The Soluble Granulocyte-Macrophage Colony-Stimulating Factor Receptor's Carboxyl-Terminal Domain Mediates Retention of the Soluble Receptor on the Cell Surface through Interaction with the Granulocyte-Macrophage Colony-Stimulating Factor Receptor β-Subunit†

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ABSTRACT: The hematopoietic cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) mediates its activity through binding to cell-surface receptors. The high-affinity GM-CSF receptor (GMR) consists of two transmembrane-anchored subunits: a ligand-specific, low-affinity subunit (GMRα); and a signal-transducing β-subunit (GMRβ). The human GMRα subunit also exists in a soluble isoform (SOLα) which antagonizes GM-CSF activity in vitro. Previous studies by us have shown that coexpression of SOLα and a mutated GMRβ in BHK cells results in retention of SOLα on the cell surface and the formation of an intermediate affinity binding complex (K_d approximately 300 pM). This paper investigates the mechanism of the retention of SOLα on the cell surface. The data demonstrate that SOLα is anchored by a direct, ligand-independent interaction with GMRβ which also occurs when SOLα is coexpressed with wild-type GMRβ. However, SOLα and wild-type GMRβ form a complex which binds GM-CSF with high affinity (K_d = 39 pM), indistinguishable from the binding characteristics of the TMα/GMRβ complex. The experiments further reveal that the interaction between SOLα and GMRβ is abrogated by removal of the unique 16 amino acid carboxyl-terminal domain of SOLα. Specific mutation of cysteine 323 in this carboxyl-domain to alanine also eliminates the cell-surface retention of SOLα identifying this residue as being necessary for the formation of the SOLα/GMRβ complex.

GM-CSF[1] is a 22 kDa hematopoietic cytokine, produced by several cell types including activated T lymphocytes, endothelial cells, and fibroblasts (reviewed in *1*), that plays a role in growth, differentiation, and the functional activity of neutrophils, macrophages, and eosinophils (*2*−*8*). All GM-CSF effects are mediated by a highly specific cell-surface receptor (*9*, *10*) expressed in low numbers on responding cells. As currently understood, the functional high-affinity GM-CSF receptor (GMR) is composed of two different membrane-spanning subunits: a ligand-specific α-subunit (TMα) which binds GM-CSF with low affinity (K_d = 2−8 nM) (*11*); and a β-subunit (GMRβ) which is incapable of binding GM-CSF on its own but interacts with TMα in the presence of GM-CSF to form a high-affinity receptor complex (K_d = 50−100 pM) capable of signal transduction (*12*−*17*). Both receptor subunits are members of the cytokine receptor molecular superfamily characterized by conserved extracellular structures (*18*). In addition, the β-subunit is utilized by the IL-3 (*14*) and IL-5 (*15*) receptors, leading to its designation as β-common (βc) and possibly accounting for the overlap in cytokine function between GM-CSF, IL-3, and IL-5 (reviewed in *19*, *20*).

Soluble isoforms of many of the cytokine receptors have been identified (reviewed in *21*). Most of the soluble receptors represent variants of the ligand-specific, low-affinity α-subunits. Alternative mRNA splicing events give rise to the majority of the soluble receptors, but the soluble CNTF receptor and perhaps the soluble IL-6 receptor arise by cleavage of the cell-surface-associated forms. Several functions have been proposed for soluble cytokine receptors including protection or facilitation of their ligands, receptor down-regulation, or inhibition of ligand function. In addition, some soluble receptors such as solIL-2R and solIL-6R may represent biological markers of disease activity.

A soluble form of the GMRα (SOLα) has been identified by several groups (*22*−*25*). This soluble isoform arises by

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[1] Abbreviations: GM-CSF; granulocyte-macrophage colony-stimulating factor; GMR, GM-CSF receptor complex; GMRα, α-subunit of the GMR complex; TMα, transmembrane spanning isoform of GMRα; SOLα, soluble isoform of GMRα; GMRβ, β-subunit of the GMR complex; βc, wild-type isoform of GMRβ; βmut, GMRβ containing two mutations in the extracellular domain; IL-6R; interleukin-6 receptor; CNTFR, ciliary neurotrophic factor receptor; LIFR, leukemia inhibitory factor receptor.

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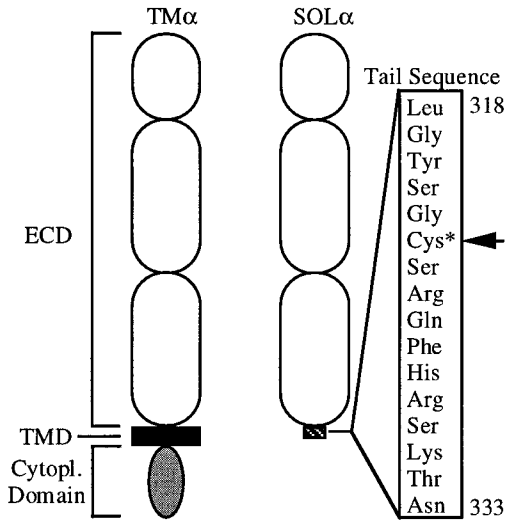

FIGURE 1: Comparison of the domain structure of TMα and SOLα. ECD = extracellular domain, TMD = transmembrane domain, Cytopl. = cytoplasmic. Numbers denote the position of the amino acid relative to the first residue of the mature polypeptide. The position of cysteine 323 within the C-terminal "tail" sequence of SOLα is indicated by an asterisk and arrow.

an alternative splicing event (*23*, *26*). Examination of the genomic structure of GMRα (*26*) reveals that exon 11 encodes only the transmembrane domain and is exactly 97 base pairs in length, the precise sequence deleted in the SOLα message which distinguishes it from the full-length TMα mRNA. The deletion causes a frame shift which, while maintaining the extracellular domain intact, encodes the removal of the transmembrane domain and the creation of a new stop codon within 51 base pairs. This eliminates the normal cytoplasmic domain and leaves the new soluble molecule with a unique and significantly shortened 16 amino acid "tail" containing a cysteine 11 amino acids from the C-terminal end (Figure 1). The RNA message for SOLα has been demonstrated in a variety of primary cells and tissues (*25*) as well as several human myeloid leukemic cell lines (*27*), and its expression appears to be regulated (*28*). Sasaki et al. (*29*) have identified SOLα in the supernatant of a human choriocarcinoma cell line, JEG-3, and recent work in our lab has shown the presence of the SOLα protein in supernatant conditioned by human leukemic cell lines, neutrophils, and hematopoietic progenitor cells and also in normal plasma (J. Prevost and C. B. Brown, unpublished experiments).

Recombinant SOLα is a 55−60 kDa glycoprotein which binds to GM-CSF in solution with low affinity (*30*), and although it appears to form homooligomers in solution, GM-CSF binding activity is restricted to the monomeric form (*31*). When added exogenously in vitro to GM-CSF-dependent assays of hematopoiesis, SOLα antagonizes the biological activity of GM-CSF (*25*, *30*).

In addition to its expected behavior as a secreted, soluble protein, previous studies by our group have also shown that coexpression of human SOLα and βmut, a human GMRβ containing two amino acid substitutions in extreme opposite ends of the extracellular domain (Glu9 → Ala, Thr420 → Ile), results in the retention of SOLα on the cell surface and the formation of an intermediate affinity GM-CSF binding complex (K_d approximately 300 pM) (*32*). Only two other soluble cytokine receptors are known to function in solution and also be retained on the cell surface, but this property has important consequences on the biological activity of their cognate ligands (*33*, *34*) emphasizing the potential significance of the cell surface anchoring of SOLα.

The purpose of the experiments reported in this paper was to elucidate the mechanism by which SOLα was anchored on the cell surface. First, we wanted to demonstrate that the retention of SOLα on the cell surface was mediated by a direct interaction of SOLα with GMRβ and furthermore was not dependent on the mutations in βmut. Second, we wanted to test the hypothesis that the unique 16 amino acid carboxy-terminal "tail" of SOLα was responsible for the formation of the SOLα/GMRβ complex.

MATERIALS AND METHODS

Receptor Subunit Cloning and Expression. The cloning of receptor subunits and the establishment of stable BHK cell lines have been previously described (*32*). The human βc cDNA was a kind gift of Dr. M. Eder, Frankfurt, Germany. The ECDα clone was generated from the human SOLα by PCR-based mutagenesis which introduced a stop codon immediately after the coding sequence for amino acid 317 at the putative junction of the extracellular domain and transmembrane domain. The mutated cDNA was cloned into the TA vector (Stratagene, Inc). The desired alteration was confirmed by full sequencing of the cDNA which was subsequently subcloned into the mammalian expression vector pRc/CMV (Invitrogen, Inc). The SOLαC323A clone was generated from the human SOLα by PCR-based site-directed mutagenesis and cloned into the pNUT vector. Transfections of ECDα and SOLαC323A into BHK cells were performed using calcium phosphate precipitation (*35*). Clonal cell lines were established by selection in G418 and/or methotrexate according to the properties of the expression vectors.

^{125}I-GM-CSF Receptor Binding Assays. Cell-surface-associated receptor binding assays, soluble receptor binding assays, and Scatchard and Dixon analyses of binding kinetics were performed as previously described (*30*, *32*).

Cell Culture Supernatant Blots. Cell culture supernatants from BHK cells expressing SOLα, ECDα, or SOLαC323A were generated by culturing cells in Opti-MEM I medium (Gibco BRL, Life Technologies, Inc., Mississauga, Canada) in the absence of serum for 48 h. Supernatants were centrifuged at 1500*g* for 10 min at 4 °C to remove intact cells and cellular debris and concentrated approximately 15-fold using an Ultrafree-15 centrifugal filter device (30 000 dalton molecular mass cutoff; Millipore, Mississauga, Canada) according to the manufacturer's instructions. Concentrated supernatants were mixed with equal amounts of 2 × reducing buffer (62.5 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.025% bromphenol blue) and boiled for 10 min or with 0.1 volume of nonreducing buffer (1 M Tris, pH 6.9, 2% SDS, 0.2% bromophenol blue, 10% sucrose). Samples were electrophoresed on 8% polyacrylamide−SDS gels, transferred to a PVDF membrane, and probed as described with the immunoprecipitation procedure.

Flow Cytometry. Flow cytometry was performed as previously described (*32*). Mouse anti-human GMRα antibody CDw116 17-A was purchased from PharMingen (Mississauga, Ontario, Canada) and inhibits GM-CSF binding.

Mouse anti-human GMRα antibody 8G6 and mouse anti-human GMRβ antibody 1C1 were a gift from Dr. Angel Lopez (Hanson Centre for Cancer Research, Adelaide, Australia).

Immunoprecipitation. Cells were washed in 1 × 5 mL of cold phosphate buffered saline (PBS, without Ca^{2+} or Mg^{2+}) and lysed on tissue culture plates in 500 μL of NP-40 lysis buffer [50 mM Tris, pH 8.0, 10% glycerol, 1% Nonidet P-40, 137 mM NaCl, 10 μg/mL leupeptin, 100 KIU/mL Trasylol (aprotinin), 1 mM PMSF, 1 mM NaF, 2 mM Na_3VO_4] on ice for 10 min. Tissue culture plates were scraped with a cell scraper, and cell lysates were precleared with washed protein A−Sepharose (4 Fast Flow; Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) for 30 min at 4 °C. Insoluble material and protein A−Sepharose were removed by centrifugation at 12000*g* for 10 min at 4 °C. Cleared cell lysates were incubated with 1 μg of primary antibody (mouse anti-human GMRα antibodies 8G6 for TMα expressing cell lines, CDw116 17-A for SOLα or SOLαC323A expressing cell lines, or mouse anti-human GMRβ antibody 1C1) for 2 h at 4 °C. Protein A−Sepharose washed in NP-40 lysis buffer was added, and the mixture was incubated for an additional 1 h. Immune complexes were washed 3 times with cold lysis buffer and boiled for 10 min in 2 × reducing buffer (125 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 2% 2-mercaptoethanol, 0.02% bromophenol blue). Samples were centrifuged to pellet the Sepharose, and the supernatants were electrophoresed on 8% polyacrylamide−SDS gels using a mini-Protean II electrophoresis system (Bio-Rad Laboratories, Ltd., Mississauga, Canada) and then transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore) using a mini trans-blot electrophoretic transfer cell for 1 h at 200 mA at 4 °C. The protein blots were air-dried, rewet in methanol, and blocked overnight at 4 °C by incubation in Tris-buffered saline (TBS: 20 mM Tris, pH 7.6, 137 mM NaCl) containing 2% bovine serum albumin. Blots were washed extensively in TBS containing 0.2% Tween 20 and incubated for 2 h at room temperature in primary antibody (equal mix of anti-GMRα antibodies 8G6 and CDw116 17-A or anti-GMRβ antibody 1C1) diluted in TBS−Tween 20. Blots were washed and incubated for 1 h at room temperature in rabbit anti-mouse IgG peroxidase-conjugated secondary antibody (Bio/Can Scientific, Mississauga, Canada) diluted to 1/25 000 in TBS−Tween 20. Blots were washed extensively in TBS−Tween 20 and were visualized using enhanced chemiluminescence detection reagents (ECL, Amersham Life Science, Oakville, Ontario, Canada) and exposure to X-ray film. For samples stimulated with GM-CSF, 30 μL of a 100 μg/mL solution of GM-CSF was added to cells in 10 mL of media on a tissue culture plate for 10 min at 37 °C prior to the initial wash with PBS.

Subcellular Fractionations. Cells were washed twice in cold PBS and removed from the tissue culture plates using Puck's solution (5.4 mM KCl, 140 mM NaCl, 4.2 mM $NaHCO_3$, 5 mM dextrose, 10 mM Hepes, 1 mM EDTA). Cells were then suspended in hypotonic lysis buffer [10 mM Tris, pH 8.0, 10 mM KCl, 1 mM EDTA, 0.1% 2-mercaptoethanol, 10 μg/mL leupeptin, 100 KIU/mL Trasylol (aprotinin), 1 mM PMSF, 1 mM NaF, 2 mM Na_3VO_4] and were allowed to swell for 20 min on ice. Cells were lysed with 30 strokes in a Dounce homogenizer. Nuclei and any remaining intact cells were removed by low-speed centrifugation (10 min at 1000*g*). Membranes were isolated from the cleared lysate by high-speed centrifugation (50,000*g*) for 1 h at 4 °C. The supernatants (cytosol) were removed, membrane pellets were resuspended in NP-40 lysis buffer, and immunoprecipitations were carried out as described above.

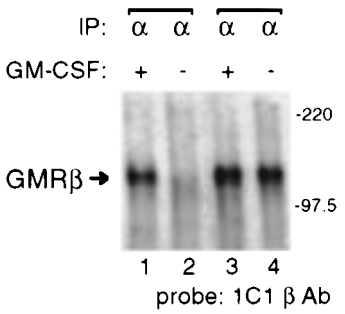

FIGURE 2: Coimmunoprecipitation of GMRβ with GMRα. BHK cells engineered to permanently coexpress GM-CSF receptor subunits were incubated with (+) or without (−) GM-CSF. Proteins were subsequently immunoprecipitated from whole cell lysates with anti-GMRα monoclonal antibody 8G6. Bands were visualized by probing with anti-GMRβ monoclonal antibody 1C1. Shown is a representative blot of repeated experiments ($n = 3$). Lanes 1, 2 = TMα/βmut; lanes 3, 4 = SOLα/βmut. The position of the molecular mass standards in kDa is indicated.

RESULTS

SOLα Is Retained on the Cell Surface by a Direct Ligand-Independent Interaction with GMRβ. To test whether SOLα and GMRβ are physically associated on the cell surface, immunoprecipitations using anti-GMRα antibody 8G6 were done in the absence or presence of GM-CSF, seeking evidence for the coprecipitation of GMRβ. In keeping with previous work (*36*) suggesting that the preligand association of TMα and GMRβ is minimal but is subsequently stabilized at least 1000-fold by the addition of GM-CSF, GMRβ could only be coimmunoprecipitated from TMα/βmut-coexpressing cell lines with an anti-GMRα antibody in the presence of GM-CSF (Figure 2, lanes 1 and 2). In contrast, GM-CSF stimulation was not necessary for the coimmunoprecipitation of GMRβ from the SOLα/βmut-coexpressing cells nor did the presence of GM-CSF augment the precipitation of GMRβ (Figure 2, lanes *3*, *4*).

SOLα Forms a Cell-Surface Complex with Wild-Type GMRβ. βmut has been found to have two amino acid substitutions in the extracellular domain (Glu9 → Ala, Thr420 → Ile). To assess whether these mutations were responsible for the retention of the SOLα on the cell surface, SOLα and wild-type GMRβ (βc) cDNA constructs were transfected into BHK cells. Flow cytometric analysis demonstrated that SOLα was also retained on the cell surface when coexpressed with βc (data not shown), and all subsequent experiments were therefore undertaken utilizing βc.

Our previous work had shown that SOLα/βmut form a binding complex with an affinity for GM-CSF of approximately 300 pM (*32*), intermediate between that of TMα/βmut or TMα/βc (50−100 pM) (*12*, *32*) and that of TMα alone on the cell surface (2−8 nM) (*11*) or SOLα in solution (approximately 2 nM) (*30*). In the current studies, receptor binding assays were again performed to compare the affinities of TMα/βc, SOLα/βc, and SOLα/βmut. The results of hot saturation experiments were analyzed using the LIGAND

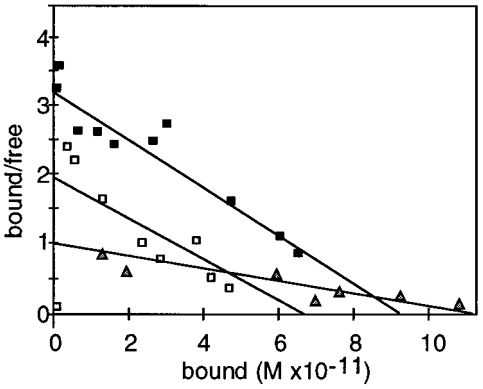

FIGURE 3: Comparison of the ligand binding affinities of cell-surface GM-CSF receptor complexes. Shown are Scatchard analyses of representative hot saturation binding experiments of BHK cells coexpressing TMα/βc (closed squares), SOLα/βc (open squares), or SOLα/βmut (triangles).

software program (RADLIG v4.0, Biosoft Inc., Cambridge, U.K.), and representative Scatchard plots are shown in Figure 3. The TMα/βc cells exhibited a single class of high-affinity binding sites (K_d = 59 ± 43 pM, n = 2) similar to our previously reported value for TMα/βmut (K_d = 64 ± 9 pM, n = 2) (*32*). However, the SOLα/βc cells also displayed high-affinity binding (K_d = 38 ± 14 pM, n = 2). This represents an approximately 10-fold higher ligand affinity than that observed for SOLα/βmut (K_d = 331 ± 56 pM, n = 4) (*32*).

In an effort to understand the basis for the affinity difference between the SOLα/βc and SOLα/βmut complexes, the two cell lines were evaluated by flow cytometry using two anti-GMRα antibodies, 8G6 and CDw116 17-A (Figure 4). Antibody CDw116 17A has been characterized by Nicola et al. (*36*) and inhibits GM-CSF binding. Both antibodies recognize TMα when expressed alone or with βc or βmut. However, only antibody CDw116 17A was found capable of detecting SOLα on both SOLα/GMRβ cell lines. Antibody 8G6 was not capable of detecting cell-surface SOLα on the βc-expressing cells unless GM-CSF was also present.

Role of the Carboxyl-Terminal Domain in the Function of SOLα. The splicing event which gives rise to the soluble isoform of GMRα replaces the transmembrane and cytoplasmic domains of TMα with a unique 16-amino acid sequence (Figure 1). To test the hypothesis that residues in this carboxyl-terminal "tail" were responsible for the ability of SOLα to interact with GMRβ and to be retained on the cell surface, we removed this 16-amino acid domain by PCR-based site-directed mutagenesis to create a GMRα mutant which consists of only the extracellular domain (ECDα). Whether transfected alone or with βc, the ECDα cDNA directs the expression and secretion of a soluble GM-CSF binding protein which, as shown in Figure 5A,B, has a solution phase affinity for GM-CSF (K_d = 3.3 ± 1.2 nM, n = 3) which is virtually identical to wild-type SOLα (K_d = 3.6 ± 1.8 nM, n = 3). However, despite the expression of large amounts of βc, receptor binding assays and flow cytometric analysis of the ECDα/βc-coexpressing cells could detect no evidence of cell-surface retention of ECDα (data not shown), supporting our hypothesis that the carboxyl-terminal tail is responsible for the interaction between SOLα and GMRβ.

Cys323 in the Tail of SOLα Is Necessary for the Interaction of SOLα with GMRβ. Inspection of the sequence of the 16 amino acid tail of SOLα reveals a cysteine at position 323, 11 amino acids from the carboxyl terminus (Figure 1). With evidence that the carboxyl-terminal tail of SOLα was responsible for the interaction between SOLα and GMRβ, this cysteine residue within the tail domain seemed a likely candidate for specific mediation of the intermolecular interaction. To test the contribution of Cys323 to the formation of the SOLα/GMRβ cell-surface complex, PCR-based site-directed mutagenesis was utilized to change the cysteine to alanine, creating SOLαC323A. This construct was introduced into BHK cells, alone and in combination with βc. Using soluble receptor binding assays, it was determined that these cell lines expressed a GM-CSF binding protein in the cell culture supernatant whether transfected alone or with βc. However, analysis of the binding data (Figure 5C) indicated that the mutation of Cys323 to Ala resulted in a 10-fold decrease in the affinity of SOLαC323A (K_d = 43 ± 7 nM, n = 3) compared to SOLα or ECDα.

The SOLαC323A/βc cell lines were then tested for the retention of SOLαC323A on the cell surface. The expression of βc was confirmed by flow cytometry (data not shown), and receptor binding assays were performed (Figure 6A). Cells expressing SOLα or SOLαC323A by themselves showed no evidence of GM-CSF binding while the SOLα/βc line was able to bind GM-CSF. However, SOLαC323A/βc showed no evidence of GM-CSF binding despite expressing equivalent amounts of soluble receptor (Figure 6B).

To examine whether SOLαC323A is retained on the cell surface by interaction with βc but does not form a functional ligand binding complex, we analyzed the SOLαC323A/βc cells by flow cytometry using anti-GMRα antibody CDw116 17A (Figure 6C). While SOLα/βc cells were positive for GMRα, the SOLαC323A/βc cells showed no reactivity with the anti-GMRα antibody. This is not merely because of an inability of antibody CDw116 17A to recognize SOLαC323A since, in receptor binding assays, we showed that CDw116 17A equally blocks the interaction of SOLα and SOLαC323A with GM-CSF (data not shown).

Finally, we wondered whether SOLαC323A was able to interact with βc but the Cys323Ala mutation precluded transport to and expression on the cell surface. To investigate this possibility, whole cell and membrane preparations were collected from the appropriate cell lines, and immunoprecipitations were performed using anti-GMRα monoclonal antibodies CDw116 17A and 8G6. Precipitated products were then probed for the presence of βc using anti-GMRβ monoclonal antibody 1C1 in an effort to determine if SOLαC323A and βc were still able to interact but were sequestered in the cytoplasm. Figure 7 demonstrates that, while βc could easily be coimmunoprecipitated with SOLα both from whole cell and from membrane preparations (panel A, lanes 7, 8), and despite ample intracellular presence of SOLαC323A (panel B, lane 6) and βc (panel C, lane 3) in the SOLαC323A/βc cell line, no evidence was found for the coassociation of SOLαC323A and βc in either whole cell or membrane preparations (panel A, lanes 9, 10). This further suggests that the Cys323Ala mutation abrogates the formation of the SOLα/βc complex and does not merely interfere with the function or presentation of the complex on the cell surface.

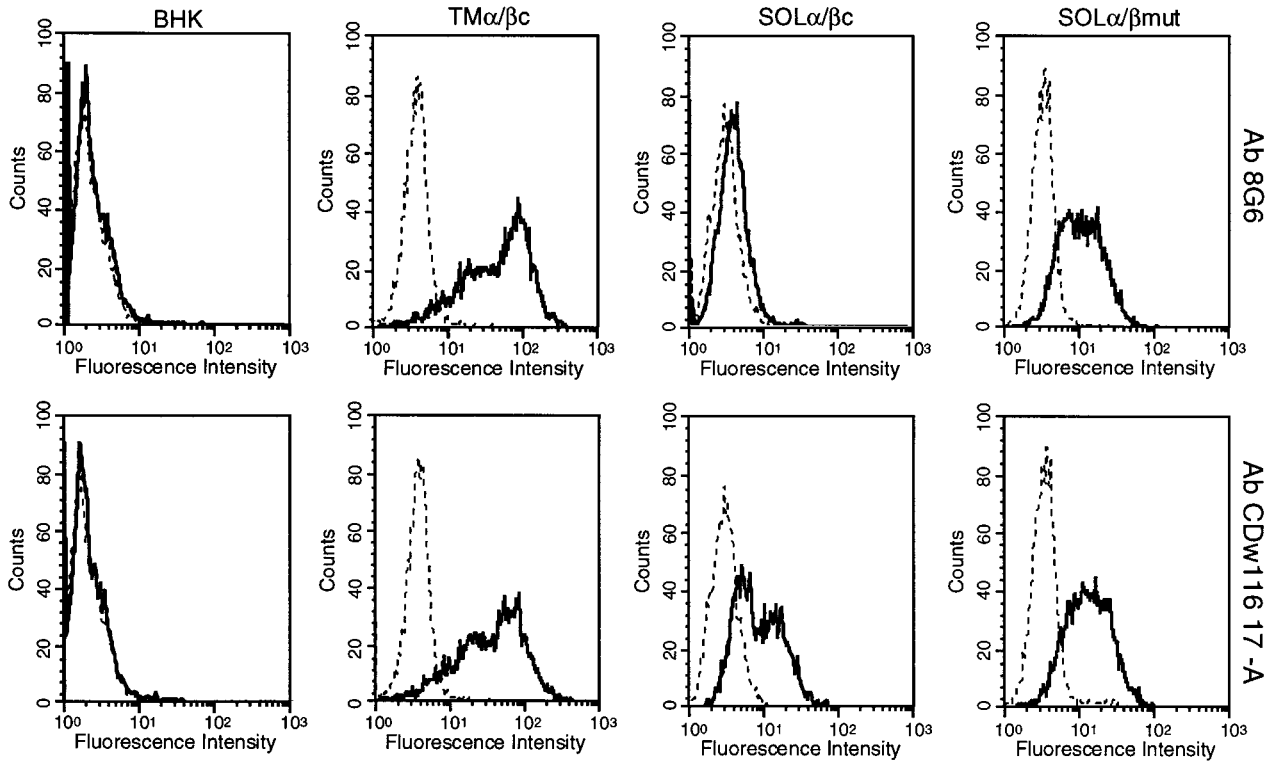

FIGURE 4: Comparison of the binding profiles of two anti-GMRα monoclonal antibodies to GMRα constructs. Untransfected BHK cells or BHK cells coexpressing GM-CSF receptor subunits as indicated were flow cytometrically analyzed for their ability to bind anti-GMRα antibodies 8G6 (upper panels) or CDw116 17-A (lower panels) using a fluorescein isothiocyanate-labeled goat anti-mouse Ig secondary antibody. Broken lines = secondary antibody alone; solid lines = primary + secondary antibodies. Results are representative of repeated experiments ($n = 3$).

DISCUSSION

Soluble receptor variants of the ligand-specific subunits exist for virtually all of the cytokine receptors. However, only three of these soluble isoforms, the soluble IL-6R, the soluble CNTFR, and the soluble GM-CSF receptor, have been demonstrated to also be able to anchor themselves on the cell surface (*32*−*34*). In earlier work, we reported that SOLα can be tethered on the cell surface when it is coexpressed with GMRβ and established that the cell-surface anchoring was a ligand independent event (*32*). In this paper, we have investigated the mechanism by which SOLα is retained on the cell surface. Our data suggest that SOLα is anchored through a direct physical interaction with GMRβ. This interaction is not dependent upon previously documented mutations in the extracellular domain of GMRβ as the SOLα/GMRβ complex is also formed with the wild-type GMRβ. The current experiments confirm that the interaction is ligand-independent and further demonstrate that the tethering of SOLα to GMRβ is mediated by the unique carboxyl-terminal tail of SOLα which is created by the mRNA splicing event giving rise to the SOLα message from the full-length TMα message. Specifically, Cys323, within the carboxyl tail, is required for the ability of SOLα to interact with GMRβ.

The experiments demonstrate that the interaction is not dependent on mutations in the extracellular domain of GMRβ but occurs equally with the wild-type GMRβ. Of note, however, is the significant difference in ligand affinity between SOLα/βmut ($K_d = 331 \pm 56$ pM) and SOLα/βc ($K_d = 38 \pm 14$ pM). The high affinity of the SOLα/βc complex is indistinguishable from that of TMα/βc ($K_d = 59 \pm 43$ pM) and suggests that it can no longer be taken for granted that the identification of a high-affinity GM-CSF binding complex on the surface of a cell is synonymous with the exclusive presence of TMα and βc.

The finding of a 10-fold difference in affinities between SOLα/βmut and SOLα/βc was surprising since we had already demonstrated that TMα/βmut forms a high-affinity complex ($K_d = 64 \pm 9$ pM) identical to that of TMα/βc ($K_d = 59 \pm 43$ pM). The difference in affinities would suggest a fundamental difference in how SOLα and TMα interact with GMRβ. These functional clues are corroborated by our immunological data. In Figure 4, we show that two different anti-GMRα monoclonal antibodies, CDw116 17A and 8G6, recognize GMRα on the surface of the TMα/βc cell lines but only CDw116 17A recognizes SOLα/βc. In addition, in an attempt to strengthen our coimmunoprecipitation data showing a strong, ligand-independent interaction between SOLα and βc (Figures 2 and 7), we undertook reciprocal cross-immunoprecipitations using our anti-GMRβ antibody as the precipitating agent. However, in our attempts at such experiments, we were unable to successfully coimmunoprecipitate SOLα using the anti-GMRβ antibody. This occurred despite our clear ability to immunoprecipitate GMRβ with this same antibody whether GMRβ was expressed alone or when coexpressed with SOLα or TMα and despite the fact that the inclusion of GM-CSF in the experiments performed with the TMα/GMRβ cells did not preclude the immunoprecipitation of GMRβ and facilitated the coimmunoprecipitation of TMα (data not shown). Taken together, the

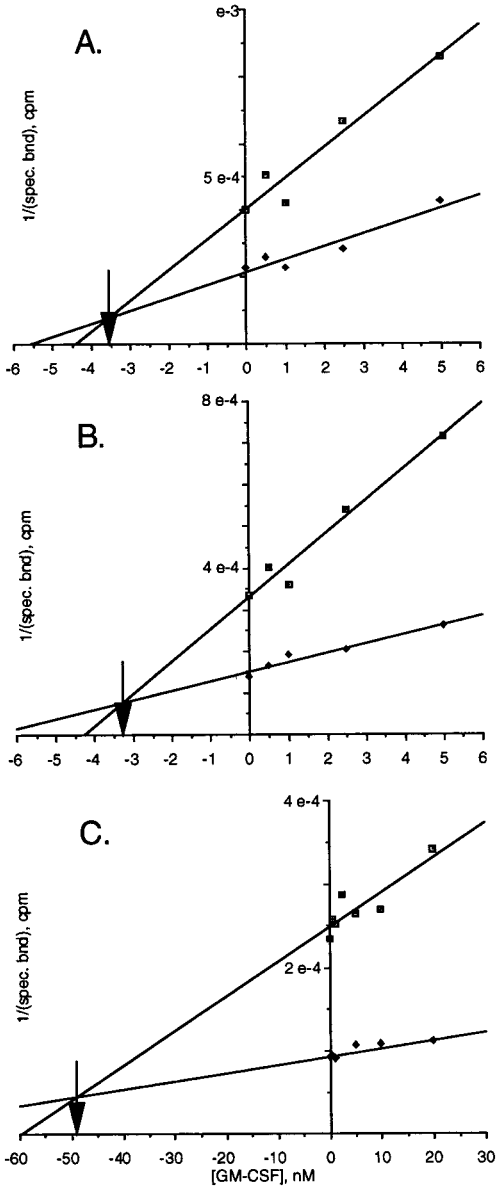

FIGURE 5: Solution phase ligand binding characteristics of soluble GMRα constructs. Concentrated cell culture supernatant conditioned by BHK cells transfected with either wild-type SOLα, ECDα, or SOLαC323A was utilized in cold saturation solution phase receptor binding assays. Shown are Dixon analyses of representative experiments for (A) SOLα, K_d = 3.6 nM; (B) ECDα, K_d = 3.3 nM; and (C) SOLαC323A, K_d = 49 nM. Box data points indicate 125 pM ^{125}I-GM-CSF, and diamond data points indicate 300 pM ^{125}I-GM-CSF. Arrowheads indicate the points of intersection extrapolated to the *x* axis (K_d).

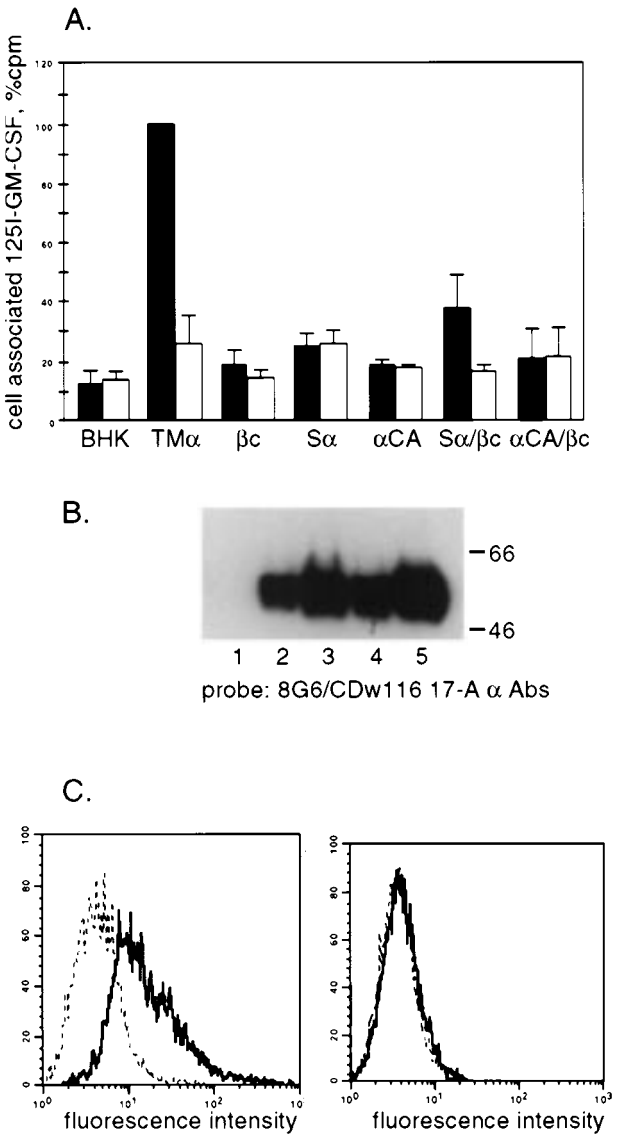

FIGURE 6: Cell-surface expression of GMRα. (A) A minimum of two independent clones of BHK cells transfected with the various GM-CSF receptor constructs were tested for the ability to bind GM-CSF using a cell-surface receptor binding assay. Cells were examined for ^{125}I-GM-CSF binding in the absence (solid bars) or presence (open bars) of a >50-fold excess of unlabeled GM-CSF (n = 5). All results are compared to a single clone of BHK cells expressing TMα alone. Results represent means ± SD. Sα = SOLα, αCA = SOLαC323A. (B) Equal volumes of supernatant conditioned by each of the cell lines were size-fractionated by polyacrylamide gel electropheresis and subjected to Western analysis using anti-GMRα antibodies. Lane 1 = untransfected BHK cells, lane 2 = SOLα, lane 3 = SOLαC323A, lane 4 = SOLα/βc, lane 5 = SOLαC323A/βc. (C) Evidence for the cell-surface expression of SOLαC323A in the presence or absence of GMRβ was sought by flow cytometry using anti-GMRα monoclonal antibody CDw116 17-A and a fluorescein isothiocyanate-labeled goat anti-mouse Ig secondary antibody. Left panel = SOLα/βc, right panel = SOLαC323A/βc. Broken lines = secondary antibody alone, solid lines = primary + secondary antibodies.

data suggest that the 8G6 epitope on GMRα and the 1C1 epitope on GMRβ are exposed when TMα and βc interact but are buried when SOLα and βc interact.

The ability of SOLα to be tethered to GMRβ in a ligand-independent manner is clearly a property unique to SOLα and is not dependent on domains common between SOLα and TMα. The splicing event giving rise to SOLα endows the soluble variant with a unique 16 amino acid carboxyl-terminal tail (Figure 1), and our experiments demonstrate that removal of the tail, while having no effect on the affinity of SOLα for GM-CSF in solution (Figure 5), eliminates the retention of SOLα on the cell surface. The specific mutation of the single cysteine in the tail domain to alanine also eliminates the interaction between SOLα and GMRβ, identifying this particular residue, Cys323, as necessary for the ability of SOLα and GMRβ to physically associate when coexpressed. The experiments in this paper took precaution to demonstrate that the Cys to Ala mutation specifically affected the interaction between SOLα and βc and did not

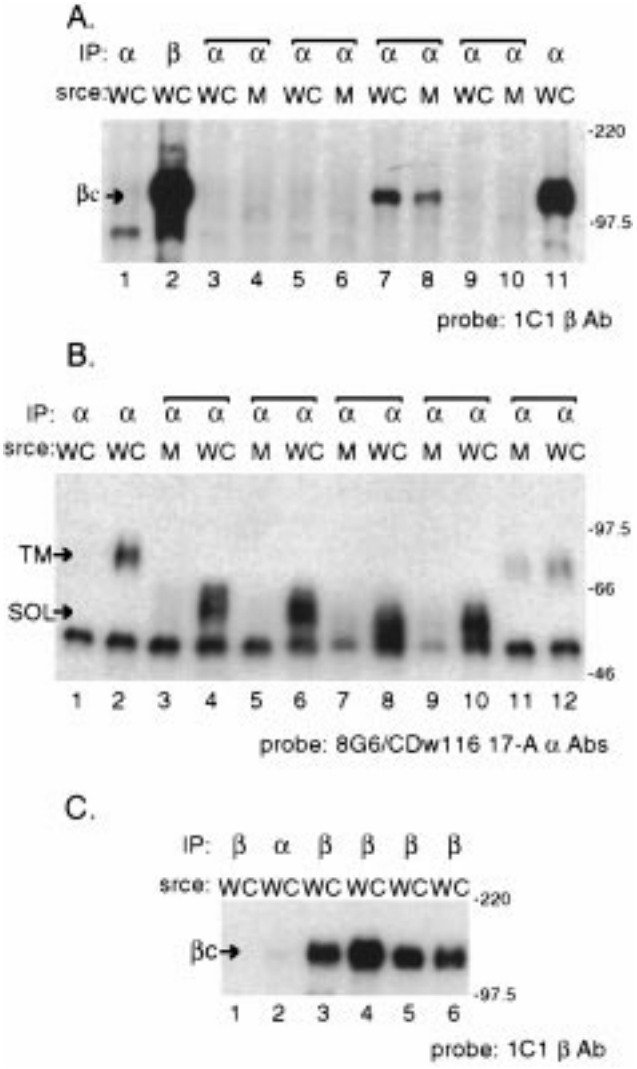

FIGURE 7: Coimmunoprecipitation of GMRβ and GMRα from cellular compartments. WC = whole cell lysates, M = membrane preparations. (A) Coimmunoprecipitation of GMRβ using anti-GMRα monoclonal antibodies (CDw116 17-A or 8G6) from BHK cells transfected with various GM-CSF receptor subunits. Lane 1 = untransfected BHK cells, lane 2 = βc, lanes 3, 4 = SOLα, lanes 5, 6 = SOLαC323A, lanes 7, 8 = SOLα/βc, lanes 9, 10 = SOLαC323A/βc, lane 11 = TMα/βc + GM-CSF. (B) Immunoprecipitation of GMRα using anti-GMRα monoclonal antibodies (CDw116 17-A or 8G6). Lane 1 = untransfected BHK cells, lane 2 = TMα, lanes 3, 4 = SOLα, lanes 5, 6 = SOLαC323A, lanes 7, 8 = SOLα/βc, lanes 9, 10 = SOLαC323A/βc, lanes 11, 12 = TMα/βc + GM-CSF. (C) Immunoprecipitation of GMRβ using monoclonal anti-GMRβ antibody 1C1. Lane 1 = untransfected BHK cells, lane 2 = TMα, lane 3 = SOLαC323A/βc, lane 4 = SOLα/βc, lane 5 = TMα/βc, lane 6 = βc.

merely abrogate the ligand binding function of the SOLα/βc complex (Figure 6C) or hinder its transport from the cytoplasm to the cell surface (Figure 7). This is not meant to imply that Cys323 is the only residue in the carboxyl tail involved in the interaction with βc. Such a conclusion could only be drawn after careful examination of the role of all the residues in the tail, a task not undertaken in this paper. While the involvement of a cysteine residue in the interaction of the two molecules implies a covalent, disulfide bond linkage between SOLα and βc, we have not formally proven this, and such an interaction should not be assumed without further experimentation.

The importance of the tail domain in the formation of the SOLα/βc complex and the immunological data suggesting a basic difference in how SOLα and TMα interact with βc lead us to recommend caution in extrapolating the findings of our past and present experiments with the wild-type SOLα in order to draw conclusions about the interaction of TMα and βc on the cell surface (*37*). On the other hand, removal of the tail domain has no effect on the affinity of the soluble receptor for GM-CSF, implying that ECDα is a more useful molecule than wild-type SOLα as a model in experiments investigating GMRα/βc interactions (*38*). In this context, it is curious to find that the mutation of Cys323 to Ala had such a profound effect on the affinity of SOLα while ECDα had none. It would suggest that Cys323 imparts structural properties to the tail domain that, when altered, lead to interference with ligand binding domains on other parts of the molecule by the short, 16 amino acid tail sequence.

Two other soluble cytokine receptors, solIL-6R (*33*) and solCNTFR (*34*), have also been demonstrated to be able to anchor themselves on the cell surface. Like SOLα, the tethering of both solIL-6R and solCNTFR occurs via an interaction with the membrane-spanning signaling subunits of their respective receptors (gp130 for IL-6R and gp130/LIFRβ for CNTFR). However, these two soluble receptors otherwise differ significantly from SOLα in the mechanism of their cell-surface retention. First, solIL-6R and solCNTFR are able to interact with their membrane-bound receptor components without trafficking through the same intracellular machinery while SOLα can only interact with βc if the two are produced in the same cell. That is, an exogenous source of solIL-6R and solCNTFR can interact with free membrane-spanning receptor components while an exogenous source of SOLα cannot interact with βc (*32*). Second, the interaction of the soluble and membrane-bound components of the IL-6R and CNTFR is ligand-dependent while the formation of the SOLα/βc complex is completely ligand-independent. Finally, the interaction of the soluble IL-6R and soluble CNTFR with their respective ligands and membrane-spanning receptor components results in successful signal transduction. The signaling capacity of SOLα/βc is currently unknown and was not addressed in the experiments described in this paper. However, the determination of the influence of the SOLα/βc complex on GM-CSF signaling is one of the critical next steps in understanding the significance of this complex.

The other major goal regarding the SOLα/βc cell-surface complex is to seek evidence for its existence in vivo. These experiments pose special challenges. Our current experiments suggest that the TMα/βc and SOLα/βc complexes have identical ligand affinities on the cell surface such that simple binding experiments will not distinguish between the two. In addition, available antibodies recognize domains common to both TMα and SOLα, making immunological strategies for identification of SOLα/βc difficult. Nevertheless, efforts are underway to define the signaling capacity and cellular distribution of SOLα/βc in order to better understand the potential physiological and pathophysiological relevance of this unique cytokine receptor complex.

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