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Molecular Assembly of the Activated Granulocyte-Macrophage Colony-Stimulating Factor Receptor

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ABSTRACT

Human granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine that controls the normal production and functional activation of myeloid blood cells and has been implicated in diseases including myeloid leukemia, asthma and arthritis. GM-CSF mediates its effects by binding to specific cell surface receptors expressed on responsive cells. GM-CSF binds to a ligand-specific α-subunit (GMRα) with low affinity, and high affinity binding and signal transduction is achieved by the recruitment of the common β chain (hβc) to the GM-CSF:GMRα complex. Despite GM-CSF's central role in normal hematopoiesis, inflammation and disease, the mechanism of assembly and composition of the activated GM-CSF receptor (GMR) complex was unknown prior to this body of work.

Using both cell surface expressed receptors and soluble extracellular domains of the GMR, the receptor assembly required for the activation of the GMR is shown here. The hβc was shown to form a functional dimer in activated cell surface expressed GMR using a functional assay for receptor homodimerization where the extracellular and transmembrane regions of the hβc were fused to the cytoplasmic domain of the functionally homodimeric erythropoietin receptor (EPO-R).

A previously unreported and uncharacterised interspecies interaction between exogenous hβc and endogenous mouse βc was revealed when the human GMR (hGMR) was expressed in mouse cell lines. This interspecies interaction was responsible for dramatically influencing hGMR signalling events, including assembly and activation, in a misleading manner. This highlights the critical importance of using fully homologous experimental systems when studying human hematopoietic cytokines and their receptors. These findings question the validity

of previous reports where heterologous systems were used, and suggest that homologous GM-CSF systems should be used to reach meaningful conclusions.

Reported GMR stoichiometry studies using full-length surface receptors produced conflicting ratios for receptors within the activated GMR. Here we produced and purified soluble GMR components, sGMRα and sβc, and formed soluble GMR complexes in solution and determined the stoichiometric ratio of these complexes in the absence of membranes. Analysis of the individual soluble receptor components revealed that sGMRα exists as a monomer, whereas sβc exists as a dimer. Soluble GMRα was found to bind GM-CSF with a stoichiometry of 1:1. Importantly GM-CSF, but not a GM-CSF analogue (E21R), formed a ternary complex composed of one sβc dimer with a single molecule of each sGMRα and GM-CSF. These studies revealed a functionally relevant, direct interaction between GM-CSF and hβc extracellular domain that had been anticipated but never observed. A similar receptor assembly is likely to be conserved in the related IL-3 and IL-5 receptors. Interestingly the soluble GMR studies revealed both an inducible complex and a preformed complex, where the extracellular regions of sGMRα and sβc could interact in the absence of ligand.

In summary, my research characterised how the individual GMR components assemble into an active receptor complex and determined a stoichiometry of 1:1:2 for GM-CSF:sGMRα:sβc. Importantly, this functionally relevant soluble GMR ternary complex led to X-ray crystallography studies where the structure of the GMR ternary complex was ultimately solved.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for joint-awards of this degree.

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Barbara McClure

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ABBREVIATIONS

Akt	protein kinase B
AML	acute myeloid leukemia
Ba/F3	pro-B murine cell line
BION-1	anti-hβc neutralising monoclonal antibody
CML	chronic myeloid leukemia
COS	African green monkey fibroblast cell line
CRM	cytokine receptor module
CTLL-2	cytotoxic T lymphocyte murine cell line
E21R	GM-CSF containing point mutation where glutamic acid at position 21 is substitute with an arginine
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
EPO-R	erythropoietin receptor
FCDP-1	murine bone marrow cell line
G-CSF	granulocyte-colony stimulating factor
GH	growth hormone
GHR	growth hormone receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMR	granulocyte-macrophage colony-stimulating factor receptor
GMRα	granulocyte-macrophage colony-stimulating factor receptor alpha chain
gp130	glycoprotein 130
Grb-2	growth factor receptor-bound protein 2
hβc	human common beta chain

IL-3	interleukin 3
IL-5	interleukin 5
IL-3Rα	interleukin 3 receptor alpha chain
IL-5Rα	interleukin 5 receptor alpha chain
IL-6	interleukin 6
JAK	janus kinase
JMML	Juvenile myelomonocytic leukemia
Kd	dissociation constant
MAP-K	mitogen-activated protein kinase
mGM-CSF	murine granulocyte-macrophage colony-stimulating factor
mIL-3Rα	murine interleukin 3 receptor alpha chain
mIL-5Rα	murine interleukin 5 receptor alpha chain
mβc	murine common beta chain
$m\beta c_{IL-3}$	murine beta chain specific for IL-3
Mpl	thrombopoietin receptor
PAP	pulmonary alveolar proteinosis
PTB	phosphotyrosine binding domain
sGMRα	soluble granulocyte-macrophage colony-stimulating factor receptor alpha chain
sβc	soluble human granulocyte-macrophage colony-stimulating factor receptor beta chain
SCF	stem cell factor
TPO	thrombopoietin
SH2	src-homology domain
STAT	signal transducer and activator of transcription protein

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GENERAL INTRODUCTION

It is nearly 30 years since the initial identification of the hematopoietic cytokine receptor GMRα. Less than a decade later in 1996, when the work described here was initiated, there were many unknowns about how the GMR subunits assembled in response to GM-CSF binding and initiated receptor activation.

At this time the GM-CSF receptor subunits GMRα and hβc had been identified, and together shown to provide high affinity GM-CSF binding and signal transduction. The interaction of GM-CSF with GMRα and hβc, and the ligand contacts required had been modelled on the well-characterised structure of Growth Hormone bound to its homodimeric receptor. While this was crucial in revealing individual residues required for GM-CSF binding and receptor activation, the stoichiometry of the active GMR complex was unclear. The nature of the individual subunits GMRα and hβc had not been characterised, and conflicting reports proposed the minimum assembly required for GMR signal transduction to be either simple receptor heterodimers or higher order receptor complexes, but the exact stoichiometry remained unresolved.

Using both cell surface expressed receptors and soluble extracellular domains of the GMR, the receptor assembly required for the activation of the GMR is shown here. The work presented in this thesis provided the path forward from structural homology modelling, and provided the initial studies that ultimately led to the resolution of the GMR atomic structure. Defining the molecular mechanisms of GMR receptor assembly will ultimately provide targets for therapeutic agents for the treatment of GM-CSF implicated diseases including leukemia and chronic inflammatory diseases.

CHAPTER 1:

Introduction

INTRODUCTION

1.1 The regulatory roles of hematopoietic cytokines.

The communication between cells within the body is an exquisitely regulated process that allows complex coordinated events such as maintenance of hematopoiesis, and the control of growth and development of the growing body. Hematopoiesis is the dynamic process that regulates steady state blood cell numbers, increases of blood cell numbers following blood loss and regulates the immune response to clear infections. These processes are achieved by precise regulation of required blood cell numbers and their maturation and activation when necessary.

Important regulatory molecules called cytokines facilitate communication between cells within the body [1]. These small, secreted glycoproteins regulate hematopoiesis by promoting the lineage commitment of stem cells, stimulating their expansion and differentiation and ultimately inducing the effector functions of mature blood cells (see *Figure 1*). Multiple cell types produce cytokines, including fibroblasts, myeloid cells, endothelial cells, T cells and bone marrow stroma. Cytokines may exert their functions locally in a paracrine manner on cells within close proximity, or influence cell populations at sites distant from the point of cytokine production.

In addition to the regulatory role cytokines play in hematopoiesis they are also instrumental in the co-ordination of the inflammatory response. Inflammation involves various immune cells and many mediators that act to clear sites of infection within the body. Cytokines, in addition to other mediators including chemokines, act as messengers to promote leukocyte numbers and their trafficking to sites of infection, recruitment of neutrophils and macrophages to kill and clear microbial pathogens.

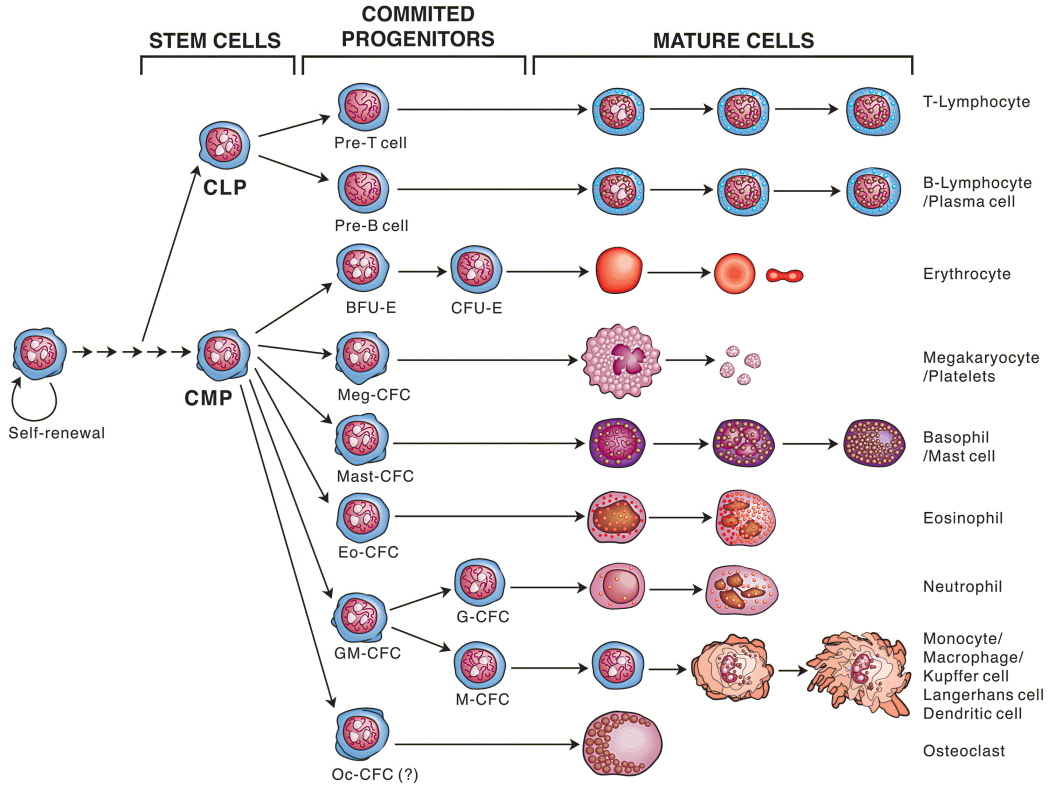

Figure 1.
The process of hematopoiesis derives the eight major blood lineages from self-renewing multipotent stem cells. CMP (common myeloid progenitor), CLP (common lymphoid progenitor), CFC (colony forming cell). (From Bloodlines by Don Metcalf Alpha Med Press 2007 www.bloodlines.stemcells.com/chapters.html).

Cytokines mediate their functions by binding to specific surface glycoproteins, cytokine receptors, that are expressed on responsive cells at low levels (100-1000 per cell) [2]. When cytokines engage their receptors, signals are transduced within the cell resulting in activation of a biochemical cascade that results in a biological response. These cytokine stimulated signalling processes are vital to ensure the appropriate growth, survival and functional activity of responsive cells.

Through understanding how different cytokines control the process of hematopoiesis, light has been shed on how a stem cell is directed to follow a particular commitment pathway and how mature cells are activated to perform a biological task. Cytokines can act independently to

influence cell survival or function, or may act in conjunction with other cytokines. Examples of hematopoietic cytokines include thrombopoietin (TPO), erythropoietin (EPO) and granulocyte-colony stimulating-factor (G-CSF) which stimulate the production of platelet and megakaryocytes, red blood cells and neutrophils, respectively, while stem cell factor (SCF), an essential growth factor in normal hematopoiesis, exerts potent synergistic effects when combined with other cytokines [3].

1.2 Myeloid cell production and function

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), Interleukin-3 (IL-3) and Interleukin-5 (IL-5) are a distinct family of cytokines that regulate myeloid cells, including neutrophils, macrophages and eosinophils. The GM-CSF, IL-3 and IL-5 cytokine subfamily act specifically to promote survival, proliferation, differentiation and effector functions of myeloid cells (see *Figure 1*). These three cytokines are closely related as seen by their close proximity of chromosomal location, the structure of their genes and how they transduce a signal in responsive cells. In addition, these cytokines show some degree of overlapping biological activities on certain hematopoietic lineages, whilst maintaining distinct biological actions. GM-CSF, IL-3 and IL-5 are principally produced by activated T cells and act to modulate survival and effector functions of cells that express receptors for these cytokines [4]. GM-CSF exerts its effector functions on myeloid progenitors and mature myeloid cells including neutrophils, monocytes, eosinophils, basophils and dendritic cells [5]. IL-3 influences early hematopoietic progenitors and myeloid progenitors, eosinophils and basophils, while the role of IL-5 is restricted to regulation of eosinophils [6] and B cells [7].

The presence of the GM-CSF, IL-3 or IL-5 receptor (GMR, IL-3R or IL-5R) on the surface of a cell confers the responsiveness of that cell to its cognate ligand. These three cytokines bind to

receptors that are composed of two subunits, a ligand specific α chain and a shared β chain (hβc). The α chains GM-CSFRα [8], IL3Rα [9], and IL5Rα [10] bind their cognate ligand with low affinity (1-10nM[8], 10-100nM[11] and 100-500nM[12], respectively) but this interaction is insufficient for signalling. The subsequent recruitment of the common signalling receptor subunit hβc, shared by all three cytokines, leads to high affinity binding and signal transduction [13].

1.3 Granulocyte-Macrophage Colony-Stimulating Factor

GM-CSF is a heterologous glycoprotein, approximately 28000Da that is principally produced by activated T-cells, macrophages and endothelial cells. Macrophages and granulocytes rise in numbers and exhibit a prolonged life-span and enhanced effector function in response to GM-CSF [14-17], properties that have encouraged its use initially in infectious diseases such as those associated with AIDS [18, 19] and clinically as an adjunct in cancer therapy to correct anaemia, particularly neutropenia which results from ablative chemotherapy [20]. GM-CSF has also been used to mobilise stem cells from the bone marrow into the peripheral blood such that these cells can be easily harvested and used for autologous transplantation following chemotherapy treatment [21]. GM-CSF also controls dendritic cell production, differentiation and function and potentiates responses of CD4+ T cells *in vivo* [22, 23].

Interestingly, mice that are null for GM-CSF show no perturbations in steady state hematopoiesis suggesting GM-CSF is redundant in basal hematopoiesis. GM-CSF knockout mice have a distinct pathology where surfactant and lipids accumulate in the alveolar space of the lung, a condition similar to a human condition known as pulmonary alveolar proteinosis (PAP) [24]. This suggested GM-CSF is critical for pulmonary homeostasis and resistance to local infection [25]. While the absence of GM-CSF can lead to PAP, the excess production of GM-CSF, IL-3 or

IL-5 leads to other disease conditions where pathology arises as the result of inappropriate chronic inflammatory responses. These include asthma [26, 27], multiple sclerosis [28, 29] and rheumatoid arthritis [30]. The production of GM-CSF by chondrocytes has been implicated in the inflammatory pathology associated with arthritis as GM-CSF knockout mice are protected from collagen induced arthritis [31]. Persistent cytokine production, including GM-CSF, has been directly measured from synovial fibroblasts and its production may play an important role in the recruitment and activation of inflammatory cells in arthritic joints [32]. GM-SF is also drives arthritic and inflammatory pain [33]. The development of a human monoclonal antibody that neutralises GM-CSF signalling, mavrilimumab, has shown good efficiency treating rheumatoid arthritis in clinical trials [34] suggesting targeting GMR can provide a therapeutic benefit.

In addition to the pathological role GM-CSF can play in inflammatory diseases it has also been implicated in myeloid leukaemia's including acute myeloid leukemia (AML), juvenile myelomonocytic leukemia (JMML) and chronic myeloid leukemia (CML) where it is believed to enhance the growth and survival of leukemic blasts [35, 36]. JMML, a lethal childhood disease, is a prime example of this as malignant cells produce GM-CSF in an autocrine fashion to promote the spontaneous proliferation of malignant cells and *in vitro* progenitors show hypersensitivity to GM-CSF [37, 38].

Understanding how GM-CSF promotes cell signalling through its specific receptor interactions will aid the development of molecules that can prevent inappropriate GM-CSF signals [4]. Such molecules that target the GMR have shown potential for treatment in both inflammatory and leukemic diseases [39, 40]. To aid in the design of specific inhibitors a clear insight into how the ligand and receptor come together to form an active receptor complex is required.

The crystal structure of GM-CSF has been solved [41]. It comprises a core structure of four alpha-helical bundles with a double overhanded topology, a fold which is common to other hematopoietic cytokines including IL-5 [42] and growth hormone (GH) [43]. This highlights the structural conservation between cytokines despite displaying a low level of sequence similarity. While the atomic structure of GM-CSF is known, the molecular interactions required to interact with its receptor components are still being determined.

1.4 The GM-CSF Receptor

GM-CSF receptors are expressed on cells of the myeloid lineage including progenitor and mature neutrophils, eosinophils, macrophages monocytes and mast cells [44-46]. As with other hematopoietic cytokines, activation of the GM-CSF receptor in response to GM-CSF is followed by oligomeric receptor formation and receptor phosphorylation. Unlike homodimeric receptor systems (eg.GH and EPO-R) the human GM-CSF receptor is a heterodimer composed of 2 distinct subunits, a receptor α chain (GMRα) [8] that provides ligand specificity and hβc [13], and is communal to the IL-3 and IL-5 receptors. Both GMRα and hβc are transmembrane proteins of apparent molecular weight of 84 and 130kDa respectively and are glycosylated on their extracellular domains. The presence of asparagine-linked glycosylation on the extracellular domains of GMRα (11 potential sites) and hβc (3 potential sites) is crucial for both low and high affinity binding [47].

Both GMRα and hβc are members of the type I cytokine receptor family, identified by the presence of conserved cytokine receptor modules (CRM) in their extracellular domain. The common structural motif within the CRM is the presence of at least 2 fibronectin like folds, a domain first identified in the Growth Hormone receptor (GHR). Each fibronectin-like domain

contains seven β-strands (A-G and A'-G' respectively) interspersed by loop regions. In addition, each CRM contains 4 spatially conserved cysteine residues required for correct protein folding and a transmembrane proximal tryptophan-serine motif (WSXWS) [48] which is essential for receptor surface expression [49]. EPO-R, G-CSFR, IL-4R, IL-6R, IL-7R and IL-2β also belong to the type I cytokine receptor family. The hβc extracellular domain contains two CRM modules, whereas the GMRα contains a single CRM (as do IL3Rα and IL5Rα). The GMRα chain has a unique N-terminal domain that only shares homology with IL3Rα and IL5Rα, characterising these three alpha chains as a distinct receptor family.

GMRα binds GM-CSF with low affinity (equilibrium dissociation constant, Kd = 1-10nmol/L) and is unable to mediate signalling alone, although some reports have suggested a role for GMRα alone in glucose transport [50]. The hβc has not been shown to detectably bind GM-CSF alone, but forms a high affinity receptor (Kd = 20-100pmol/L) with the GMRα subunit.

Given the importance of GMR in signals that regulate immune responses, hematologic recovery, and in some cases, leukemia, a significant amount of work has been devoted to structure-function analysis of the cytokine-receptor systems. Characterisation of this family of receptors sought to identify functional epitopes responsible for receptor activation and identified regions in the cytoplasmic domain of hβc that couple to specific signalling molecules such as janus kinases (JAKs), STATs and the ras/MAP Kinase pathway [51-53]. However results from these studies have in some cases been ambiguous or even conflicting.

Structural and dimerization requirements exist for the GMR to assemble upon ligand binding and for subsequent signalling to occur, some of which have been defined. While the stoichiometry of the active GMR remains unresolved, extensive structure-function analysis has identified several

residues involved in GM-CSF, GMRα and hβc protein interaction and biological activity. For example, the binding of GM-CSF to GMRα appears to involve an electrostatic interaction between Asp^{112} in the fourth alpha helix of GM-CSF and Arg^{280} in the F-G loop of GMRα [54, 55]. The biological activities and high-affinity binding of GM-CSF are exquisitely dependent on Glu^{21} in the first alpha helix of GM-CSF implying an interaction with hβc, although direct contact has not been demonstrated. Substitution of this amino acid with arginine generates a GM-CSF analogue, E21R, which exhibits only low-affinity binding and is unable to stimulate cellular proliferation and mature cell functions [56]. Importantly E21R generated as recombinant protein in *E. coli* is able to antagonise GM-CSF binding and function [57], however, the molecular basis of this antagonism is not fully understood. In hβc, residues in the B-C loop (Tyr^{365}, His ^{367}, Ile^{368}) and F-G loop (Tyr^{421}) of domain 4 are crucial for GM-CSF high-affinity binding and function [58-61]. Furthermore, the monoclonal antibodies directed against the hβc epitope encompassing these loops blocks GM-CSF binding and biological activities [62, 63].

The regions of GMR targeted by site-directed mutagenesis were based on the structural homology with the GH homodimeric receptor:ligand complex, a homodimeric type 1 cytokine receptor whose structure had been solved by crystallography. While the GHR structure is a useful tool to guide the selection of potential ligand:receptor and receptor:receptor interactions within the GMR, it is important to remember that GHR is a simple homodimeric receptor and may not completely reflect the heterodimeric nature of the GMR.

Heterodimerization of GMRα and hβc is recognised as a crucial step for the activation of the GM-CSF receptor complex, however, the exact composition of the ternary complex remains unclear. Receptor dimerization is a common theme among the cytokine receptor superfamily and

is usually a pre-requisite for receptor activation. For example, IL-6 induces oligomerization of IL-6Rα and gp130 [64] that results in the assembly of a hexameric complex containing two IL-6 molecules, two IL-6Rα subunits and a gp130 homodimer [65]. Formation of this higher order complex is required for receptor phosphorylation and cell signalling in response to IL-6 [66]. Similarly, erythropoietin induces receptor dimerization and subsequent receptor phosphorylation [67, 68]. The IL-3 receptor, which is closely related to the GMR [69], binds IL-3 and induces receptor heterodimerization between IL-3Rα and hβc, followed by intermolecular covalent disulphide bridging between their N-terminal domains [70]. The structural similarities and functional overlap between the GM-CSF and IL-3 receptor systems have suggested that the activation of the GMR may follow a similar pattern of events. Indeed, GM-CSF has been shown to induce co-association of the GMRα and hβc [71], and a general mechanism has been noted that involves disulphide bridging between receptor α chain and a cysteine motif in the hβc that is essential for activation of GM-CSF, IL-3 and IL-5 receptors [72] (and unpublished observations). Despite exhibiting some common features of activation with other receptors, the GMR also appears to exhibit some distinct features. For example, mutant forms of the GMR that are deficient in GM-CSF binding when expressed alone on cells are able to support binding when co-expressed with hβc [49, 55], suggesting the hβc can compensate for losses in binding affinity.

Receptor chimera studies suggest that simple heterodimerization of GMRα and hβc is sufficient to activate the GM-CSF receptor (Figure 2) [71]. However, both crosslinking and dominant negative studies using surface expressed receptors suggest the formation of higher order GM-CSF receptor complexes are required for receptor activation (Figure 2) [70, 73]. Dimerization of at least two hβc in particular has also been suggested to be an important and necessary step for

receptor activation [74] which this work confirmed. This may reflect the need to bring into close proximity the cytoplasmic domains of two hβc molecules associated with JAK-2, resulting in JAK transphosphorylation and receptor phosphorylation. Interestingly, hβc has been shown to exist as a preformed homodimer on the cell surface [70, 74] and the crystal structure of the complete extracellular domain of hβc has recently also shown it to be a dimer [75]. However, the exact stoichiometry of the active GM-CSF receptor complex had not been resolved until the work presented here.

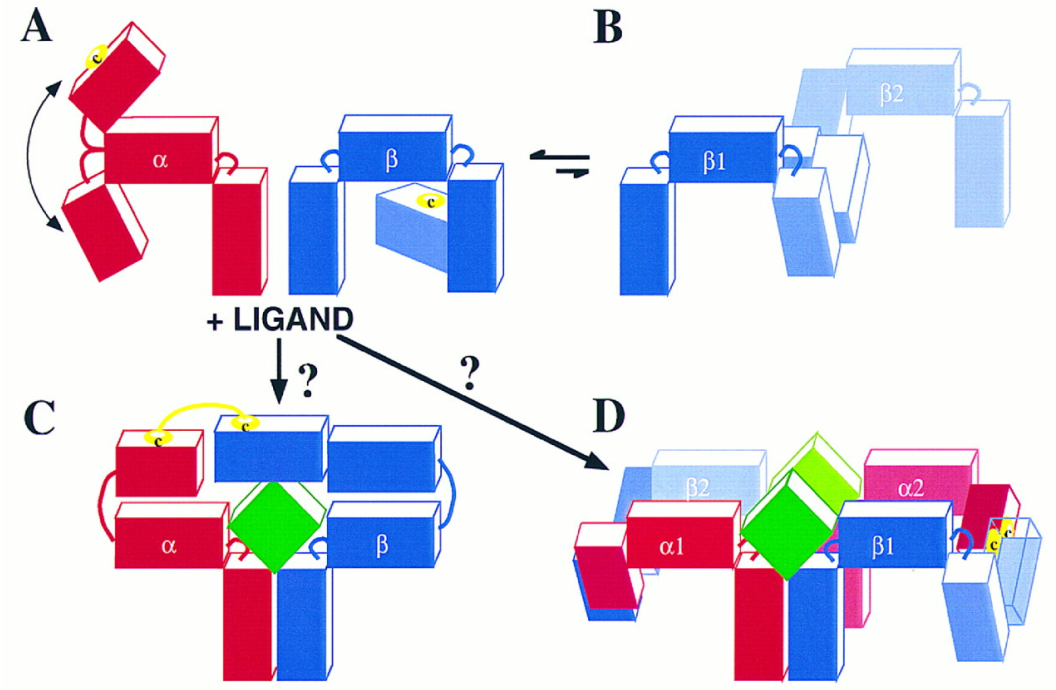

Figure 2.
Schematic presentation of possible GM-CSF receptor complexes*. (A) GMRα and hβc in absence of GM-CSF and (B) potential reversible βc homodimerization. Following GM-CSF binding GMR subunits may assemble to form a heterodimer composed of GM-CSF:GMRα:hβc with stoichiometry of 1:1:1(C) or (D) a higher order complex with GM-CSF:GMRα:hβc stoichiometry of 2:2:2 [72].*

1.5 GM-CSF receptor signal transduction

The biological actions of GM-CSF, include proliferation, differentiation and functional activation. These actions occur in response to GMR activation and are the result of a tightly

regulated intracellular signalling cascade influencing gene expression. Receptor signalling in response to GM-CSF emanates from the intracellular domain of the hβc subunit through protein/protein interactions resulting in the activation of the JAK/STAT pathway, the mitogen-activated protein (MAP) kinase pathway, and the PI-3 kinase pathway. The hβc achieves this despite a lack of intrinsic tyrosine kinase activity within the receptor [76, 77].

The critical first step in GM-CSF signalling, following receptor oligomerization, is the activation of the tyrosine kinase JAK-2. The requirement for two molecules of JAK-2 to be brought into close proximity for activation suggests the requirement that at least two hβc be present in the activated receptor. Following JAK-2 activation, a rapid and reversible phosphorylation of many cellular proteins occurs including the hβc receptor, but not GMRα. JAK-2 activation is essential for *c-myc* induction (supporting a proliferative cellular response) and the activation, dimerization and nuclear translocation of STAT-5 that regulates several GM-CSF inducible genes including *pim-1*, *oncostatin M* and *Id-1* [78]. JAK-2 constitutively associates with a proline-rich membrane proximal region of βc, termed box 1, and disruption of this region leads to lack of binding and activation of JAK-2 [53, 74].

The tyrosine residues within the intracellular region of hβc (at positions 450, 452, 577, 612, 695, 750, 806 and 866) are targets for phosphorylation by JAK-2 and these phosphorylated residues then act as docking sites allowing signalling complexes to assemble through src-homology (SH2) and phosphotyrosine binding (PTB) domains. A mutation of these tyrosine residues has enabled distinct pathways emanating from each residue to be discerned. Residues Tyr577, Tyr612 and Tyr695 of hβc are implicated in the activation of the ras/MAP kinase pathway [79]. Tyr577 recruits SHC through its PTB domain [52, 80, 81] domain whilst Tyr577 and Tyr612 are important for the activation of Grb2 [52]. Both SHC and Grb-2 are upstream mediators within

the ras/MAP kinase pathway. The PI 3-kinase pathway is also activated by GM-CSF [79, 82, 83] despite the lack of direct interaction between PI3-kinase and the hβc. A 14-3-3 binding motif surrounding the serine at position 585 of hβc has been identified [84]. This has been shown to recruit PI 3-kinase allowing activation of effector molecules of this pathway including Akt, resulting cell survival through the activation of survival genes including *bcl-2* [85, 86]. Interestingly, when all eight tyrosine residues in the hβc intracellular domain are conservatively mutated to phenylalanine, preventing tyrosine phosphorylation, the hβc is still capable of transmitting a survival signal in response to ligand [52]. This suggests the signalling through regulation of the motif surrounding Ser585 is sufficient to elicit a survival response in the absence of JAK/STAT and ras/MAP kinase pathway activation. Constitutive phosphorylation of Ser585 is seen in CML and AML blasts [87, 88] potentially contributing to leukemic blast survival.

Despite the large number of cellular proteins that are modified following GM-CSF receptor activation, the GMRα chain is not a target of phosphorylation nor has it been shown to directly recruit signalling molecules. Despite this, the presence of the short intracellular domain is absolutely required to support GM-CSF induced signalling through hβc [53]. Whilst the pathways that emanate from the GM-CSF receptor (and IL-3R and IL-5R) have been extensively mapped the stoichiometry of the receptor components remained unclear until this body of work. Resolution of the GMR stoichiometry and the identification of interfaces through which it assembles will provide novel targetable regions.

1.6 Inhibitors of GM-CSF/IL-3/IL-5 signalling

The involvement of GM-CSF (and IL-3 and IL-5) in chronic inflammatory diseases has driven the design of molecules that can neutralise the action of the cytokines and their receptors. These

molecules can be potential therapeutic agents and also useful tools for mapping epitopes important for receptor activation and assembly. Both anti-ligand neutralising antibodies [89] and antagonists that sequester ligand, such as soluble GMRα (sGMRα) [90], have been shown to prevent ligand-binding the receptor and subsequent receptor activation. An alternate approach to prevent activation of ligand specific receptors has been to prevent ligand from interacting with the α chain. This has been achieved by generating anti-IL-3Rα chain blocking antibodies [70] or by engineering specific ligand mutants such as the GM-CSF mutant E21R that behaves as a specific GM-CSF antagonist [57]. More recently the generation of an antibody that binds an epitope that encompasses the ligand-binding site of hβc can, not only inactivate the GMR, but also simultaneously inactivate signal transduction through the IL-3R and IL-5R [63]. Similarly the signalling of these three cytokines can be inhibited by oligodeoxynucleotides targeting the hβc [91]. Increasingly, synthesised compounds and mimetic peptides are being generated and screened to prevent receptor activation and have the potential for therapeutic use [92, 93].

In addition to specific engineered antagonists of GM-CSF, other surface receptors can interact with and modulate GM-CSF signal transduction. The laminin receptor (LR) has also been reported to interact with the intracellular portion of GMRα and hβc and this acts to inhibit GMR complex formation and subsequent signalling [94]. However, when laminin or fibronectin bind the LR this prevents the association of LR with hβc and allows GMR to complex and signal. This may enhance cell responsiveness at transendothelial migration sites to GM-CSF.

1.7 Experimental systems for analysis of GM-CSF receptor assembly and activation.

Central to the analysis of cytokine and receptor mutants is the choice of experimental system used. To date this has relied on mouse myeloid and lymphoid cell lines (Ba/F3, CTLL and FDCP-1) that can be readily transfected with human cytokine receptor subunits. The expression

of endogenous receptors for the murine cytokines can potentially pose a problem as the human and mouse receptors show high structural homology. In the murine system there are two βc subunits, mβc that are analogous to hβc, transducing signals by mGM-CSF, mIL-3 or mIL-5, and $m\beta c_{IL\text{-}3}$, that is specific for mIL-3 [95, 96]. Analysis of hGMR introduced into cell lines expressing endogenous mouse GMR components have led to misleading interpretations of receptor functions. For example, transfection of hGMRα alone in murine FDCP-1 cells was initially reported to be sufficient to mediate a proliferative signal despite only displaying low affinity hGM-CSF binding [97]. However, it was later shown that functional reconstitution of hGMR required both hGMRα and hβc subunits [13], and that the initial observation with hGMRα alone was confounded by the recruitment of endogenous mβc [98]. Likewise, interaction of an extracellular point mutant of hβc with an endogenous mGMRα in FDCP-1 cells has been shown to lead to factor-independent proliferation [99] and chimeras between a constitutively active erythropoietin receptor with the cytoplasmic domain of GMRα promoted proliferation but only in presence of mβc [100].

The homology between the human and mouse GM-CSF receptors, which allow cross-species interactions, have led to the misinterpretation of the role of receptor subunits and their functional regions. While a cellular system is required for investigation of the cellular signalling and functional responses of cytokines, a cell free system using soluble forms of receptor components is emerging as a powerful tool for investigating the assembly, stoichiometry and ultimately atomic structure of hematopoietic cytokines bound to their receptors.

1.8 GM-CSF Soluble receptors

Soluble GMRα components occur naturally and have been exploited to probe receptor organisation. Initial studies describing the primary binding characteristics of GM-CSF receptors

and the process of ligand-complex formation were investigated on surface expressed receptors and in solution where solubilised membranes were isolated. GM-CSF binding in solution to solubilised membranes that contained GMR lacked high affinity binding (Kd= 500pM), but GM-CSF could be cross-linked within a 110- and 200kDa receptor ligand-complex [101], presumably to GMRα and hβc respectively [56]. This technique not only allowed the isolation and characterisation of GMR from a variety of cell types in a cell free environment, but also suggested that stable GM-CSF:GMR complexes could be formed in solution. Analysis of the GMR in solution had the potential to be further exploited not only to investigate binding characteristics of the GMR, but also for the investigation of the receptor complexes that were formed upon ligand binding.

While full length soluble GMR could be generated by solubilising membrane *in vitro*, it was also becoming apparent that the GMRα could be produced not only in a full length transmembrane form, but also as a secreted soluble form *in vivo* [90, 102]. Expression of the sGMRα isoform occurs through an alternative mRNA splicing of the GMRα gene product [47] and ectodomain shedding [103]. This soluble receptor binds GM-CSF with low affinity (Kd = 3.8nmol/L) akin to cell surface expressed GMRα and can act as an antagonist to both surface expressed low and high affinity binding GMR *in vitro* and to biological activity [90]. The naturally occurring sGMRα isoform is not only present in the supernatant of cell lines that express GMR but is also secreted from monocytes [103] and is a normal constituent of human plasma [104]. Interestingly, GM-CSF and inflammatory mediators up regulate the naturally occurring sGMRα secretion, which may act to down modulate inflammatory responses [103]. In addition to their innate biological activity, soluble receptors can be used as building blocks *in vitro* to investigate how receptor complexes come together. Such studies can reveal important information such as the stoichiometry of receptor components and the sites of interaction between them. Ultimately

soluble receptor complexes can be used to reveal the atomic structure through x-ray crystallography.

Crystal structures of ligands alone or in complex with soluble receptors have been elucidated, such as GH, only the atomic structure of GM-CSF from this sub-family has been determined [41]. The heterodimeric complex GM-CSF forms with GMRα and βc was initially modelled based on homology to the homodimeric GH receptor. Insights into the stoichiometry of another hematopoietin receptor family member, the IL-6 receptor, were achieved using soluble receptor components of IL-6R and the shared signalling subunit gp-130. These studies reveal that IL-6 binds with low affinity to the IL-6R with a stoichiometry of 1:1, whereas the high affinity complex is hexameric containing two molecules of each IL-6, IL-6R and gp-130 [105]. The use of an analogous approach assembling complexes of the soluble receptor extracellular domains of both GMRα and βc could therefore give insight into the progression of assembly and stoichiometry of the GMR.

1.9 Thesis Aims:

GM-CSF plays an important role in the proliferation, differentiation and survival of myeloid precursors and mature effector cell functions, and has been implicated in the pathology of myeloid leukemias and chronic inflammatory diseases. Recent advances in the signalling pathways that emanate from the activated GMR have revealed the regulatory pathways that are utilised to mediate GM-CSF signalling through defined regions of the hβc. Despite these findings it was unclear how this receptor family assemble upon ligand engagement, if functional intermediate complexes were formed or how receptor assembly may be selectively modulated to prevent signal transduction. It also remained unclear if the GMR is composed of a simple heterodimeric receptor or a higher order complex to achieve GM-CSF signal transduction. This

was addressed by characterising the assembly of the GMR subunits in the presence and absence of ligand, in both a soluble form and when expressed on the cell surface. Cell systems were developed and critically analysed to investigate the assembly of full length GMRα, hβc and functionally dimeric receptor chimeras with GM-CSF. The development of these systems aimed to enable the determination of the GMR stoichiometry to reveal insights into the molecular mechanism of GMR assembly. These studies are critical preliminary steps required for crystallisation studies of the GMR complex to solve its atomic structure and understand how signalling is achieved.

CHAPTER 2

The human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor exists as preformed receptor complex that can be activated by GM-CSF, interleukin-3 or interleukin-5

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THE HUMAN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF) RECEPTOR EXCISTS AS A PREFORMED RECEPTOR COMPLEX THAT CAN BE ACTIVATED BY GM-CSF, INTERLEUKIN-3, OR INTERLEUKIN-5.

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Molecular assembly of the activated GM-CSF receptor *B. J. McClure*

STATEMENT OF AUTHORSHIP

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Blood 1997;Volume 90 (8): 3005-3017.

WOODCOCK, J.M.
Generated the soluble form of GMRα, performed binding assay on eosinophils, analysed binding kinetics, supervised development of work, interpreted data and wrote manuscript.

Signed... .....Date....20/12/2016

McCLURE, B.J. (Candidate) 60% Contribution
Generated the soluble form of βc, and performed all experiments on the soluble forms of the GM-CSF receptor, including biochemical analysis, co-immunoprecipitation, binding studies, data interpretation and wrote sections of manuscript.

Signed .....Date.....19/12/2016

STOMSKI, F.C.
Performed co-immunoprecipitation experiments on endogenous surface expressed receptors and cell lines.

Signed.... .....Date.....4/1/2017

ELLIOT, M.J.
Performed association binding assays.

Signed..... .....Date.....4/1/17.....

BAGLEY, C.J.
Helped in data interpretation, experimental design and manuscript evaluation.

Signed..... .....Date.....18-Dec-2016.....

LOPEZ, A.F.
Supervised development of work, and helped in data interpretation and manuscript evaluation.

Signed..... .....Date.....4/1/17.....

The Human Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Receptor Exists as a Preformed Receptor Complex That Can Be Activated by GM-CSF, Interleukin-3, or Interleukin-5

By Joanna M. Woodcock, Barbara J. McClure, Frank C. Stomski, Michael J. Elliott, Christopher J. Bagley, and Angel F. Lopez

The granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor is expressed on normal and malignant hematopoietic cells as well as on cells from other organs in which it transduces a variety of functions. Despite the widespread expression and pleiotropic nature of the GM-CSF receptor, little is known about its assembly and activation mechanism. Using a combination of biochemical and functional approaches, we have found that the human GM-CSF receptor exists as an inducible complex, analogous to the interleukin-3 (IL-3) receptor, and also as a preformed complex, unlike the IL-3 receptor or indeed other members of the cytokine receptor superfamily. We found that monoclonal antibodies to the GM-CSF receptor α chain (GMRα) and to the common β chain of the GM-CSF, IL-3, and IL-5 receptors (β_c) immunoprecipitated both GMRα and β_c from the surface of primary myeloid cells, myeloid cell lines, and transfected cells in the absence of GM-CSF. Further association of the two chains could be induced by the addition of GM-CSF. The preformed complex required only the extracellular regions of GMRα and β_c, as shown by the ability of soluble β_c to associate with membrane-anchored GMRα or soluble GMRα. Kinetic experiments on eosinophils and monocytes with radiolabeled GM-CSF, IL-3, and IL-5 showed association characteristics unique to GM-CSF. Significantly, receptor phosphorylation experiments showed that not only GM-CSF but also IL-3 and IL-5 stimulated the phosphorylation of GMRα-associated β_c. These results indicate a pattern of assembly of the heterodimeric GM-CSF receptor that is unique among receptors of the cytokine receptor superfamily. These results also suggest that the preformed GM-CSF receptor complex mediates the instantaneous binding of GM-CSF and is a target of phosphorylation by IL-3 and IL-5, raising the possibility that some of the biologic activities of IL-3 and IL-5 are mediated through the GM-CSF receptor complex.

GRANULOCYTE-MACROPHAGE colony-stimulating factor (GM-CSF) is a pleiotropic cytokine that exhibits its effects on most cell types in the hematopoietic compartment.[1,2] GM-CSF exhibits overlapping biologic activities with interleukin-3 (IL-3) on several hematopoietic cells owing to a similar pattern of receptor expression and to the sharing of a communal signal transducing receptor subunit that is also shared with IL-5.[3] Indeed, on eosinophils that express GM-CSF, IL-3, and IL-5 receptors, these three cytokines stimulate the same functions with very similar potency.[4] The functional receptors for GM-CSF, IL-3, and IL-5 are closely related and are composed of two subunits: a ligand-specific α chain and the communal β chain (β_c).[5-7] The receptor α chains bind their cognate cytokine ligands with low affinity but are largely unable to mediate signalling alone, although some reports have suggested a role for GM-CSF receptor α chain (GMRα) in glucose transport.[8] The communal β chain, β_c, is unable to bind any cytokine alone, but confers high-affinity binding on a ligand: α chain complex (kd ~100 pmol/L) and is required for receptor signalling.[5-7] 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 coexpressing cytokine-specific α chains and β_c[9-11]; 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 among the cytokine receptor superfamily and is usually a prerequisite for receptor activation. For example, IL-6 induces IL-6Rα and gp130 dimerization[12] with homodimerization of gp130 causing receptor phosphorylation.[13] Similarly, ciliary neurotrophic factor induces receptor dimerization and subsequent receptor activation.[14] In the case of the IL-3 receptor that is closely related to the GM-CSF receptor,[15] IL-3 induces receptor α:β_c heterodimerization followed by covalent disulphide bridging between receptor α chain and β_c.[16] The structural similarities and functional overlap between the GM-CSF and IL-3 receptor systems have suggested that activation of the GM-CSF receptor may follow a similar pattern of events. Indeed, GM-CSF has been shown to induce coassociation of GMRα with β_c,[17] and a general mechanism has been noted that involves disulphide bridging between receptor α chain and a cysteine motif in β_c that is essential for activation of GM-CSF, IL-3, and IL-5 receptors (Bagley et al[18] and unpublished observation). 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 GMRα that are deficient in GM-CSF binding when expressed alone on cells are able to support binding when coexpressed with β_c,[19,20] suggesting that β_c can compensate for losses in binding af-

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finity. 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.[21] Lastly, a recent report showed that a naturally occurring soluble form of GMRα is retained at the cell surface when coexpressed with β_c, although coimmunoprecipitation of the two subunits could only be demonstrated in the presence of GM-CSF.[22]

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 (MoAbs) specific for the GM-CSF receptor α and β_c, we found that both subunits could be coimmunoprecipitated in the absence of GM-CSF regardless of 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 show 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 β_c associated with GMRα. A model is proposed in which IL-3 and IL-5 recruit the performed GM-CSF receptor into a high order complex, raising the possibility that some of the biologic activities of IL-3 and IL-5 are mediated indirectly through activation of the preformed GM-CSF receptor complex.

MATERIALS AND METHODS

Cell lines. Chronic myelogenous leukemia (CML) cells were obtained as described previously[16] and cultured in RPMI supplemented with 10% fetal calf serum (FCS). Mo7e cells and Ba/F-3 cells expressing GMRα/β_c were maintained in DMEM (GIBCO, Melbourne, Australia) supplemented with 20 mmol/L HEPES supplemented with 10% FCS and 5 or 2 ng/mL GM-CSF, respectively. TF-1.8 cells were maintained in RPMI supplemented with 10% FCS and 2 ng/mL GM-CSF. Factor dependent cells were routinely starved of growth factor overnight before cytokine treatment. Chinese hamster ovary (CHO) cells were maintained in F12 medium supplemented with 10% FCS and transfected as described previously.[21]

Plasmid construction. The cDNA for the human β_c was cloned by polymerase chain reaction (PCR) from cDNA prepared from the KMT-2 cell line.[23] A soluble form of the β_c (sβ_c) was created by PCR using the following synthetic oligonucleotides: (1) 5′-TGA-ATTCGCCTGTCCAGAGCTGACCAGGG-3′ that starts 25 nucleotides 5′ of the ATG and contains an engineered *Hin*dIII site and (2) 5′- ATACACTCTATATCACGACTCGGTGTCCCAGGAGCG -3′ that contains an inframe termination codon immediately 5′ of the transmembrane region followed by an engineered *Xba* I site. The PCR product obtained from these primers was subcloned into the Neomycin resistance conferring expression vector pRc/CMV (Invitrogen Corp, San Diego, CA) giving rise to sβ_cpRc/CMV. A soluble form of the human GMRα (sGMRα) was made in a similar fashion using the following synthetic oligonucleotides: (1) 5′-ATA-CACAAGCTTAGCACCATGCTTCTCCTGGTG-3′ that starts 18 nucleotides 5′ of the ATG and contains an engineered *Hin*dIII site and (2) 5′-ATACACTCTAGATCACCCGTCGTCAGAACCAAA-TTC-3′ that contains an inframe termination codon immediately 5′ of the transmembrane region followed by an engineered *Xba* I site. The PCR product obtained from this set of primers was subcloned into pRc/CMV to produce sGMRαpRc/CMV.

To allow for dual stable transfection of two receptors, pRc/CMV was engineered such that the neomycin resistance gene (Neo^R) was replaced with the puromycin resistance gene (*pac*) from pRuf puro.[24] Briefly, the 1.5-kb *Kpn* I-*Bam*HI fragment from pRc/CMV containing Neo^R and its flanking SV40 early promoter and poly-adenylation region was subcloned into pUC19. The Neo^R gene was removed by *Eco*RV-*Nae* I digestion and *pac* introduced as a *Sal* I-*Cla* I fragment from pRuf puro. The puromycin resistance gene plus flanking SV40 early promoter and poly-adenylation region was excised from pUC19 as a *Kpn* I-*Bam*HI fragment and subcloned into *Kpn* I-partial *Bam*HI digested sβ_cpRc/CMV, resulting in sβ_cpRc/CMVpuro. Subsequently, full-length β_c cDNA was introduced in on an *Eco*RI-*Xba* I fragment thereby generating β_cpRc/CMVpuro.

Construction of stable CHO cell lines. The CHO cell lines, sβ_cCHO and sGMRα CHO, were developed as described previously for the GMRα CHO cell line, A9/C7.[21] CHO lines expressing sGMRα or GMRα were subsequently cotransfected with either β_cpRc/CMVpuro or sβ_cpRc/CMVpuro by the same method and selected in 2.5 μg/mL Puromycin (Calbiochem, La Jolla, CA). Cell surface expression of transfected receptors was confirmed by flow cytometry as described previously[16] and analyzed on an EPICS Profile II Flow Cytometer (Coulter Electronics, Hialeah, FL).

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.[25] Monocytes were purified from the peripheral blood of normal donors obtained from the Adelaide Red Cross Transfusion Service as described previously.[26]

Antibodies. MoAbs directed against GMRα, IL-3Rα, or β_c were generated as previously described[27] and purified and characterized as detailed elsewhere.[16,27,28] The MoAbs 8E4 and 4F3 were selected for their ability to specifically immunoprecipitate β_c, 8G6 for GMRα, and 9F5 for IL-3Rα. The MoAb 1C1 and an antipeptide polyclonal rabbit antibody (against residues 131-241 of β_c) were used for immunoblotting β_c and an MoAb 8D10 for immunoblotting GMRα. Phosphotyrosine residues were detected by immunoblot using directly horseradish peroxidase-conjugated PY20 antibody (Sapphire Bioscience, Alexandria, New South Wales, Australia). MoAbs 4F3, 8G6, and 6H6 were used for cell surface expression staining for β_c, GMRα, and IL-3Rα, respectively. The anti-β_c antibody, 3D7,[28] was used for affinity purification of sβ_c protein. The MoAbs were purified from ascites as described.[27] A rabbit polyclonal anti–GM-CSF antibody was used for immunoprecipitating GM-CSF.[21]

Purification of recombinant soluble human β_c receptor. Soluble β_c protein was purified from conditioned medium from CHO cells stably expressing the protein using a 3D7 anti-β_c MoAb affinity column. Bound soluble β_c was eluted with a linear gradient from 3 to 1 mol/L KSCN in 10 mmol/L Tris-HCl, pH 8.0, and subsequently buffer exchanged into phosphate-buffered saline (PBS) containing 0.02% (vol/vol) Tween 20 [polyoxyethylene (20)-sorbitan monolaurate].

^{125}I-surface labeling and immunoprecipitation conditions. Cells were cell-surface labeled with ^{125}I by the lactoperoxidase method as described previously.[29] Approximately 10^8 cells were labeled with 1 mCi ^{125}I (NEN, Boston, MA) in PBS. Cells were lysed in lysis buffer consisting of 137 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), 10% glycerol, and 1% nonidet P-40 (NP40) with protease and phosphatase inhibitors (10 μg/mL leupeptin, 2 mmol/L phenylmethlsulphonyl fluoride, 10 μg/mL aprotonin, and 2 mmol/L sodium vanadate) for 30 minutes at 4°C followed by centrifugation of the lysate for 15 minutes at 4°C. After 1 hour of preclearance with protein A-sepharose (Pierce, Rockford, IL) at 4°C, the supernatant was incubated for 18 hours with 10 μg/mL antibody. Protein-Ig complexes

were captured by incubation for 1 hour with protein A-sepharose followed by 6 subsequent washes in lysis buffer and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoprecipitation of proteins from conditioned medium was performed similarly.

Deglycosylation conditions. Deglycosylation of proteins was performed after immunoprecipitation with the protein still attached to the protein A-sepharose beads. The immunoprecipitated protein was first incubated in 200 mmol/L sodium cacodylate, pH 7.0, 0.1% SDS and then in 0.75% NP40 with neuraminidase, O-glycanase (Genzyme, Castle Hill, New South Wales, Australia), and N-glycanase (New England Biolabs, Arundel, Australia) for 18 hours at 37°C before separation by SDS-PAGE.

SDS-PAGE and silver staining. Immunoprecipitated proteins were analyzed by SDS-PAGE on 7.5% or 10% polyacrylamide gels as stated. Samples were boiled for 10 minutes in either the presence or absence of 2-mercaptoethanol (ie, reducing or nonreducing) before separating immunoprecipitated proteins by SDS-PAGE. Molecular weights (MW) were estimated using SeeBlue Pre-Stained Standards (Novex, San Diego, CA). Radiolabeled proteins were visualized using an ImageQuant Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Silver staining of gels was performed as described previously.[30]

Immunoblotting and enhanced chemiluminescence (ECL) detection. Immunoprecipitated proteins separated by SDS-PAGE were transferred to nitrocellulose membrane by electroblotting. Nitrocellulose membranes were routinely blocked in a solution of PBS, 0.05% Tween 20 (vol/vol) (PBT) containing 5% skim milk (wt/vol) or in 10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.05% Tween 20 (vol/vol) (TNT) containing 5% bovine serum albumin (wt/vol) and probed with antibody, followed where appropriate by either rabbit antimouse horseradish peroxidase (Dako, Carpenteria, CA) or goat antirabbit horseradish peroxidase (Dako). Immunoreactive proteins were detected by chemiluminescence using the ECL kit (Amersham, Little Chalfont, UK) following the manufacturer's instructions. Stripping of membranes was performed by incubating nitrocellulose membrane for 30 minutes at 50°C in 100 mmol/L 2-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris-HCl, pH 6.7, followed by two sequential washes in PBT or TNT. Membranes were reblocked for 1 hour before reprobing.

Production and radio-iodination of GM-CSF, IL-3, and IL-5. Recombinant GM-CSF was produced in *Escherichia coli* as described previously.[21] For the kinetic experiments, recombinant GM-CSF, IL-3, and IL-5 were produced in yeast as described previously.[31] Radioiodination of cytokines was performed by the iodine monochloride method[32] and the iodinated proteins separated from iodide ions on a Sephadex G-25 PD-10 column (Pharmacia, Uppsala, Sweden) and eluted with PBS containing 0.02% Tween 20 and stored at 4°C for up to 4 weeks. The yeast derived radio-iodinated cytokines were purified before use as described previously.[31]

Saturation binding assays. Binding assays were performed on CHO cells grown to confluency in 96-well plates over a concentration range of 10 pmol/L to 10 nmol/L ^{125}I-labeled GM-CSF in binding medium (RPMI containing 0.5% [wt/vol] bovine serum albumin and 0.1% [wt/vol] sodium azide) with nonspecific binding determined at each concentration with excess unlabeled GM-CSF. After incubation at room temperature for 2 hours, radioligand was removed and the wells were 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 γ-counter (Cobra Auto Gamma; Packard Instruments Co, Meridien, CT). Dissociation constants were calculated using the EBDA and LIGAND programs[33] (Elsevier Biosoft, Cambridge, UK).

Binding assays were performed on soluble receptors in solution in a similar fashion to soluble receptor assays described previously.[34] Aliquots of soluble receptor (100 μL) were incubated with ^{125}I-labeled GM-CSF (10 μL) over a concentration range of 0.5 to 20 nmol/L. An excess of unlabeled GM-CSF was added to assays to determine nonspecific binding. Assays were incubated at room temperature for 1 hour, and then Con A-sepharose (10 μL of 50% slurry in PBS) was 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 the tubes were centrifuged to pellet the beads. Pelleted beads were washed once in PBS and then the radioactivity was determined by counting on a γ-counter.

Kinetic binding assays. Association kinetics were determined at 4°C with eosinophils and monocytes using radio-iodinated cytokines at 200 pmol/L. Cells (2 to 4 $\times$ 10^6 per tube) were incubated in 0.15 mL of binding medium containing radioligand with or without 100-fold excess unlabeled 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 seconds at maximum speed in a Beckman microfuge (Beckman, Gladesville, New South Wales, Australia). The visible cell pellet was removed by cutting and the radioactivity in the pellet determined on the γ-counter. The apparent association rate (K_{obs}) was calculated using the KINETIC program (Elsevier Biosoft) 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: $K_{obs} = K_{on}[L] + K_{off}$.

RESULTS

GMRα and β_c are preassociated on the cell surface. During the course of our studies on IL-3 receptor complex formation, we previously observed coimmunoprecipitation of an 80,000 MW protein with β_c from ^{125}I-surface–labeled primary CML cells in the absence of exogenous stimuli.[16] The size of this protein suggested it could be the GMRα. To examine this possibility, we conducted immunoprecipitation of ^{125}I-surface–labeled CML cells either in the presence or absence of GM-CSF or IL-3 with anti-GMRα, anti–IL-3Rα, or anti-β_c antibodies. Immunoprecipitation of unstimulated cells with anti-GMRα antibody 8G6 immunoprecipitated a protein of 80,000 MW, consistent with the size of GMRα (Fig 1A). A second protein of 120,000 MW, corresponding in size to β_c, coimmunoprecipitated with GMRα in the absence of GM-CSF and its level did not increase with the addition of GM-CSF (Fig 1A). Reciprocally, immunoprecipitation with anti-β_c antibody 4F3 immunoprecipitated both the 120,000 MW β_c protein and the 80,000 MW GMRα protein in the presence or absence of GM-CSF (Fig 1A). Coimmunoprecipitation of GMRα with β_c with either anti-GMRα or anti-β_c antibodies could be the result of these antibodies recognizing similar epitopes on both receptor chains. However, in previous studies, we have shown that these antibodies are absolutely specific for their respective receptor chains and show no cross-reactivity.[16, 28]

In contrast to the coimmunoprecipitation seen with GMRα and β_c, coimmunoprecipitation of IL-3Rα and β_c by either anti–IL-3Rα or anti-β_c antibodies was only seen in the presence of IL-3 (Fig 1B), as shown previously.[16] The phosphorimage signal for the IL-3 receptor (Fig 1B) is strong relative to the signal obtained for GM-CSF receptor (Fig 1A) owing to the high level of IL-3 receptor expression

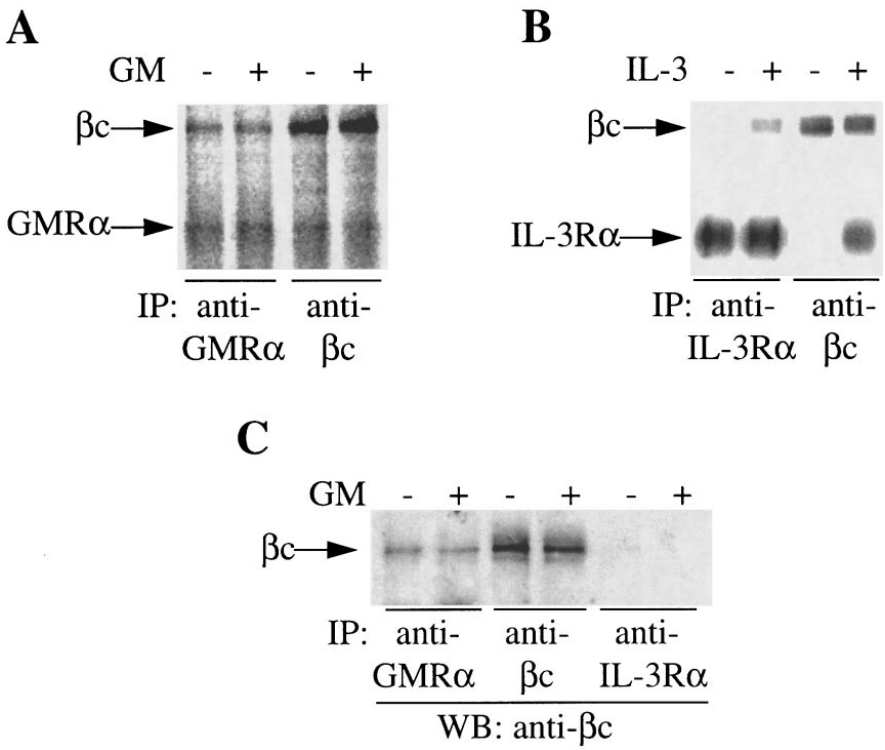

Fig 1. Coimmunoprecipitation of GMRα and β_c from primary CML cells. (A and B) CML cells were ^{125}I–surface-labeled, treated with (+) or without (–) GM-CSF or IL-3 (6 nmol/L) for 5 minutes at 4°C and immunoprecipitation was performed either with anti-GMRα (8G6), anti–IL-3Rα (9F5), or anti-β_c (4F3) MoAb. Immunoprecipitated proteins were separated on 7.5% SDS-PAGE and visualized by phosphorimager and are presented at exposure levels appropriate for the specific signal obtained. (C) Proteins immunoprecipitated from CML cells with different antireceptor antibodies either in the presence (+) or absence (–) of GM-CSF were subjected to Western transfer and immunoblotted using a polyclonal anti-β_c antibody.

relative to GM-CSF receptor on these cells.[35] As stated previously, a protein of 80,000 MW, consistent in size with GMRα, coimmunoprecipitated with β_c in either the presence or absence of IL-3, although at much weaker intensity than either β_c or IL-3Rα[16] and is hence not visible at the exposure shown (Fig 1B).

To confirm the identity of the 120,000 MW protein coimmunoprecipitated by anti-GMRα antibody 8G6, we performed immunoprecipitations with unlabeled cells before and after treatment with GM-CSF using anti-GMRα (8G6), anti-β_c (4F3), and anti–IL-3Rα (9F5) antibodies. After Western transfer, an immunoblot with anti-β_c antibody was performed. An 120,000 MW protein was clearly detected in the presence or absence of GM-CSF in both GMRα and β_c immunoprecipitates but not in the IL-3Rα immunoprecipitate (Fig 1C). This indicates that β_c is associated with GMRα but not with IL-3Rα in the absence of added cytokine on these primary CML cells.

One possible explanation for the preassociation of GMRα with β_c was the autocrine production of GM-CSF by the CML cells. However, we were unable to detect either GM-CSF protein by enzyme-linked immunosorbent assay or GM-CSF mRNA by Northern analysis or reverse transcription-PCR (data not shown). Nevertheless, to confirm the GM-CSF–independent association between GMRα and β_c and to determine the generality of this observation, we performed immunoprecipitation experiments 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 GMRα and β_c maintained in GM-CSF were starved overnight before GM-CSF stimulation. Cells were ^{125}I-surface labeled and proteins were immunoprecipitated with anti-GMRα (8G6) or anti-β_c (8E4) before and after treatment with GM-CSF. We observed coimmunoprecipitation of the 120,000 MW β_c protein and the 80,000 MW GMRα protein with either antibody in the presence or absence of GM-CSF (Fig 2A and B), 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 β_c with GMRα (Fig 2A and B), indicating that only a proportion of GM-CSF receptors are preformed in these cells. To confirm that the 120,000 MW protein coimmunoprecipitated from Ba/F-3 cells with GMRα was human β_c and not a mouse β chain protein, an immunoblot was performed on the immunoprecipitates with anti-β_c antibody. The anti-β_c antibody was highly immunoreactive against the 120,000 MW protein immunoprecipitated by anti-GMRα antibody in either the presence or absence of GM-CSF, confirming the 120,000 MW protein as human β_c (Fig 2C). Reprobing the immunoblot with antiphosphotyrosine antibody PY20 showed that the β_c was phosphorylated only after treatment of the Ba/F-3 cells with GM-CSF (Fig 2C), indicating that the preformed GMRα:β_c complex is not

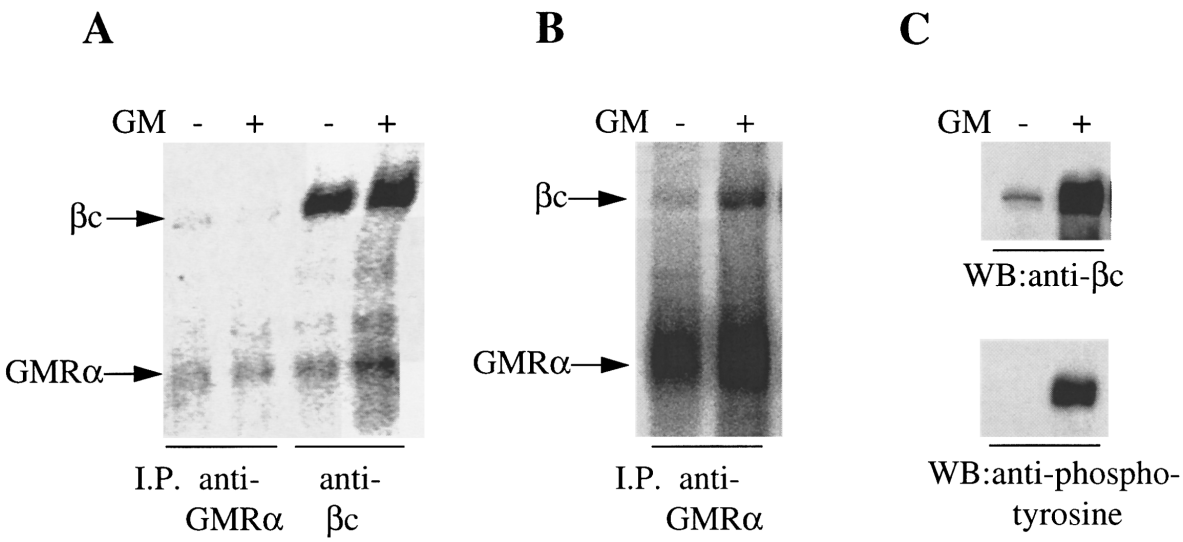

Fig 2. Coimmunoprecipitation of GMRα and β_c from Mo7e and hGMRα/hβ_c-expressing Ba/F-3 cells. (A) Mo7e cells were starved overnight and ^{125}I–surface-labeled and treated with (+) or without (–) GM-CSF (6 nmol/L) for 5 minutes and immunoprecipitation was performed either with anti-GMRα MoAb (8G6) or anti-β_c MoAb (4F3). Immunoprecipitated proteins were separated on 7.5% SDS-PAGE under reducing conditions and the gel was exposed to phosphorimager. (B) hGMRα/hβ_c-expressing Ba/F-3 cells were starved overnight and ^{125}I–surface-labeled and treated with (+) or without (–) GM-CSF (6 nmol/L) for 5 minutes and immunoprecipitation was performed with anti-GMRα MoAb (8G6). Immunoprecipitated proteins were separated on 7.5% SDS-PAGE under reducing conditions and the gel was exposed to phosphorimager. (C) Proteins immunoprecipitated from hGMRα/hβ_c-expressing Ba/F-3 cells with anti-GMRα MoAb (8G6) either in the presence (+) or absence (–) of GM-CSF were subjected to Western transfer and immunoblotted using anti-β_c antibody 1C1 (upper panel) or antiphosphotyrosine antibody PY20 (lower panel).

activated and that this complex was not the result of residual cytokine on the cells after overnight factor depletion. These findings strongly suggest that GMRα and β_c are associated at the cell surface in the absence of GM-CSF as a preformed complex.

A soluble form of β_c interacts with cell surface expressed GMRα. To determine whether the extracellular portions of GMRα and β_c are sufficient for ligand-independent GMRα:β_c interaction, we made a construct encoding a soluble form of β_c (sβ_c) comprising the entire extracellular domain but lacking the transmembrane and cytoplasmic regions and examined its ability to associate with GMRα. Initial characterization of sβ_c was performed by transfection into CHO cells and affinity purification of conditioned medium on an anti-β_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 visualized on a reducing SDS-PAGE gel by silver staining (Fig 3A). These two proteins were also detected after Western transfer by immunoblotting with anti-β_c antibody (1C1; Fig 3B), implying that they represent two forms of sβ_c protein. Intriguingly, when the eluted sβ_c fractions were run on SDS-PAGE under nonreducing conditions, proteins of 120,000 MW and higher were seen by silver staining (Fig 3A) and also by anti-β_c immunoblotting (Fig 3B), suggesting that the sβ_c forms disulphide-linked dimers and higher order complexes. A similar phenomenon was observed with a soluble form of the mouse IL-3–specific β chain, sAIC2A,[36] and may relate to the ability of β_c to spontaneously form dimers, as previously noted.[16,18,37]

The association of sβ_c with GMRα was studied by transfecting the sβ_c construct into CHO cells expressing GMRα and monitoring sβ_c retention at the cell surface with anti-β_c MoAb. Initial flow cytometric analysis showed specific binding of anti-β_c MoAb on the surface of CHO cells coexpressing sβ_c and GMRα but not on CHO cells expressing sβ_c alone (data not shown). Importantly, the specific association of sβ_c with GMRα on the surface of CHO cells could be also demonstrated by coimmunoprecipitation experiments. In these experiments we also sought to establish that the retained β_c reactivity detected on the GMRα-expressing CHO cells was indeed sβ_c and not another protein with a common epitope or a fusion protein produced by an anomalous transfection event. To examine surface expressed β_c specifically and avoid involvement of β_c from intracellular compartments, CHO cells expressing either full-length or soluble β_c with or without GMRα were surface labeled with ^{125}I and β_c protein immunoprecipitated using an anti-β_c antibody (8E4). A single ^{125}I-labeled protein of 120,000 MW was immunoprecipitated from CHO cells expressing full-length β_c (Fig 4A). Two ^{125}I-labeled proteins of 55,000 and 65,000 MW were immunoprecipitated from CHO cells expressing GMRα and sβ_c (Fig 4A) that corresponded in size to the sβ_c proteins detected in cell supernatants (Fig 3). No labeled protein was immunoprecipitated from CHO cells expressing sβ_c alone (data not shown), indicating that the

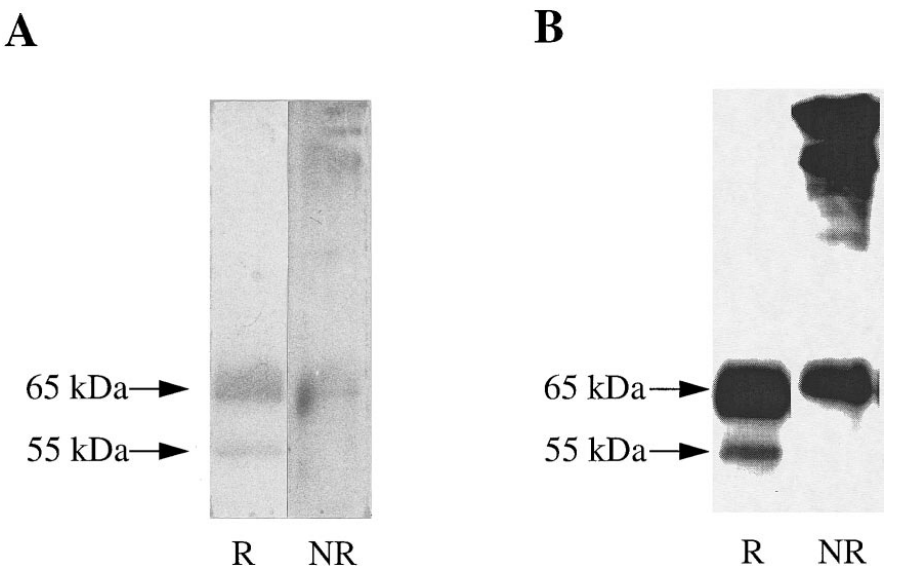

Fig 3. Soluble β_c protein was purified from conditioned medium from CHO transfectants. Soluble purified β_c protein was run under reducing (R) or nonreducing (NR) conditions on 10% SDS-PAGE and either (A) silver stained or (B) subjected to Western transfer and immunoblotted with anti-β_c antibody (1C1).

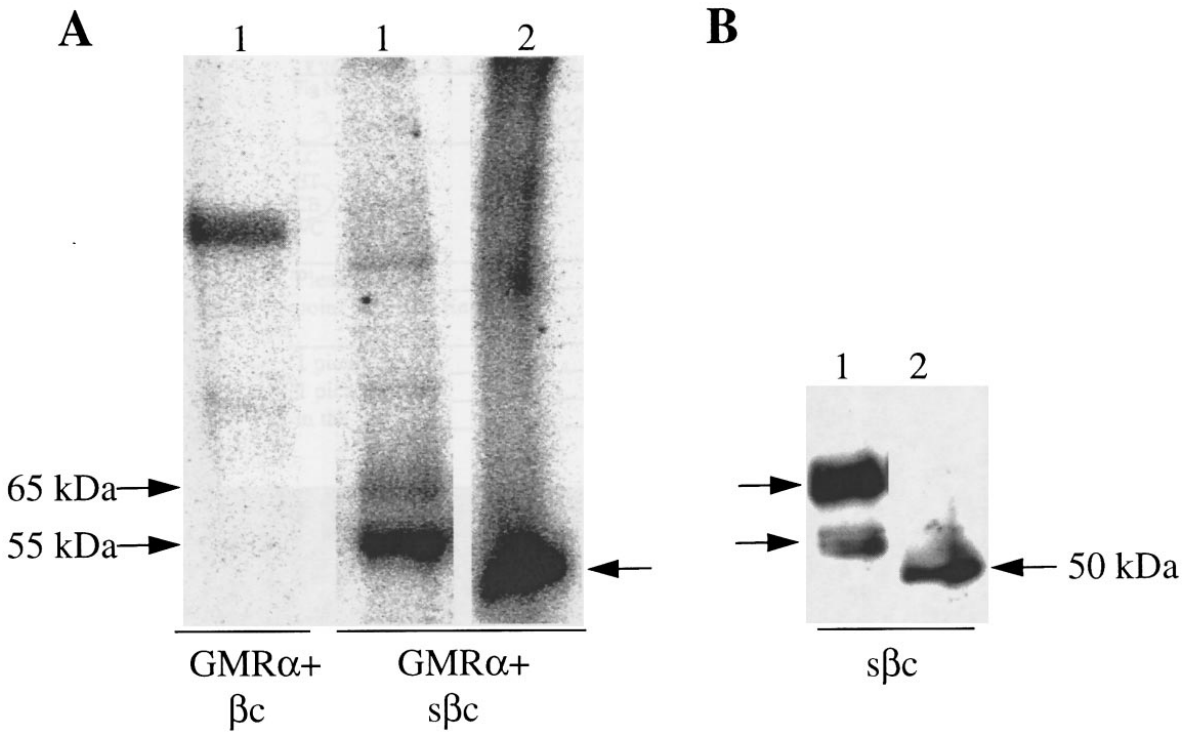

Fig 4. Soluble β_c is retained on the surface of GMRα-expressing CHO cells. (A) CHO cells expressing GMRα and either full-length β_c (β_c) or soluble β_c (sβ_c) were ^{125}I–surface-labeled and immunoprecipitation was performed with anti-β_c MoAb (8E4). The immunoprecipitated proteins were then either incubated with (2) or without (1) deglycosylating enzymes and subsequently separated on 7.5% SDS-PAGE under reducing conditions and visualized by phosphorimager. (B) Soluble β_c was immunoprecipitated from the medium of CHO cells coexpressing GMRα and soluble β_c (sβ_c) and the immunoprecipitated proteins were either subjected to enzymatic deglycosylation (2) or not (1) and subsequently separated on 7.5% SDS-PAGE. Western transfer was then performed and immunoblotting with anti-β_c antibody 1C1.

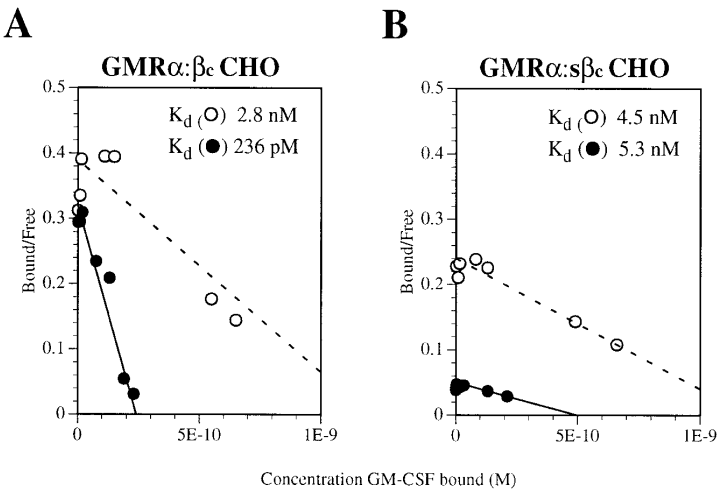

Fig 5. Scatchard transformation of saturation binding studies performed on CHO cells coexpressing (A) GMRα and full-length β_c or (B) GMRα and soluble β_c (sβ_c). Binding assays were performed with ^{125}I-labeled GM-CSF over a concentration range of 10 pmol/L to 10 nmol/L. After binding had reached equilibrium, the cells were washed briefly and either lysed directly (○) or subjected to five 15-minute washes to remove ligand bound with low affinity (●). The dashed line indicates the best fit for nondissociated binding and the solid line indicates the best fit for dissociated binding.

sβ_c retained on the surface of GMRα-expressing cells does not represent sβ_c protein in the process of secretion but is specifically retained by GMRα.

To investigate the nature of the sβ_c protein doublet detected on GMRα-expressing CHO cells, in vitro deglycosylation was performed on the immunoprecipitated protein before SDS-PAGE. The two ^{125}I-labeled sβ_c proteins were both rendered to a 50,000 MW protein (Fig 4A). Similarly, the two 55,000 and 65,000 MW forms of sβ_c immunoprecipitated from conditioned medium were converted to a 50,000 MW protein after in vitro deglycosylation as seen by immunoblot using anti-β_c antibody (1C1; Fig 4B). This shows that the 55,000 and 65,000 MW proteins represent differentially glycosylated forms of sβ_c, as has previously been observed with the full-length β_c,[36] and that both forms are retained on GMRα-expressing cells.

To determine whether the GMRα:sβ_c complex is able to bind GM-CSF with high affinity, saturation binding assays were performed on GMRα CHO cells coexpressing a similar amount of either sβ_c or full-length β_c. Because of the very high level of GMRα chain expression on these transfectants (5×10^5 sites per cell, as determined by Scatchard analysis) no high-affinity sites could be detected directly from either transfectant (Fig 5A and B). To reduce this interference, dissociation of weakly bound radioligand was performed after binding, thereby removing ligand interacting with low-affinity receptors. Using this approach, high-affinity binding sites (kd 236 pmol/L) were detectable on GMRα cells coexpressing full-length β_c (Fig 5A) but not on those coexpressing sβ_c (kd 5.3 nmol/L; Fig 5B). This finding implies that the sβ_c protein is unable to confer full high-affinity binding on the GM-CSF:GMRα complex, a function that may require a conformational change facilitated by the transmembrane and cytoplasmic regions of β_c.

Soluble GMRα and β_c can exist as a complex and bind GM-CSF. Based on our demonstration of a preformed complex between GMRα and β_c on the cell surface and also the retention of sβ_c by cells expressing GMRα chain, we suspected that it may be possible to observe coassociation of a soluble form of GMRα and sβ_c in solution. To test this idea, we constructed a soluble carboxy-truncated form of GMRα that comprised only the extracellular portion of the receptor, termed sGMRα. By immunoprecipitation and immunoblotting using a GMRα chain specific antibody (8G6), we detected a 65,000 MW protein in the medium of CHO cells transfected with this construct, indicating that the soluble GMRα protein was expressed and was able to bind GM-CSF specifically with low affinity (kd 13.7 nmol/L; data not shown).

We then cotransfected the sGMRα construct together with the sβ_c encoding cDNA into CHO cells. Both soluble proteins were detectable by immunoprecipitation and Western blotting with appropriate antibodies in the cell medium of cotransfected cells (data not shown). Significantly, sβ_c protein was detected by immunoblot when immunoprecipitated not only with anti-β_c (4F3) but also anti-GMRα antibody (8G6) but not an irrelevant antibody (9F5) (Fig 6A). This suggests that some but not all sGMRα is associated with sβ_c in solution. Immunoprecipitation of a mixture of conditioned medium from cells expressing sGMRα and sβ_c separately did not result in coimmunoprecipitation of the two chains (data not shown). This is consistent with the retention of sβ_c on GMRα-expressing CHO cells in that it appears that coexpression of the two soluble receptor chains is required for the association to occur.

To determine whether the sGMRα:sβ_c complex is capable of binding ligand, conditioned medium from cells expressing sGMRα and sβ_c was incubated with GM-CSF and subsequently immunoprecipitation was performed with anti–GM-CSF antibody. Immunoblotting of the precipitated material showed that sβ_c was associated with the anti–GM-CSF immunoprecipitated complex when conditioned medium from cells coexpressing the two receptor proteins was used, but not when conditioned medium from cells expressing the two chains separately was mixed (Fig 6B). This implies that the association of sβ_c with GM-CSF is dependent on its interaction with sGMRα in conditioned medium from cells coexpressing sGMRα and sβ_c.

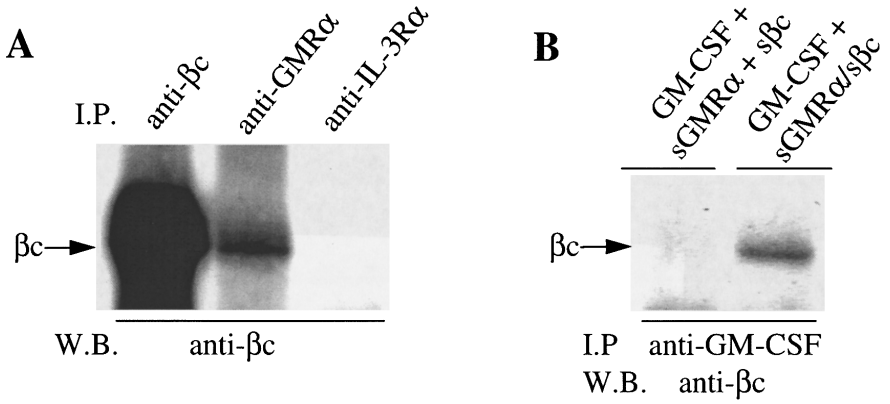

Fig 6. Soluble forms of GMRα and β_c spontaneously associate when coexpressed and bind GM-CSF. (A) Conditioned medium from CHO cells coexpressing soluble GMRα and soluble β_c was immunoprecipitated using either anti-β_c (4F3), anti-GMRα (8G6), or a control antibody (9F5); and the proteins were separated on 10% SDS-PAGE, Western transferred, and immunoblotted with anti-β_c antibody 1C1. (B) Conditioned medium from CHO cells either coexpressing soluble GMRα and soluble β_c (sGMRα/sβ_c) or a mixture of conditioned medium from CHO cells expressing the soluble proteins separately (sGMRα + β_c) were incubated with GM-CSF and immunoprecipitation was performed with anti–GM-CSF antibody. Proteins were separated on 10% SDS-PAGE, Western transferred, and then immunoblotted with anti-β_c antibody 1C1.

GM-CSF exhibits rapid receptor association compared with IL-3 and IL-5. To examine whether a preformed GM-CSF receptor complex may influence the kinetics of GM-CSF binding, we examined the kinetics of association of ^{125}I–GM-CSF to primary human eosinophils and monocytes. We used these cells because they express IL-3 receptors and, in the case of eosinophils, IL-5 receptors as well as GM-CSF receptors, thus allowing a comparison between different receptor systems. The association of GM-CSF was compared with IL-3 and IL-5 on human eosinophils in binding studies performed at 4°C with 200 pmol/L ^{125}I-labeled cytokine in which specific binding was determined over a 24-hour time course (Fig 7A). We found that GM-CSF binding approached equilibrium faster than IL-3 and IL-5 and that binding was detected at very early time points. Curve fitting analysis showed that a significantly improved fit was obtained for GM-CSF association when binding was resolved into two classes of binding site (Table 1): one site exhibiting a rapid approach to equilibrium about 1,000-fold faster than IL-3 or IL-5 and the other exhibiting similar apparent association kinetics to IL-3 and IL-5 (Table 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 1). In previous studies, we have shown that eosinophils exhibit only high-affinity binding sites for GM-CSF, IL-3,[38] and IL-5.[31] 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 were rapid and approached equilibrium faster than IL-3 binding (Fig 7B). We have previously shown that the approach to equilibrium by GM-CSF is approximately 10 times faster than IL-3.[39] The rate of approach to equilibrium of IL-3 on monocytes is comparable to that seen for IL-3 and

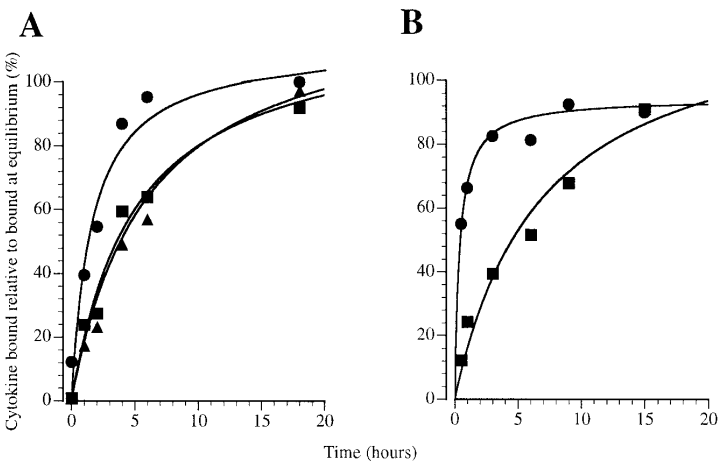

Fig 7. Association kinetics of ^{125}I-labeled cytokines binding to (A) eosinophils and (B and C) monocytes at 4°C with 200 pmol/L ^{125}I-CSF: (●) GM-CSF, (■) IL-3, and (▲) IL-5.

Table 1. Kinetic Parameters for ^{125}I-CSF Interaction With Eosinophils

^{125}I-CSF	K^*_{obs} (min^{-1})	No. of Sites†
GM-CSF‡	2.5 ± 1.2	15
	0.0061 ± 0.0015	105
IL-3	0.0071 ± 0.0024	90
IL-5	0.005 ± 0.002	160

Kinetic parameters determined as described in the Materials and Methods.

* Apparent association rate.

† Number of binding sites exhibiting K_{obs}.

‡ Statistical fit of 1 versus 2 sites ($P = .005$).

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.

The preformed GMRα:β_c can be phosphorylated in response to IL-3 and IL-5. The functional significance of the preformed GMRα:β_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, β_c becomes phosphorylated in response to ligand binding,[10,40] a process that requires the ligand-specific α chain. We have examined the phosphorylation of β_c induced by cytokines in Mo7e and TF-1.8 cells and have found that phosphorylated β_c can be detected by antiphosphotyrosine (PY20) immunoblot after treatment with GM-CSF and immunoprecipitation with either anti-β_c (8E4) or anti-GMRα (8G6) antibody (Fig 8A and B). Similarly, treating Mo7e cells with IL-3 also resulted in β_c phosphorylation that was immunoprecipitable by either anti-β_c (8E4) or anti–IL-3Rα antibody (9F5) (Fig 8A). However, strikingly, we found that anti-GMRα antibody also immunoprecipitated phosphorylated β_c in cells treated with IL-3, indicating that GMRα is associated with the IL-3–induced receptor complex (Fig 8A). Similar results were obtained in TF-1.8 cells, with the addition that anti-GMRα antibody also immunoprecipitated β_c phosphorylated in response to IL-5 (Fig 8B). However, treatment of TF-1.8 cells with erythropoietin did not result in β_c phosphorylation (data not shown), indicating that β_c phosphorylation is specific to GM-CSF, IL-3, and IL-5 and not a general activation event. The involvement of GMRα in the IL-3– and IL-5–induced receptor complexes is specific to GMRα and may be mediated by the preformed GMRα:β_c complex. Thus, these findings raise the possibility that the preformed GMRα:β_c complex can be recruited into an active receptor complex induced not only by GM-CSF but also by IL-3 or IL-5.

DISCUSSION

We show here the existence of a GMRα:β_c complex that is formed in the absence of GM-CSF. We have observed this ligand-independent association between GMRα and β_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 GMRα:β_c complexes observed on cells varied from cell to cell. In some cases, all of the GMRα and β_c chains were apparently coassociated and no further association was induced by GM-

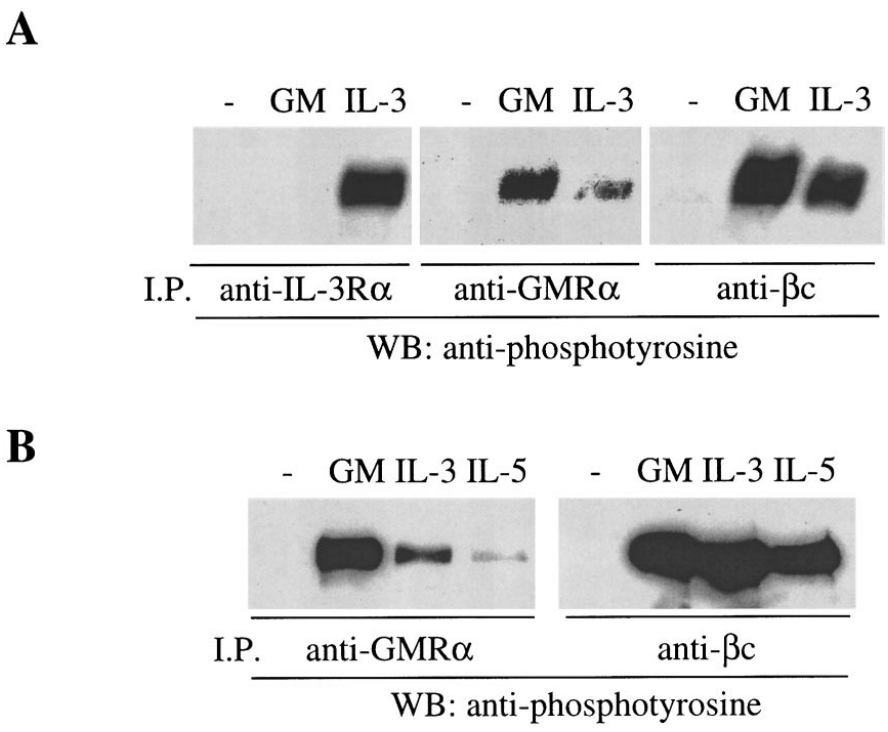

Fig 8. β_c phosphorylated in response to GM-CSF, IL-3, or IL-5 is immunoprecipitable by anti-GMRα antibody. (A) Mo7e cells were starved overnight and then treated with either 6 nmol/L GM-CSF, IL-3, or medium for 5 minutes at 4°C and then immunoprecipitated with either anti–IL-3Rα (9F5), anti-GMRα (8G6), or anti-β_c (8E4) antibody. (B) TF-1.8 cells were starved overnight and then treated with either 6 nmol/L GM-CSF, IL-3, or IL-5 or medium for 5 minutes at 4°C and then immunoprecipitated with either anti-GMRα (8G6) or anti-β_c (8E4) antibody. Immunoprecipitated proteins were separated on 7.5% SDS-PAGE, Western transferred, and then immunoblotted with antiphosphotyrosine antibody (PY20).

CSF treatment, whereas on other cells only a component of GMRαs and β_cs were preassociated 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 showing that GM-CSF induces GMRα and β_c association[17] and reconciles this observation with that of Ronco et al,[19] who suggested that the GM-CSF receptor may exist as a preformed complex. This possibility was raised by the inability of a mutant GMRα to bind GM-CSF unless it was coexpressed with β_c. This was interpreted as β_c preassociated with GMRα compensating for the loss of GM-CSF binding on the mutant GMRα. In an analogous manner, a GM-CSF helix D mutant showed no detectable binding to GMRα alone, yet could bind to cells expressing both GMRα and β_c,[21] possibly reflecting the effect of a GMRα:β_c preformed complex.

By using soluble receptor constructs, we were able to demonstrate the formation of sGMRα:sβ_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 the addition of sβ_c to GMRα expressing cells nor combining separately expressed sGMRα and sβ_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 sβ_c by cells expressing GMRα did not result in a detectable increase in affinity for GM-CSF, in contrast to full-length β_c that confers high-affinity binding on the GM-CSF:GMRα complex. Under the dissociation conditions used it is possible that binding of intermediate affinity was lost and so we can only conclude that sβ_c is unable to confer full high-affinity binding on GMRα-expressing cells. This deficiency in binding with sβ_c may be due to the β_c lacking transmembrane and extracellular portions. Our findings are consistent with recent studies in which a naturally occurring soluble form of GMRα was found to be retained on the cell surface when coexpressed with full-length β_c on BHK cells.[22] The soluble GMRα conferred GM-CSF binding on the cells albeit with intermediate affinity, indicating some deficit in the interaction with β_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 GMRα and β_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,[41] the A-B loop and the E strand in the fourth domain of β_c appear to be good candidates for interaction with the second domain of the cytokine receptor module of GMRα. It is worth noting that insertions, deletions, and point mutations in this domain of β_c lead to factor-independent activation.[42] It is possible that various perturbations of an already preformed complex may result in receptor activation. In our hands we did not observe receptor activation, as measured by antiphosphotyrosine reactivity of the performed complex (Fig 2C). However, it would be interesting to examine this possibility with β_c mutants and indeed in human leukemias.

In seeking to determine the functional significance of the preformed GMRα:β_c complex, we performed kinetic analysis for GM-CSF association. Using normal cells expressing GM-CSF receptor, we 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 we have shown that eosinophils exhibit only high-affinity binding sites for GM-CSF, IL-3,[38] and IL-5.[31] This suggests that there are sufficient β_cs 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 composed of free GMRαs and β_cs 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[39] and induces their adhesion faster than IL-3.[43] The presence of preformed GMRα:β_c complexes may also account for these kinetic differences 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[26,38,44] and leukemia cells.[45,46] The molecular basis of this phenomenon is the competition between GM-CSF:GMRα, IL-3:IL-3Rα, and IL-5:IL-5Rα for β_c interaction. The proposed preformed GMRα:β_c complex might be expected to have an effect on this phenomenon, sequestering β_c for the exclusive binding of GM-CSF. However, cross-competition experiments performed previously on eosinophils[38] and CML cells[35] show that IL-3 is able to compete for ^{125}I–GM-CSF binding effectively, with up to 90% competition. This suggests that the β_c associated with GMRα in the preformed complex is in equilibrium with free β_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 β_c. Thus, cells that express excess β_c and thus exhibit high-affinity binding sites only may have relatively more preformed sites compared with cells that express limiting amounts of β_c.

The stoichiometry of the active GM-CSF receptor is not known and may involve a GMRα:β_c ratio of 1:1 or a 2:2 complex. Because of the disulphide-linked GMRα:β_c heterodimer and molecular modelling of the extracellular region of β_c, we favor the second possibility.[18] This is also consistent with the observations that at least two molecules of GMRα are required for receptor activation[47] and that phosphorylation of β_c dimers[36] and disulphide-linked β_c con-

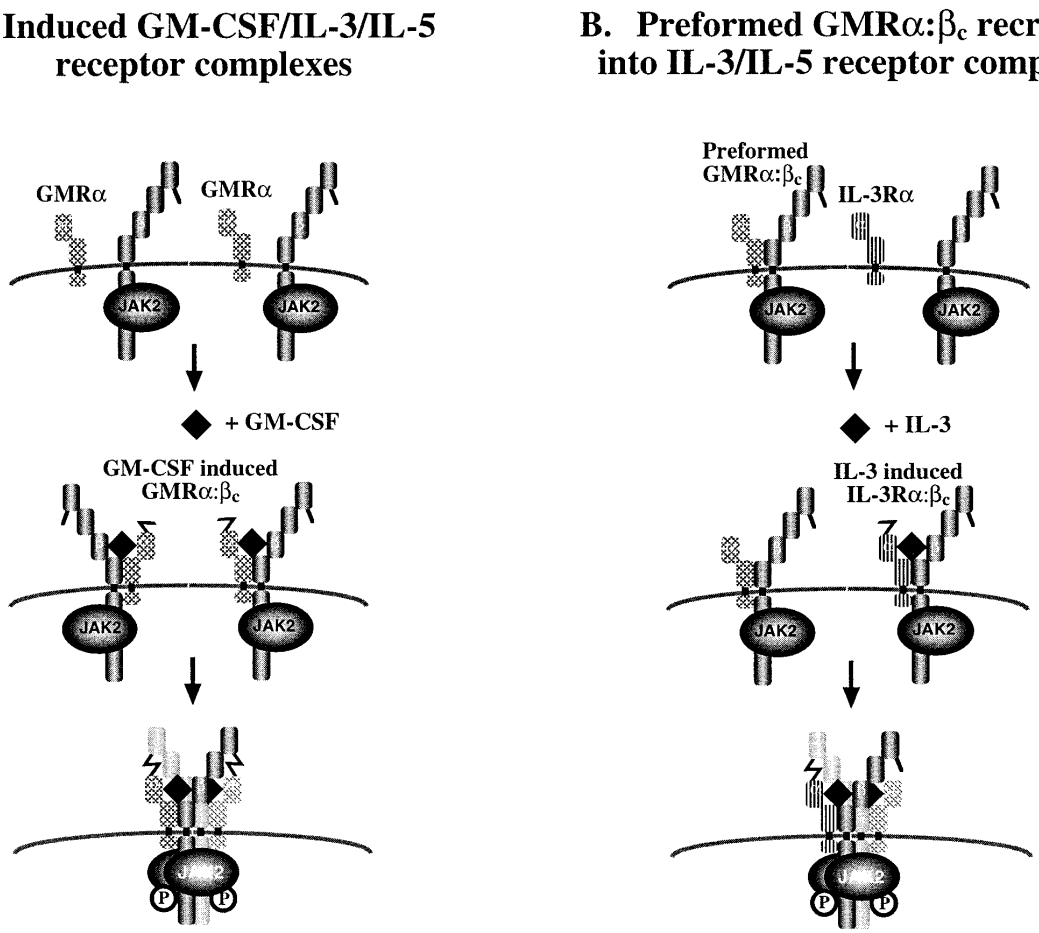

Fig 9. Proposed models for assembly of (A) GM-CSF–, IL-3–, and IL-5–induced receptor complexes and (B) preformed GM-CSF receptor complexes into activated receptors. In (A), GMRα, IL-3Rα, or IL-5Rα are in close proximity to (although not associated with) β_c on the cell surface. Ligand binding to the appropriate a chain induces α:β_c heterodimerization and a conformational change in a chain that allows its disulphide linkage to β_c. Modelling of β_c suggests that this bridging would only be possible if the unpaired cysteines in the α chain of receptor 1 formed a disulphide bridging with cysteine of β_c in receptor 2.[18] The bringing together of two β_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 α chain and β_c triggers a conformational change in the α subunit analogous to model (A). However, in this case, a disulphide bridge can be formed between the free cysteine in the IL-3Rα or IL-5Rα and a cysteine in β_c that is already noncovalently associated with GMRα chain in a preformed complex. This interaction may be sufficient to bring together two β_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 GMRα:β_c complex.

taining complexes[18] occurs in response to GM-CSF. In this model, binding of ligand may render cysteine residues in GMRα and β_c reactive, leading to disulphide bond formation across two receptors in a hexameric configuration, thus bringing together two β_c-associated JAK-2 molecules, thereby inducing receptor activation (Fig 9A). Given the observation of the preformed GMRα:β_c, we speculate that it could be recruited into an IL-3 or IL-5 receptor complex (Fig 9B). Consistent with this possibility, we found that anti-GMRα antibodies immunoprecipitated phosphorylated β_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 β_c associated with IL-3Rα (Fig 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-downmodulation experiments in the mouse in which IL-3 was found to trans-downmodulate GM-CSF, macrophage colony-stimulating factor (M-CSF), and granulocyte colony-stimulating factor (G-CSF) receptors, but GM-CSF or G-CSF were unable to trans-downmodulate the mouse IL-3 receptor.[48,49] The transphosphorylation of GMRα-associated β_c we observe 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 hematopoietic lineage and, intriguingly, most cells that express either IL-3 or IL-5 receptors also express GM-CSF receptors. The data presented here suggest that IL-3 and IL-5 are able

to activate preformed GM-CSF receptors, thus raising the possibility that the biologic 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. However, the presence of preformed complexes may theoretically reduce the number of ligand-induced receptor heterodimers required to produce a functional signal. These possibilities are currently being investigated.

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CHAPTER 3

GM-CSF binding to its receptor induces oligomerisation of the common beta-subunit

CHAPTER 3

GM-CSF BINDING TO ITS RECEPTOR INDUCES OLIGOMERISATION OF THE COMMON BETA-SUBUNIT.

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McCLURE, B.J. (Candidate) 85% Contribution
Developed C-terminally truncated GMRα variant, generated all cell lines, performed flow cytometry analysis ta and wrote manuscript.

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D'ANDREA, R.J.
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SHORT COMMUNICATION

GM-CSF BINDING TO ITS RECEPTOR INDUCES OLIGOMERISATION OF THE COMMON BETA-SUBUNIT

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The stoichiometry of the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor complex is still unresolved. We have utilised a sensitive, functional assay for receptor homodimerisation to show that GM-CSF induces dimerisation of the common signalling subunit, hβ$_c$. We generated a chimeric cytokine receptor in which the extracellular and transmembrane domains of hβ$_c$ are fused to the cytoplasmic domain of erythropoietin receptor (EPO-R). Given that to induce EPO-R activation and mitogenic signalling there is a requirement for formation of a specific homodimeric complex, we reasoned that the cytoplasmic domain of EPO-R could be utilised as a highly sensitive reporter for functional homodimer formation. We show that, in the presence of a cytoplasmically truncated GM-CSF α-subunit, the hβc–EPO receptor chimera transduces a mitogenic signal in BaF-B03 in response to GM-CSF. This is consistent with formation of a hβ$_c$ homodimer following GM-CSF binding and implies that ligand stimulation induces formation of a higher order complex that contains the hβ$_c$ homodimer.

The granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor complex is comprised of a ligand-specific α-subunit (GMRα) and a signalling β subunit (hβ$_c$) that is shared with the IL-3 and IL-5 receptors.[1] Both subunits belong to the cytokine receptor (CR) superfamily. Triggering of cytoplasmic janus-activated kinases (JAKs) associated with the intracellular portions of cytokine receptors (CRs) is the critical first step in signalling.[2] A major unresolved issue, due to contradictory reports, regards the stoichiometry of the active GMR complex.

While there are significant questions relating to the role of hβ$_c$ dimerisation in GM-CSF activation other CRs are well characterised as homodimeric complexes. One of the best-characterised of this class of receptors is erythropoietin receptor (EPO-R), in which case multiple studies have demonstrated that homodimerisation is critical for signalling.[3–5] The most compelling evidence that activation results in formation of an EPO-R homodimer with a specific conformation is provided by recent studies using a protein-fragment complementation system. This demonstrated that formation of the active homodimer follows a ligand-induced conformational change, involving a switch from a unique unliganded, homodimeric structure, to the ligand-bound, activated, homodimeric complex.[6,7]

Given that to induce EPO-R activation and mitogenic signalling there is a requirement for formation of a specific homodimeric complex, we reasoned that the cytoplasmic domain of EPO-R could be utilised as a reporter for functional homodimer formation. Specifically, we wished to determine whether stimulation with GM-CSF leads to formation of a complex containing a hβ$_c$ homodimer. We generated a chimeric CR in which the extracellular and transmembrane domains of hβ$_c$ are fused to the cytoplasmic domain of EPO-R. We show that in the presence of the chimeric receptor and GMRα, GM-CSF-induces mitogenic signalling in BaF-B03 cells that is independent of the cytoplasmic domain of GMRα. This implies that ligand stimulation induces

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formation of a higher order complex that contains at least an $h\beta_c$ homodimer.

RESULTS AND DISCUSSION

To address the question of whether homodimerisation of $h\beta_c$ occurs during GMR signal transduction a receptor chimera was generated that will only function in response to GM-CSF upon homodimerisation. This chimeric receptor comprises extracellular and transmembrane domains of $h\beta_c$ fused to the intracellular domain of EPO-R. We determined the ability of this receptor to mediate proliferation, when co-expressed with GMRα on BaF-B03 cells and stimulated with GM-CSF. BaF-B03 cells were infected with GMRα in combination with $h\beta_c$ or $h\beta_c$/EPO-R. Receptor expression in the resulting cell pools was determined by flow cytometry. Figure 1 shows that pools expressing GMRα and $h\beta_c$ (A); and GMRα and $h\beta_c$/EPO-R (B) display similar levels of receptors. A control population infected with GMRα alone also expressed similar levels of receptor (data not shown). The proliferative potential of each population in response to GM-CSF was determined. As expected BaF-B03 cells expressing GMRα or $h\beta_c$/EPO-R alone cannot respond to GM-CSF (Fig. 2A and data not shown). EPO-R signalling is normal in BaF-B03 cells, as a population infected with a full-length EPO-R retrovirus construct is capable of responding to EPO with an ED_{50} of 0.3 U/ml (data not shown). Cells expressing both subunits of the GM-CSF receptor can respond to GM-CSF with an ED_{50} of 0.03 ng/ml (Fig. 2B). Importantly, cells expressing both $h\beta_c$/EPO-R and GMRα were able to respond to GM-CSF (Fig. 2C).

Signalling through the chimeric receptor in response to GM-CSF is consistent with the induction of dimerisation of $h\beta_c$/EPO-R. However, we wished to rule out the possibility that the GMRα and EPO-R cytoplasmic domains were interacting leading to activation in the absence of dimerisation of the EPO-R cytoplasmic domain. This was important as the GMRα cytoplasmic domain contributes to signalling in the context of normal GMR.[8] For this we employed a truncated GMRα that lacked cytoplasmic sequences (GMRαΔCOOH). BaF-B03 cells were infected with GMRαΔCOOH together with $h\beta_c$ or $h\beta_c$/EPO-R. Figure 1 shows that the receptor expression of pools expressing GMRαΔCOOH and $h\beta_c$ (C); or GMRαΔCOOH and $h\beta_c$/EPO-R (D) were similar. A control population infected with GMRαΔCOOH alone expressed similar receptor levels (data not shown) and did not respond to GM-CSF (Fig. 2D). Scatchard analysis revealed that GMRαΔCOOH could bind GM-CSF with high affinity when expressed with $h\beta_c$ (data not shown). When $h\beta_c$ is co-expressed with

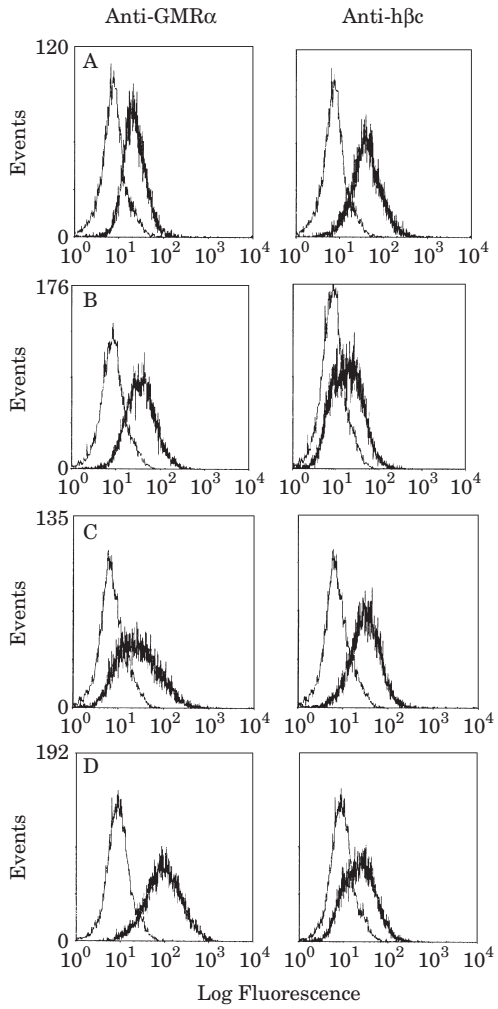

Figure 1. Flow cytometric analysis of transfected BaF-B03 cell lines.

Expression of cells transfected with GMRα+$h\beta_c$ (A); GMRα+$h\beta_c$/EPO-R (B); GMRαΔCOOH+$h\beta_c$ (C); or GMRαΔCOOH+$h\beta_c$/EPO-R (D) was determined as described in Materials and Methods. Thin line indicates reactivity with an isotype-matched control (A14). Thick line represents receptor expression of $h\beta_c$ or $h\beta_c$/EPO-R with 1C1 monoclonal antibody (right panels) and GMRα with 4H1 monoclonal antibody (left panels).

GMRαΔCOOH, BaF-B03 cells no longer exhibit a proliferative response to GM-CSF (Fig. 2E) consistent with the importance of the GMRα cytoplasmic domain for GM-CSF mediated signalling. Importantly, however cells expressing the GMRα cytoplasmic truncation (GMRαΔCOOH) and $h\beta_c$/EPO-R chimera responded to GM-CSF (Fig. 2F) consistent with GM-CSF induced homodimerisation of $h\beta_c$. Thus it is likely that in response to GM-CSF a complex is formed that contains $h\beta_c$ dimers.[9,10] This may occur via an intermediate αβ complex with some signalling capacity, as chimeric molecules, which permit forced heterodimerisation of α and β subunits, are sufficient to generate a mitogenic signal under some circumstances.[11,12] We

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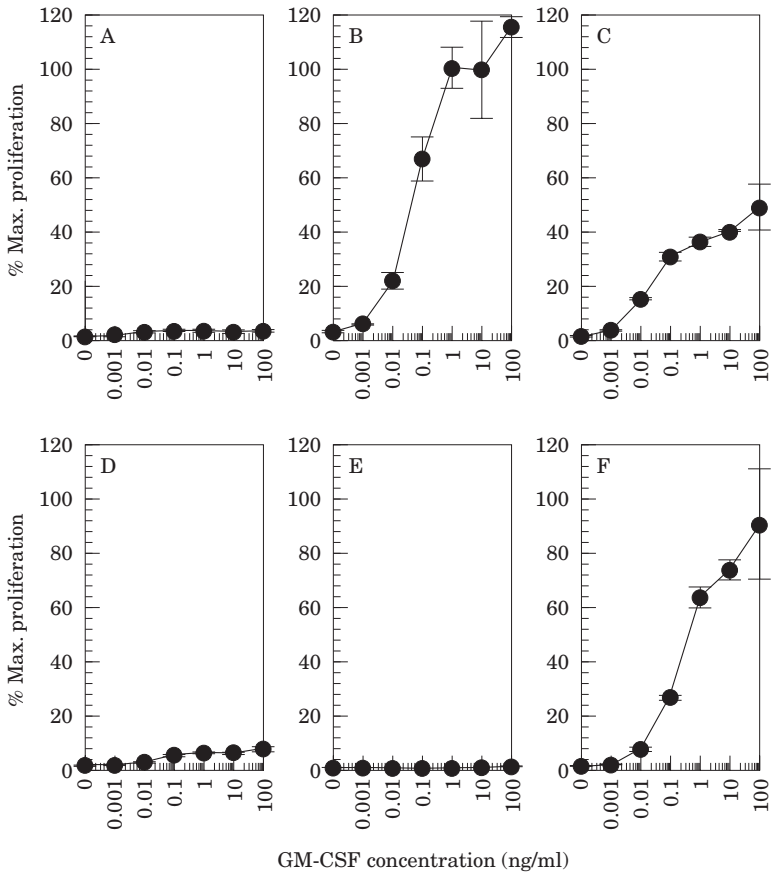

Figure 2. Dimerisation of hβ$_c$ is required for signalling in response to GM-CSF.

Following an overnight starvation, BaF-B03 cells expressing either GMRα alone (A); GMRα+hβ$_c$ (B); GMRα+hβ$_c$/EPO-R (C); GMRαΔ COOH alone (D); GMRαΔDCOOH+hβ$_c$ (E); or GMRαΔCOOH+hβ$_c$/EPO-R (F), were cultured with increasing concentrations of hGM-CSF for 48 h and proliferation measured by the incorporation of ^{3}H-thymidine. The results are expressed as a percentage of maximal proliferation of that seen with murine IL-3 and each point represents the mean of triplicate determination with error bars representing one standard deviation.

have proposed previously that formation of an αβ heterodimer may be an intermediate step in formation of the higher order complex comprising at least an hβ$_c$ dimer.[9,10] Activation of the GM-CSF-induced hβ$_c$ homodimer requires the cytoplasmic domain of the GMRα subunit for mitogenic signalling. This is an interesting observation as it suggests that the hβ$_c$ and EPO-R cytoplasmic domains are functionally different with respect to activation. The differential requirement for the GMRα cytoplasmic domain may reflect subtle differences in the structural requirements for formation of functional hβ$_c$ and EPO-R homodimers. Alternatively, the GM-CSF-induced hβ$_c$ dimer requires additional GMRα-mediated events for activation of proximal signalling molecules. The system outlined here will be potentially useful for studying critical differences in activation of the signalling subunits in response to GM-CSF and EPO-R.

In summary we have described a highly sensitive functional assay for receptor dimerisation to show induced oligomerisation of hβ$_c$ in response to GM-CSF. In this respect activation of the GM-CSF signalling subunit is reminiscent of many other receptors that utilise JAK2 (EPO, thrombopoietin, growth hormone and prolactin receptors). We are currently using chimeric hβ$_c$/EPO-R constructs containing activating mutations[10,13] to establish further the role of homodimerisation in hβ$_c$ activation.

MATERIALS AND METHODS

Generation of chimeric hβ$_c$ and truncated GMRα cDNAs

The extracellular and transmembrane regions of hβ$_c$ and the cytoplasmic domain of EPO-R (kindly provided by Dr L. Jolliffe, J&J Research, Ratitan, New Jersey, USA) were generated by PCR and then cloned into the retroviral vector pRufNeo. An AvrII site was introduced to facilitate cloning and the resulting hβ$_c$/EPO-R chimeric cDNA encoded VLLALRPRRRALK at the junction between the hβ$_c$ transmembrane and EPO-R cytoplasmic domains. The

cytoplasmic deletion of GMRα (GMRαΔCOOH) was generated by PCR to introduce an inframe termination codon 4 residues after the transmembrane domain. Both wild-type GMRα and GMRαΔCOOH cDNA were cloned into the retroviral expression vector pLXSN.

Generation of cell lines, flow cytometry and proliferation assays

Purified plasmid DNA was used to transfect the ecotropic packaging cell line, φ2 and virus from the G418-resistant φ2 cells was used to infect the murine IL-3 dependant pro-B cell line BaF-B03 by co-cultivation. Receptor expression was confirmed by flow cytometry using an XL flow cytometer (Coulter Electronics, Hialaeh, FL, USA). Monoclonal antibodies 1C1,[14] for detection of wild-type and chimeric hβc, and 4H1 and A14 (GMRα-specific and isotype control) were produced as described.[14] Following cytokine starvation overnight, the proliferation of BaF-B03 populations in response to GM-CSF was determined as previously described.[15]

Acknowledgements

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CHAPTER 4

Perverted responses of the human granulocyte-macrophage colony-stimulating factor receptor in mouse cell lines due to a cross-species β-subunit association

CHAPTER 4

PERVERTED RESPONSES OF THE HUMAN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR RECEPTOR IN MOUSE CELL LINES DUE TO CROSS-SPECIES β-SUBUNIT ASSOCIATION.

B.J. McClure, F.C. Stomski, A.F. Lopez and J.M. Woodcock.

This chapter contains a brief report published in *Blood.*

Blood **2001;Volume 98 (10): 3165-3168.**

Molecular assembly of the activated GM-CSF receptor *B. J. McClure*

STATEMENT OF AUTHORSHIP

PERVERTED RESPONSES OF THE HUMAN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR RECEPTOR IN MOUSE CELL LINES DUE TO CROSS-SPECIES β-SUBUNIT ASSOCIATION.

Blood 2001;Volume 98 (10): 3165-3168.

McCLURE, B.J. (Candidate) 85% Contribution
Performed all experiment (except on UT7 cells), interpreted data and wrote manuscript.

Signed. .....Date.....19/12/2016.....

STOMSKI, F.C.
Performed the experiment showing βc tyrosine phosphorylation of UT7 cells.

Signed.. .....Date.....4/1/2017.....

LOPEZ, A.F.
Supervised development of work, and helped in data interpretation and manuscript evaluation.

Signed..... .....Date.....20/12/2016.....

WOODCOCK, J.M.
Supervised development of work, acted as corresponding author and helped in data interpretation and manuscript evaluation.

Signed... .....Date.....20/12/2016.....

Brief report

Perverted responses of the human granulocyte-macrophage colony-stimulating factor receptor in mouse cell lines due to cross-species β-subunit association

Barbara McClure, Frank Stomski, Angel Lopez, and Joanna Woodcock

Transfected murine cell lines are commonly used to study the function of many human cytokine or receptor mutants. This study reports the inappropriate activation of the human granulocyte-macrophage colony-stimulating factor (hGM-CSF) receptor by the human GM-CSF antagonist, E21R, when the human receptor is introduced into the murine cell line BaF-B03. E21R-induced proliferation of the BaF-B03 cells is dependent on transfection with both hGM-CSF receptor α and β_c subunits. Studies on the underlying mechanism revealed constitutive association between human and mouse β_c and GM-CSF receptor-α, tyrosine phosphorylation of mouse and human β_c, and association of phosphorylated mouse β_c into an activated human GM-CSF receptor complex in response to E21R and GM-CSF. This interspecies receptor cross-talk of receptor signaling subunits may produce misleading results and emphasizes the need to use cell lines devoid of the cognate endogenous receptors for functional analysis of ligand and receptor mutants. (Blood. 2001;98:3165-3168)

Introduction

Human granulocyte-macrophage colony-stimulating factor (hGM-CSF) is a pleiotropic cytokine that stimulates the proliferation, differentiation, and survival of myeloid precursors and induces the effector functions of mature myeloid cells.[1,2] These multiple functions are mediated by binding to high-affinity receptors that comprise a GM-CSF–specific α chain (hGMR-α) and an affinity-converting, signal-transducing β subunit ($h\beta_c$), which is shared with the interleukin-3 (IL-3) and IL-5 receptors.[3,4] In the murine system there are 2 β subunits, $m\beta_c$, which is analogous to $h\beta_c$, transducing signals induced by mGM-CSF, mIL-3 or mIL-5, and $m\beta_{IL\text{-}3}$, which is specific for mIL-3.[5,6] Mouse myeloid cell lines, for instance FDCP-1 and BaF-B03, express both mouse β subunits.

Given the importance of $h\beta_c$ in transducing signals that regulate immune responses, hematologic recovery, and, in some cases, leukemia, a significant amount of work has been devoted to structure-function analysis of these cytokine-receptor systems. Studies have led to the engineering of cytokine analogs with unique properties such as the hGM-CSF mutant E21R that behaves as a specific hGM-CSF antagonist.[7] Characterization of the receptors has sought to identify functional regions involved in receptor activation and has identified regions in the cytoplasmic domain of $h\beta_c$ that couple to specific signaling molecules such as JAKs, STATs, and the ras/MAP kinase pathway.[8-10] However results from these studies have in some cases been ambiguous or even conflicting.

Central to the analysis of cytokine and receptor mutants is the choice of experimental system. Predominantly, mouse myeloid cell lines, which are readily transfected with human receptor subunits, have been used. A major problem is the expression of endogenous receptors for these cytokines in these cell lines. For example, transfection of hGMR-α alone in murine FDCP-1 cells was initially reported to be sufficient to mediate a proliferative signal despite only displaying low-affinity hGM-CSF binding.[11] However, it was later shown that functional reconstitution of hGMR required both hGMR-α and $h\beta_c$ subunits,[12] and that the initial observation with hGMR-α alone was confounded by the recruitment of endogenous $m\beta_c$.[13] Likewise, interaction of an extracellular point mutant of $h\beta_c$ with an endogenous mGMR-α has been shown to lead to factor-independent proliferation[14] and chimeras between a constitutively active erythropoietin receptor with the cytoplasmic domain of GMR-α promoted proliferation but only in presence of $m\beta_c$.[15] Here we show an abnormal response of the human GM-CSF antagonist E21R in transfected mouse cell lines and the molecular basis for human-mouse GM-CSF receptor cross-talk. These results emphasize the need for caution when interpreting data from experiments using transfectants and the desirability of using homologous systems.

Study design

Cell lines and proliferation assays

The human erythroleukemic cell line TF1.8, the megakaryocytic leukemia human cell line, UT7, and the murine pro-B-cell line, BaF-B03 (transfected with human GMR-α and $h\beta_c$), were grown as previously described.[16,17] Following cytokine starvation overnight, the proliferation of cell lines in response to hGM-CSF or E21R was determined as previously described.[18] E21R, a single point mutant of hGM-CSF (Gln21Arg) was donated by Bresagen (Adelaide, Australia).

Antibodies

Murine monoclonal antibodies 1C1 and 8E4, for detection of $h\beta_c$, and 4H1 for immunoprecipitating hGMR-α were produced as previously described.[16] 1C1 was biotinylated using a cellular labeling and immunoprecipitation kit (Boehringer Mannheim, Rose Park, SA, Australia) and streptavidin–horseradish peroxidase (HRP) was purchased from Amersham Life Science

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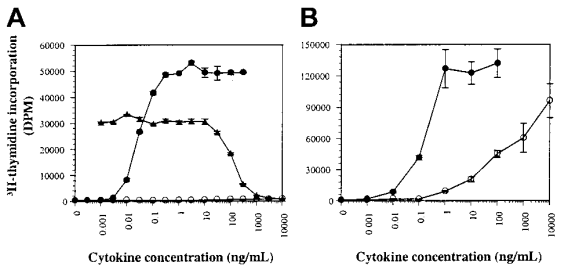

Figure 1. The human GM-CSF antagonist E21R behaves as an agonist on mouse cell lines. Factor-deprived human TF1.8 (A) and mouse hGMR BaF-B03 cells (B) were stimulated with hGM-CSF (●) or E21R (○). Functional antagonism of 0.03 ng/mL GM-CSF with increasing concentrations of E21R on human TF1.8 cells is also shown (▲). Cells were cultured with cytokine for 48 hours and the resulting proliferation was measured by the incorporation of ^{3}H-thymidine. The results are expressed in disintegrations per minute (dpm) and each point represents the mean of triplicate determination with error bars representing 1 SD.

(Little Chalfont, United Kingdom). Anti-mβ_c rabbit polyclonal K-17 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated antibodies specific for mouse or rabbit immunoglobulins were purchased from Pierce (Rockford, IL) and Dako (Botany, NSW, Australia), respectively. The HRP-conjugated antiphosphotyrosine monoclonal antibodies, PY20 and 4G10, were obtained from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology (Lake Placid, NY), respectively.

Immunoprecipitation, sodium dodecylsulfate–polyacrylamide gel electrophoresis, and immunoblotting

Analysis of ligand-induced receptor complexes and their phosphotyrosine status was determined using starved cells that were stimulated with factors for 5 minutes at room temperature at indicated concentrations. Cells were lysed and immunoprecipitated proteins were run on reducing sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblotting as previously described.[19]

Results and discussion

Human GM-CSF supports the proliferation of human cells by activating endogenous hGMR.[7] The antagonistic hGM-CSF mutant, E21R, however, cannot mediate a proliferative response in human cells and is unable to bind hGMR with high affinity.[7] We observed that the biologic activity of E21R is strikingly different when analyzed in hGMR-transfected murine cell lines. First, the biologic activity of E21R was determined in comparison to wild-type hGM-CSF on the human cell line, TF1.8, and the murine cell line BaF-B03 transfected with both hGMR-α and hβ_c (hGMR BaF-B03). Human GM-CSF induces proliferation of TF1.8 cells with an effective dose (ED_{50}) of 0.03 ng/mL, whereas E21R is unable to elicit a proliferative response and antagonizes the activity of wild-type GM-CSF (Figure 1A).[7] Surprisingly, however, E21R induced a proliferative response in the hGMR BaF-B03 cell line at concentrations of E21R above 10 ng/mL (Figure 1B).

It has been previously described that hGMR-α can interact with either hβ_c or mβ_c to transmit a growth signal in response to hGM-CSF in BaF-B03 cells.[13] However, the interaction of hGMR-α with mβ_c, unlike hβ_c, does not form a high-affinity receptor. The proliferation induced by E21R in hGMR BaF-B03 cells is also the result of a low-affinity interaction with a dissociation constant of approximately 4 nM (data not shown) as determined by Scatchard analysis of saturation binding,[18] identical to the affinity of E21R measured on human neutrophils.[7]

The abnormal proliferative response of hGMR BaF-B03 cells to E21R differs from the previously described cross-talk phenomenon between hGMR-α and mβ_c because it is dependent on the coexpression of both hGMR-α and hβ_c. This is highlighted by the inability of BaF-B03 cells expressing hGMR-α alone to respond to E21R, even at concentrations of 100 μg/mL (data not shown). Because the abnormal agonism was only observed in cell lines where mβ_c is endogenously expressed, it suggested that the unexpected activity demonstrated by E21R may be the result of a novel cross-species interaction, involving both hβ_c and mβ_c.

Together with the inability of E21R to mediate a proliferative response in human cells, it cannot stimulate tyrosine phosphorylation of hβ_c. The tyrosine phosphorylation status of hβ_c was investigated to determine if E21R can activate hβ_c in this murine system. Tyrosine phosphorylation of hβ_c was readily detected in the murine cell line hGMR BaF-B03 after stimulation with E21R (Figure 2B), but not the human cell line expressing endogenous hβ_c, UT7 (Figure 2A), and TF1.8 (data not shown). In comparison, tyrosine phosphorylation of hβ_c was detected following stimulation

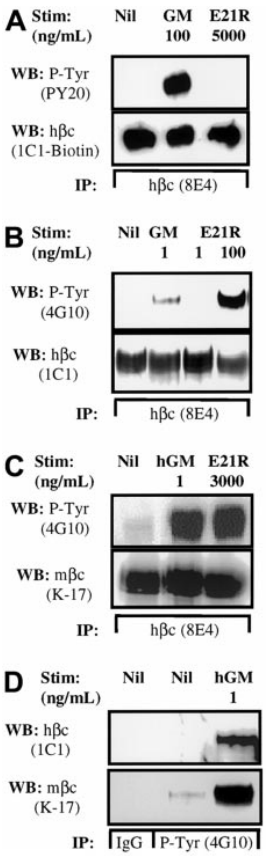

Figure 2. Transfected hβ_c spontaneously associates with endogenous mβ_c and the β_c complex is phosphorylated inappropriately in response to E21R. Factor-deprived human UT7 cells (A) or mouse hGMR BaF-B03 cells (B-D) were stimulated as indicated for 5 minutes at 25°C. Cells were lysed and hβ_c was immunoprecipitated with 8E4 anti-hβ_c antibody (A-C). The presence of tyrosine phosphorylated hβ_c was detected using an antiphosphotyrosine antibody, PY20 (A) or 4G10 (B,C). Filters were then stripped and reprobed for the presence of hβ_c with 1C1 anti-hβ_c antibody (A,B). The presence of mβ_c following hβ_c immunoprecipitation was determined on a duplicate filter using anti-mβ_c antibody, K-17 (C). Tyrosine phosphorylated proteins were immunoprecipitated with the 4G10 antiphosphotyrosine antibody (D) and the presence of hβ_c detected using 1C1 antibody. The filter was then stripped and reprobed for the presence of mβ_c with K-17.

with hGM-CSF in both human UT7 cells and murine hGMR BaF-B03 cells (Figure 2A,B). The dose response of E21R-induced tyrosine phosphorylation of $h\beta_c$ is consistent with the proliferation data where responsiveness occurs at a concentration of E21R above 10 ng/mL, with no response at 1 ng/mL (Figure 1B).

Because $m\beta_c$ functions when recruited to a hGM-CSF–hGMR-α complex, we investigated if this molecule plays a role in facilitating the tyrosine phosphorylation of $h\beta_c$ in response to hGM-CSF or E21R in hGMR BaF-B03 cells. Stimulation of hGMR BaF-B03 cells with either GM-CSF or E21R resulted in tyrosine phosphorylation of $h\beta_c$ (Figure 2C). Significantly, $m\beta_c$ was associated with $h\beta_c$ and this interaction appears to occur regardless of stimulation (Figure 2C). It is interesting to note that dimerization of a least 2 $h\beta_c$ subunits is required for hGMR activation.[19,20] The interaction between mouse and human β_c may provide the molecular basis to support the agonistic activity of E21R and mediate this perverted receptor response observed in this murine cell line.

Phosphotyrosine immunoprecipitations were performed to address if preassociation between the human and murine β_c subunits allows $m\beta_c$ to be associated with an activated hGMR complex. Human β_c was immunoprecipitated by an antiphosphotyrosine antibody following stimulation of hGMR BaF-B03 cells with hGM-CSF but not when left unstimulated or with an isotype-matched control antibody (Figure 2D). Mouse β_c appears to be weakly phosphorylated in the absence of stimulation, but interestingly was strongly coimmunoprecipitated with antiphosphotyrosine antibody after stimulation with hGM-CSF (Figure 2D), suggesting an increase in phosphorylation of $m\beta_c$ on stimulation with the human ligand.

The hGMR subunits GMR-α and $h\beta_c$ have previously been shown to exist as a preformed complex on human cells,[20,21] and we have now shown that $m\beta_c$ and $h\beta_c$ are also associated prior to ligand stimulation on hGMR BaF-B03 cells. To determine whether $m\beta_c$ is also a component of a preformed complex on hGMR BaF-B03 cells, hGMR-α was immunopreciptated from nonstimulated and GM-CSF–stimulated cells. Immunoblotting revealed that both $h\beta_c$ and $m\beta_c$ were associated with hGMR-α regardless of stimulation (data not shown) suggesting that $m\beta_c$ chain may be a component of a preformed hGMR receptor on hGMR BaF-B03 cells. Therefore activation of the receptor by GM-CSF and E21R may be mediated by a preformed hGMR-α–$h\beta_c$–$m\beta_c$ complex in these cells.

The ability of human and mouse β_c subunits to interact and influence responses of a hGM-CSF variant introduces a new level of complexity in the analysis of ligand-receptor interaction and subsequent signaling capabilities. It also raises the question of the influence this interaction has had on previous hGMR studies that have been performed in murine cell lines. Clearly $m\beta_c$ can become associated within an active hGMR complex, and it is important to consider its contribution to the receptor's biologic response. This is of concern especially when investigating the activation of downstream signaling molecules.[22] In light of the interaction between $h\beta_c$ and $m\beta_c$ it will be difficult to discriminate which signaling molecules have emanated from the $h\beta_c$ alone.[23] In addition, the associated $m\beta_c$ subunit may facilitate signaling by an otherwise inactive $h\beta_c$ mutant. This may explain the surprising result seen where $h\beta_c$ deficient in intracellular tyrosines is still capable of responding to hGM-CSF.[10] In addition, the observation that a cytoplasmically truncated $h\beta_c$ can still respond to hGM-CSF when expressed in BaF-B03 cells may be potentiated via signaling through $m\beta_c$.[24] The contribution this interspecies subunit interaction plays when distinguishing regions in hGMR responsible for differentiation or proliferation in murine myeloid cell lines remains unclear. It has been demonstrated previously that $h\beta_c$ can form homodimers that are activated in response to hGM-CSF.[20,21] The association between $m\beta_c$ and $h\beta_c$ must result in a complex that has different properties to $h\beta_c$ homodimers because E21R is unable to activate the latter.

A number of receptors belonging to the cytokine receptor family show cross-species specificity between human and murine components. Because receptor dimerization is a common theme in receptor activation, it cannot be ruled out that interaction of interspecies signaling components may obscure results when studying a wide range of human receptors in murine cells and similar precautions may need to be taken. In addition to our findings that the use of transfected murine cell lines may not be the best approach when studying hGM-CSF ligand variants, other cytokines show similar discrepancies. An analogous situation has been reported for a human IL-4 mutant that can mediate either agonistic or antagonistic responses when studied in either a murine or human cell line, respectively.[25] Similarly, mutational analysis of thrombopoietin shows conflicting data in the identification of residues functionally important in interaction with its receptor when screened on Mpl-transfected BaF-B03 cells compared to enzyme-linked immunosorbent assay or Biacore analysis.[26,27]

The demonstration of an inherent association between mouse and human β_c shown here highlights the need for a careful selection of appropriate systems. In the case of the hGMR the availability of β_c knockout mice permits the use of cells from these animals for receptor reconstitution experiments as a better background to analyze structural and functional outcomes with the hGMR.

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CHAPTER 5

Molecular assembly of the granulocyte-macrophage colony-stimulating factor receptor complex

CHAPTER 5

MOLECULAR ASSEMBLY OF THE TERNARY GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR RECEPTOR COMPLEX.

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Molecular assembly of the activated GM-CSF receptor *B. J. McClure*

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HEMATOPOIESIS

Molecular assembly of the ternary granulocyte-macrophage colony-stimulating factor receptor complex

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic cytokine that stimulates the production and functional activity of granulocytes and macrophages, properties that have encouraged its clinical use in bone marrow transplantation and in certain infectious diseases. Despite the importance of GM-CSF in regulating myeloid cell numbers and function, little is known about the exact composition and mechanism of assembly of the GM-CSF receptor complex. We have now produced soluble forms of the GM-CSF receptor α chain (sGMRα) and β chain (sβc) and utilized GM-CSF, the GM-CSF antagonist E21R (Glu21Arg), and the βc-blocking monoclonal antibody BION-1 to define the molecular assembly of the GM-CSF receptor complex. We found that GM-CSF and E21R were able to form low-affinity, binary complexes with sGMRα, each having a stoichiometry of 1:1. Importantly, GM-CSF but not E21R formed a ternary complex with sGMRα and sβc, and this complex could be disrupted by E21R. Significantly, size-exclusion chromatography, analytical ultracentrifugation, and radioactive tracer experiments indicated that the ternary complex is composed of one sβc dimer with a single molecule each of sGMRα and of GM-CSF. In addition, a hitherto unrecognized direct interaction between βc and GM-CSF was detected that was absent with E21R and was abolished by BION-1. These results demonstrate a novel mechanism of cytokine receptor assembly likely to apply also to interleukin-3 (IL-3) and IL-5 and have implications for our molecular understanding and potential manipulation of GM-CSF activation of its receptor. (Blood. 2003;101: 1308-1315)

Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine produced by many cells in the body that regulates the production, effector cell function, and survival of myeloid cells.[1-4] Macrophages and granulocytes rise in numbers and exhibit a prolonged life span and enhanced effector function in response to GM-CSF,[5,6] properties that have encouraged its use in bone marrow transplantation[7] and infectious diseases such as those associated with AIDS.[8] In addition, GM-CSF controls dendritic cell production, differentiation, and function and potentiates responses of $CD4^+$ T cells in vivo.[9,10] This dual action of GM-CSF has encouraged its utilization in different vaccination strategies.[11] On the other hand, these same properties have implicated GM-CSF in myeloid leukemia and several inflammatory conditions such as asthma[12] and rheumatoid arthritis.[13]

The actions of GM-CSF are mediated by specific receptors composed of 2 different subunits, a receptor α chain (GMRα),[14] which provides specificity and the major binding contact, and a β chain (βc),[15] which is common with the interleukin-3 (IL-3) and IL-5 receptors, promotes affinity conversion, and acts as the major signal transducer. For this complex to be assembled and to signal, there exist structural and dimerization requirements, some of which have been defined. Extensive structure-function analysis has identified several residues involved in GM-CSF, GMRα, and βc protein interaction and biologic activity. For example, the binding of GM-CSF to GMRα involves an electrostatic interaction between Asp112 in the fourth α helix of GM-CSF and Arg280 in the F-G loop of GMRα.[16,17] The biologic activities and high-affinity binding of GM-CSF are exquisitely dependent on Glu21 in the first α helix of GM-CSF, although direct contact with βc has not been demonstrated. Substitution of this amino acid with arginine generates a GM-CSF analog, E21R (Glu21Arg), which exhibits only low-affinity binding and is unable to stimulate cellular proliferation and mature cell functions.[18] Importantly, E21R is able to antagonize GM-CSF binding and function[19]; however, the molecular basis of this antagonism is not fully understood. In βc, residues in the B-C loop (Tyr365, His367, Ile368) and F-G loop (Tyr421) of domain 4 are involved in GM-CSF high-affinity binding and function.[20-23] The monoclonal antibody (mAb) BION-1, which binds an area in βc encompassing these loops, blocks GM-CSF binding and biologic activities.[24]

Dimerization of the α and βc subunits of this family of cytokine receptors is recognized as a crucial step for their activation; however, the exact composition of the assembled complex remains unclear. A number of studies suggest that simple heterodimerization is sufficient to activate the GM-CSF receptor,[25] whereas both cross-linking and dominant-negative studies using surface-expressed receptors suggest that the formation of higher-order GM-CSF receptor complexes is required for receptor activation.[26,27] Dimerization of βc in

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particular has also been shown to be an important and necessary step for receptor activation,[28,29] probably reflecting the need to bring into close proximity the cytoplasmic domains of 2 βc molecules associated with Janus kinase-2 (JAK-2), resulting in JAK transphosphorylation and receptor phosphorylation. Interestingly, βc has been shown to crystallize as a dimer[30] and to exist as a preformed homodimer on the cell surface.[26,28] Despite these findings, little is known about the full assembly of this family of receptors, the intermediate steps in their formation, and how receptor assembly may be selectively modulated.

In this paper we show for the first time the full assembly of the human GM-CSF receptor in solution. This shows a novel mode of cytokine receptor assembly in which 1 molecule of GM-CSF associates with 1 molecule of GMRα and 2 molecules of βc. In addition, these studies reveal an essential, direct interaction between GM-CSF and βc and provide a molecular understanding of GM-CSF antagonism by E21R or BION-1. This novel mode of receptor assembly may also apply to the IL-5 and IL-3 receptors.

Materials and methods

Human GM-CSF and GM-CSF analogs

Soluble wild-type human GM-CSF was produced in *Escherichia coli* and recovered from the periplasmic space by osmotic shock as described previously.[19] Crude periplasmic extracts were adjusted to 25 mM N-ethylmorpholine HCl (NEM), pH 7.0, loaded onto Q Sepharose Fast Flow (Amersham Biosciences, Sydney, Australia) equilibrated in 25 mM NEM, pH 7.0, and a linear gradient of 0 to 600 mM NaCl in 25 mM NEM, pH 7.0, used to elute the bound proteins. GM-CSF purified by anion exchange was further purified by reversed phase high-performance liquid chromatography (HPLC), lyophilized, dissolved in phosphate-buffered saline (PBS) as previously described,[19] and sterile-filtered (0.45 μm). The E21R analog of GM-CSF (BresaGen, Adelaide, South Australia) contains a glutamate to arginine substitution at residue 21 and a modified 12–amino acid leader peptide, MFATSSSTGNDG, to facilitate expression in *E coli*.[31]

Radiolabeling of human GM-CSF

To enable phosphorylation of GM-CSF under mild conditions, we made the GM-CSF analog, SGMKIN, in which the amino acids from alanine at position 3 to proline at position 6 were replaced by the peptide sequence RRASV, which is recognized by the catalytic subunit of cyclic adenosine monophosphate (cAMP)–dependent protein kinase from heart muscle.[32] Complementary oligonucleotides were used to create a *Hin*dIII/*Nco*I fragment encoding the N-terminal 12 amino acids of SGMKIN. This fragment was ligated with an *Nco*I/*Bam*HI fragment encoding the C-terminal 116 amino acids of human GM-CSF (hGM-CSF) into *Hin*dIII/*Bam*HI–digested pIN-III-OmpH3 expression vector[19] to create the plasmid, pSGMKIN. Soluble SGMKIN was expressed in *E coli* and purified as described for wild-type GM-CSF. The final product was at more than 95% purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the SGMKIN analog displayed biologic activity indistinguishable from the wild-type GM-CSF (data not shown). Labeling of SGMKIN with ^{32}P used a protocol adapted from Kaelin et al.[32] Fifty micrograms of purified SGMKIN was incubated in a 200-μL reaction mix containing 20 mM Tris (tris(hydroxymethyl)aminomethane) HCl, pH 7.5; 100 mM NaCl; 12 mM $MgCl_2$; 10 mM β-mercaptoethanol; 1 μCi/μL (0.037 MBq/μL) [γ-^{32}P]adenosine triphosphate ([γ-^{32}P]ATP) (3000 Ci/mmol [111 000 GBq/mmol]; Geneworks, Adelaide, South Australia), and 1 U/μL of the protein kinase catalytic subunit (Sigma, Castle Hill, Australia). The reaction proceeded at 4°C for 30 minutes, was terminated by the addition of 200 μL of 100 mM EDTA (ethylenediaminetetraacetic acid), adjusted to 0.1% (vol/vol) trifluoroacetic acid (TFA; Auspep, Parkville, Australia), 1% (vol/vol) acetic acid, and loaded onto a Sep-Pak C18 reversed phase cartridge (Waters, Rydalmere, Australia) equilibrated in 0.01% TFA. The cartridge was washed with 0.01% TFA and bound SGMKIN eluted using 10 mL of 50% (vol/vol) acetonitrile in the presence of 0.01% TFA. Ten equal fractions were collected, and those containing the peak of eluted radioactivity were pooled and concentrated using a Speed-vac (Savant Instruments, Farmingdale, NY) to a final volume of approximately 100 μL.

Production of recombinant soluble GM-CSF receptor subunits

DNA fragments encoding the soluble extracellular domains of GMRα (sGMRα) or βc (sβc) were generated by PCR using the primers 5′-CTGACCGGATCCATGCTTCTCCTGGTGACAAGCC-3′ and 5′-GTACACGGATCCGAATTCTTACCCGTCGTCAGAACCAAATTC-3′ for sGMRα and 5′-CTGACCGGATCCATGGTGCTGGCCCAGGGGCTGC-3′ and 5′-CAGCACGGATCCGAATTCTTACGACTCGGTGTCCCAGGAGCG-3′ for sβc, with *Eco*RI and *Bam*HI restriction sites underlined. Stop codons were inserted immediately prior to the transmembrane domain for each receptor molecule, following Gly at position 320 for GMRα and Ser at position 438 for βc. The PCR products were digested with *Bam*HI and *Eco*RI and cloned into the baculovirus transfer vector BacPAK9 (Clontech, Palo Alto, CA) and the sequence of the cloned inserts were verified by cycle sequencing with BigDye chemistry (Applied Biosystems, Foster City, CA). The cDNA encoding sGMRα and sβc was introduced into the genome of *Bsu*36I-digested BacPAK6 viral DNA (Clontech) by homologous recombination following the manufacturer's instructions. Expression of recombinant protein is under the control of the strong polyhedrin promoter. Large-scale expression of sGMRα or sβc was performed by infection of Sf21 cells, grown in serum-free Ex-Cell 420 medium (JRH Biosciences, Brooklyn, Australia), with recombinant baculovirus at a multiplicity of infection of 0.3. Supernatant containing soluble receptor was harvested following incubation at 27°C for 5 to 7 days.

Purification of soluble GMRα and sβc

Conditioned media containing sGMRα (20 L) or sβc (9 L) were concentrated to less than 1 L using tangential flow filtration cartridges (10 000 molecular weight cutoff, 0.23 m^2) (Millipore, Northryde, Australia) operated at 80 kPa and 4°C. Insoluble material in the concentrate was pelleted at 3000*g* for 30 minutes and the resulting supernatant filtered (3 μm) prior to affinity chromatography. Affinity matrices were prepared by coupling E21R or the anti-βc mAb, BION-1,[24] to cyanogen bromide (CNBr)–activated Sepharose 4B (Amersham Biosciences) following the manufacturer's instructions. Recombinant soluble receptor was bound to the affinity matrix, washed extensively in PBS containing 0.01% (vol/vol) polyoxyethylene 20 sorbitan monolaurate (Tween 20), and bound proteins eluted with 100 mM NaCl, 100 mM sodium acetate (pH 4.0). The eluate fractions were immediately neutralized using 2 M Tris and analyzed for the presence of soluble receptor by SDS-PAGE. Fractions containing purified soluble receptor were pooled and concentrated using a stirred-cell device with a 10 000 molecular weight cutoff, low protein-binding membrane (YM10; Millipore) operated at 300 kPa and 4°C. Concentrated soluble receptor was dialyzed extensively into PBS, sterile-filtered (0.2 μm), and stored at 4°C.

SDS-PAGE

Samples were analyzed on 10% or 12.5% polyacrylamide gels containing 38:1 acrylamide/bisacrylamide under reducing or nonreducing conditions as specified. Bands were visualized by staining with either Coomassie brilliant blue R-250 or silver.[33]

Mass spectrometry

Electrospray ionization mass spectrometry was performed using a PE/Sciex API100 mass spectrometer (Perkin-Elmer Sciex Instruments, Ontario, Canada). Protein samples were desalted in-line using a 1 × 10 mm reversed phase column eluted with 60% (vol/vol) acetonitrile in the presence of 0.04% (vol/vol) TFA and the primary mass spectrum transformed to give a true-mass profile using instrument software.

Protein analyses by size-exclusion chromatography

Size-exclusion chromatography was initially used to quantify purified soluble receptors and their ligands. Samples were chromatographed on a

SMART system with a Superdex 200PC 3.2/30 (3.2 mm × 300 mm) column (Amersham Biosciences) operated at 40 μL/min at 25°C using 150 mM NaCl, 50 mM sodium phosphate, pH 7.0, as running buffer. The area under the protein peak was integrated using the extinction coefficient (absorbance units × mL^{-1} × mg^{-1}) calculated for each protein: GM-CSF, 0.95; E21R, 0.88; sGMRα, 1.17; sβc, 1.95.

To analyze protein-protein interactions, individual proteins and protein complexes were prepared in a final volume of 50 μL, adjusted with PBS as required, and incubated at 25°C for at least 1 hour. Samples were analyzed by size-exclusion chromatography using the SMART system as described above with data presented from representative experiments (n = 5). The dependence of elution time on the $\log_{10}$ (MW) of protein standards was used to calibrate the column and to generate a trend line for each set of standards. External standards included myoglobin, MW 17 kDa; ovalbumin, MW 44 kDa; γ-globulin, MW 158 kDa; and thyroglobulin, MW 670 kDa (Biorad Laboratories, Hercules, CA). Internal standards were GM-CSF, MW 14.5 kDa; E21R, MW 15.7 kDa; sGMRα, MW 43 kDa; and sβc, MW 101 kDa as determined by mass spectrometry and SDS-PAGE. Soluble βc was found to be a dimer by size-exclusion chromatography consistent with previous reports.[30] Calibration curves constructed from the external and internal standards were essentially parallel (see Figure 2A). The calibration curve for the internal standards was extrapolated to higher mass (670 kDa) because this was found to be the limit of the linear range for the external standards.

Analytical ultracentrifugation

The molecular weights of GM-CSF, E21R, sGMRα, sβc, and the binary and ternary complexes were determined by sedimentation equilibrium. Individual proteins and protein complexes were isolated by size-exclusion chromatography using a fast protein liquid chromatography (FPLC) system with a Superdex 200 10/30 (10 mm × 300 mm) column (Amersham Biosciences) operated at 0.5 mL/min at 25°C using 150 mM NaCl, 50 mM sodium phosphate, pH 7.0, as running buffer. Pooled fractions were concentrated using Centricon 10 microconcentrators (Amicon, Beverly, MA). Sedimentation equilibrium experiments were performed using a Beckman XL-A analytical ultracentrifuge equipped with a Ti60 rotor (Beckman, Palo Alto, CA) and filled epon centerpieces (12-mm path length). Sedimentation equilibrium profiles were obtained at 20°C using the rotor speeds indicated. Equilibrium distributions were fitted by nonlinear regression analysis to obtain best-fit values for the M (1-νρ), where M is the molecular weight and ν the partial specific volume of the sedimenting species and ρ the solution density. The compositional molecular weights of the proteins and the partial specific volumes of GM-CSF and E21R were calculated from their amino acid sequences. Partial specific volumes for the glycosylated forms of sGMRα and sβc were calculated assuming these proteins were monomer and dimmer, respectively. A value of 0.622 mL/g was assumed for the partial specific volume of carbohydrate. The experimental value of M (1-νρ) and the molecular weight (M_p) and partial specific volume of the protein component were then used to solve for the weight fraction of bound carbohydrate and hence the partial specific volume of the carbohydrate-bound protein. Values for the partial specific volumes of the GM-CSF/sGMRα and E21R/sGMRα complexes were calculated assuming a 1:1 complex and no volume change on association. A value of 0.72 mL/g was assumed for the GM-CSF/sGMRα/sβc complex.

Cross-linking experiments

Stable cross-linking of sβc or soluble complexes of ligand with sβc was performed by incubation of 2.6 μg sβc with either 2.4 μg GM-CSF or E21R for 1 hour at 25°C followed by addition of BS^3 cross-linker (Pierce, Rockford, IL) at a final concentration of 0.1 mg/mL for 10 minutes. The reaction was then stopped by addition of ethanolamine HCl, pH 8.0, to a final concentration of 100 mM. Cross-linked proteins were subjected to reducing SDS-PAGE and compared with non–cross-linked material. Antibody Fab fragments of BION-1 (anti-βc fourth domain blocking mAb) and 2H1 (anti-βc fourth domain control mAb) used in cross-linking experiments were generated by digestion with ficin using the Immunopure IgG_1 Fab Preparation Kit (Pierce) following the manufacturer's instructions. Fab fragment (18 μg) was preincubated with 2.6 μg sβc for 30 minutes at 25°C prior to the addition of 2.4 μg GM-CSF in a final volume of 20 μL for a further hour. Cross-linking was then performed as above followed by SDS-PAGE analysis.

Results

Production, purification, and analysis of GM-CSF soluble receptor components

Complementary DNA fragments encoding the extracellular domains of GMRα and βc (sGMRα and sβc) were generated by PCR and cloned into a baculovirus transfer vector. Following introduction into a baculovirus expression system by homologous recombination, the soluble receptor components were generated by infection of Sf21 cells. Purification of the soluble receptors was achieved by affinity chromatography using immobilized ligand for sGMRα and immobilized mAb BION-1[24] for sβc. Purified soluble receptors were recovered at more than 95% purity as assessed by silver-stained SDS-PAGE under reducing conditions (Figure 1A) with an apparent molecular weight (MW) of approximately 43 kDa for sGMRα and 55 kDa for sβc. Importantly, these MWs determined for sGMRα and for sβc did not alter when analyzed under nonreducing conditions (Figure 1B), indicating the absence of disulfide-linked dimers. A small amount of disulfide-aggregated sβc was visible by nonreducing SDS-PAGE (Figure 1B) and as an early, minor peak during size-exclusion chromatography (Figure 2C). The absence of detectable disulfide-linked dimers in sβc was confirmed by ion-spray mass spectrometry, which demonstrated that the protein preparation had a major species of 50.623 kDa with several minor species representing glycosylation variants.

The physical properties of sGMRα and sβc were further characterized by size-exclusion chromatography. We initially determined the retention times of sGMRα (Figure 2B), sβc (Figure 2C), GM-CSF (Figure 2D), and E21R (Figure 2G). The individual proteins eluted at 39.17 minutes for sGMRα, 34.75 minutes for sβc, 44.56 minutes for GM-CSF, and 44.11 minutes for E21R. External MW standards for calibration of the size-exclusion chromatography (Figure 2A) indicated that sGMRα, GM-CSF, and E21R were monomeric but that sβc was dimeric. The dimeric nature of sβc was confirmed by cross-linking experiments with purified sβc, which produced a covalent dimer with a MW of 100 kDa as determined by SDS-PAGE (see Figure 8B). The observation that sβc exists as a dimer is consistent with a recent report describing the structure of the extracellular domain of βc expressed in insect cells.[30,34] We observed that both the ligands and the receptor components eluted from size-exclusion chromatography

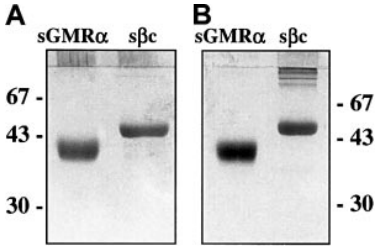

Figure 1. SDS-PAGE analysis of purified sGMRα and sβc. Soluble GMRα and sβc were produced by Sf21 cells infected with recombinant baculovirus encoding appropriate cDNA and affinity purified from the supernatant as described in "Materials and methods." Soluble GMRα (1 μg) and sβc (0.5 μg) were fractionated by 10% SDS-PAGE under reducing (A) and nonreducing (B) conditions and silver stained. The positions of molecular weight markers are shown in kilodaltons.

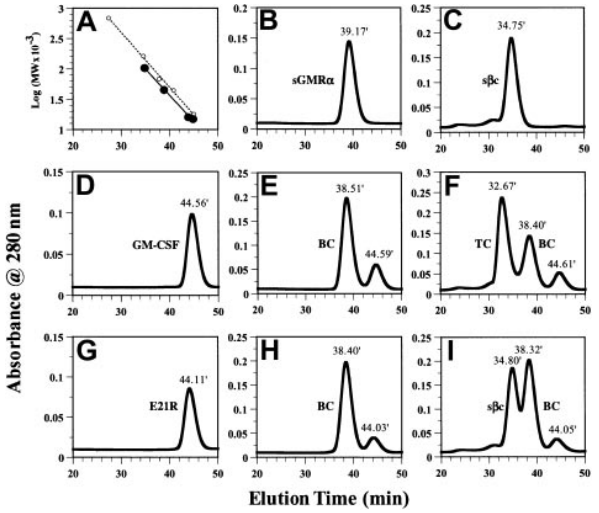

Figure 2. GM-CSF but not the GM-CSF analog E21R induces the assembly of the ternary GM-CSF receptor complex in solution. The presence and molecular weight of individual proteins and protein complexes were determined using size-exclusion chromatography as described in "Materials and methods." (A) Linear regression of $\log_{10}$ (MW $\times 10^{-3}$) versus elution times using external (○) and internal (●) standards for calibration of the column. (B-I) Individual proteins sGMRα (B), sβc (C), GM-CSF (D), and E21R (G) were applied separately. Mixtures of sGMRα (6 μM) and GM-CSF (12 μM) (E); sβc (3 μM), sGMRα (6 μM), and GM-CSF (12 μM) (F); sGMRα (6 μM) and E21R (12 μM) (H); sβc (3 μM), sGMRα (6 μM), and E21R (12 μM) (I) were incubated for 1 hour before being applied to the column. The number above each peak represents elution time. Peaks containing binary (BC) or ternary (TC) complexes are indicated.

earlier than expected from the elution times of the external MW standards (Figure 2A). We chose to use the proteins of interest as internal MW standards and constructed a calibration curve for the internal MW standards that is parallel to that constructed from the external MW standards (Figure 2A). This is expected to provide a superior estimate of the masses of the receptor complexes.

Soluble GMRα interactions with GM-CSF and E21R

Purified sGMRα (6 μM) was incubated with GM-CSF (12 μM) and fractionated on a Superdex 200 column, producing a modest shift (from 39.17 minutes to 38.51 minutes) in the elution time of sGMRα (Figure 2E). The shifted peak, with an apparent MW of 48 kDa, contained both GM-CSF and sGMRα as determined by SDS-PAGE analysis of fractions (data not shown). The MW of the GM-CSF/sGMRα binary complex is consistent with a stoichiometry of 1:1 as has previously been described.[35] The complete peak shifts observed when sGMRα binds GM-CSF suggest that all of this soluble receptor is competent to bind ligand. Saturation binding experiments revealed that GM-CSF bound to sGMRα with a dissociation constant (K_d) of 1.5 to 9 nM, similar to that seen with cell surface–expressed GMRα (data not shown).

Purified sGMRα (6 μM) was incubated with E21R (12 μM) and fractionated on a Superdex 200 column, producing a modest shift (from 39.17 minutes to 38.40 minutes) in the elution time of sGMRα (Figure 2H). The shifted peak, with an apparent MW of 49 kDa, contained both E21R and sGMRα as determined by SDS-PAGE analysis of fractions (data not shown). The MW of the E21R:sGMRα binary complex is consistent with a stoichiometry of 1:1.

The sβc induces the formation of a GM-CSF ternary complex

Purified sβc (3 μM) was incubated with sGMRα (6 μM) plus GM-CSF (12 μM) and fractionated on a Superdex 200 column, producing a complete shift in the elution time of sβc (from 34.75 minutes to 32.67 minutes) as well as peaks corresponding to the binary complex at 38.40 minutes and free ligand at 44.61 minutes (Figure 2F). The peak eluting at 32.67 minutes had an apparent MW of 155 kDa and contained GM-CSF, sGMRα, and sβc as determined by SDS-PAGE analysis of fractions (Figure 3A). The MW of this ternary GM-CSF receptor complex is consistent with a stoichiometry of 1 GM-CSF:1 sGMRα:2 sβc.

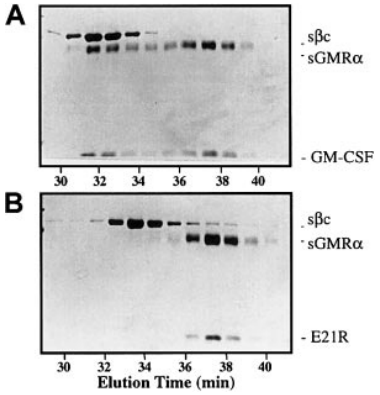

Figure 3. SDS-PAGE analysis of the ternary GM-CSF receptor complex. Mixtures of sβc, sGMRα, and either GM-CSF (A) or E21R (B) were analyzed by size-exclusion chromatography as described for Figure 2F and I. Fractions were collected at 1-minute intervals, fractionated by 12.5% SDS-PAGE under reducing conditions, and silver stained.[33] The positions of individual components are indicated.

In contrast, no ternary complex was observed when purified sβc (3 μM) was incubated with sGMRα (6 μM) plus E21R (12 μM) and fractionated on a Superdex 200 column (Figure 2I). Whereas the binary complex eluting at 38.32 minutes contained E21R and sGMRα, the peak at 34.80 minutes contained sβc but no sGMRα or E21R as determined by SDS-PAGE analysis of fractions (Figure 3B).

E21R disrupts the formation of the ternary GM-CSF receptor complex

To investigate whether the formation of a binary complex was an intermediate step in the formation of the ternary GM-CSF receptor complex, we tested the effect of E21R in this process. Purified sβc (3 μM) was incubated with sGMRα (6 μM) and GM-CSF (12 μM) for 1 hour. A 100-fold molar excess of E21R was then added, and after a further 1-hour incubation the mixture was fractionated on a Superdex 200 column. In the absence of E21R the ternary GM-CSF receptor complex eluted at 33.12 minutes (Figure 4). Significantly, in the presence of a 100-fold molar excess of E21R (Figure 4) there was a reduction in the amount of ternary GM-CSF receptor complex and an increase in its elution time (34.10 minutes), more comparable with the elution time of free sβc (34.75 minutes). The

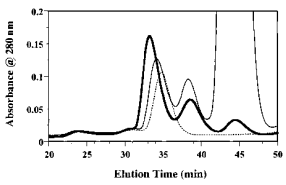

Figure 4. E21R prevents formation of the ternary GM-CSF receptor complex. Following formation of the GM-CSF/sGMRα/sβc ternary complex using a 1:2:4 molar ratio, a 100-fold molar excess of E21R over GM-CSF was added for a further hour at 25°C before size-exclusion chromatography. The chromatogram shows the A_{280} profile of a GM-CSF/sGMRα/sβc mixture in the absence (thick line) or presence of E21R (thin line) or in sβc alone (dashed line).

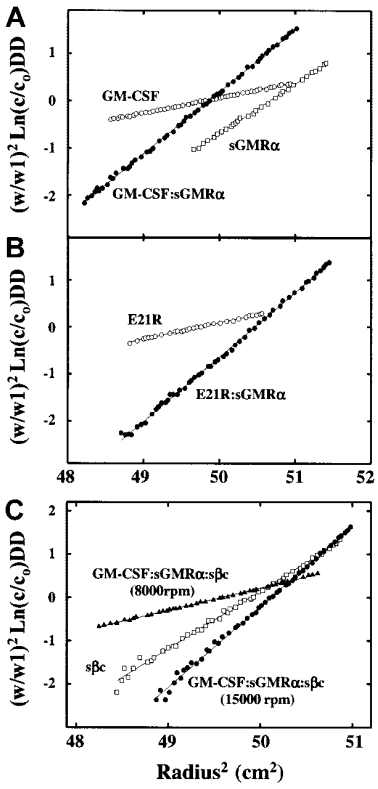

Figure 5. Analyses of the ternary GM-CSF receptor complex by sedimentation equilibrium. The individual proteins or protein complexes in 150 mM NaCl/50 mM sodium phosphate, pH 7.0, were centrifuged at 20°C at angular velocity, W rpm, for 16 hours. The equilibrium profiles are presented as $(W/W1)^2$ $Ln(c/c_o)$ versus the square of the radial distance, where c/c_o is the optical density at 280 nm divided by the initial optical density and W1 is 20 000 rpm. For a single species, this plot is linear with a slope proportional to the molecular weight of the sedimenting species. The initial concentrations were in the range 0.40 to 0.47 mg/mL, and samples were centrifuged at 20 000 rpm except for E21R, where the initial concentration was 0.2 mg/mL and the angular velocity 15 000 rpm. Panel A samples: GM-CSF (○), sGMRα (□), GM-CSF/sGMRα complex (●). Panel B samples: E21R (○), E21R/sGMRα complex (●). Panel C samples: purified sβc (□) was centrifuged at 15 000 rpm with an initial concentration of 0.45 mg/mL, whereas the GM-CSF/sGMRα/sβc ternary complex at an initial concentration of 0.47 mg/mL was centrifuged at either 8000 rpm (▲) or 15 000 rpm (●) for 16 hours at 20°C.

reduction in the amount of ternary complex along with an increased amount of binary complex (38.40 minutes) and free ligand (44.00 minutes) is consistent with sGMRα preferentially forming a binary complex with E21R, which is unable to recruit sβc into a ternary complex.

Stoichiometry of the ternary GM-CSF receptor complex

To confirm the 1 GM-CSF:1 sGMRα:2 sβc stoichiometry of the ternary GM-CSF receptor complex obtained by size-exclusion chromatography, we utilized 2 other complementary and independent methods. In one of these the molecular weights of the individual proteins and of the binary and ternary complexes were determined by sedimentation equilibrium. The results showed (Figure 5; Table 1) values similar to those obtained by gel filtration. The estimates of the molecular weight of the binary complexes GM-CSF/sGMRα, 52.7 kDa (Figure 5A; Table 1), and E21R/sGMRα, 54.8 kDa, (Figure 5B; Table 1), are consistent with a 1:1 stoichiometry. The molecular weight of the ternary GM-CSF/sGMRα/sβc complex was determined to be 135 kDa (Figure 5C and Table 1). This value is consistent with a model where one sβc dimer (97.4 kDa) associates with one GM-CSF/sGMRα binary complex (52.7 kDa) (Table 1) with a theoretical molecular weight of 150.1 kDa.

In a separate approach, we used radiolabeled GM-CSF as a tracer molecule. Purified sβc (0 to 7 μM) was titrated against a mixture of sGMRα (3.2 μM) and cold GM-CSF (7.3 μM) spiked with the GM-CSF analog ^{32}P-SGMKIN and subjected to size-exclusion chromatography as above. Addition of sβc to the GM-CSF/sGMRα mixture led to the dose-dependent formation of the ternary complex and depletion of the binary complex (Figure 6A). Once the concentration of sβc saturated the available binary complex, a shoulder appeared on the trailing edge of the ternary complex peak, presumably reflecting the presence of free sβc. Formation of the ternary complex was associated with a dose-dependent accumulation of radioactivity at the appropriate elution time of the ternary complex and was accompanied by a reduction of radioactivity at the elution time of the binary complex (Figure 6B). Titration of sβc did not lead to a reduction of radioactivity at the elution time of free ligand, although a modest shift at the leading edge of the free ligand peak was observed. We then determined the distribution of ^{32}P-SGMKIN into the ternary complex and expressed it as a percentage of total label in the ternary and binary complexes versus proportion of sβc present (Figure 6C). When compared with the theoretical distribution predicted for a ternary complex with a GM-CSF/sGMRα/sβc ratio of 1:1:2 or 2:2:2, the observed distribution was consistent with a 1:1:2 stoichiometry. The observed distribution only departed from the modeled linear distribution as the concentration of binary complex became limiting.

The use of radiolabeled GM-CSF also allowed us to investigate whether the presence of sβc in the ternary complex led to affinity conversion. GM-CSF spiked with the GM-CSF analog ^{32}P-SGMKIN was titrated against an equimolar mixture of sGMRα and sβc, allowed to equilibrate, and fractionated by size-exclusion chromatography. For each GM-CSF concentration point, radioactivity bound in the binary and ternary complexes was determined and the proportion in each complex was expressed as a percentage of total bound counts. We found (Figure 6D) a 4-fold preferential distribution of ^{32}P-SGMKIN into ternary complexes at subsaturating concentrations of ligand, indicating that the

Table 1. Sedimentation equilibrium analysis of the molecular weights of GM-CSF, E21R, sGMRα, sβc, and their complexes

Species	M (1-$\nu\rho$)	ν	MW	Predicted MW	Predicted stoichiometry
GM-CSF	3 590	0.734	13 700	—	—
E21R	4 050	0.734	14 300	—	—
sGMRα	11 800	0.706	40 700	—	—
sβc	26 600	0.723	97 400	—	—
sGMRα plus GM-CSF	14 600	0.718	52 700	54 400	1:1
sGMRα plus E21R	15 400	0.714	54 800	55 000	1:1
sβc plus sGMRα plus GM-CSF	37 300	0.72	135 300	151 800	2:1:1

The buffer used was 150 mM NaCl/50 mM sodium phosphate, pH 7.0, and the temperature was 20°C. The complexes formed between GM-CSF and E21R with sGMRα and between GM-CSF, sGMRα, and sβc were isolated by gel filtration. The initial concentration used for all samples was between 0.40 and 0.47 mg/mL except for E21R, where the starting concentration was 0.2 mg/mL. The reduced molecular weights of the samples, M (1-$\nu\rho$), were determined by direct fitting of the sedimentation data presented in Figure 5. These values were used to calculate the molecular weight (MW) of the sedimenting species using the partial specific volumes (ν) indicated.

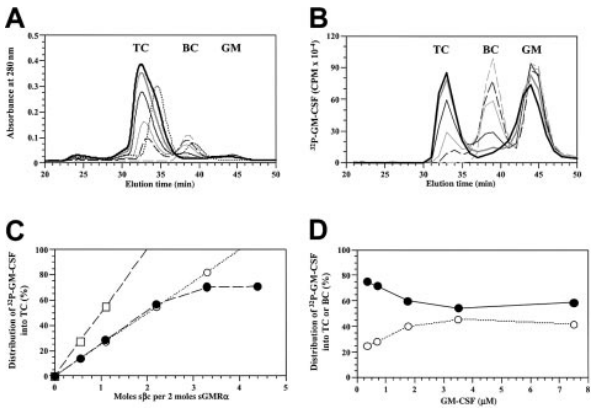

Figure 6. Radiolabeled GM-CSF differentially partitions to the ternary GM-CSF receptor complex. (A-C) A titration of purified sβc (0 to 7.03 μM) against a mixture of 3.2 μM sGMRα and 7.3 μM GM-CSF spiked with ^{32}P-labeled SGMKIN. Reaction mixes were set up with 0 (dashed gray), 0.88 μM (dashed black), 1.76 μM (thin gray), 3.52 μM (thin black), 5.27 μM (thick gray), or 7.03 μM (thick black) sβc and incubated at 25°C for 1 hour before size-exclusion chromatography. Fractions were collected at 1-minute intervals. A control reaction was also prepared with 5.27 μM sβc and 3.2 μM sGMRα but no GM-CSF (dashed black). (A) Chromatogram of A_{280} profiles for each sample with the location of the ternary complex (TC), binary complex (BC), and free ligand (GM) indicated. (B) Distribution of radioactivity among the ternary complex, binary complex, and free ligand for the reactions described in panel A. (C) Radioactive GM-CSF distributed into the ternary complex, expressed as a percentage of the total radioactive GM-CSF in ternary and binary complexes; comparing experimentally observed values for the reactions described in panel A (●) with a theoretical distribution based on 1GM:1α:2β (○) and 2GM:2α:2β (□) models. (D) Titration of GM-CSF (0 to 7 μM) spiked with ^{32}P-labeled SGMKIN against a mixture of 3.5 μM sGMRα and 3.5 μM sβc. Reaction mixes were allowed to reach equilibrium at 25°C for at least 2 hours before being fractionated by size-exclusion chromatography. The distribution of radioactivity among ternary (●) and binary (○) complexes was determined and the radioactivity in each complex was expressed as a percentage of total bound counts where counts in TC plus counts in BC is 100%.

presence of sβc in the ternary complex induces a measurable degree of affinity conversion.

GM-CSF binds sβc in the absence of sGMRα

Initial chromatography experiments at approximately equimolar concentrations indicated that GM-CSF was unable to form a complex with sβc in the absence of sGMRα. However, close inspection of the elution profile of radiolabeled GM-CSF in the presence of free sβc (Figure 6B) revealed a modest decrease in the elution time of GM-CSF suggestive of a weak interaction between sβc and GM-CSF. To investigate this further and to determine the specificity of this interaction, we titrated sβc against GM-CSF or the E21R analog (Figure 7). Titration of sβc against GM-CSF had a dose-dependent effect on GM-CSF peak height with a concomitant spreading of the GM-CSF profile to earlier elution times (Figure 7A). Titration of sβc against E21R had no effect on E21R elution time or profile (Figure 7B). These results show that GM-CSF directly interacts with sβc through the functionally important Glu21

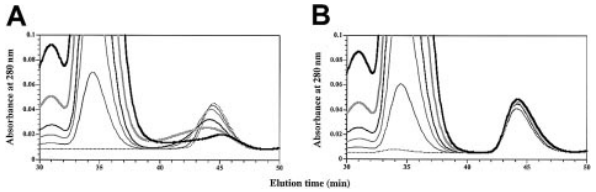

Figure 7. GM-CSF binds directly to βc. Purified sβc was titrated (0 to 20 μM) against 5 μM GM-CSF (A) or 5 μM E21R (B). Reaction mixes were set up with 0 (dashed black), 1 μM (thin black), 2.5 μM (medium gray), 5 μM (medium black), 10 μM (thick gray), or 20 μM (thick black) sβc, incubated at 25°C for 2 hours, and fractionated by size-exclusion chromatography.

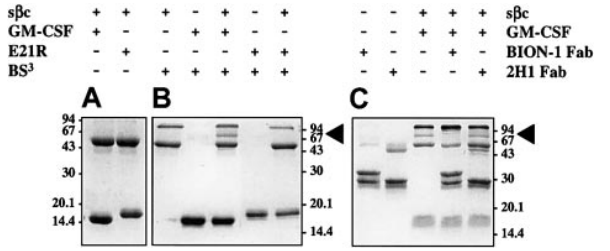

Figure 8. Discrete regions in GM-CSF and βc mediate their direct interaction. Purified sβc was incubated at 25°C for 1 hour alone or in the presence of either GM-CSF or E21R. Samples were left untreated (A) or were treated for 10 minutes with BS^3 cross-linker (B). To determine if GM-CSF was interacting with the cytokine-binding site in the fourth domain of βc, purified sβc was preincubated with a Fab fragment of the neutralizing anti-βc mAb, BION-1, or the nonneutralizing control anti-βc mAb, 2H1. GM-CSF was then allowed to bind and the mixture and individual mAb treated with BS^3 cross-linker (C). Samples were analyzed on 12.5% (A) or 10% (B-C) SDS-PAGE gels and stained with Coomassie. The positions of molecular weight markers are shown in kilodaltons, and the position of sβc cross-linked to GM-CSF is indicated by ◀.

residue and that substitution of this residue makes a qualitative difference to the GMRα-independent recognition of βc by GM-CSF.

A second approach confirmed the direct interaction of GM-CSF with βc and extended these findings to the identification of the reciprocal region in βc. We incubated purified sβc with a 3-fold molar excess of GM-CSF or E21R, treated with the BS^3 cross-linker and analyzed the mixture by SDS-PAGE under reducing conditions (Figure 8). No covalent interactions between sβc and GM-CSF or E21R were observed in the absence of cross-linker (Figure 8A). GM-CSF and E21R were not dimerized by cross-linker under the conditions used, whereas the sβc dimer was partially cross-linked, yielding a band of MW 100 kDa (Figure 8B). Significantly, when GM-CSF was incubated with sβc and cross-linked, a unique band of MW 70 kDa was observed (Figure 8B). Western blotting with anti-βc or anti–GM-CSF antibodies showed that this band contains both sβc and GM-CSF (data not shown). E21R could not be cross-linked directly to sβc as seen by the absence of the 70 kDa band (Figure 8B), thus confirming that Glu21 of GM-CSF is necessary for direct contact with βc. Neither the structurally related cytokine human growth hormone nor sGMRα was able to be cross-linked to sβc under these conditions (data not shown).

To determine if the interaction between sβc and GM-CSF was occurring through a functionally relevant region of βc, we used BION-1, a mAb that blocks GM-CSF, IL-3, and IL-5 binding and signaling through βc.[24] BION-1 recognizes a discrete region in the fourth domain of βc associated with high-affinity GM-CSF binding and function.[20,22,36] Preincubation of sβc with BION-1 Fab fragment prevented GM-CSF from being cross-linked to sβc, as seen by the absence of the 70-kDa band (Figure 8C). The Fab fragment of a mAb that binds to the fourth domain of βc but does not block cytokine binding was unable to perturb the cross-linking of sβc to GM-CSF (Figure 8C).

Discussion

We report here the first demonstration of a fully assembled GM-CSF/GM-CSF receptor ternary complex in solution and describe the molecular interactions required for its formation. It is shown that the ternary complex exhibits a novel mode of cytokine receptor assembly that comprises 1 molecule of GM-CSF and 1 molecule of GM-CSF receptor α chain interacting monovalently with a noncovalently linked dimer of βc. In addition, a direct interaction between GM-CSF and βc in the absence of the receptor

α chain could be demonstrated. The recruitment of βc as a preformed dimer may facilitate receptor activation and may also represent a mechanism utilized by the related IL-3 and IL-5 receptors. The GM-CSF ternary complex was demonstrated by gel filtration and sedimentation equilibrium analyses to have a molecular weight of between 135 kDa and 156 kDa, consistent with a GM-CSF/sGMRα/sβc stoichiometry of 1:1:2. In addition, the relative distribution of radiolabeled GM-CSF fitted a ternary complex with a 1:1:2 stoichiometry. The preferential distribution of radiolabeled GM-CSF into the ternary complex is indicative of sβc-mediated, affinity conversion. No disulfide linkages between receptor subunits were observed; there were no differences seen when the ternary complex was analyzed by SDS-PAGE under either reducing or nonreducing conditions or when the free cysteine groups in sβc were blocked with iodoacetamide (data not shown). These results are consistent with previous reports suggesting that GM-CSF receptor heterodimerization is required to activate the GM-CSF receptor,[25] the dimeric nature of βc observed both on the cell surface and in solution,[26,28,30] the affinity conversion afforded by βc,[15,20] and the requirement of at least a βc dimer for function and activation of downstream signaling molecules.[28,29] The intermediate binding affinity for GM-CSF in the ternary complex is consistent with a report describing the low-affinity binding of murine GM-CSF to detergent-solubilized GM-CSF receptors extracted from a murine cell line.[37] In addition, these results do not rule out the formation of higher-order complexes on the cell surface,[27,38] which may lead to further affinity conversion and disulfide linkage required for receptor stabilization, activation, or internalization purposes. The assembly of the human GM-CSF receptor shown here is different from that seen for the IL-6[39] and LIF[40] receptors, which exhibit a stoichiometry of 2:2:2 and 1:1:1, respectively. Interestingly, the dynamics of the GM-CSF receptor assembly are analogous to the IL-6 receptor in that following the binding of ligand to the major binding subunit (α chain) there is recruitment of the signaling subunit (βc or gp130). However, although dimerization of gp130 requires a second IL-6/IL-6Rα chain binary complex, this is not the case with βc, which is recruited to a single GM-CSF/sGMRα binary complex as a preformed dimer. Despite the dimeric nature of sβc and even in the presence of a 2-fold molar excess of the GM-CSF/sGMRα binary complex, we saw no evidence for the formation of a ternary complex with a stoichiometry of 2:2:2. The functional monovalency of sβc may be due to conformational changes within the sβc dimer, induced by the binding of one GM-CSF/sGMRα binary complex that prevents the binding of a second binary complex.

The recruitment of sβc to the GM-CSF/sGMRα binary complex occurs through functionally relevant sites in GM-CSF and βc itself. This is demonstrated by the inability of the GM-CSF analog E21R to form the ternary complex and by the inhibition of sβc cross-linking to GM-CSF by the anti-βc mAb BION-1, which blocks the high-affinity binding of GM-CSF.[24] Given that there is an homologous glutamic acid in IL-3 (position 22) and in IL-5 (position 13) and the fact that BION-1 also blocks high-affinity binding of IL-3 and IL-5, it is possible that this mode of receptor assembly will also apply to the IL-3 and IL-5 receptors. The recruitment of dimerized βc and associated JAK-2 molecules may facilitate receptor phosphorylation and activation in this subfamily of receptors. Using a soluble receptor system we could detect for the first time a direct interaction between GM-CSF and βc in the absence of the GM-CSF receptor α subunit. We observed this by gel filtration (Figure 7) and cross-linking studies (Figure 8). The interaction was sensitive to the E21R substitution and the mAb BION-1, indicating that the direct interaction observed between GM-CSF and sβc is chemically and spatially equivalent to the interaction that occurs with the cell membrane–anchored receptor. Considering that all βc-interacting cytokines do so through a chemically and structurally conserved mechanism,[36] it is likely that a direct interaction between βc and IL-3 or IL-5 will also exist. The relative affinity of the direct βc interaction for each cytokine may help to explain differences in βc-mediated affinity conversion in the high-affinity binding of IL-3, GM-CSF, or IL-5. Despite the direct interaction of βc with GM-CSF seen in the soluble system, this may not be sufficient to activate the receptor in vivo given the very high concentrations of both receptor and ligand needed to detect this weak interaction (in the micromolar range) and the fact that GMRα intracellular domain has been previously shown to be crucial for GM-CSF signaling.[41]

In the IL-4 system, a high-affinity (K_d = 0.15 nM) interaction between IL-4 and the IL-4 receptor α chain[42] utilizes a chemically and structurally homologous mechanism, suggesting that the type of direct interaction we observed between GM-CSF and βc may be conserved among other cytokines. The direct interaction we detected between GM-CSF and sβc also suggests that conformational changes in the GM-CSF/sGMRα binary complex may not be necessary for the recruitment of βc. However, the monovalent binding of the GM-CSF/sGMRα binary complex to sβc suggests the possibility of an induced conformational change within the extracellular domain of sβc. Conformational changes in the cytoplasmic region of βc may be induced by the assembly of the ternary complex to promote βc/JAK-2 proximity and receptor activation as shown for the erythropoietin receptor.[43] The assembly of the human GM-CSF receptor system in solution described herein also provides a useful tool for investigating its dynamics and structural requirements. The initial event in activation of the GM-CSF receptor is the binding of ligand to the GMRα with low affinity prior to recruitment of βc. The soluble system used here revealed a 1:1 stoichiometry of binding between the sGMRα chain and GM-CSF with a K_d equivalent to that seen with the full-length GMRα on the cell surface. We were able to show that E21R, a GM-CSF analog defective in high-affinity binding and a specific GM-CSF antagonist currently in phase 2 clinical trials, also binds sGMRα with a 1:1 stoichiometry. Importantly, E21R is incapable of forming a ternary receptor complex and when present in excess is able to prevent the formation of the ternary GM-CSF receptor complex, thus explaining its antagonistic activity. This set of experiments also demonstrates that the assembly of the GM-CSF receptor is a sequential process that involves first the formation of a binary complex. In structural terms it will be interesting to use single point mutants of βc to examine the residues that participate in direct contact with GM-CSF or the GM-CSF receptor α chain. This may be also a useful system for the identification of small molecules that prevent the formation of the ternary complex. Finally, the assembly of the human GM-CSF ternary complex in solution should aid in its crystallization and ultimately in the solving of its structure.

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CHAPTER 6:

Discussion

DISCUSSION

The papers presented here describe the nature of the GM-CSF receptor components and illustrate their stoichiometric progression upon GM-CSF binding from a low affinity interaction with GMRα, through to the recruitment of the signalling subunit hβc into a higher order complex [106-108]. This clarified the stoichiometry of the active GMR which had been unclear as the composition had only been inferred as higher order complexes or simple heterodimers through dominant negative receptor studies [73], and leucine zipper-mediated heterodimerization of GMRα and hβc intracellular domains, respectively [109].

This was achieved by investigating the assembly of the GMR subunits in both a cell based system, where full length receptor components assemble and signal; and using soluble receptor system, where extracellular receptor domains assemble in solution and complex stoichiometry monitored directly in the absence of a cell membrane. The use of both a cell based and cell free system enabled investigation of different aspects of GMR assembly, and together revealed the formation of a higher order GMR complex upon receptor activation. This thesis also provided cautionary observations that can confound the identification of critical interactions between GM-CSF and GMR components, which may be relevant to other type I cytokine receptors, by revealing potentially misleading interspecies interactions between homologous receptors [110].

A common feature of type I cytokine receptor activation is receptor oligomerization as prerequisite for the JAK2 homodimerization for initiation of signal transduction [72]. To determine if one or more hβc interact with GMRα for receptor activation we developed a chimeric receptor system that would only permit signal transduction if at least two hβc interacted within the active GMR. The chimeric receptor used comprised the extracellular and transmembrane domains of hβc fused to the intracellular domain EPO-R that can only become

activated by receptor homodimerization [111]. When chimeric hβc/EPO-R receptor was expressed with GMRα on cells a GM-CSF response was demonstrated. This suggested the homodimerization of at least two hβc subunits was required for GMR activation and signal transduction. The GM-CSF responsiveness was attributed to hβc dimerization alone as hβc/EPO-R responsiveness to GM-CSF was retained in the presence of a C-terminally truncated GMRα, excluding any contribution from the GMRα cytoplasmic domain in this system. This confirmed that an interaction between at least two molecules of signalling subunit hβc is required in the active GMR, akin to the signal transducing subunit of the IL-6 R, gp130 [66]. Dimerization of hβc is potentially required for IL-3 and IL-5 receptor as these cytokines also share hβc for signal transduction. This supported other lines of evidence that suggested that active GMR is more than just a simple heterodimeric complex of GMRα and βc [73, 74]. The formation of hβc homodimers within the active receptor may bring two JAK2 molecules, which are constitutively associated with hβc, into close proximity allowing them to transphosphorylate each other as the preliminary step for initiating receptor signal transduction. While these results indicated that the GMRα:hβc heterodimer alone does not signal, it did not indicate the number of GMRα subunits required for receptor activation or if multiple hβc dimers interact.

While cell based systems are useful tools for the investigation of how receptors assemble and signal in response to cytokines our studies of the hGM-CSF antagonistic mutant, E21R, revealed the problematic influence that endogenous cytokine receptors can have [110]. We observed that the biological activity of E21R is strikingly different when analysed in hGMR transfected murine cell lines compared to its actions on human cells expressing endogenous GMR. In human cells E21R cannot mediate a proliferative response and is unable to bind hGMR with high affinity [57]. Surprisingly on murine cells expressing human GMR E21R exhibited agonistic activity that

was promoted by the tyrosine phosphorylation of transfected hβc, an event that does not occur in an endogenous human system. In addition, we found a ligand independent pre-association between the mouse and human βc. The inappropriate signalling detected in this mixed species system may be the result of receptor activation following the recruitment of oligomers formed between the homologous mβc and hβc [110].

Receptor dimerization is a common theme in receptor activation, and the potential for inter-species interaction of signalling components obscuring results when studying human receptors in murine cells must be controlled for. An analogous situation has been reported for a human interleukin-4 mutant that can mediate either agonistic or antagonistic responses when studied in either a murine or human cell line respectively [112]. Similarly, analysis of TPO mutants showed conflicting data in the identification of residues functionally important in interaction with its receptor when screened on Mpl transfected Ba/F3 cells compared to ELISA or Biacore analysis [113, 114]. These findings highlighted that the use of transfected murine cell lines expressing endogenous murine GMR may not be the best approach when studying hGM-CSF ligand variants. The use of lymphoid cell lines, such as CTLL-2, or cell lines made from mβc/$m\beta_{IL\text{-}3}$ knockout mice will avoid any potential contribution from endogenous murine GMR components [115]. Similarly receptor reconstitution experiments in mβc/$m\beta_{IL\text{-}3}$ knockout mice may provide a better background to analyse functional outcomes of hGMR activation. The analysis of soluble GMR component assembly avoided potential interspecies interactions and facilitated the analysis of the receptor components in isolation.

Receptor assembly studies using full-length receptors do not discern if the hβc homodimer assembles through interactions between the extracellular domains, intracellular domains or a combination of the two. A cell free system has been utilised to look directly at the extracellular

interactions between IL-6 and soluble cytokine receptors revealing its composition [105, 116]. The production of soluble receptors provided an alternative system for elucidating ligand and receptor interactions in defined systems free of intracellular interactions. The stoichiometry of assembled soluble receptors can be determined directly by techniques such as size exclusion chromatography and analytical ultracentrifugation in the absence of heterologous receptor chains.

Soluble forms of the GMR receptor were generated by over expression of secreted C-terminally truncated forms in COS cells [107]. This system allowed the initial characterisation of the soluble receptor components, sGMRα and sβc, and investigation of their interactions with each other or full-length receptors when co-expressed. Under certain circumstances, on the cell surface and as soluble receptors, the GMRα and βc could be found to co-associate in the absence of ligand. This indicated that these proteins directly interact through an as yet unidentified binding interface. Our soon to be published GMR structure (Appendix 1) reveals an interaction between the membrane proximal GMRα and βc from two adjacent ternary complexes. Although data showing the ligand independent association between GMRα and sβc was generated, the system used for these studies proved to be not optimal. Expression levels of the individual soluble receptors in the COS cell system were too low to enable purification of amounts required for receptor assembly studies. In addition, the majority of sβc produced in transiently transfected COS cells formed disulphide linked aggregates which could not be used in assembly studies.

Production of cytokine receptor extracellular domains, such as IL-6R, in insect cells have been successfully used in receptor assembly studies [117]. Expression of human proteins in insect cell lines, such as Sf21, gives high yields of receptor protein with glycosylation that resembles the native receptor. By changing our soluble GMRα and βc production to insect cells we improved

our yield dramatically and also overcame the problem of di-sulphide linked sβc aggregates, producing material suitable for receptor complex assembly. A high yield expression system subsequently established and enabled the large-scale production of receptor components for structural studies of GMR complexes.

Both sGMRα and sβc were successfully produced in Sf21 insect cells using a baculovirus expression system. Characterisation of the purified soluble receptor components revealed sGMRα to be monomeric with low affinity GM-CSF binding characteristics that mimic the full-length receptor. The sGMRα was free of di-sulphide linked aggregates that have been previously described for a naturally occurring truncated form of sGMRα [118]. Soluble GMRα was found to bind GM-CSF in a 1:1 stoichiometric ratio, confirming previous studies [118]. Soluble βc was found to exist as a preformed homodimer as shown previously of full length cell surface approaches [74] and in the apo-crystal structure [75]. During our characterisation of the sβc we tried to revert sβc dimer into a monomer using a gentle caotropic agent, however a stable monomeric complex could not be formed (unpublished data). The homodimeric nature of sβc suggests the hβc is a non-functional dimer in the absence of ligand and GMRα chain, how hβc dimer then becomes activated following interaction with a ligand:GMRα is still unclear. The recruitment of hβc as a preformed dimer may facilitate receptor activation, and may also represent a mechanism utilised by the related IL-3 and IL-5 receptors.

This work showed the first demonstration of a GMR soluble complex that contains the sGMRα, sβc and GM-CSF and the stoichiometry of this complex. The interaction of these three soluble components is reflective of the assembly seen on the cell surface utilising the same residues that

are critical for GM-CSF binding and signalling. This was clearly demonstrated by the direct interaction revealed by crosslinking studies of GM-CSF, but not E21R, with the sβc.

Interaction between GM-CSF and hβc has not been previously described on cell surface expressed receptors. In addition the blocking antibody that targets the ligand-binding site of hβc, BION-1 [63, 119], can prevent GM-CSF interacting with cell surface hβc, mimicking the neutralising antibody's ability to prevent high affinity binding on the cell surface and subsequent signalling. This was the first description of a direct physical interaction between hβc and GM-CSF that occurs through a functionally relevant epitope in the absence of GMRα. Unlike GM-CSF, E21R in unable to interact with sβc demonstrating that E21 on GM-CSF can directly interact with βc.

Previously our model, based on computer modelling, proposed the GMR contained a stoichiometry of 2GM-CSF:2hβc:2GMRα, based on the structural homology to the GH receptor and inclusion of the role of disulphide linkages between receptor component [72]. The ability of IL-3 to trans-phosphorylate GMRα associated hβc support the formation of a higher order complex that contains two alpha-receptors [107]. Our studies revealed that at least two hβc extracellular domains are for signalling and that the extracellular domains of hβc exist as a preformed dimer, both findings support the proposal of a higher order GMR complex. However, size exclusion chromatography revealed the stoichiometry of the sβc:GMRα:GM-CSF soluble receptor ternary complex was found to be 2:1:1. This may represent an assembly intermediate, as this complex did not bind GM-CSF with high affinity. Although improved affinity was seen following the addition of hβc to the GMRα:GM complex, we were unable to demonstrate high affinity binding comparable to that achieved with full-length receptors on the cell surface

(unpublished observations). This suggests the cell membrane, transmembrane or intracellular interactions may be required for full affinity conversion, potentially through stabilising the complex or membrane anchoring required for conformational constraints. Our subsequent gel filtration studies using higher protein concentrations for complex formation suggest that the system can be pushed to produce a larger complex, possibly 2:2:2 (Appendix 1).

The crystal structure of hβc extracellular domain revealed a stable interlocking dimer [75] that revealed a new binding epitope in domain 1, and functional activity confirmed by mutagenesis studies [120]. Domain 1 of hβc appears to contribute loop residues, in addition to those previously described in domain 4, to the ligand-binding site. This had not been predicted by our previous modelling based on the GHR structure. While the structural determination of hβc extracellular domain in isolation is an important step forward in understanding the nature of the GMR, it does not address how the GMR assembles. This structure described the membrane proximal domains to be 120 angstroms apart and this presumably reflects a similar separation of the cytoplasmic domains. It remains difficult to understand how the interlocking hβc dimer would allow the trans-phosphorylation of associated JAK2 to occur when a 120 angstrom space exists between the intracellular hβc domains. While it is useful to determine the structure of the individual components that comprise the GMR, alone they are unable to illustrate the assembly of receptor components that occurs upon ligand binding.

The production of a soluble GMR ternary complex, as described in this thesis, was the initial requirement needed to resolve the molecular structure of this receptor complex. Structural analysis of the GMR is paramount to reveal critical molecular interactions required for complex formation upon GM-CSF high affinity binding. The identification of critical epitopes required for GMR assembly will provide targets therapeutic inhibitors to limit inappropriate receptor

activation, an important advance in for diseases including asthma, arthritis and some leukemias given GM-CSF's pathogenic role [121]. These studies also highlight that any such drugs generated must be analysed on human cells, or in *in vitro* systems that are not confounded by non-human endogenous receptor chains, which can lead to inappropriate GMR activation by interaction with heterologous receptors.

This work lead to the large-scale production of the individual extracellular GMR components in the Sf21 insect cell line expression system. These cell lines enabled the production of milligram quantities of the individual GMR receptor components. Individual components were then purified by affinity chromatography, ternary complexes were assembled and isolated using size exclusion chromatography, and analysed in crystallographic studies (Appendix 1).

In summary, this thesis described the nature of the components of the GMR using both full-length receptors in cell based systems and soluble extracellular domains, and describes the formation and stoichiometry of a soluble GMR ternary complex. This provided important preliminary data that lead to successful crystallographic studies and the subsequent resolution of the GMR ternary complex that and brought insight into how this multi-subunit type I cytokine receptor is activated.

CHAPTER 7:

Concluding Remarks

CONCLUDING REMARKS

The work presented here were fundamental for the determination of the structure of the GM-CSF receptor complex (Appendix 1). The atomic structure of the GM-CSF ternary complex was subsequently determined and revealed a hexamer assembly of two hβc, two GMRα chains and two GM-CSF molecules (Figure 3). This stoichiometry differed from the observed 2:1:1 described in Chapter 5. This is likely due to our consequent findings that GM-CSF ternary complex formation showed a protein concentration dependant shift in molecular weight (Appendix 1). The 2:1:1 stoichiometry may represent a receptor assembly intermediate.

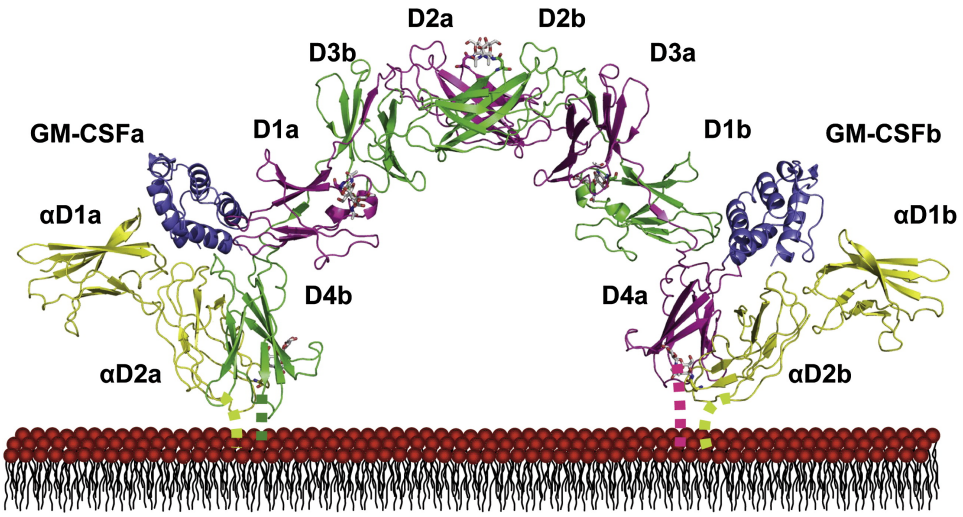

Figure 3.
The structure of the GM-CSF Receptor Ternary Complex as it would sit on the cell membrane*. GM-CSF is highlighted in blue and GMRα in yellow. One monomer of hβc is shown in magenta (chain a) and the other in green (chain b). Labels correspond to domain names (Appendix 1).*

The hexameric GM-CSF ternary complex showed an arrangement similar to other type I cytokine receptors that use three sites of interaction, and the hβc formed an intertwined homodimer akin to the apo-hβc structure [75]. Interaction of GM-CSF with GMRα and hβc

within the structure were consistent with previous mutagenesis studies that reported the functional importance of GM-CSF D112 interaction with GMRα, and GMRα residues Y248 and R302 interaction with GM-CSF. Similarly E21 of GM-CSF hydrogen bonds with Y421 of hβc, consistent with the key importance of this residue in GM-CSF binding and function.

Additionally, an unexpected novel higher-order dodecamer complex was observed in the crystal lattice (Appendix 1). The GMR dodecamer assembled via a novel interaction site, termed Site 4, which brought together the membrane proximal GMRα and βc domains from two adjacent hexameric complexes.

A cell line model in the murine T cell line, CTL-EN, was established to investigate the functional relevance of the Site 4 interface. Both WT and mutated GMR were expressed in this *in vitro* system that importantly lacked endogenous mGMR/mIL3R. This ensured that interspecies receptor interactions did not confound functional activity of GMR receptor mutants. Mutation of residues on hβc at the dodecamer interface did not alter high affinity binding suggesting that a GMR hexamer is sufficient for high affinity binding. While high affinity binding is retained in dodecamer interface mutants a reduction in GM-CSF mediated proliferation was observed, along with greatly reduced tyrosine phosphorylation. This suggests that following a high affinity GMR hexamer forming that dodecamer formation is required for full GM-CSF receptor activation (Figure 4). Dodecamer assembly can provide the close proximity required between two JAK-2 molecules bound to hβc on adjacent hexamers, allowing transphosphorylation and subsequent downstream signal transduction to occur. Interestingly, we did not observe the formation of intermolecular covalent di-sulphide bridges that had been observed in cell-surface receptor studies.

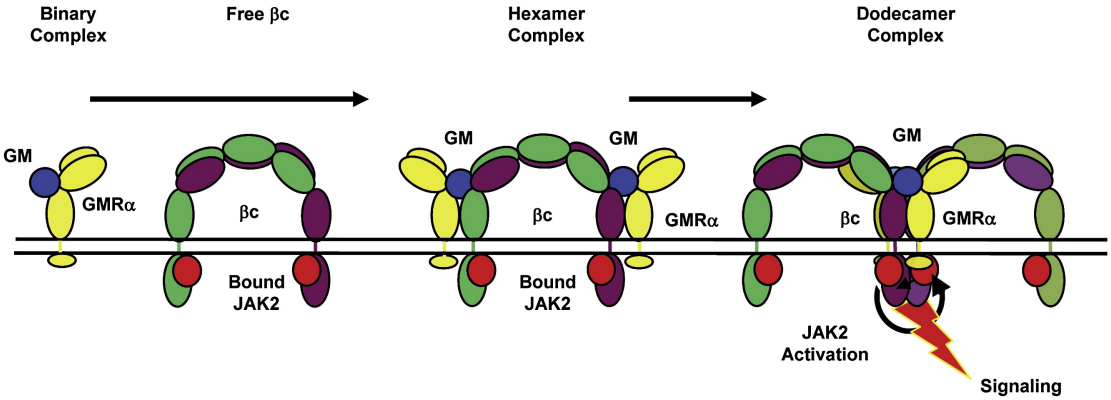

Figure 4.
Model of GM-CSF signal transduction. *The low-affinity complex consists of GM-CSF bound to GMRa. Interaction with free hβc forms the high affinity hexamer complex. Dodecamer (or higher-order) complexes form by lateral aggregation of hexamer complexes to form the fully competent signalling complex. JAK2 associated with hβc (red sphere) is able to dimerize and transphosphorylate in the dodecamer complex but not in the hexamer (Appendix 1).*

Ultimately, the findings presented in this thesis revealed the mechanism of GMR assembly and determined it's stoichiometry. The nature of the individual GMR components in the absence of ligand, the low affinity binding and high affinity GM-CSF binding complexes was shown, and subsequently enabled the GMR ternary complex structure to be solved. This structure revealed a novel mechanism of activation where GMR hexamers oligomerize into dodecameric complexes. Using appropriate *in vitro* cell line systems the dodecamer interface was shown to be required for activation of hβc associated JAK-2 and signal transduction. The structural determination of the GMR and the observed dodecameric complex formation revealed the mechanism of how GMR assembly enables the activation of hβc associated JAK-2. The GMR dodecamer interface may offer a new therapeutic target for the development of neutralising molecules for GM-CSF implicated diseases including leukemia and chronic inflammatory diseases.

APPENDIX 1:

Publications arising

Cell

The Structure of the GM-CSF Receptor Complex Reveals a Distinct Mode of Cytokine Receptor Activation

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SUMMARY

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine that controls the production and function of blood cells, is deregulated in clinical conditions such as rheumatoid arthritis and leukemia, yet offers therapeutic value for other diseases. Its receptors are heterodimers consisting of a ligand-specific α subunit and a βc subunit that is shared with the interleukin (IL)-3 and IL-5 receptors. How signaling is initiated remains an enigma. We report here the crystal structure of the human GM-CSF/GM-CSF receptor ternary complex and its assembly into an unexpected dodecamer or higher-order complex. Importantly, mutagenesis of the GM-CSF receptor at the dodecamer interface and functional studies reveal that dodecamer formation is required for receptor activation and signaling. This unusual form of receptor assembly likely applies also to IL-3 and IL-5 receptors, providing a structural basis for understanding their mechanism of activation and for the development of therapeutics.

INTRODUCTION

A common feature of cytokine receptor activation is ligand-induced receptor aggregation involving the homo or heterodimerization of two or more receptor components and their assembly into a fully functional signaling complex. Structural data for heterodimeric receptor families that utilize a common binding and signaling subunit such as the IL-2 and IL-6 receptor systems have provided unique insights into their functional activation (Boulanger et al., 2003; Wang et al., 2005; Stauber et al., 2006). The GM-CSF, IL-3, and IL-5 family of receptors remains the last major group of class I hematopoietic receptor systems to be structurally and functionally elucidated.

The GM-CSF, IL-3, and IL-5 family of cytokines regulates the survival, proliferation, differentiation, and functional activation of hematopoietic cells (Guthridge et al., 1998) with GM-CSF also controlling dendritic cell (Mellman and Steinman, 2001) and T cell function (Barouch et al., 2002), thus linking innate and acquired immunity. While on the one hand GM-CSF offers therapeutic promise to bolster antitumor immunity (Sun et al., 2002; Fleetwood et al., 2005) and innate immunity for the treatment of Crohn's disease (Korzenik et al., 2005), on the other hand abnormalities in GM-CSF production or receptor function have been implicated in multiple pathologies such as rheumatoid arthritis (Cook et al., 2001), juvenile myelomonocytic leukemia (Birnbaum et al., 2000), chronic myelomonocytic leukemia (Ramshaw et al., 2002), and alveolar proteinosis (Dirksen et al., 1998). Furthermore, the GM-CSF receptor may also be important in the pathogenesis of chronic myeloid leukemia and myeloproliferative diseases by propagating survival and proliferation signals promoted by the abnormal expression of Bcr-Abl and JAK2 mutations, respectively (Wilson-Rawls et al., 1996; James et al., 2005). The receptors for GM-CSF, IL-3, and IL-5 are expressed at very low levels (100–1000 per cell) on the surface of hematopoietic cells and comprise a cytokine-specific α subunit and the βc subunit that is common to all three receptors (Guthridge et al., 1998). Each α subunit binds cytokine with low affinity (K_D = 0.2–100 nM) but the presence of βc converts this to high affinity (K_D = 100 pM) causing dimerization of both subunits and receptor activation (Stomski et al., 1996). Structure-function studies of GM-CSF, IL-3, and IL-5 and their receptors have noted regions of importance for ligand binding and biological activity; however the composition, assembly, and underlying mechanisms of receptor activation have remained elusive.

Cell

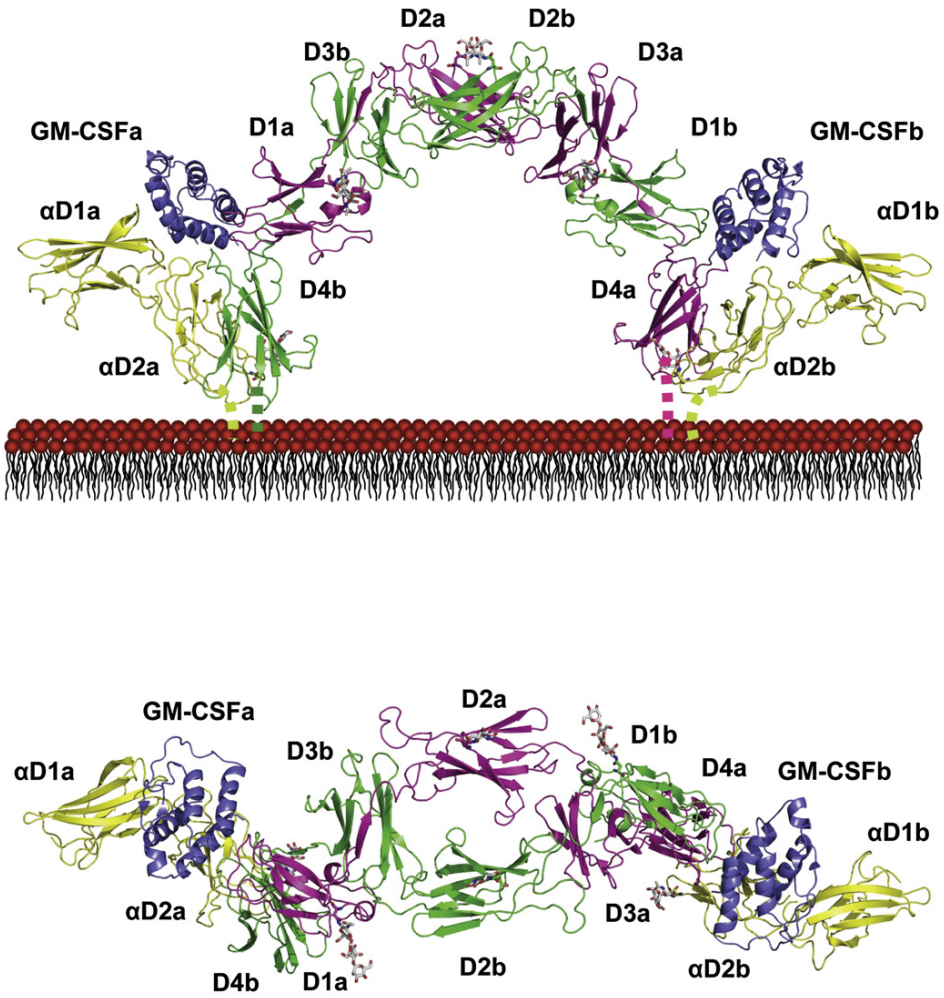

Figure 1. Structure of the GM-CSF Receptor Ternary Complex

GM-CSF is highlighted in blue and GMRα in yellow. One monomer of βc is shown in magenta (chain a) and the other in green (chain b). Labels correspond to domain names. Orthogonal views show how the complex would sit on the membrane surface. The bottom panel shows the view of the receptor when looking toward the membrane and the top panel shows a side-on view with the molecule sitting on a membrane surface. Observed N-linked carbohydrates are shown as sticks. Disordered peptides that connect the C termini of each chain to the membrane are shown as dashed lines. This and the following figures were prepared with PyMOL (DeLano, 2002).

Although GM-CSF receptor activation follows general rules that invoke receptor dimerization and tyrosine transphosphorylation of the cytoplasmic domains (Schlessinger, 2000), it is not clear how this is achieved. The GM-CSF receptor does not have intrinsic tyrosine kinase activity but associates with the tyrosine kinase JAK2, which is required for βc transphosphorylation and the initiation of signaling and biological activity. The cytoplasmic domains of both GMRα and βc are essential for receptor activation (Sakamaki et al., 1992; Muto et al., 1995), but it appears that mainly βc associates with JAK2 (Brizzi et al., 1994; Quelle et al., 1994; Lilly et al., 2001). Since the crystal structure of isolated βc revealed a dimer in which the membrane-proximal domains were 120 Å apart (Carr et al., 2001), a distance too great to allow transphosphorylation of βc by their associated JAK2 kinases, it has remained an enigma how activation of the GM-CSF, IL-3, and IL-5 family of receptors could be achieved. We show here that the crystal structure of the GM-CSF ternary complex assembles into an unexpected dodecamer arrangement. Functional analyses show that this dodecamer or higher-order complex brings two βc dimers into close proximity and provides for the functional dimerization and activation of the GM-CSF receptor.

RESULTS

The Structure of the GM-CSF Receptor Complex Reveals a Hexameric Assembly

We have determined the structure of the GM-CSF ternary complex revealing a 2:2:2 hexamer consisting of two βc chains, two GMRα chains, and two GM-CSF molecules (Figure 1). This differs from the 2:1:1 stoichiometry (2 βc: 1 GMRα: 1 GM-CSF)

we observed in solution (McClure et al., 2003). This is likely to be a result of differences in the total protein concentration at which the complex is formed as we observe a protein concentration-dependent shift in molecular weight and hence stoichiometry of GM-CSF receptor complex (Figure S1 and Table S2 available online). The 2:1:1 complex probably represents a relatively stable intermediate in the assembly of higher-order 2:2:2 GM-CSF receptor complexes. The structure adopted by GM-CSF in the ternary complex appears very similar to the structure of the isolated cytokine (Rozwarski et al., 1996). We also observe the same intertwined βc homodimer previously seen in the structure of the isolated βc subunit (Carr et al., 2001). In our case the dimer is generated by a crystallographic two-fold axis that runs through the center of the hexameric complex. A comparison of the βc chain in the complex compared to the structure of the isolated molecule (Carr et al., 2006) reveals that domain 4 has rotated 3° toward the crystallographic diad that runs through the center of the hexameric complex (Figure S2). The hinge region about which the rotation has occurred is located close to domain 1 in the linker region connecting domains 3 and 4, although the conformation of the linker region closely resembles that seen in the isolated molecule. Each βc chain consists of two cytokine receptor homology modules (CRMs), which in turn consist of two fibronectin type III (FnIII) domains. The GMRα chain consists of an N-terminal "knob" domain followed by one CRM. However, we only observe good electron density for the C-terminal FnIII domain of GMRα (GMRα domain 2). Although the electron density for N-terminal FnIII domain (GMRα domain 1) is of poorer quality, it was sufficient to recognize core β sheet enabling a partial model to be built and refined.

GM-CSF Interactions with GMRα—Site 1

GM-CSF binds to the elbow region defined by domains 1 and 2 of GMRα, reminiscent of the binding mode seen in other class I cytokine receptors (Wells and de Vos, 1996). There is a small interaction surface between GM-CSF and GMRα domain 2 of ~240 Å^2 per molecule with a surface complementarity of 0.66, a value within the range of normal protein-protein interaction surfaces (Figure 2, Site 1). Additional surface area would be provided by domain 1. The loop residues 241 to 251 and 299 to 305 of GMRα interact with residues 11 to 23 (helix A) and residues 112 to 118 (helix D) of the cytokine. This is consistent with mutagenesis studies showing that D112 of GM-CSF interacts with GMRα (Hercus et al., 1994b) and that GMRα residues Y248 and R302 are important for GM-CSF binding to GMRα (Rajotte et al., 1997; Haman et al., 1999).

GM-CSF Interactions with βc—Site 2

Recruitment of βc to the GM-CSF:GMRα binary complex converts the GM-CSF binding to high affinity and leads to receptor activation (Hayashida et al., 1990; Bagley et al., 1997). Surprisingly, our structure reveals a composite GM-CSF binding site at the elbow region between domain 1 (A-B and E-F loops) of one βc chain and domain 4 (B-C and F-G loops) of the second βc chain (Figure 2, Site 2), an arrangement not seen previously in cytokine-cytokine receptor complex structures (see Discussion). The involvement of these loops in cytokine binding is consistent with previous mutagenesis data (Lock et al., 1994; Woodcock et al., 1994, 1996; Murphy et al., 2003). The interaction at Site 2 buries ~570 Å^2 of surface area per molecule with a surface complementarity value of 0.62. Helix A of GM-CSF nestles into a surface crevice of βc formed by the E-F loop (residues 100 to 107) of βc domain 1 and the B-C (residues 360 to 369) and F-G (residues 417 to 423) loops of domain 4 (Figure 2). All the loops of βc that interact with cytokine adopt conformations very similar to that seen in isolated βc. In βc domain 1, Y39 of the A-B loop forms pi-pi interactions with Y421 in the F-G loop of domain 4. Residues V104, V105, and T106 of the E-F loop of domain 1 nestle into a hydrophobic pocket formed by helices A and C of GM-CSF. The side chain of the adjacent F103 residue is sandwiched between E-F and A-B loops of domain 1 stabilizing the conformation of this region in the Site 2 interface. D107 potentially forms a salt bridge interaction with K72 of GM-CSF. Y365 and H367 of the B-C loop of domain 4 are positioned on either side of E21 of GM-CSF. In the F-G loop of βc domain 4, Y421 hydrogen bonds with E21 of GM-CSF (Figure 2, Site 2). These observations are consistent with mutagenesis studies showing that the principal cytokine interaction with βc occurs through a conserved glutamate in helix A of GM-CSF (E21) (Lopez et al., 1992; Hercus et al., 1994a), IL-3 (E22) (Barry et al., 1994), or IL-5 (E13) (Tavernier et al., 1995) with E21 of GM-CSF making direct contact with βc (McClure et al., 2003) and with alanine substitution of βc at Y39, F103, Y365, H367, I368, or Y421 abolishing GM-CSF binding (Lock et al., 1994; Woodcock et al., 1994, 1996; Murphy et al., 2003). Phenylalanine substitution of Y421 ablates high-affinity binding of GM-CSF and IL-3 (Figure S3), highlighting the importance of the phenolic hydroxyl group in ligand binding.

Interactions between GMRα and βc—Site 3

Domain 2 of GMRα forms an extensive interaction with domain 4 of βc resulting in ~680 Å^2 of buried surface area per molecule with a surface complementary value of 0.52. The interface, involving residues 231, 232, 259, 266–270, and 280–286 of GMRα and residues 350, 353, 366–369, 389–400, and 418 of βc, is predominantly hydrophobic with a rim of charged residues (Figure 2, Site 3). We previously reported two surface hydrophobic patches, denoted H1 and H2, in our crystal structure of βc domain 4 and speculated that they might be involved in interaction with other parts of βc and GMRα, respectively (Rossjohn et al., 2000). The structure reported here shows that residues in H1 contact the A-B loop of βc domain 1 whereas residues of H2, located at the edge of the beta strands D and E of βc domain 4, are involved in the formation of the interface with GMRα. The Site 3 interface provides additional interacting surfaces between GMRα and βc, thereby enhancing the overall binding affinity of GM-CSF for its receptor.

The GM-CSF Receptor Complex Assembles into a Dodecamer

The crystal lattice generates an unexpected dodecamer complex, an assembly not previously observed in structures of cytokine receptor complexes, consisting of two hexameric complexes related by a crystallographic two-fold axis (Figure 3A). Intriguingly, the dodecamer assembles in a head-to-head orientation bringing the C-terminal tails of neighboring βc domain 4′s

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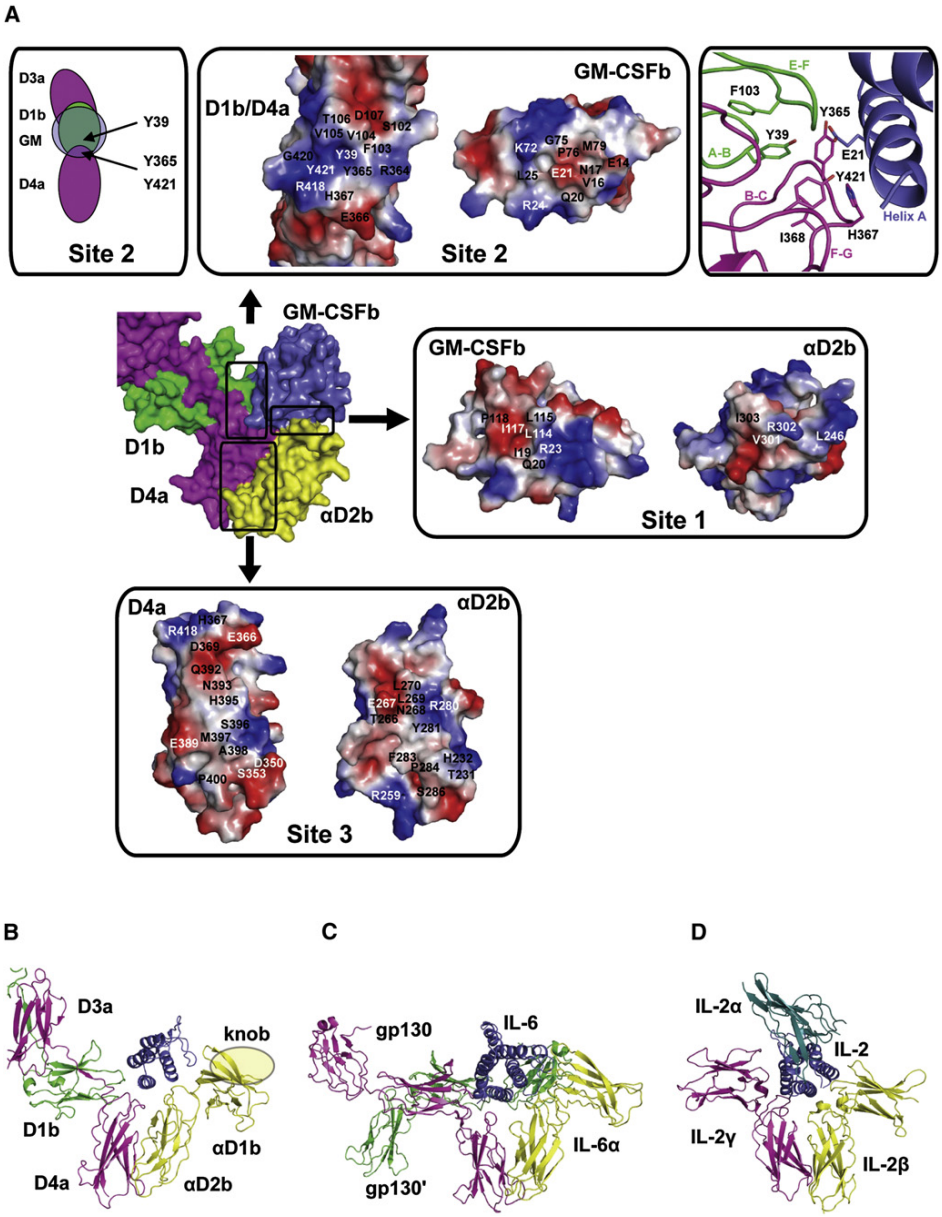

Figure 2. Peeled-away Interaction Surfaces between Components of the GM-CSF Receptor and Comparison of Related Class I Heteromeric Cytokine Receptor Systems

(A) The surfaces are colored by residue property: blue is basic and red is acidic. Residues involved in the interfaces are labeled. The top panel shows how βc interacts with GM-CSF (Site 2), the right middle panel shows the interaction between cytokine and GMRα (Site 1), and the bottom panel shows the interaction surfaces between βc and GMRα (Site 3). The composite nature of the GM-CSF binding Site 2 surface of βc is highlighted by a cartoon of the βc:GM-CSF interaction shown to the left of the Site 2 surfaces. A detailed view of GM-CSF E21 and interacting residues of βc is shown to the right of the Site 2 surfaces.

(B) Ribbon representation of the GM-CSF receptor complex. The panel shows a blown-up view of part of the ternary complex in the same orientation presented in the top panel of Figure 1. Also shown is the likely location of the GMRα knob domain.

(C) The IL-6 receptor complex (Boulanger et al., 2003). For clarity only the unique components of the hexameric complex are shown with the exception of the second gp130 molecule where the N-terminal domain interacts with the cytokine molecule.

(D) The IL-2 receptor complex (Wang et al., 2005). Related receptor components in each of the three systems shown in (A)–(D) are highlighted by the same color.

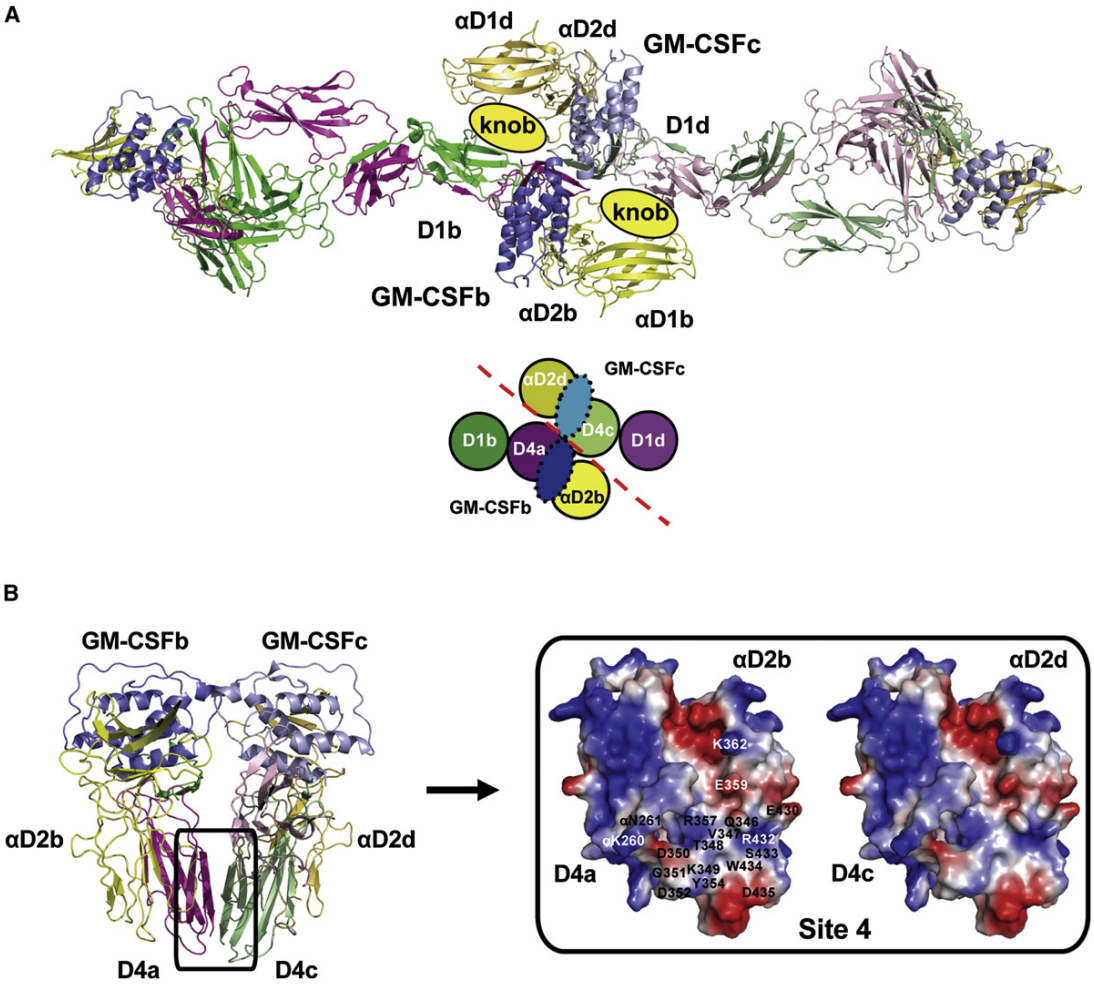

Figure 3. The Dodecamer Complex

(A) View of the dodecamer when looking toward the membrane surface. Coloring as in Figure 1 with second hexamer in lighter shade. (A model of the complete domain 1 of GMRα [labeled αD1] was superimposed onto the partial model derived from the crystallographic data.) Also shown is the likely location of the GMRα knob domain. In the lower panel a simplified version of the upper panel highlights the arrangement of chains and domains. The chains of βc are labeled a to d and domains denoted with a "D."

(B) Side-on (with respect to the membrane) view of the complex, highlighting peeled-away interaction surfaces between GMRα domain 2 and βc domain 4 of each hexamer (Site 4).

and GMRα domain 2′s into close proximity, suggesting a physiological relevance to the assembly (Figure 3B). The interaction surface, that we term Site 4, is large with ~770 Å^2 being buried per hexamer with major contributions between βc domain 4 of each hexamer (~520 Å^2 per molecule) and the rest contributed from an interaction between the GMRα of one hexamer and βc domain 4 of the other. The mostly polar interface involves residues 260 and 261 of GMRα and residues 346–354, 357, 359, 362, and 430–435 of βc with a high surface complementarity value of 0.63. The βc domain 4 dodecamer interface includes the mutated N-linked glycosylation site, N346Q. Complex sugar chains are readily accommodated at the interface with only the first couple of sugars emanating from N346 located at the interface while the rest protrude out into solution (Figure S4).

Dodecamer Complex Provides a Mechanism of Activation for JAK2 Associated with βc

The JAK/STAT pathway is the principal signal transduction pathway used by cytokine receptors. JAK activation occurs by ligand-induced dimerization and transphosphorylation of receptor-bound JAK molecules that subsequently phosphorylate the receptor itself, STATs, and other signaling molecules recruited to the receptors (Rawlings et al., 2004). JAK2 activation is a feature of GM-CSF receptor activation (Quelle et al., 1994; Watanabe et al., 1996) and plays a critical and nonredundant role in GM-CSF signaling (Watanabe et al., 1996; Parganas et al., 1998). Although JAK2 has been seen to specifically associate with βc (Brizzi et al., 1994; Soldi et al., 1997; Lilly et al., 2001) its association with GMRα is much less clear (Quelle et al., 1994).

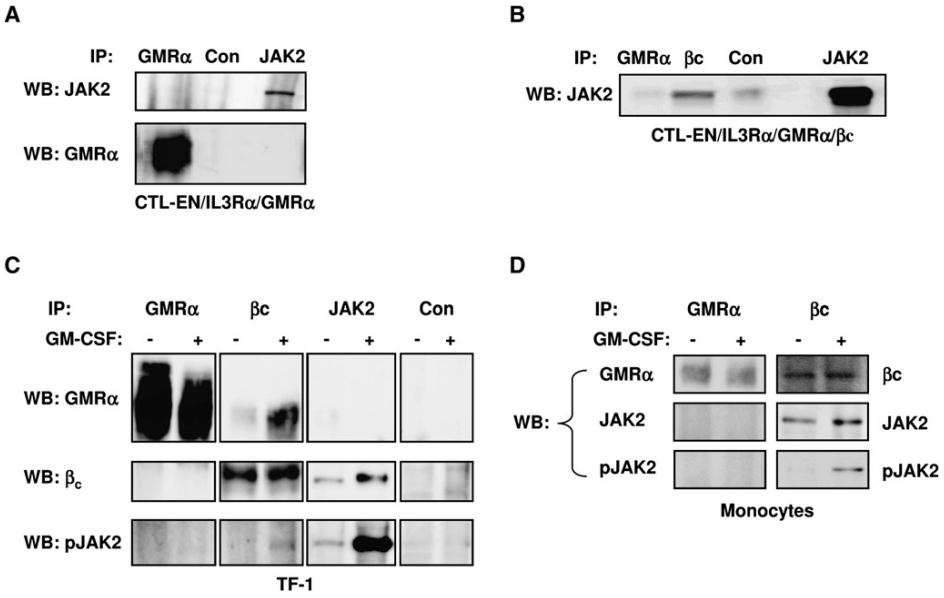

Figure 4. Association of JAK2 with βc but Not GMRα

(A) JAK2 does not associate with GMRα in the CTL-EN/IL3Rα/GMRα cell line. CTL-EN/IL3Rα/GMRα were lysed and immunoprecipitated with antibodies specific for GMRα, for JAK2, or for an irrelevant control antigen. Immunoprecipitates were analyzed by western blotting using an antibody specific for JAK2 or GMRα.

(B) JAK2 associates with βc but not GMRα in the CTL-EN/IL3Rα/GMRα/βc cell line. CTL-EN/IL3Rα/GMRα/βc were lysed and immunoprecipitated with antibodies specific for GMRα, βc, JAK2, or an irrelevant control antigen. Immunoprecipitates were analyzed by western blotting using an antibody specific for JAK2.

(C) Phosphorylated JAK2 associates with βc but not GMRα in the hematopoietic cell line TF-1. TF-1 cells starved of GM-CSF for 18 hr were treated with 0 or 150 ng/ml GM-CSF for 5 min then lysed and immunoprecipitated with antibodies specific for GMRα, βc, JAK2, or an irrelevant control antigen. Immunoprecipitates were analyzed by western blotting using antibodies specific for GMR, βc, or phosphorylated JAK2.

(D) JAK2 associates with βc but not GMRα in normal human monocytes and is phosphorylated only in the presence of GM-CSF. Cells were treated with 0 or 150 ng/ml GM-CSF for 5 min then lysed and immunoprecipitated with antibodies specific for GMRα or βc. Immunoprecipitates were analyzed by western blotting using antibodies specific for GMRα, βc, total JAK2, and phosphorylated JAK2 (pJAK2).

This is an important point as the selective association of JAK2 with βc would support the notion that juxtaposition of neighboring βc domain 4′s as seen in the dodecamer complex (but not the hexameric complex) (Figure 3B) would be required for JAK2 transphosphorylation and subsequent receptor activation.

First, we coimmunoprecipitated JAK2 with each receptor subunit using CTL-EN cell lines stably expressing GMRα alone (Figure 4A) or coexpressing GMRα and βc (Figure 4B) and observed that only βc coimmunoprecipitated JAK2. Second, we used the human erythroleukemic TF-1 cell line that endogenously expresses both receptor subunits and proliferates in response to GM-CSF to perform reverse experiments where JAK2 immunoprecipitation brought down βc but not GMRα (Figure 4C). Furthermore, following stimulation with GM-CSF, immunoprecipitation of βc brought down GMRα as well as phosphorylated JAK2, indicating that the JAK2 preassociated with βc only becomes phosphorylated after cytokine stimulation (Figure 4C). Finally, we studied human monocytes, a key effector cell type of the immune system and also observed JAK2 association with βc but not with GMRα (Figure 4D). Association of JAK2 with βc was observed in the absence of GM-CSF stimulation, and JAK2 binding and phosphorylation were upregulated following GM-CSF treatment. Similar results were observed with cells from a patient with acute myeloid leukemia and from purified human neutrophils (Figure S5). The exclusive association of JAK2 with βc suggests that GMRα:βc heterodimerization is not sufficient for receptor activation and that JAK2 activation by GM-CSF requires functional βc dimerization as provided by the dodecamer.

Interfering with Site 4 Profoundly Affects Receptor Signaling

The dodecamer structure makes two important predictions that have physiological consequences for how GM-CSF engages its receptor and activates intracellular signaling. First, disruption of the Site 4 interaction is predicted to disturb dodecamer assembly and impede biological responses. Second, such disruption of Site 4 interactions should have little impact on hexamer assembly and so high-affinity binding should remain intact. We tested these possibilities by first mutating specific βc residues in Site 4 that were identified in the structure to make contact between hexamers. CTL-EN/IL3Rα/GMRα cells were stably transfected with plasmids encoding wild-type or mutant βc (Figure 5A) and then sorted for βc expression. The resulting stable pools were assayed for GM-CSF- and IL-3-mediated proliferation (Figure 5B). All cell lines showed an equivalent response to murine IL-2 (data not shown) that was used to normalize the proliferation responses. Mutation of residues 430–436 (M1) reduced

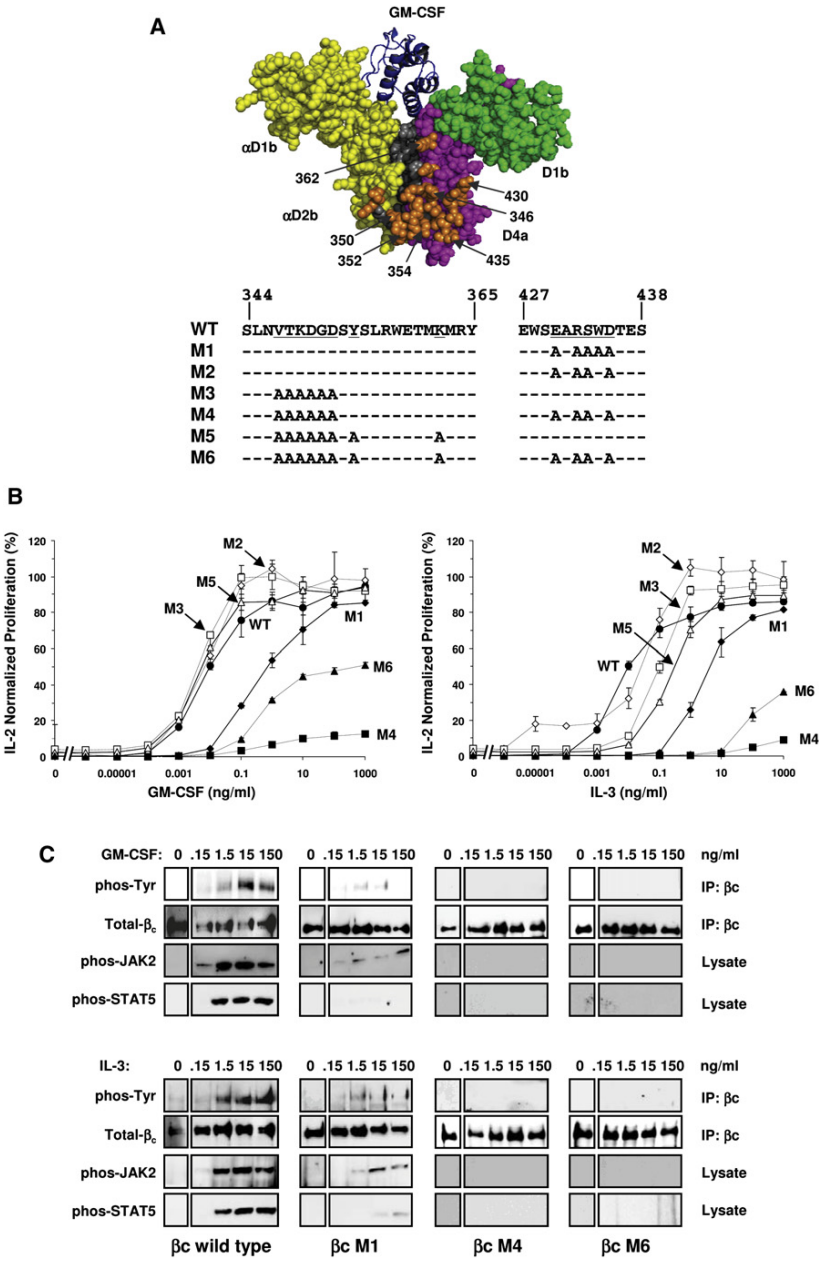

Figure 5. Mutation of βc at the Dodecamer Interface Reduces GM-CSF- and IL-3-Mediated Cell Proliferation and Signaling

(A) Dodecamer interface of one hexamer showing Site 4 residues colored in orange, Site 3 residues colored in gray, and the remaining residues colored yellow for GMRα and magenta (domain 4a) or green (domain 1d) for βc. The alanine substitutions of Site 4 residues are listed under the figure and some of the mutated residues are labeled.

(B) GM-CSF- and IL-3-mediated cell proliferation is reduced by mutation of βc Site 4 contact residues. Proliferation of cells expressing wild-type βc (filled circle), βcM1 (filled diamond), βcM2 (open diamond), βcM3 (open square), βcM4 (filled square), βcM5 (open triangle), or βcM6 (filled triangle) in response to GM-CSF (left panel) or IL-3 (right panel) was normalized against the proliferation of each cell line in response to murine IL-2. Each point is determined in triplicate and error bars represent one standard deviation. Representative data are shown from multiple experiments.

(C) GM-CSF- and IL-3-mediated cell signaling is reduced by mutation of βc Site 4 contact residues. CTL-EN/IL3Rα/GMRα cells expressing wild-type βc or the βc M1, M4, or M6 mutants were stimulated for 5 min with GM-CSF (upper panel) and IL-3 (lower panel) at 0, 0.15, 1.5, 15, or 150 ng/ml. Cells were lysed, immunoprecipitated with antibody specific for βc, then analyzed by western blotting using an anti-phosphotyrosine antibody (phos-Tyr) or an antibody specific for βc (Total βc). Cell lysates were analyzed by western blotting using antibodies specific for phosphorylated JAK2 (phos-JAK2) or phosphorylated STAT5 (phos-STAT5).

proliferation in response to GM-CSF (50-fold) and IL-3 (450-fold). Restoration of the buried tryptophan residue at 434 (M2) increased GM-CSF and IL-3 function, suggesting that the W434A substitution in M1 caused some degree of structural perturbation contributing to the loss of function. Mutation of residues 347–352, alone (M3) or combined with mutation of residues 354 and 362 (M5), had no effect on GM-CSF-mediated proliferation but caused a modest reduction in IL-3-mediated proliferation (10-fold). Combination of M2 and M3 (M4) virtually abolished proliferation in response to GM-CSF and IL-3, but additional mutation of residues 354 and 362 (M6) partially restored GM-CSF and IL-3 function (Figure 5B). Importantly, the strongest effect was observed with M4 that encompassed both targeted regions of the large area that forms Site 4 (Figure 5A). The IL-3 response is consistently more sensitive to mutation of Site 4 residues in βc than the GM-CSF response, but the overall hierarchy in mutant function is conserved between the two cytokines. CTL-EN cell lines expressing the βc mutants support high-affinity binding sites for GM-CSF and IL-3 (Table S3), indicating that functional βc Sites 2 and 3 are retained by these mutants and that they are expressed on the cell surface. The reduced proliferation observed for cells expressing βcM1, βcM4, and βcM6 was accompanied by a greatly reduced ability of GM-CSF and IL-3 to stimulate tyrosine phosphorylation of βc, JAK2, and STAT5 (Figure 5C) and a substantial loss of colony-forming capacity (βcM1) in bone marrow cells stimulated with GM-CSF (Figure S6). Functional disruption of Site 4 required multiple mutations, probably reflecting the large surface area of this protein-protein interface. Together, these data indicate that Site 4 residues predicted from the structure to be essential for dodecamer formation affect GM-CSF receptor

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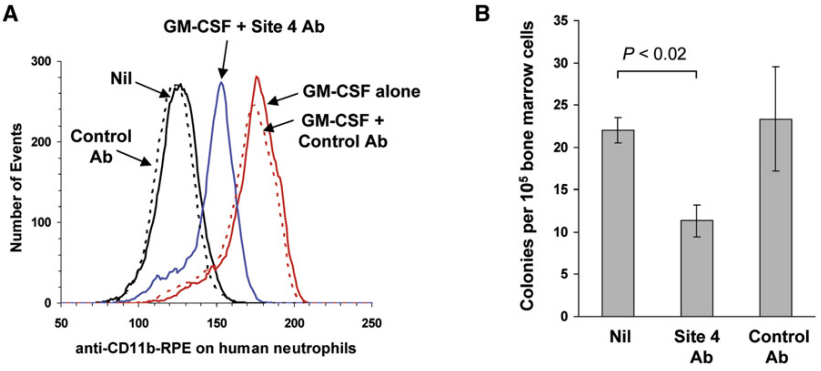

Figure 6. Antibodies to βc Site 4 Inhibit GM-CSF-Mediated Functions in Normal Cells and in Leukemic Cells

(A) Affinity-purified antibodies to βc Site 4 residues inhibit upregulation of CD11b in human neutrophils treated with GM-CSF. Histograms shows relative fluorescence for CD11b staining on neutrophils either unstimulated (black) or GM-CSF-stimulated in the presence of nil (red) or 4 μg/ml (blue) of affinity-purified βc Site 4 antibody. Preincubation with 1 μg/ml of an irrelevant control antibody (dashed line) had no effect on CD11b expression by unstimulated (black) or GM-CSF-stimulated (red) neutrophils.

(B) Affinity-purified antibodies to βc Site 4 residues inhibit GM-CSF-stimulated colony formation by bone marrow cells from a patient with chronic myelomonocytic leukemia (CMML). Bone marrow mononuclear cells (1×10^5) from a patient with CMML were plated in 2 ng/ml GM-CSF plus medium (Nil), or plus 10 μg/ml βc Site 4 antibody (Site 4 Ab), or plus 0.5 μg/ml of a control βc antibody (Control Ab). Data show average of triplicate plates and error bars represent the standard error of the mean (SEM). The two-tailed p value was generated using an unpaired t test.

activation. Furthermore, our results show that high-affinity binding (and hexamer assembly) is not sufficient for biochemical or functional receptor outputs and suggest that formation of a higher-order dodecamer complex is an obligate step for full receptor activation.

To determine the physiological significance of the dodecamer complex we generated antibodies specific to a portion of βc (residues 427–440) within Site 4 and assessed their ability to interfere with normal GM-CSF functions. Affinity-purified antibodies against the βc Site 4 peptide recognized the immunizing peptide as well as the soluble extracellular domains of βc (Figure S7). We first examined GM-CSF upregulation of CD11b in neutrophils, a key component of the CD11b/CD18 (Mac-1) integrin responsible for integrin activation, neutrophil adhesion, leukocyte extravasation, and phagocytosis (Fagerholm et al., 2006). Significantly, anti-βc Site 4 antibodies inhibited GM-CSF-mediated upregulation of CD11b expression in human neutrophils (Figure 6A). Furthermore, anti-βc Site 4 antibodies inhibited GM-CSF-mediated colony formation from bone marrow cells of a patient with chronic myelomonocytic leukemia (CMML), a condition where myeloid cells display a hypersensitive response to GM-CSF (Figure 6B). These results indicate that Site 4 is involved in normal as well as in immunopathological functions mediated by GM-CSF.

DISCUSSION

We present here the structure of a ligand:receptor complex in the GM-CSF/IL-3/IL-5 family of receptors and provide structural and functional evidence for an unexpected form of cytokine receptor assembly, namely a dodecamer, that can explain for the first time how these receptors are activated. The structure of the GM-CSF receptor complex reveals a hexamer with a stoichiometry of 2 GM-CSF:2 GMRα:2 βc, in an arrangement analogous to other heteromeric cytokine receptors and using three sites of interaction (Figure 2). Site 1 is formed largely by helix D of GM-CSF interacting with domains 1 and 2 of GMRα. Site 2 is largely provided by helix A of GM-CSF interacting with a composite βc surface comprising the A-B and E-F loops of domain 1 from one βc chain and the B-C and F-G loops of domain 4 of the second βc chain. This elbow region resembles the ligand-binding interface conserved in the simpler two-domain hematopoietin receptors, despite domains 1 and 4 being noncontiguous. The composite nature of the interface formed between GM-CSF and the βc dimer is highly unusual. In other ternary cytokine-cytokine receptor complexes different regions of the cytokine interact with just one receptor chain (Figure 2). More generally, we haven't been able to identify known examples of nonobligate heterotrimers that involve an obligate dimer in which different surfaces of each component interact with each other. The key to the Site 2 interaction is a hydrogen bond formed between E21 of GM-CSF and Y421 in βc that is consistent with mutagenesis studies that highlight the importance of these residues for GM-CSF binding and function (Hercus et al., 1994a; Woodcock et al., 1996). Mutagenesis studies suggest that structurally similar interactions are likely to exist between Y421 of βc and E22 of IL-3 and E13 of IL-5 (Barry et al., 1994; Tavernier et al., 1995: Woodcock et al., 1996). Site 3 is formed between domain 2 of GMRα and domain 4 of βc and serves to stabilize the cytokine-mediated heterodimerization.

We observed a higher-order dodecamer complex composed of two hexamers that interact through a Site 4 surface and bring the membrane-proximal domains of βc and GMRα into close proximity (Figure 3). The functional significance of the dodecamer complex is supported first by the observation that JAK2, which is essential for GM-CSF signaling, is associated with βc and not GMRα (Figure 4). This could be seen in transfected cells, in hemopoietic cell lines that respond to GM-CSF using endogenous GM-CSF receptors, and, most importantly, in primary human monocytes and neutrophils, essential leukocytes that participate in immunity. Thus JAK2-associated βc as a homodimer in the unliganded receptor (Muto et al., 1996; Stomski et al., 1996; Carr et al., 2001) or as a heterodimer with GMRα in the hexamer complex (Figure 1) would be too far apart (~120 Å) to transphosphorylate the receptor. The dodecamer structure (Figure 3) on the other hand, brings JAK2-associated βc close enough (~10 Å) to allow functional dimerization and transphosphorylation of the receptor and initiation of signal transduction. Second,

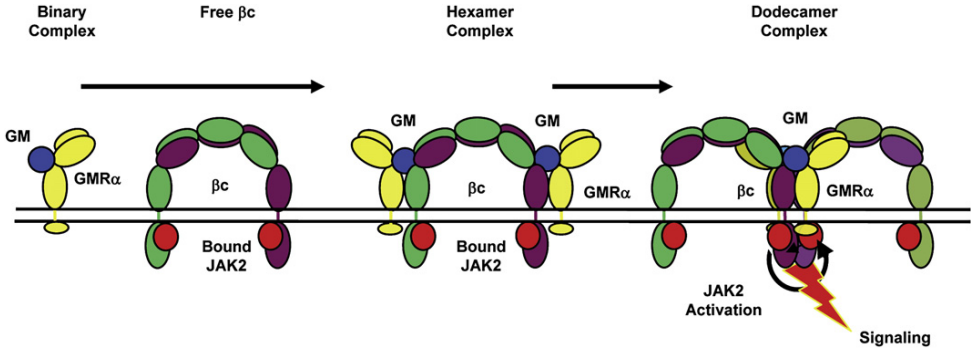

Figure 7. Model of Signal Transduction

The low-affinity complex consists of GM-CSF bound to GMRα. Interaction with free βc forms the high-affinity hexamer complex. Dodecamer (or higher-order) complexes form by lateral aggregation of hexamer complexes to form a fully competent signaling complex. JAK2 associated with βc (shown as red spheres) is able to dimerize and transphosphorylate in the dodecamer complex but not in the hexamer complex.

several mutants across the Site 4 interface decreased biological activity and signaling with M4 showing an almost complete elimination of function while not affecting high-affinity binding (Figure 5). Third, antibodies directed against part of βc Site 4 significantly reduced GM-CSF-mediated upregulation of the integrin CD11b in human neutrophils and stimulation of the growth and differentiation of leukemic cells (Figure 6).

Assembly of the dodecamer complex also promotes the interaction of two GMRα chains. While βc is the principal signaling component of the GM-CSF/IL-3/IL-5 receptor family, it is clear that the cytoplasmic domains of alpha chains are essential for signal transduction (Muto et al., 1995; Lia et al., 1996; Lilly et al., 2001). The observation that GMRα lacking a cytoplasmic domain acts in a dominant-negative fashion (Lia et al., 1996) indicates that the signaling complex must contain at least two GMRα chains. In the dodecamer complex the GMRα cytoplasmic tails are ~30 Å from the βc cytoplasmic tails of each neighboring hexamer (Figure 3A), thus also providing a structural explanation for how the cytoplasmic domain of GMRα can participate in signaling.

In analyzing the role of Site 4 in receptor activation we focused largely on two regions of βc, residues 344–365 and 427–438, because their side chains are located in close proximity on the surface of βc (Figure 5A). Interestingly, alanine substitution mutants targeting only one of these regions at a time had little impact on GM-CSF function (Figure 5B). The mutations with the greatest effect on GM-CSF function, M4 and M6, encompassed both regions of Site 4, suggesting that many residues in Site 4 contribute to the function of this interacting surface.

While we have not examined the effect of βc Site 4 mutations on IL-5 function, the IL-5 dimer can be docked into our dodecamer structure (Figure S8), suggesting that IL-5 is capable of assembling a dodecamer complex. This particular assembly may also allow the simultaneous incorporation of different cytokines bound to the cognate alpha chain, potentially explaining how stimulation of cells with IL-3 or IL-5 can result in phosphorylation of the GM-CSF receptor (Woodcock et al., 1997). The structural and functional data shown here support a model of GM-CSF/IL-3/IL-5 receptor activation where the hexamer complex provides for high-affinity cytokine binding but the dodecamer complex is required for receptor activation and signal transduction (Figure 7). Interestingly, the different mutations of βc at the Site 4 interface greatly reduced signaling and function not only from GM-CSF but also from IL-3 (Figure 5), suggesting that dodecamer formation may be a conserved mechanism utilized by the whole GM-CSF/IL-3/IL-5 receptor family. As such it may offer new avenues to interfere with receptor activation and facilitate the development of new molecules, which by simultaneously blocking GM-CSF, IL-3, and IL-5 function may be of clinical use in diseases where all these cytokines play a pathogenic role.

EXPERIMENTAL PROCEDURES

Expression and Purification of Receptor Components

Soluble human GM-CSF was produced in *Escherichia coli* (Hercus et al., 1994a). The soluble extracellular domains of GMRα or βc were expressed in Sf21 insect cells and purified by affinity chromatography (McClure et al., 2003).

Structure Determination of the Complex

Ternary complex consisting of GM-CSF, sGMRα, and sβc was concentrated to 5 mg/ml in 10 mM HEPES (pH 7.0). The best crystals were grown from a solution containing 100 mM HEPES buffer (pH 7.0), 6% (v/v) PEG3350, 0.2 M proline at 20°C using the hanging drop technique. The crystals belonged to the space group $P6_322$ with unit cell dimensions $a = b = 166.4$ Å and $c = 212.2$ Å. Diffraction data were collected on the BioCARS beamline 14-BM-C at the Advanced Photon Source (Chicago, IL, USA) (Table S1). The structure was determined by molecular replacement using the known structure of isolated βc (Carr et al., 2001) and refined at 3.3 Å resolution (Supplemental Data).

Mutagenesis and Generation of Cell Lines

Mutations of βc were generated by site-directed mutagenesis using the Altered site system (Promega). Wild-type and Site 4 mutant βc cDNAs were subcloned into the expression vector pRcCMVpuro (Woodcock et al., 1997). Murine CTL-EN cells (Jenkins et al., 1999) stably expressing human IL3Rα and GMRα chains (CTL-EN/IL3Rα/GMRα) were grown routinely in RPMI 1640 medium supplemented with 10% v/v FCS, 2 mM glutamine, 50 μM mercaptoethanol, and 100 U/ml mIL-2. TF-1 cells were maintained as previously described (Hercus et al., 1994a).

CTL-EN/IL3Rα/GMRα cells (5×10^6) were electroporated with 20 μg of pRcCMVpuro DNA encoding wild-type or Site 4 mutant βc at 960 μF with 270 V and selected in 2–5 μg/ml puromycin 48 hr after transfection. Following selection, pools of cells were sorted for βc expression by flow cytometry using indirect immunofluorescence staining as previously described (Woodcock et al., 1996) on a FACSAria (Becton Dickinson). Cells were starved of growth

factor overnight prior to use, and for biochemical studies, starve medium included 0.5% v/v FCS.

Receptor Binding Assays

Radio-iodinated GM-CSF and IL-3 binding assays to CTL-EN/IL3Rα/GMRα cell lines stably expressing wild-type βc or βc Site 4 mutants were as previously described (Woodcock et al., 1994). COS cells were cotransfected with plasmids encoding GMRα and IL3Rα together with wild-type or βc Site 2 mutants by electroporation and saturation binding assays with radio-iodinated cytokine carried out in 24 well plates as previously described (Woodcock et al., 1996). Dissociation constants were determined using Prism (Graphpad Software).

Cell Proliferation Assays

Starved CTL-EN/IL3Rα/GMRα cell lines stably expressing wild-type βc or βc Site 4 mutants were incubated (1×10^4 cells per well) with growth factors over a range of concentrations for 40 hr. Cells were pulsed with 0.25 μCi/well of [6-^{3}H]-thymidine (GE-Healthcare) for 4 hr, harvested onto glass fiber filters, and counted in liquid scintillation fluid on a Top Count NXT (Perkin Elmer).

Immunoprecipitations and Immunoblotting

Human mononuclear cells from normal donors were isolated by Ficoll-Hypaque density-gradient centrifugation and resuspended in PBS containing 0.1% human albumin (CSL). CTL-EN/IL3Rα/GMRα and CTL-EN/IL3Rα/GMRα/βc cells were lysed and immunoprecipitated using 4H1 anti-GMRα monoclonal antibody (Stomski et al., 1998), 1C1 anti-βc monoclonal antibody (Stomski et al., 1996), anti-JAK2 monoclonal antibody (Cell Signaling), or 12CA5 anti-influenza hemagglutinin monoclonal antibody. Samples from 2×10^7 cells were subjected to western blotting with antibodies specific for GMRα or total JAK2. Human monocytes, starved TF-1 cells, or starved CTL-EN/IL3Rα/GMRα/βc cells were stimulated with 0 or 150 ng/ml GM-CSF for 5 min, lysed, and immunoprecipitated using 4H1, 1C1, anti-JAK2, or 12CA5 monoclonal antibodies. Samples were subjected to western blotting with antibodies specific for GMRα, βc, total JAK2, or tyrosine-phosphorylated JAK2. Starved CTL-EN/IL3Rα/GMRα cell lines stably expressing wild-type βc or βc Site 4 mutants were stimulated with 0, 0.15, 1.5, 15, or 150 ng/ml GM-CSF or IL-3 for 5 min, lysed, and immunoprecipitated using the 1C1 anti-βc monoclonal antibody. Samples of βc immunoprecipitates were subjected to western blotting with 1C1 anti-βc or 4G10 anti-phosphotyrosine monoclonal antibodies (Upsate Cell Signaling Solutions). Samples of cell lysates were subjected to western blotting with antibodies specific for phosphorylated JAK2 or phosphorylated STAT5.

Generating and Functional Testing of Polyclonal Antibodies against βc Site 4

Antibodies directed to βc Site 4 were generated by immunizing New Zealand white rabbits with a peptide (EWSEARSWDTESVL) spanning residues 427–440 of βc (Mimotopes) and conjugated to keyhole limpet antigen. Peptide-specific polyclonal antibody was affinity purified using the EWSEARSWDTESVL peptide coupled to Sepharose. CD11b expression on neutrophils was monitored as previously described (Guthridge et al., 2006) but included a 30 min preincubation with antibody at 4°C followed by a 30 min incubation with 15 ng/ml GM-CSF at 37°C. The 12CA5 monoclonal antibody was used as a negative control. Bone marrow colony assays where performed as previously described (Ramshaw et al., 2002) with the 1C1 monoclonal antibody used as a negative control.

ACCESSION NUMBERS

Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession code 3CXE.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, eight figures, and three tables and can be found with this article online at http://www.cell.com/cgi/content/full/134/3/496/DC1/.

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Supplemental Data

The Structure of the GM-CSF Receptor Complex Reveals a Distinct Mode of Cytokine Receptor Activation

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Supplemental Experimental Procedures

Expression and Purification of Receptor Components

Soluble human GM-CSF was produced in *Escherichia coli* and purified by anion exchange chromatography and reversed phase HPLC (Hercus et al., 1994). DNA fragments encoding the soluble extracellular domains of GMRα (sGMRα) or βc (sβc) were cloned into the pIBV5-His expression vector (Invitrogen) and transfected into Sf21 insect cells using Cellfectin (Invitrogen). Permanently transfected Sf21 cell lines were selected by incubation with Blasticidin at 50 μg/ml (Invitrogen) and maintained in serum-free ExCell 420 medium (JRH Biosciences) at 27°C. Soluble receptors were purified from conditioned medium by affinity chromatography (McClure et al., 2003).

Human βc contains three N-linked glycosylation sites and elimination of the third site at N346 by glutamine substitution has previously been demonstrated to improve the diffraction properties of crystallized sβc (Gustin et al., 2001). We expressed and purified sβc N346Q from Sf21 cells and observed a modest reduction in molecular weight compared to the

wild-type sβc but no difference in its ability to form a ternary complex with sGMRα and GM-CSF (results not shown).

Crystallization of the Complex

Ternary complex consisting of GM-CSF, sGMRα and sβc N346Q was isolated by gel filtration chromatography using a Superdex 200 column (26mm x 600mm, GE Healthcare) operated at 2 ml/min at 4°C with 150 mM NaCl, 50 mM sodium phosphate pH 7.0, as running buffer. For crystallization the complex was concentrated to 5 mg/ml in 10 mM HEPES pH 7.0. A number of crystal forms of the wild-type ternary complex were grown but none diffracted beyond 8 Å resolution using synchrotron radiation. We subsequently grew a number of crystal forms of the ternary complex using the sβc N346Q construct. However, only one out every 100 crystals diffracted beyond 4 Å resolution. Complete details of the crystallization trials will be published elsewhere. Briefly, using the hanging drop technique, crystals appeared after 1 week and grew to full size (up to 0.8 x 0.8 x 0.4 mm) within 2 weeks The best crystal was obtained mixing 1 µl of ternary GM-CSF receptor complex at a concentration of ~5 mg/ml with 1 µl reservoir solution containing 100 mM HEPES buffer pH 7.0, 6% (v/v) PEG3350, 0.2 M proline at 20ºC. The crystal belonged to the space group $P6_322$ with unit cell dimensions $a = b = 166.4$ Å and $c = 212.2$ Å.

Structure Determination of the Complex

Diffraction data were collected at 100 K and recorded on an ADSC Quantum-315 CCD detector on BioCARS beam line 14-BM-C at the Advanced Photon Source (Chicago, USA). The wavelength was set to 0.9 Å. Data were processed using MOSFLM and SCALA (CCP4, 1994) (Table S1). The best crystal diffracted to a resolution of 3.3 Å. The structure was

determined by molecular replacement using PHASER (Storoni et al., 2004). The search models were GM-CSF βc chain (Carr et al., 2001; PDB id: 1GH7) and GM-CSF (Rozwarski et al., 1996; PDB id: 2GMF). Molecular replacement solutions for GM-CSF βc and GM-CSF gave Z-scores of 51.6 and 6.3 to a resolution of 3.3 Å, respectively. Difference electron density maps calculated with phases obtained after a few rounds of refinement clearly showed unexplained density close to domain 4 of the βc chain. A homology model of domain 2 of the alpha chain (Chris Bagley, unpublished results) was built into this density. (The choice of which alpha chain domain to build into the density was straight forward as it was interacting with domain 4 of βc, the membrane proximal domain of that chain, and thus the correct choice had to be the membrane proximal alpha chain domain 2). The model was completed by iterative cycles of manual rebuilding using COOT (CCP4, 1994) and refinement with REFMAC (Murshudov et al, 1997). In the final refinement steps the βc model was replaced by a better quality structure that just became available (Carr et al., 2006; PDB id: 2GYS). During the final refinement step each subunit was treated as a rigid body for the purposes of TLS (translation, libration and screw rotation tensors) refinement to account for overall differences in displacements between the molecules and anisotropy in the data (Winn et al., 2001; Painter and Merritt, 2006). Despite the low resolution of our data, the availability of refined structures of two of the components (βc dimer at 2.7 Å resolution (Carr et al., 2006) and GM-CSF at 2.4 Å resolution (Rozwarski et al., 1996), combined with rigidity of the structural modules (< 1.75 Å rmsds from their unliganded structures), suggests we have a more accurate model than the resolution would indicate. For example, electron density corresponding to the F-G loop in domain 4 of βc, which includes the essential residue Y421, indicated a different conformation of this loop from that described in the original structure of βc (Carr et al., 2001), although the initial maps were calculated using this model. Instead our

interpretation agrees with that in the more recently refined higher resolution structure (Carr et al., 2006).

The final model includes residues 25-436 of βc. The sequence of the published structure of βc, a variant of the one used in this work, has a six residue insertion in the C-D loop of domain 3. Density for parts of this loop were not observed in the published structure (Carr et al., 2006) but is well defined in the complex. Additionally, several loops including residues 157 to 161 (B-C loop of domain 1 in both chains), residues 349 to 351 (A-B loop in domain 4, chain A) and residues 361 to 368 (B-C loop in domain 4, chain A) were missing in the published structure but are well defined in our model. As observed in the apo structure of βc, two carbohydrate chains are covalently linked to N58 (built as NAG-NAG-MAN) and N191 (built as NAG).

The model of GM-CSF starts at residue 14 and ends at residue 118. Although electron density for GMRα is generally weaker in comparison to the rest of the structure, large parts of domain 2 could be traced (residues 218 - 316) including a portion of a carbohydrate chain covalently linked to N229 (modeled as NAG). In general the main chain of GMRα in this region is well defined by electron density. Density modification, sharpening of structure factors and calculation of composite omit maps did not lead to any significant improvement of electron density maps. Although the electron density for N-terminal FnIII domain (GMRα domain 1) is of poorer quality, it was sufficient to build a partial polyalanine model including a two-stranded beta-sheet. A homology model of the complete GMRα domain 1 could be superimposed onto the partial structure. SDS-PAGE gels of washed crystals confirm that all 3 domains of GMRα are present indicating the N-terminal knob domain is disordered in the crystals.

The stereochemical quality of the final model correlates well with structures at similar resolutions with 96.8% residues in the allowed regions of the Ramachandran plot and only 18 residues in the disallowed regions. Other stereochemical parameters are all better than or within the allowed ranges defined by PROCHECK (Laskowski et al., 2003).

Immunoprecipitations and Immunoblotting

Human monocytes, neutrophils, mononuclear cells from an acute myeloid leukaemia patient, starved TF-1 cells, or starved CTL-EN/IL3Rα/GMRα/βc cells were stimulated with 0 or 10 ng/ml GM-CSF for 5 min and lyzed in NP40 lysis buffer (10 mM Tris-HCl (pH 7.4), 137 mM NaCl, 10% v/v glycerol, 1% v/v Nonidet P-40) containing 2 mM phenylmethylsulfonyl fluoride, 4 mM sodium vanadate, 10 mM sodium fluoride, 10 mM β-glycerol phosphate, 5 μg/ml leupeptin (Sigma), 8.5 μg/ml aprotinin (Sigma). Lysates were immunoprecipitated using 4H1 anti-GMRα monoclonal antibody (Stomski et al., 1998), 1C1 anti-βc monoclonal antibody (Stomski et al., 1996), anti-JAK2 monoclonal antibody (Cell Signaling) or 12CA5 anti-influenza hemagglutinin monoclonal antibody as a negative control. Immune complexes were captured on Protein A-Sepharose beads, washed extensively in lysis buffer and eluted by boiling in SDS sample buffer. Samples from 2-4x10^7 cells were fractionated on 7.5% v/v SDS-PAGE and analyzed by Western blotting with 1 μg/ml 4H1, 1 μg/ml 1C1, 1 μg/ml anti-JAK2 monoclonal antibody or anti-phospho-JAK2 polyclonal antibody (Cell Signaling) at a dilution of 1:1000. Bound antibodies were detected using appropriate horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibodies (Pierce) and enhanced chemiluminescence (GE-Healthcare).

Starved CTL-EN/IL3Rα/GMRα cell lines stably expressing wild-type βc or βc Site 4 mutants were stimulated with 0, 0.01, 0.1, 1 or 10 ng/ml GM-CSF or IL-3 for 5 min and the

cells lyzed in NP-40 lysis buffer. Lysates were immunoprecipitated using 1C1 anti-βc monoclonal antibody then fractionated on 7.5% v/v SDS-PAGE and analyzed by Western blotting with 1 μg/ml 4G10 anti-phosphotyrosine monoclonal antibody (Upstate Cell Signaling Solutions) or 1 μg/ml 1C1. Samples of cell lysate were fractionated by SDS-PAGE and analyzed by Western blotting with anti-phospho-JAK2 polyclonal antibody or 1 μg/ml anti-phospho-STAT5 monoclonal antibody (Zymed).

Supplemental Data

Intermolecular Disulfide bonds in the GM-CSF Receptor Complex

IL-3, IL-5 and GM-CSF receptors have been shown to undergo covalent dimerization of their respective α chains with βc in the presence of their cognate ligand and this appears to be important for receptor activation (Stomski et al., 1996; Stomski et al., 1998). Modeling studies suggested the disulfide forms between domain 1 of βc and the knob domain of the α chain. It is not possible to homology model the knob region into the crystal structure of the hexamer complex so that it gets sufficiently close to domain 1 of the βc chain. However, the knob domain can be fitted snugly between domain 1 of GMRα and domain 1 of βc from the second hexamer molecule in the dodecamer structure (Figure 3A) providing further credence for the physiological relevance of the latter structure. This suggests a role for the knob in stabilizing the higher order signaling complexes for receptor signaling.

Stoichiometry of the GM-CSF Receptor Ternary Complex

We previously characterized the formation of the soluble GM-CSF receptor ternary complex with a 2:1:1 stoichiometry comprising two βc chains, one GMRα chain and one GM-CSF molecule (McClure et al., 2003). These studies utilized protein concentrations in the low micromolar range but considerably higher protein concentrations (~30 μM) were used for preparation of material used in crystallization studies. We investigated the impact of protein concentration on the stoichiometry of the GM-CSF receptor complex. Purified soluble βc was titrated alone or in the presence of equimolar soluble GMRα and a 1.5x molar excess of GM-CSF and the mixture fractionated by size exclusion chromatography (Figure S1). The elution time of the ternary complex (TC) was markedly influenced by total protein concentration unlike the GMRα:GM-CSF binary complex (BC) or free βc. The estimated molecular weight

of the ternary complex (Table S2) at protein concentrations > 5.4 μM is consistent with a 2:2:2 complex comprising two βc chains, two GMRα chains and two GM-CSF molecules. The estimated molecular weight of the ternary complex formed at 1.1 μM is consistent with a 2:1:1 complex as previously described (McClure et al., 2003). Thus the 2:1:1 complex most likely represents a relatively stable intermediate in the assembly of the higher order 2:2:2 GM-CSF receptor complex. The analogous IL-6 receptor system also exists as either a 2:1:1 complex (2 gp130 (equivalent to βc): 1 IL-6Rα: 1 IL-6) or a 2:2:2 complex. It has been argued that both models are correct with the differences reflecting a mechanism by which complex receptor systems can respond to differing cytokine concentrations (Hermanns et al., 2005).

Docking of IL-5 dimer onto Dodecameric Complex Model

IL-5 is unique amongst cytokines utilizing βc in that it forms a disulfide-linked dimer whereby helix D of one chain combines with helices A, B and C of the other chain so that each four helix bundle of the dimer resembles the bundle observed in the monomeric GM-CSF and IL-3 molecules. The possibility that IL-5 exerts its biological activity through both monomers has been supported by the observation that engineered dimers of mouse IL-3 display increased biological activity (Ishino et al., 2004). An obvious question arises: can the IL-5 dimer dock onto the dodecamer complex where the GM-CSF "dimers" bind? The IL-5 dimer was superimposed onto GM-CSF in the dodecamer complex structure (Figure S8). Because GM-CSF packs as a head-to-tail dimer in the complex whereas the IL-5 dimer assembles as a head-to-head dimer (with respect to the N-termini), the superposition results in one IL-5 monomer pointing out into solution. Attempts were made to dock the IL-5 dimer into the dodecamer complex so that both monomers interacted with βc molecules, assuming

that at least one of the essential E13 residues interacts with domain 4 of βc in similar fashion to how GM-CSF interacts with the same region. No convincing docking solution in which the IL-5 dimer interacting with both βc chains of the dodecamer could be identified. An IL-5 monomer was then superimposed onto GM-CSF in the hexameric complex and a dodecamer complex generated using the twofold axis observed in the IL-5 dimer, but the distance between equivalent βc cytoplasmic tails would be approximately 100 Å, too far apart for signaling to occur. Thus the IL-5 dimer likely functions as a monomer in the receptor complex without compromising dodecamer formation. This suggestion is in agreement with the findings that the IL-5 dimer binds its alpha chain in a 1:1 ratio (Devos et al., 1993) and that engineered IL-5 monomers stimulate cell proliferation in IL-5 receptor positive cells with a dose dependence similar to wild-type (Li et al., 1997).

Figure S1. Stoichiometry of the GM-CSF Receptor Complex

Purified sβc N346Q was titrated (1.1 – 10.9 μM) alone or mixed with equimolar sGMRα and a 1.5x molar excess of GM-CSF, adjusted to 50 μl with 150 mM NaCl, 50 mM sodium phosphate pH 7 and incubated at 25°C for at least 1 hr. Samples were chromatographed on a SMART system using a Superdex 200PC column (3.2 mm x 300 mm, GE Healthcare) as previously described (McClure et al., 2003). Elution time and area of eluted peaks was determined using the instruments software. The molecular weight of the eluted ternary complex (TC) and binary complex (BC) was estimated using the protein components as internal molecular weight standards as previously described (McClure et al., 2003) (Table S2). Chromatograms show sβc N346Q alone (A), or mixed with equimolar sGMRα and a 1.5x molar excess of GM-CSF (B). Inset graphs plot the area of the peaks in min.mAU for ternary complex (TC), binary complex (BC) and free sβc (sβc) versus the elution time of each peak in minutes. Elution position of molecular weight standards is shown above each panel.

Figure S2. **Comparison of Apo and Complexed βc Structures**

The apo structure (Carr et al., 2006) is in light grey and the complex structure in magenta. The figure highlights a 3º rotation of domain 4 on complex formation.

Figure S3. Critical role for the Phenolic Hydroxyl Group of βc Tyr^{421} in High Affinity GM-CSF and IL-3 Binding

Scatchard plots of ^{125}I-labelled GM-CSF or IL-3 binding to COS cells expressing GMRα (left) or IL3Rα (right) with wild type βc (filled square) or the Y421F (open circle), Y421A (open triangle) βc mutants. The dashed line indicates the high affinity binding component for

wild type βc and the solid lines represents the line of best fit for the Y421F and Y421A mutants βc as determined using Prism (Graphpad Software).

Figure S4. **Accommodation of N-linked Glycosylation at βc N346 Position in Dodecamer Interaction**

Ribbon pictures of Site 4 showing two different complex sugar chains, denoted in stick fashion, linked to the glycosylation site at N346. The view and coloring scheme is the same as shown in Figure 3B. The key to the different sugar models is shown in the right panels. N-acetyglucosamine residues are shown as green squares and mannose residues shown as red circles. The sugar chains are readily accommodated in the Site 4 interface without disturbing it.

Figure S5. **Association of JAK2 with βc but not GMRα in Human AML and Normal Neutrophils**

(A) JAK2 associates with βc but not GMRα in mononuclear cells from a patient with acute myeloid leukaemia (AML). Cells were treated with 0 or 10 ng/ml GM-CSF for 5 min then lyzed and immunoprecipitated with antibodies specific for GMRα, βc, JAK2 or for an irrelevant control antigen. Immunoprecipitates were analyzed by Western blotting using antibodies specific for JAK2.

(B) JAK2 associates with βc but not GMRα in normal human neutrophils. Purified neutrophils were treated with 0 or 10 ng/ml GM-CSF for 5 min then lyzed and immunoprecipitated with antibodies specific for GMRα or βc. Immunoprecipitates were analyzed by Western blotting using antibodies specific for GMRα, βc, total JAK2 and phosphorylated JAK2 (pJAK2).

Figure S6. Reduction in GM-CSF Stimulated Colony Formation in Cells Transduced with the βcM1 Mutant

HEK293T cells were co-transfected with the pEQEco packaging plasmid (Persons et al., 1998) and a bicistronic retroviral construct (Guthridge et al., 2006) encoding GMRα and either wild type or M1 mutant βc using Lipofectamine. Retroviral supernatant was harvested after three days and used to transduce fetal liver cells from $\beta_c^{-/-}/\beta_{IL3}^{-/-}$ mice in the presence of 50 ng/ml SCF. Transduction efficiency was quantified using flow cytometry to assess GMRα expression and $1x10^5$ transduced cells were plated in 0.3% agar in a cocktail of cytokines (IL-6+G-CSF+SCF+EPO) or 10 ng/ml GM-CSF and incubated for 14 days. The histogram shows the number of colonies per $1x10^5$ cells plated and the bars represent the standard error of the mean.

Figure S7. Generating Polyclonal Antibodies against βc Site 4

(A) Dodecamer interface of one hexamer showing Site 4 residues colored in orange, Site 3 residues colored in gray and the remaining residues are colored yellow for GMRα and magenta (domain 4a) or green (domain 1d) for βc.

(B) The M1 peptide corresponding to βc residues 427-440 (colored in cyan) and including some of the Site 4 contact residues was synthesized and used to raise antibodies.

(C) Samples of sβc were fractionated on 10% v/v SDS-PAGE and analyzed by Western blotting using crude pre-immune serum (Pre), M1 peptide immune serum (Imm) or M1 peptide affinity purified immune serum (AP) at a dilution of 1:1000. Bound antibodies were detected using horseradish peroxidase-coupled anti-rabbit secondary antibodies (Pierce) and enhanced chemiluminescence (GE-Healthcare).

Figure S8. IL-5 Docking onto the GM-CSF Receptor Dodecamer Complex

Structural superposition of the IL-5 dimer onto one of the GM-CSF molecules in the complex. Shown are (A) side and (B) top views. The cartoon below explains why the IL-5 dimer does not superimpose on the GM-CSF "dimer".

Table S1 Data Collection and Refinement Statistics

	GM-CSF Receptor Complex
Data collection	
Space group	$P6_322$
Cell dimensions	
a, *b*, *c* (Å)	166.6, 166.6, 213.1
α, β, γ (°)	90, 90, 120
Resolution (Å)	3.3 (3.5-3.3)
R_{merge}	7.7 (62.1)
I / σ_I	17.7 (2.3)
Completeness (%)	99.7 (99.7)
Redundancy	4.7 (4.8)
Refinement	
Resolution (Å)	3.3
No. reflections	25439
R_{work} / R_{free} (%)	27.0 / 31.7
No. atoms	5127
B-factors after TLS refn (Å^2)	
All atoms	122.71
GM-CSF-alpha	126.34
GM-CSF-beta	122.22
GM-CSF	120.77
R.m.s. deviations	
Bond lengths (Å)	0.013
Bond angles (°)	1.5

Table S2 Calculated Molecular Weight of Ternary Complex

Concentration of sβc N346Q μM	Estimated Molecular Weight TC	BC
1.1	156,000	60,000
2.2	169,000	57,000
3.3	185,000	57,000
4.3	197,000	57,000
5.4	200,000	56,000
6.5	204,000	55,000
8.7	209,000	54,000
10.9	217,000	54,000

Molecular weight of components;

GM-CSF = 13,700

sGMRα = 40,700

sβc N346Q dimer = 97,400

Predicted Molecular Weights;

1:1 (α:GM) Binary Complex (BC) = 54,400

2:1:1 (β:α:GM) Ternary Complex (TC) = 151,900

2:2:2 (β:α:GM) Ternary Complex (TC) = 206,300

Table S3 GM-CSF and IL-3 Binding Affinities of βc Site 4 Mutants

	GM-CSF Binding			IL-3 Binding		
βc Mutant	n	Kd (pM)	Receptors/cell	n	Kd (pM)	Receptors/cell
βc wild type	8	70-200	3300-11100	4	35-145	360-2200
βcM1	7	35-100	700-2200	2	50-80	240-360
βcM2	4	190-600	7600-23000		n.d.	n.d
βcM3	1	300	5100	2	60-120	420-1800
βcM4	1	200	2200	2	150-200	1000-1100
βcM5	1	40	400	2	90-170	970-1120
βcM6	1	150	2150	2	89-91	100-240

Summary of Scatchard analysis of ^{125}I-labelled GM-CSF or IL-3 binding to CTL-EN/IL3Rα/GMRα cells expressing wild type or mutant βc.

n = number of experimental determinations, n.d.= not determined

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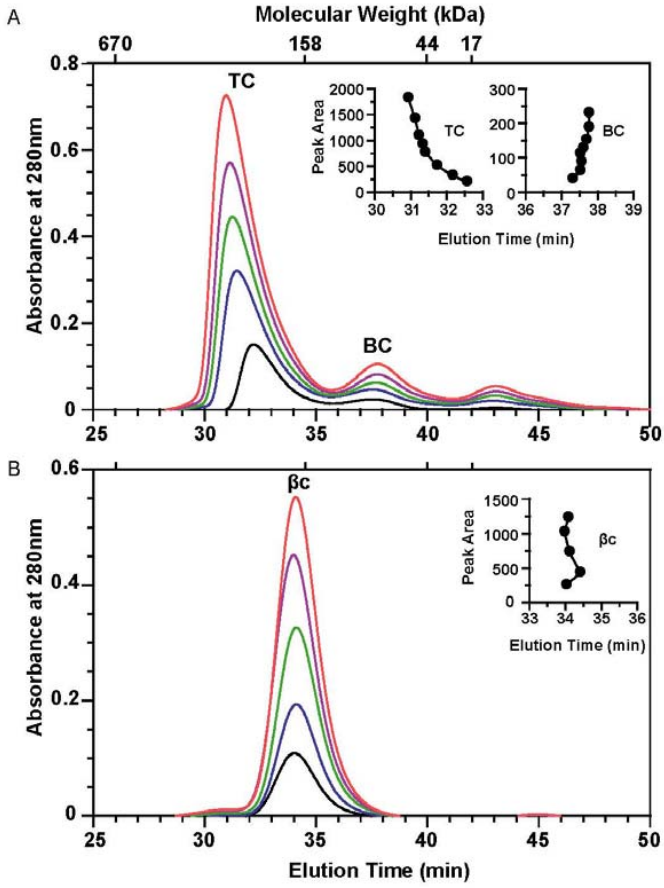
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158
44
17
TC
BC
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Peak Area
Elution Time (min)
B
βc
Absorbance at 280nm
Peak Area
Elution Time (min)

Figure S1

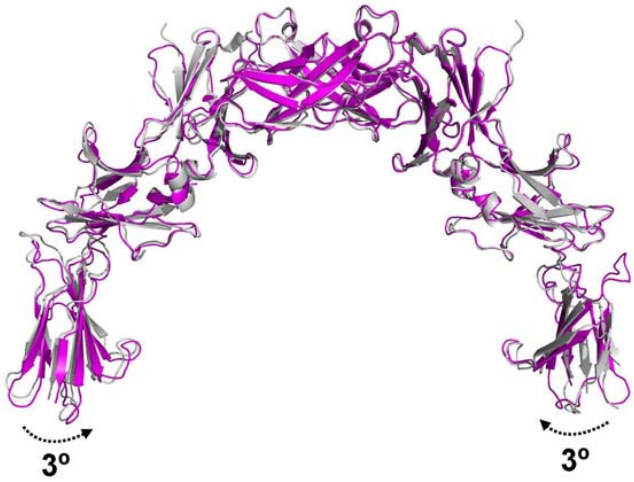
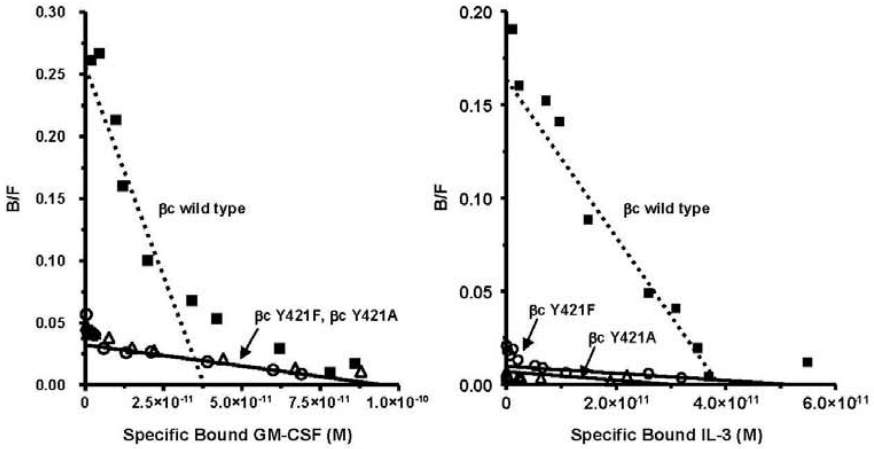


Figure S2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0.20
0.15
0.10
0.05
0.00
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βc Y421F, βc Y421A
0
2.5×10-11
5.0×10-11
7.5×10-11
1.0×10-10
Specific Bound GM-CSF (M)
B/F
0.20
0.15
0.10
0.05
0.00
βc wild type
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Specific Bound IL-3 (M)

Figure S3

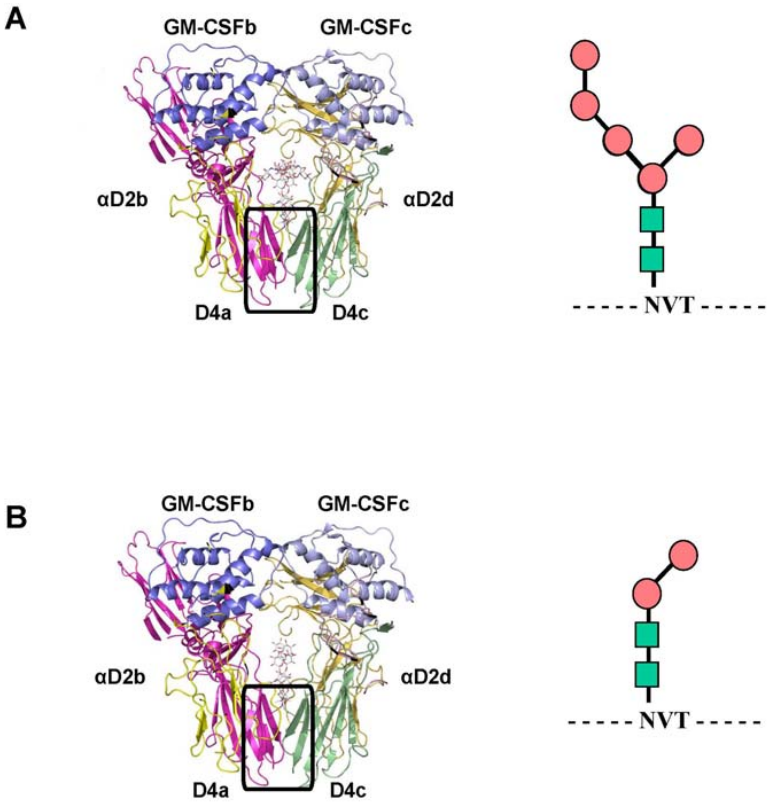
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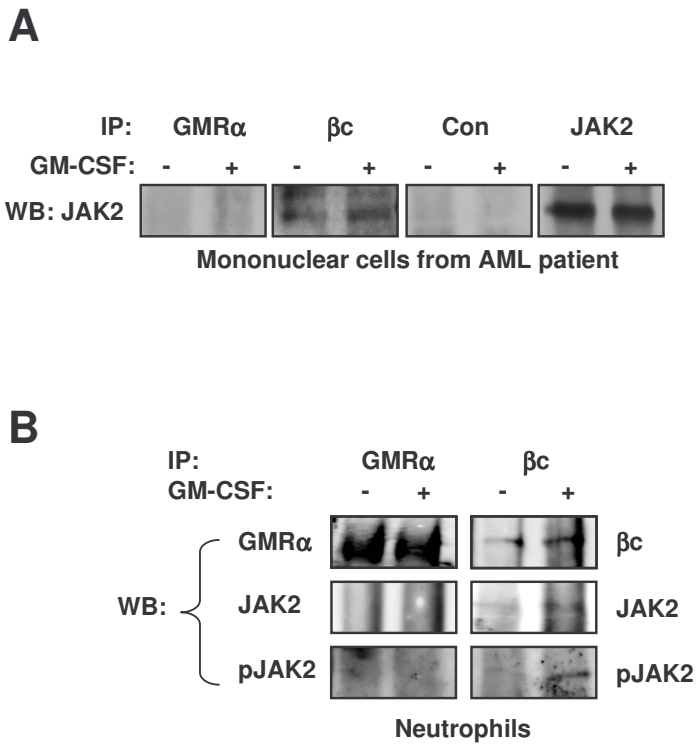
Figure S4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B
IP: GMRα βc
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WB:
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JAK2
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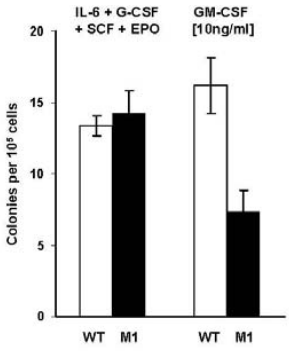
βc
JAK2
pJAK2
Neutrophils

Figure S5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+ SCF + EPO
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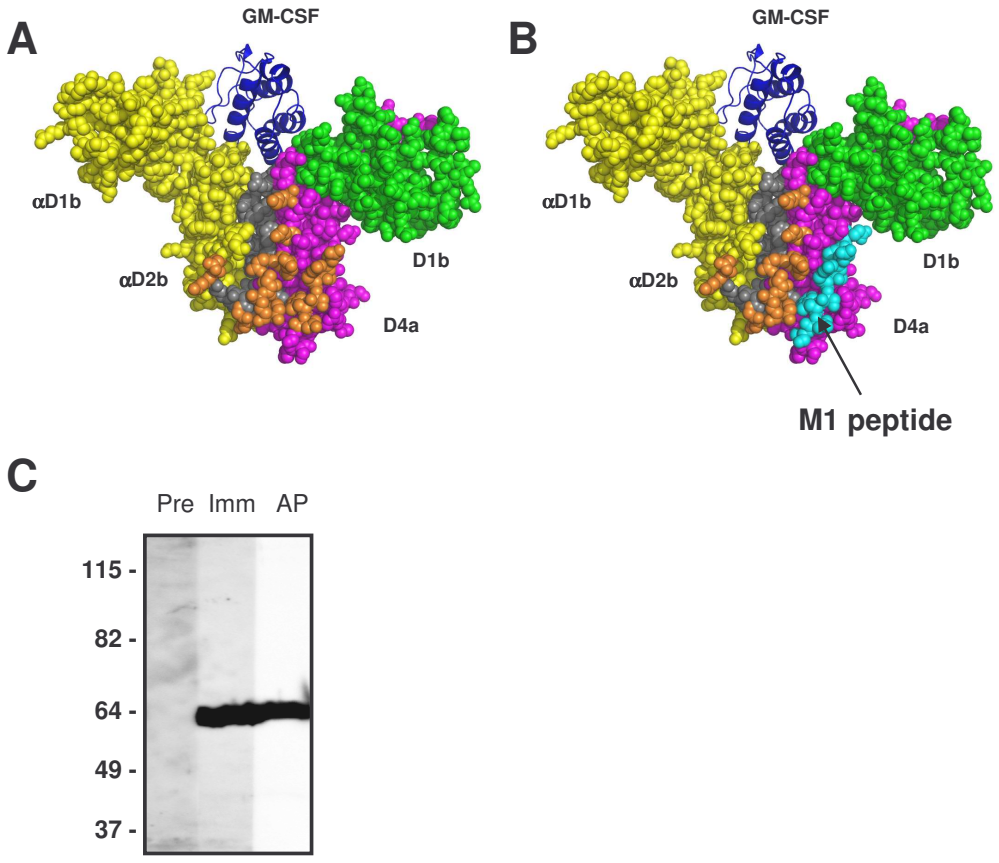
15
10
5
0
WT
M1
WT
M1

Figure S6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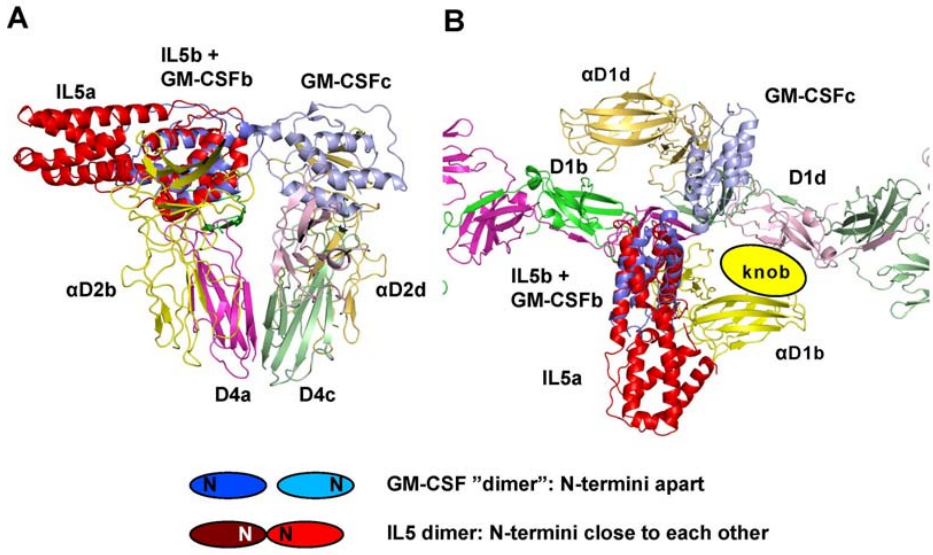
49 -
37 -

Figure S7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GM-CSFb
IL5a
GM-CSFc
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B
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D1b
D1d
knob
IL5b +
GM-CSFb
αD1b
IL5a
N
N
GM-CSF "dimer": N-termini apart
N
N
IL5 dimer: N-termini close to each other

Figure S8

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