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

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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′-TGAATTCGCCTGTCCAGAGCTGACCAGGG-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′-ATACACAAGCTTAGCACCATGCTTCTCCTGGTG-3′ that starts 18 nucleotides 5′ of the ATG and contains an engineered *Hin*dIII site and (2) 5′-ATACACTCTAGATCACCCGTCGTCAGAACCAAATTC-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] Radio-iodination 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, Meriden, 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

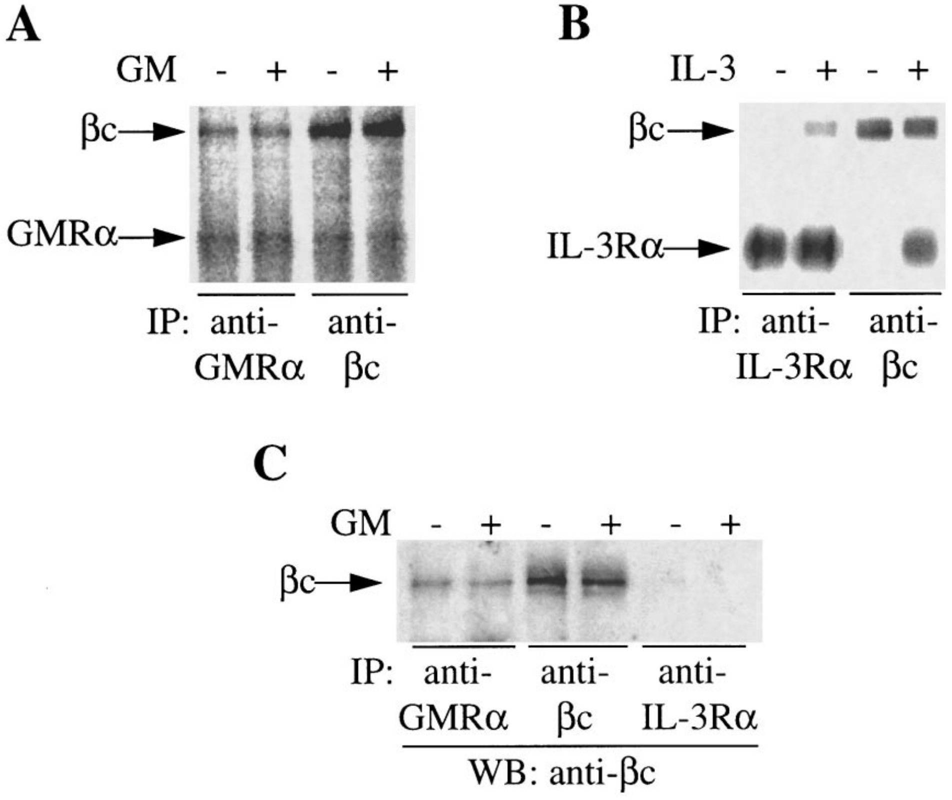

Fig 1. Coimmunoprecipitation of GMRα and β_c from primary CML cells. (A and B) CML cells were ^{125}I–surface-labeled, treated with (+) or without (−) GM-CSF or IL-3 (6 nmol/L) for 5 minutes at 4°C and immunoprecipitation was performed either with anti-GMRα (8G6), anti–IL-3Rα (9F5), or anti-β_c (4F3) 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

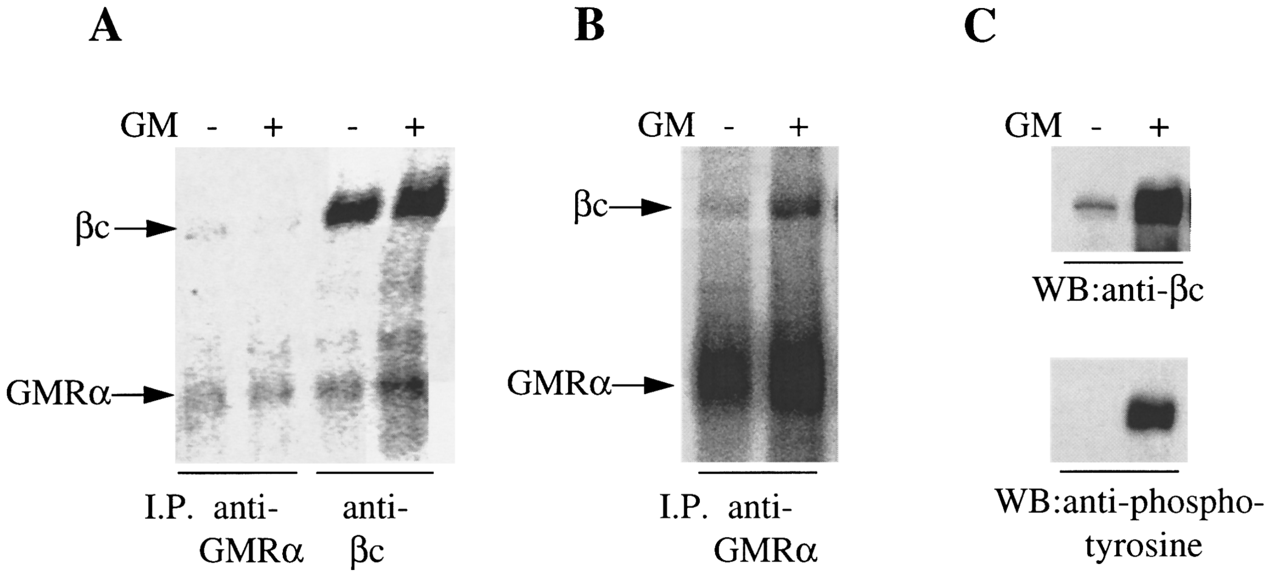

Fig 2. Coimmunoprecipitation of GMRα and β_c from Mo7e and hGMRα/hβ_c-expressing Ba/F-3 cells. (A) Mo7e cells were starved overnight and ^{125}I–surface-labeled and treated with (+) or without (−) GM-CSF (6 nmol/L) for 5 minutes and immunoprecipitation was performed either with anti-GMRα MoAb (8G6) or 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

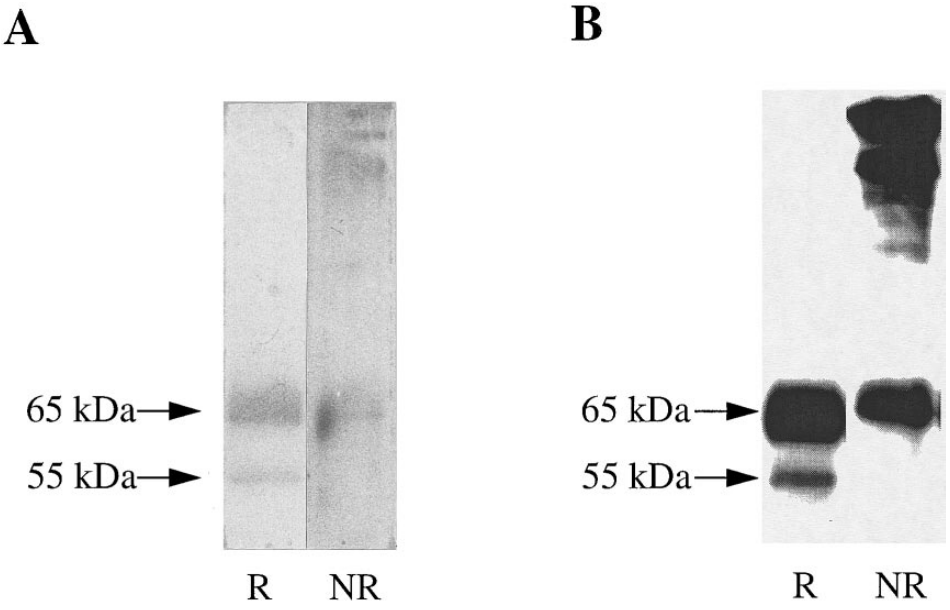

Fig 3. Soluble β_c protein was purified from conditioned medium from CHO transfectants. Soluble purified β_c protein was run under reducing (R) or nonreducing (NR) conditions on 10% SDS-PAGE and either (A) silver stained or (B) subjected to Western transfer and immunoblotted with anti-β_c antibody (1C1).

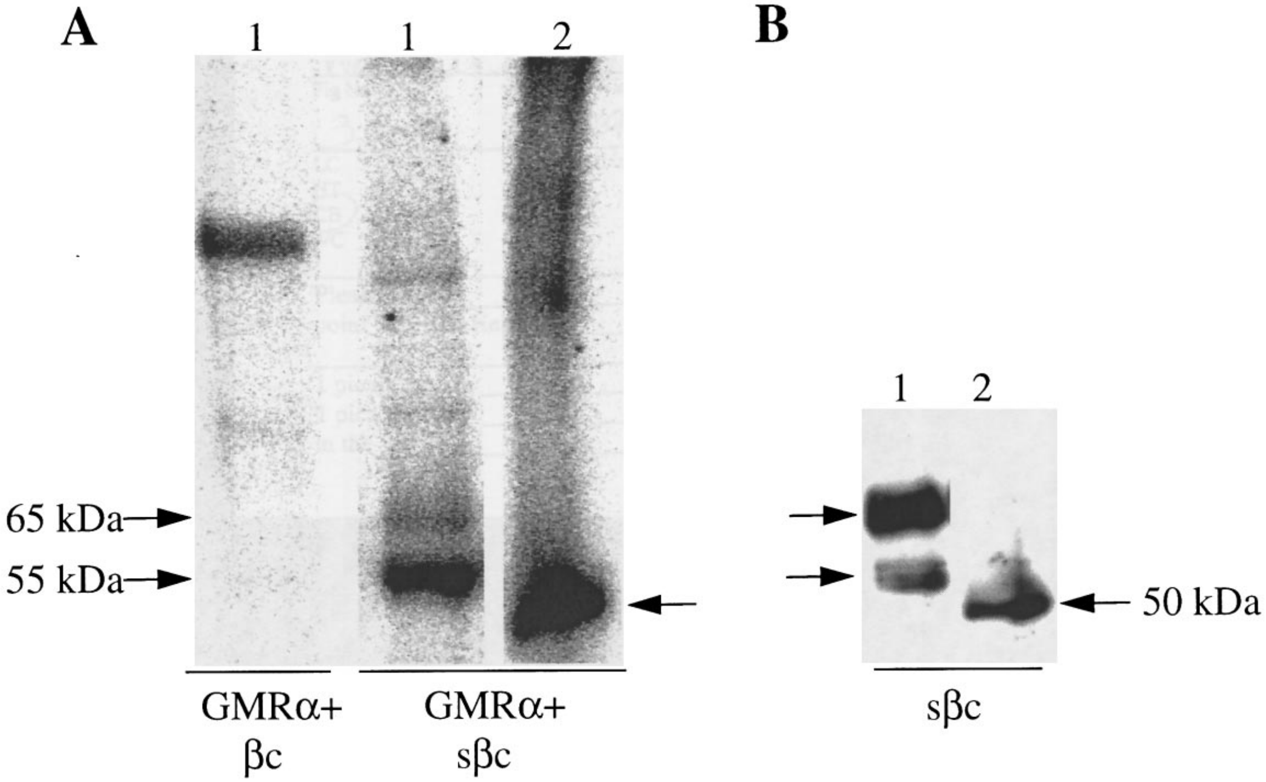

Fig 4. Soluble β_c is retained on the surface of GMRα-expressing CHO cells. (A) CHO cells expressing GMRα and either full-length β_c (β_c) or soluble β_c (sβ_c) were ^{125}I–surface-labeled and immunoprecipitation was performed with anti-β_c MoAb (8E4). The immunoprecipitated proteins were then either 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.

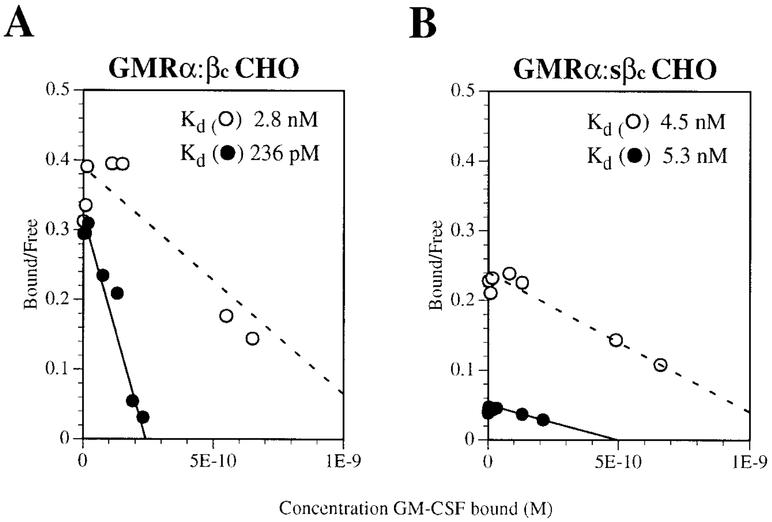

Fig 5. Scatchard transformation of saturation binding studies performed on CHO cells coexpressing (A) GMRα and full-length β_c or (B) GMRα and soluble β_c (sβ_c). Binding assays were performed with ^{125}I-labeled GM-CSF over a concentration range of 10 pmol/L to 10 nmol/L. After binding had reached 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.

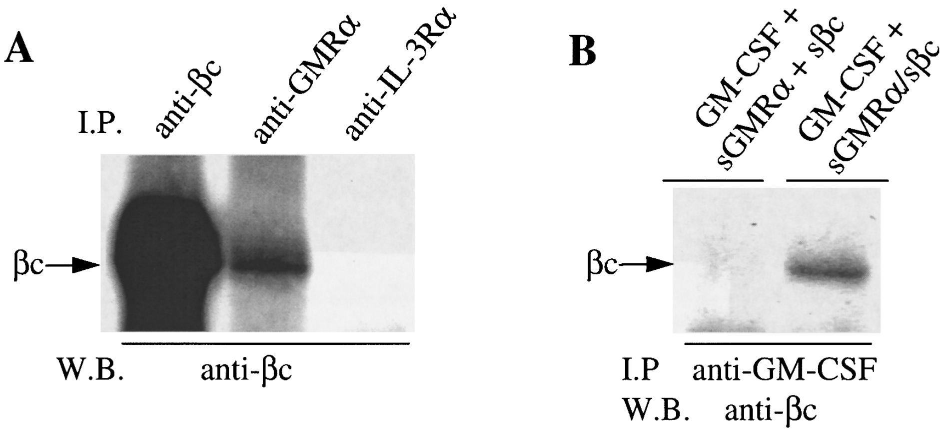

Fig 6. Soluble forms of GMRα and β_c spontaneously associate when coexpressed and bind GM-CSF. (A) Conditioned medium from CHO cells coexpressing soluble GMRα and soluble β_c was immunoprecipitated using either anti-β_c (4F3), anti-GMRα (8G6), or a control antibody (9F5); and the proteins were separated 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

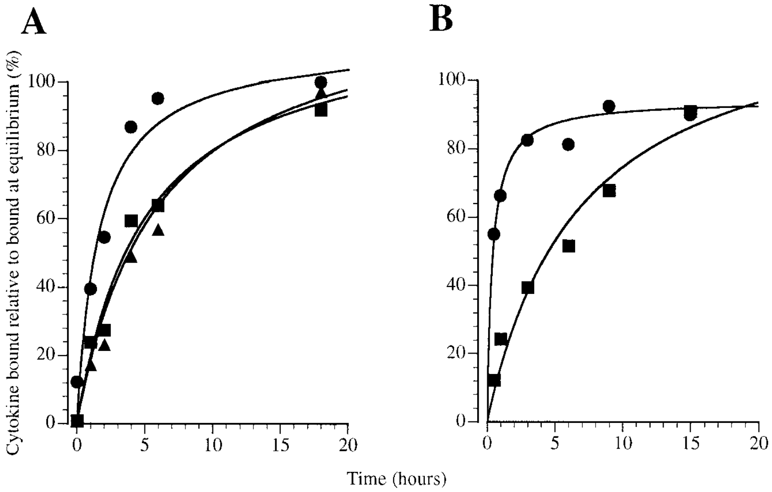

Fig 7. Association kinetics of ^{125}I-labeled cytokines binding to (A) eosinophils and (B and C) monocytes at 4°C with 200 pmol/L ^{125}I-CSF: (●) GM-CSF, (■) IL-3, and (▲) IL-5.

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-

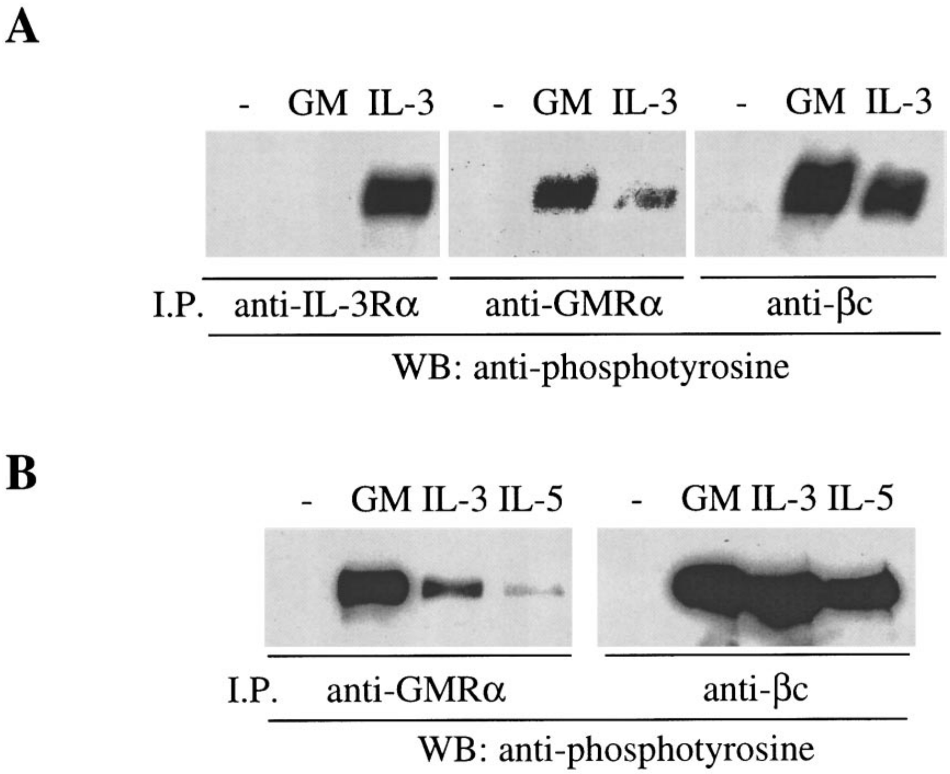

Fig 8. **β_c phosphorylated in response to GM-CSF, IL-3, or IL-5 is immunoprecipitable by anti-GMRα antibody. (A) Mo7e cells were starved overnight and then treated with either 6 nmol/L GM-CSF, IL-3, or medium for 5 minutes at 4°C and then immunoprecipitated with either anti−IL-3Rα (9F5), anti-GMRα (8G6), 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-

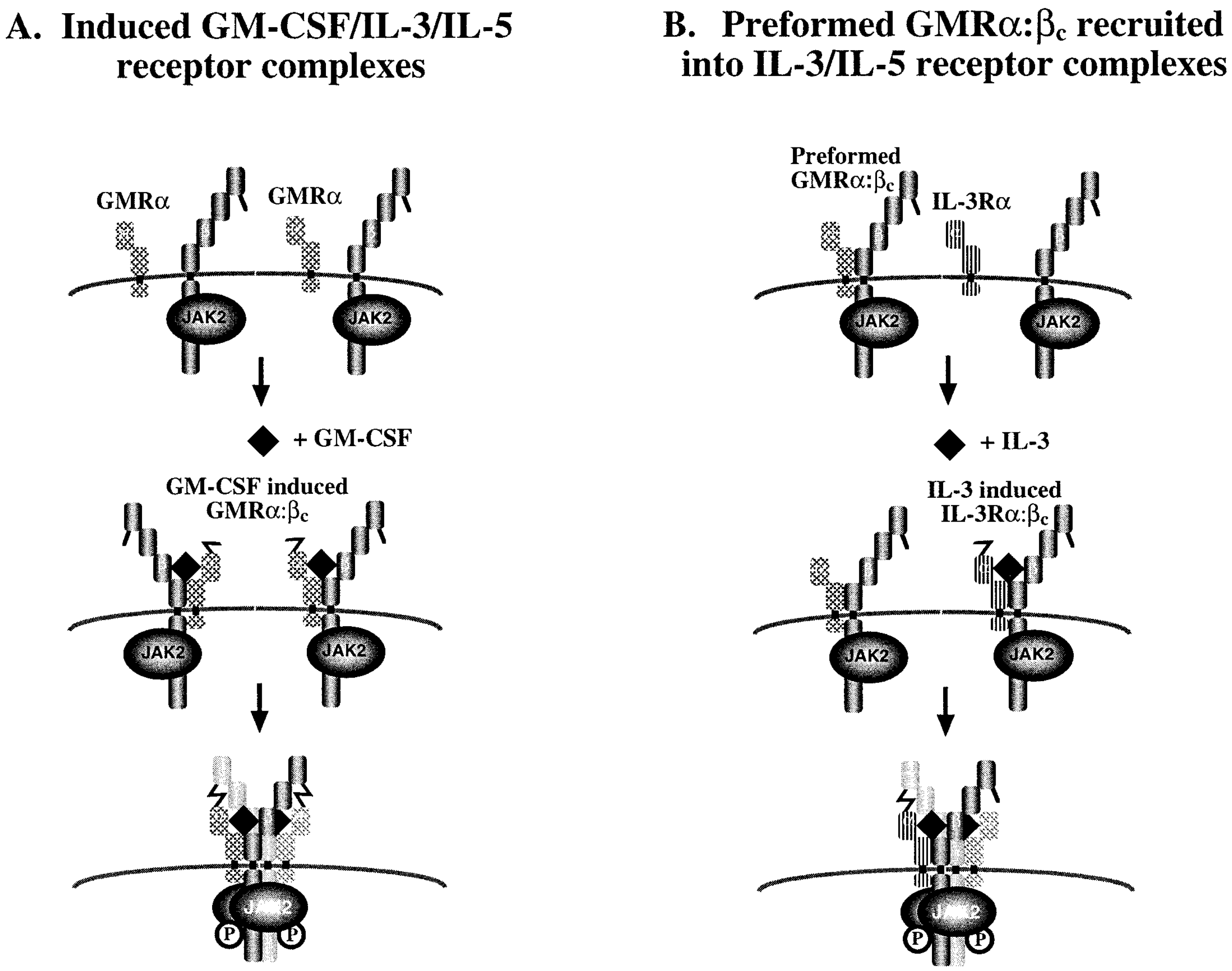

Fig 9. Proposed models for assembly of (A) GM-CSF–, IL-3–, and IL-5–induced receptor complexes and (B) preformed GM-CSF receptor complexes into activated receptors. In (A), GMRα, IL-3Rα, or IL-5Rα are in close proximity to (although not associated with) β_c on the cell surface. Ligand binding to the 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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