitrocellulose and probed with anti-phosphotyrosine antibody 3-365-10 (A, B and C). To control for the amount of  $\beta_c$  present in the filters were also probed with anti  $\beta_c$ , MAb and 1C1 (D).

**Figure 7 Ligand-induced heterodimers of IL-3R $\alpha$  and  $\beta_c$  with C86A and C91A substitution lack tyrosine phosphorylation of  $\beta_c$ .** HEK293T cells transfected with IL-3R $\alpha$  together with either wild type or mutant  $\beta_c$  were incubated with medium alone (-) or with medium containing 6.5 nM IL-3 (+IL-3) for 5 min at 4°C. After cell lysis, proteins were immunoprecipitated with MAb 9F5 (IL-3R $\alpha$ ) and the immunoprecipitates were separated under reducing conditions on a SDS-7.5% polyacrylamide gel, transferred onto nitrocellulose and probed with antiphosphotyrosine antibody 3-365-10 (A). To control for similar amounts of immunoprecipitated  $\beta_c$  the filters were also probed with MAb 1C1 anti  $\beta_c$  (B).

**Figure 8 Proposed model for assembly of GM-CSF-, IL-3- and IL-5-induced receptor complexes.** The binding of GM-CSF, IL-3 and IL-5 to GMR $\alpha$ -, IL-3R $\alpha$ - or IL-5R $\alpha$ -chain respectively induces  $\alpha$ : $\beta_c$  heterodimerization and a conformational change in  $\alpha$  chain that allows its disulfide linkage to  $\beta_c$ . Modelling of  $\beta_c$  suggests that this bridging would only be possible if the unpaired cysteines in the  $\alpha$  chain N-terminal domain (Nt) of receptor 1 formed a disulfide bridging with cysteine at position 86 or 91 in domain 1 (D1) of  $\beta_c$  in receptor 2. The bringing together of two  $\beta_c$  with their associated JAK-2 molecules would then lead to receptor activation. A cartoon model representing the side view is shown in (A) and a top view in (B)

**Table 1 Effect of alanine substitutions of Cys 86, 91, 96, 100 and 234 of  $\beta_c$  on IL-3 and GM-CSF high affinity binding**

		IL-3		GM-CSF	
		Kd1 (pM)	Number/cell	Kd1	Number/cell
Wt $\beta_c$	Exp 1	330 ± 25.3	7230 ± 409	119 ± 44.5	4700 ± 1570
	Exp2	261 ± 29.6	7110 ± 662	40.9 ± 6.0	2770 ± 265
C86A	Exp 1	41.9 ± 15.5	289 ± 60	63 ± 18	723 ± 169
	Exp 2	45.6 ± 3.5	1325 ± 71	28.5 ± 11.0	1108 ± 313
C91A	Exp 1	31.0 ± 8.9	530 ± 79	27.4 ± 3.9	421 ± 42
	Exp 2	35.8 ± 5.1	1040 ± 94	44.6 ± 123.1	1340 ± 1610
C96A	Exp 1	-	0	-	0
	Exp 2	-	0	-	0
C100A	Exp 1	277 ± 15.7	7830 ± 241	230 ± 43.3	8790 ± 1450
	Exp 2	270 ± 36.0	13500 ± 1337	25.6 ± 5.4	3310 ± 374
C234A	Exp 1	279.2 ± 22.0	10100 ± 566	164 ± 30.5	9270 ± 1450
	Exp 2	193 ± 19.3	11800 ± 710	90 ± 8.1	2650 ± 156

COS cells were transfected with IL-3R $\alpha$ , GM-CSFR $\alpha$  and either wild type  $\beta_c$  or mutated  $\beta_c$  carrying alanine substitutions of Cys at positions 86, 91, 96, 100 and 234 and subjected to saturation binding studies with <sup>125</sup>I-IL-3 and <sup>125</sup>I-GM-CSF. The radioiodinated ligand concentration for both IL-3 and GM-CSF ranged from 10 pM to 10  $\mu$ M. Non specific binding was determined in the presence of 1  $\mu$ M unlabelled ligand. Scatchard transformation of the saturation binding curves were performed and the Kd and receptor numbers determined using the LIGAND program. In the case of IL-3 due to the extreme low affinity of IL-3R $\alpha$ , the affinity was estimated to be 50 nM based on our own previous studies (25). In the case of GM-CSF binding a two site fit was statistically preferred (p<0.05) with all the  $\beta_c$  constructs except for C96A in which no high affinity sites were detected. Values from two representative experiments are shown. Experiment 2 is the same as the experiment shown in Fig 4.



Fig. 3

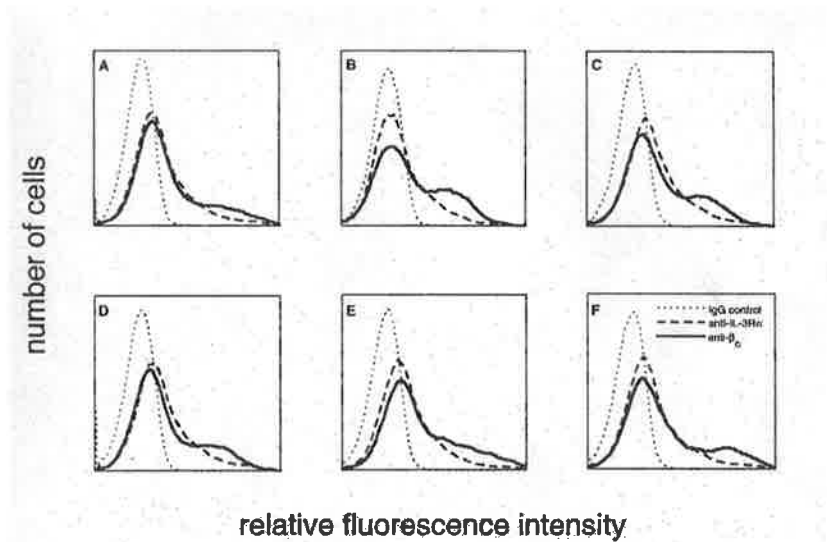


Fig. 4

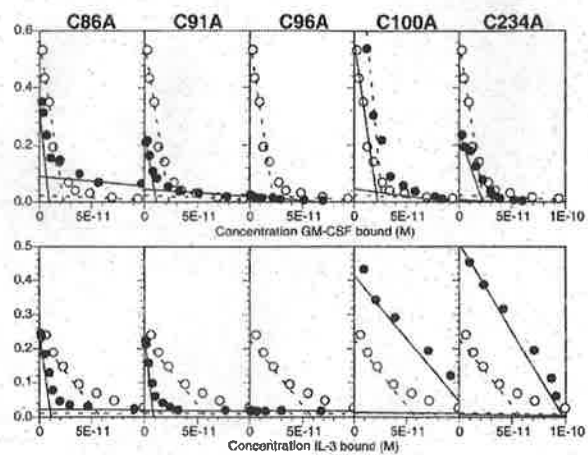


Fig. 5

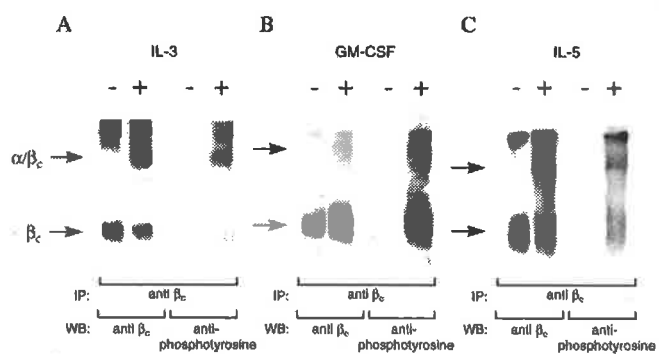


Fig. 6

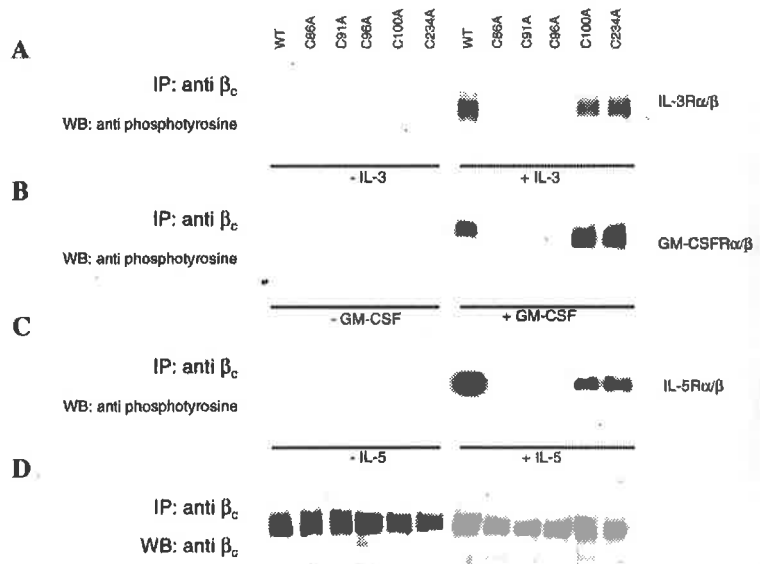


Fig. 7

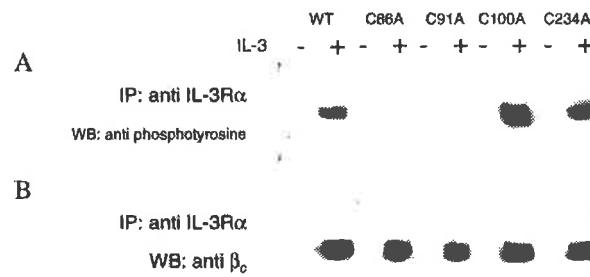


Fig. 8

