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) immunoprecipitated both GMRαs and βs 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 GMRαs and βs, as shown by the ability of soluble βs to associate with membrane-anchored GMRαs or soluble GMRαs. 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 βs. 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.

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Supported by grants from the National Health and Research Council of Australia. C.J.B. is a Rotary Peter Nelson Leukaemia Research Fellow of the Anti-Cancer Foundation of the Universities of South Australia.

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0006-4971/97/0008-0007$3.00/0
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.\textsuperscript{21}

Lastly, a recent report showed that a naturally occurring soluble form of GMR\textsubscript{a} is retained at the cell surface when coexpressed with \( \beta_1 \), although communoprecipitation of the two subunits could only be demonstrated in the presence of GM-CSF.\textsuperscript{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 \( \alpha \) and \( \beta_1 \), we found that both subunits could be communoprecipitated 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 \( \beta_1 \) associated with GMR\textsubscript{a}. A model is proposed in which IL-3 and IL-5 recruit the 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\textsuperscript{16} and cultured in RPMI supplemented with 10% fetal calf serum (FCS). Mo7e cells and Ba/F-3 cells expressing GMR\textsubscript{a} 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 started 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.\textsuperscript{21}

**Plasmid construction.** The cDNA for the human \( \beta_1 \) was cloned by polymerase chain reaction (PCR) from cDNA prepared from the KMT-2 cell line.\textsuperscript{21} A soluble form of the \( \beta_1 \) (s\( \beta_1 \)) was created by PCR using the following synthetic oligonucleotides: (1) 5’-TGA-ATTGGCCTGTTCAGCTGACGACCAGG-3’ that starts 25 nucleotides 5’ of the ATG and contains an engineered HindIII site and (2) 5’-ATACACTTATACTACGACTCGTGTCCTCAGGACCAGGCG-3’ that contains an infame 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 pRc/CMV to produce sGMR\textsubscript{a}pRc/CMV.

The PCr product obtained from this set of primers was subcloned into pRc/CMV to produce sGMR\textsubscript{a}pRc/CMV. To allow for dual stable transfection of two receptors, pRc/CMV was engineered such that the neomycin resistance gene (Neo\textsuperscript{0}) was replaced with the puromycin resistance gene (pac) from pRuf puro.\textsuperscript{24} Briefly, the 1.5-kb Kpn I-BamHI fragment from pRc/CMV containing Neo\textsuperscript{0} and its flanking SV40 early promoter and poly-adenylation region was subcloned into pUC19. The Neo\textsuperscript{0} gene was removed by EcoRV-Nae I digestion and pac introduced as a Sal I-Clu I fragment from pRuf puro. The puromycin resistance gene plus flasking SV40 early promoter and poly-adenylation region was excised from pUC19 as a Kpn I-BamHI fragment and subcloned into Kpn I-partial BamHI digested s\( \beta_1 \), pRc/CMV, resulting in s\( \beta_1 \), pRc/CMV puro. Subsequently, full-length \( \beta_1 \) cDNA was introduced in on an EcoRV-Xho I fragment thereby generating \( \beta_1 \), pRc/CMV puro.

**Construction of stable CHO cell lines.** The CHO cell lines, s\( \beta_1 \), CHO and sGMR\textsubscript{a} CHO, were developed as described previously for the GMR\textsubscript{a} CHO cell line, A9/C7.\textsuperscript{21} CHO lines expressing sGMR\textsubscript{a} or GMR\textsubscript{a} were subsequently cotransfected with either \( \beta_1 \) pRc/CMV puro or s\( \beta_1 \), pRc/CMV puro by the same method and selected in 2.5 \( \mu \)g/mL Puromycin (Calbiochem, La Jolla, CA). Cell surface expression of transfected receptors was confirmed by flow cytomotery as described previously\textsuperscript{16,26} 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.\textsuperscript{26} Monocytes were purified from the peripheral blood of normal donors obtained from the Adelaide Red Cross Transfusion Service as described previously.\textsuperscript{26}

**Antibodies.** MoAbs directed against GMR\textsubscript{a}, IL-3R, or \( \beta_1 \), were generated as previously described\textsuperscript{26} and purified and characterized as detailed elsewhere.\textsuperscript{16,26,27,28} The MoAbs 8E4 and 4F3 were selected for their ability to specifically immunoprecipitate \( \beta_1 \), 8G6 for GMR\textsubscript{a}, and 9F5 for IL-3R. The MoAb 1C1 an antipeptide polyclonal rabbit antibody (against residues 131-241 of \( \beta_1 \)) were used for immunoblotting \( \beta_1 \) and an MoAb 8D10 for immunoblotting GMR\textsubscript{a}. Phosphotyrosines 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 \( \beta_1 \), GMR\textsubscript{a}, and IL-3R, respectively. The anti-\( \beta_1 \) antibody, 3D7,\textsuperscript{26,28} was used for affinity purification of \( \beta_1 \) protein. The MoAbs were purified from ascites as described.\textsuperscript{27} A rabbit polyclonal anti-GM-CSF antibody was used for immunoprecipitating GM-CSF.\textsuperscript{21}

**Purification of recombinant soluble human \( \beta_1 \) receptor.** Soluble \( \beta_1 \) protein was purified from conditioned medium from CHO cells stably expressing the protein using a 3D7 anti-\( \beta_1 \) MoAb affinity column. Bound soluble \( \beta_1 \) was eluted with a linear gradient from 3 to 1 mol/L KCN 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).

**2D surface labeling and immunoprecipitation conditions.** Cells were cell-surface labeled with \( ^{125} \)I by the lactoperoxidase method as described previously.\textsuperscript{26,27} Approximately 10\textsuperscript{3} cells were labeled with 1 mL Cy5 (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 \( \mu \)g/mL leupeptin, 2 mmol/L phenylmethylsulphonyl fluoride, 10 \( \mu \)g/mL aprotonin, and 2 mmol/L sodium vandoate) for 30 minutes at 4°C followed by centrifugation of the lysate for 15 minutes at 4°C. After 1 hour of preclarification with protein A- sepharose (Pierce, Rockford, IL) at 4°C, the supernatant was incubated for 18 hours with 10 \( \mu \)g/mL antibody. Protein-A complexes
were captured by incubation for 1 hour with protein A-agarose 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-agarose 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.

**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 antimonospecific horseradish peroxidase (Dako, Carpenteria, CA) or goat antirabbit horseradish peroxidase (Dako). Immune-reactive 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 radioiodination of GM-CSF, IL-3, and IL-5.** Recombinant GM-CSF was produced in Escherichia coli as described previously. The recombinant GM-CSF was produced in Schwacha coli as described previously. The radioiodination of cytokines was performed by the iodine monochloride method 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 radioiodinated cytokines were purified before use as described previously.

**Saturation binding assays.** Binding assays were performed on CHO cells grown to confluence in 96-well plates over a concentration range of 10 pmol/L to 10 nmol/L. 125I-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 (Elsevier Biosoft, Cambridge, UK).

Binding assays were performed on soluble receptors in solution in a similar fashion to soluble receptor assays described previously. Aliquots of soluble receptor (100 µL) were incubated with 125I-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-agarose (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 radioiodinated cytokines at 200 pmol/L. Cells (2 to 4 × 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 radioiodinated cytokine by overlayering 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_{on}) was calculated using the KINETIC program (Elsevier Biosoft) from the specific binding data. K_{on} = \frac{K_{off} + K_{off} + K_{off} + K_{off} + K_{off} + K_{off}}{K_{off} + K_{off} + K_{off} + K_{off} + K_{off} + K_{off}}.

**RESULTS**

GMα and β, 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 β₁ from 125I-surface-labeled primary CML cells in the absence of exogenous stimuli. The size of this protein suggested it could be the GMαR. To examine this possibility, we conducted immunoprecipitation of 125I-surface-labeled CML cells either in the presence or absence of GM-CSF or IL-3 with anti-GMαR, anti-IL-3Rα, or anti-β₁ antibodies. Immune-precipitation of unstimulated cells with anti-GMαR antibody 8G6 immunoprecipitated a protein of 80,000 MW, consistent with the size of GMαR (Fig 1A). A second protein of 120,000 MW, corresponding in size to β₁, coimmunoprecipitated with GMαR in the absence of GM-CSF and its level did not increase with the addition of GM-CSF (Fig 1A). Reciprocally, immunoprecipitation with anti-β₁ antibody 4F3 immunoprecipitated both the 120,000 MW β₁ protein and the 80,000 MW GMαR protein in the presence or absence of GM-CSF (Fig 1A). Coimmunoprecipitation of GMαR with β₁, with either anti-GMαR or anti-β₁ 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.

In contrast to the coimmunoprecipitation seen with GMαR and β₁, coimmunoprecipitation of IL-3Rα and β₁ by either anti-IL-3Rα or anti-β₁ antibodies was only seen in the presence of IL-3 (Fig 1B), as shown previously. 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.
relative to GM-CSF receptor on these cells. As stated previously, a protein of 80,000 MW, consistent in size with GMRα, coimmunoprecipitated with βc in either the presence or absence of IL-3, although at much weaker intensity than either βc or IL-3Rα16 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-βc (4F3), and anti–IL-3Rα (9F5) antibodies. After Western transfer, an immunoblot with anti-βc antibody was performed. An 120,000 MW protein was clearly detected in the presence or absence of GM-CSF in both GMRα and βc immunoprecipitates but not in the IL-3Rα immunoprecipitate (Fig 1C). This indicates that βc 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 βc 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 βc, 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 βc maintained in GM-CSF were starved overnight before GM-CSF stimulation. Cells were surface labeled and proteins were immunoprecipitated with anti-GMRα (8G6) or anti-βc (8E4) before and after treatment with GM-CSF. We observed coimmunoprecipitation of the 120,000 MW βc protein and the 80,000 MW GMRα protein with either antibody in the presence or absence of GM-CSF, conﬁrming the 120,000 MW protein as human βc (Fig 2C). Reprobing the immunoblot with antiphosphotyrosine antibody PY20 showed that the βc was phosphorylated only after treatment of the Ba/F-3 cells with GM-CSF (Fig 2C), indicating that the preformed GMRα:βc complex is not
activated and that this complex was not the result of residual cytokine on the cells after overnight factor depletion. These findings strongly suggest that GMRα and βc are associated at the cell surface in the absence of GM-CSF as a preformed complex.

A soluble form of βc interacts with cell surface expressed GMRα. To determine whether the extracellular portions of GMRα and βc are sufficient for ligand-independent GMRα:βc interaction, we made a construct encoding a soluble form of βc (sβc) comprising the entire extracellular domain but lacking the transmembrane and cytoplasmic regions and examined its ability to associate with GMRα. Initial characterization of sβc was performed by transfection into CHO cells and affinity purification of conditioned medium on an anti-βc 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-βc antibody (1C1; Fig 3B), implying that they represent two forms of sβc protein. Intriguingly, when the eluted sβc 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-βc immunoblotting (Fig 3B), suggesting that the sβc 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,66 and may relate to the ability of βc to spontaneously form dimers, as previously noted.16,18,37

The association of sβc with GMRα was studied by transfecting the sβc construct into CHO cells expressing GMRα and monitoring sβc retention at the cell surface with anti-βc MoAb. Initial flow cytometric analysis showed specific binding of anti-βc MoAb on the surface of CHO cells coexpressing sβc and GMRα but not on CHO cells expressing sβc alone (data not shown). Importantly, the specific association of sβc 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 βc reactivity detected on the GMRα-expressing CHO cells was indeed sβc and not another protein with a common epitope or a fusion protein produced by an anomalous transfection event. To examine surface expressed βc specifically and avoid involvement of βc from intracellular compartments, CHO cells expressing either full-length or soluble βc with or without GMRα were surface labeled with 125I and βc protein immunoprecipitated using an anti-βc antibody (8E4). A single 125I-labeled protein of 120,000 MW was immunoprecipitated from CHO cells expressing full-length βc (Fig 4A). Two 125I-labeled proteins of 55,000 and 65,000 MW were immunoprecipitated from CHO cells expressing GMRα and sβc (Fig 4A) that corresponded in size to the sβc proteins detected in cell supernatants (Fig 3). No labeled protein was immunoprecipitated from CHO cells expressing sβc alone (data not shown), indicating that the
Fig 3. Soluble βc protein was purified from conditioned medium from CHO transfectants. Soluble purified βc 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-βc antibody (1C1).

Fig 4. Soluble βc is retained on the surface of GMRα-expressing CHO cells. (A) CHO cells expressing GMRα and either full-length βc (βc) or soluble βc (sβc) were 125I–surface-labeled and immunoprecipitation was performed with anti-βc 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 βc was immunoprecipitated from the medium of CHO cells coexpressing GMRα and soluble βc (sβc) 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-βc antibody 1C1.
sβc retained on the surface of GMRα-expressing cells does not represent sβc protein in the process of secretion but is specifically retained by GMRα.

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

To determine whether the GMRα:βc 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 sβc or full-length βc. Because of the very high level of GMRα chain expression on these transfectants (5 × 10^3 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 βc (Fig 5A) but not on those coexpressing sβc (kd 5.3 nmol/L; Fig 5B). This finding implies that the sβc 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 βc.

Soluble GMRα and βc can exist as a complex and bind GM-CSF. Based on our demonstration of a preformed complex between GMRα and βc on the cell surface and also the retention of sβc by cells expressing GMRα chain, we suspected that it may be possible to observe coassociation of a soluble form of GMRα and sβc 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 sβc 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, sβc protein was detected by immunoblot when immunoprecipitated not only with anti-βc (4F3) but also anti-GMRα antibody (8G6) but not with an irrelevant antibody (9F5) (Fig 6A). This suggests that some but not all sGMRα is associated with sβc in solution. Immunoprecipitation of a mixture of conditioned medium from cells expressing sGMRα and sβc separately did not result in coimmunoprecipitation of the two chains (data not shown). This is consistent with the retention of sβc 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:sβc complex is capable of binding ligand, conditioned medium from cells expressing sGMRα and sβc was incubated with GM-CSF and subsequently immunoprecipitation was performed with anti-GM-CSF antibody. Immunoblotting of the precipitated material showed that sβc 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 sβc with GM-CSF is dependent on its interaction with sGMRα in conditioned medium from cells coexpressing sGMRα and sβc.
Fig 6. Soluble forms of GMRα and βc spontaneously associate when coexpressed and bind GM-CSF. (A) Conditioned medium from CHO cells coexpressing soluble GMRα and soluble βc was immunoprecipitated using either anti-βc (4F3), anti-GMRα (8G6), or a control antibody (9F5); and the proteins were separated on 10% SDS-PAGE, Western transferred, and immunoblotted with anti-βc antibody 1C1. (B) Conditioned medium from CHO cells either coexpressing soluble GMRα and soluble βc (sGMRα/sβc) or a mixture of conditioned medium from CHO cells expressing the soluble proteins separately (sGMRα + sβc) 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-βc 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 125I-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 125I-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, and IL-5. From these studies it appears that the GM-CSF receptors exist 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. The rate of approach to equilibrium of IL-3 on monocytes is comparable to that seen for IL-3 and IL-5.
Table 1. Kinetic Parameters for 125I-CSF Interaction With Eosinophils

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<th>K&lt;sub&gt;on&lt;/sub&gt; (min&lt;sup&gt;−1&lt;/sup&gt;)</th>
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<tr>
<td>IL-5</td>
<td>0.007 ± 0.0024</td>
<td>90</td>
</tr>
<tr>
<td>GM-CSF&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.005 ± 0.002</td>
<td>160</td>
</tr>
</tbody>
</table>

Kinetic parameters determined as described in the Materials and Methods.

* Apparent association rate.
† Number of binding sites exhibiting K<sub>on</sub>.
‡ 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<sub>c</sub> can be phosphorylated in response to IL-3 and IL-5. The functional significance of the preformed GMRα:β<sub>c</sub> 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<sub>c</sub> becomes phosphorylated in response to ligand binding,<sup>10,40</sup> a process that requires the ligand-specific α chain. We have examined the phosphorylation of β<sub>c</sub> induced by cytokines in Mo7e and TF-1.8 cells and have found that phosphorylated β<sub>c</sub> can be detected by antiphosphotyrosine (PY20) immunoblot after treatment with GM-CSF and immunoprecipitation with either anti-β<sub>c</sub> (8E4) or anti-GMRα (8G6) antibody (Fig 8A and B). Similarly, treating Mo7e cells with IL-3 also resulted in β<sub>c</sub> phosphorylation that was immunoprecipitable by either anti-β<sub>c</sub> (8E4) or anti–IL-3Rα antibody (9F5) (Fig 8A). However, strikingly, we found that anti-GMRα antibody also immunoprecipitated phosphorylated β<sub>c</sub> 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<sub>c</sub> phosphorylated in response to IL-5 (Fig 8B). However, treatment of TF-1.8 cells with erythropoietin did not result in β<sub>c</sub> phosphorylation (data not shown), indicating that β<sub>c</sub> 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<sub>c</sub> complex. Thus, these findings raise the possibility that the preformed GMRα:β<sub>c</sub> 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<sub>c</sub> complex that is formed in the absence of GM-CSF. We have observed this ligand-independent association between GMRα and β<sub>c</sub> 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<sub>c</sub> complexes observed on cells varied from cell to cell. In some cases, all of the GMRα and β<sub>c</sub> chains were apparently co-associated and no further association was induced by GM-

![A](image1.png)

![B](image2.png)
CSF treatment, whereas on other cells only a component of GMRαs and βs 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 GMRαs and βs association and reconciles this observation with that of Ronco et al., 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 βs. This was interpreted as βs 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 βs, possibly reflecting the effect of a GMRα:βs preformed complex.

By using soluble receptor constructs, we were able to demonstrate the formation of sGMRα:βs 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 sβs to GMRα expressing cells nor combining separately expressed sGMRαs and βs, 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 sβs by cells expressing GMRα did not result in a detectable increase in affinity for GM-CSF, in contrast to full-length βs that confer 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 sβs is unable to confer full high-affinity binding on GMRα expressing cells. This deficiency in binding with sβs may be due to the βs 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 βs on BHK cells. The soluble GMRα conferred GM-CSF binding on the cells albeit with intermediate affinity, indicating some deficit in the interaction with βs. 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 βs 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, the A-B loop and the E strand in the fourth domain of βs 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 βs lead to factor-independent activation. 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 βs mutants and indeed in human leukemias.

In seeking to determine the functional significance of the preformed GMRα:βs 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, IL-3, and IL-5. This suggests that there are sufficient βs 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 βs 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 and induces their adhesion faster than IL-3. The presence of preformed GMRα:βs 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 and leukemia cells. The molecular basis of this phenomenon is the competition between GM-CSF:GMRα, IL-3:IL-3Rα, and IL-5:IL-5Rα for βs interaction. The proposed preformed GMRα:βs complex might be expected to have an effect on this phenomenon, sequestering βs for the exclusive binding of GM-CSF. However, cross-competition experiments performed previously on eosinophils and CML cells show that IL-3 is able to compete for 125I–GM-CSF binding effectively, with up to 90% competition. This suggests that the βs associated with GMRα in the preformed complex is in equilibrium with free βs and is therefore compatible 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 βs. Thus, cells that express excess βs and thus exhibit high-affinity binding sites only may have relatively more preformed sites compared with cells that express limiting amounts of βs.

The stoichiometry of the active GM-CSF receptor is not known and may involve a GMRα:βs ratio of 1:1 or a 2:2 complex. Because of the disulphide-linked GMRα:βs heterodimer and molecular modelling of the extracellular region of βs, we favor the second possibility. This is also consistent with the observations that at least two molecules of GMRα are required for receptor activation and that phosphorylation of βs dimers and disulphide-linked βs con-
The GM-CSF receptor is performed 3015

Fig 9. Proposed models for assembly of (A) GM-CSF-\(\alpha\)-, IL-3-\(\alpha\)-, and IL-5-\(\alpha\)-induced receptor complexes and (B) preformed GM-CSF receptor complexes into activated receptors. In (A), GMR\(\alpha\), IL-3R\(\alpha\), or IL-5R\(\alpha\) are in close proximity to (although not associated with) \(\beta_c\) on the cell surface. Ligand binding to the appropriate \(\alpha\) chain induces \(\alpha\beta_c\) heterodimerization and a conformational change in a chain that allows its disulphide linkage to \(\beta_c\). Modelling of \(\beta_c\) suggests that this bridging would only be possible if the unpaired cysteines in the \(\alpha\) chain of receptor 1 formed a disulphide bridging with cysteine of \(\beta_c\) in receptor 2. The bringing together of two \(\beta_c\) with their associated JAK-2 molecules would then lead to receptor activation. In (B), it is postulated that the binding of IL-3 or IL-5 to their specific \(\alpha\) chain and \(\beta_c\) triggers a conformational change in the \(\alpha\) subunit analogous to model (A). However, in this case, a disulphide bridge can be formed between the free cysteine in the IL-3R\(\alpha\) or IL-5R\(\alpha\) and a cysteine in \(\beta_c\) that is already noncovalently associated with GMR\(\alpha\) chain in a preformed complex. This interaction may be sufficient to bring together two \(\beta_c\) and two JAK-2 kinases leading to receptor activation. This model raises the possibility that some of the functions mediated by IL-3 and IL-5 are mediated inducibly through the activation of a preformed GMR\(\alpha\):\(\beta_c\) complex.

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. The transphosphorylation of GMR\(\alpha\)-associated \(\beta_c\) 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.

ACKNOWLEDGMENT

The authors acknowledge Mara Dottore for her excellent technical assistance and Dr Tom Gonda for critically reading the manuscript.

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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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