Molecular Determinants of the Granulocyte-Macrophage Colony-stimulating Factor Receptor Complex Assembly*

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The granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor (GMR) is composed of two chains that belong to the superfamily of cytokine receptors typified by the growth hormone receptor. A common structural element found in cytokine receptors is a module of two fibronectin-like domains, each characterized by seven β-strands denoted A–G and A′–G′, respectively. The α-chain (GMRα) confers low affinity GM-CSF binding ($K_d = 1–5$ nM), whereas the β-chain (βc) does not bind GM-CSF by itself but confers high affinity binding when associated with α ($K_d = 40–100$ pM). In the present study, we define the molecular determinants required for ligand recognition and for stabilization of the complex through a convergence of several approaches, including the construction of chimeric receptors, the molecular dynamics of our three-dimensional model of the GM-GMR complex, and site-directed mutagenesis. The functional importance of individual residues was then investigated through ligand binding studies at equilibrium and through determination of the kinetic constants of the GM-GMR complex. Critical to this tripartite complex is the establishment of four noncovalent bonds, three that determine the nature of the ligand recognition process involving residues Arg368 and Tyr426 of the α-chain and residue Tyr365 of the β-chain, since mutations of either one of these residues resulted in a significant decrease in the association rate. Finally, residue Tyr365 of βc serves a dual function in that it cooperates with another residue of αc, Tyr211 to stabilize the complex since mutation of Tyr365 and Tyr211 result in a drastic increase in the dissociation rate (Koff). Interestingly, these four residues are located at the B′-C’ and F’-G’ loops of GMα and of βc, thus establishing a functional symmetry within an apparently asymmetrical heterodimeric structure.

GM-CSF1 is a multifunctional growth factor (reviewed in Ref. 1) that stimulates the proliferation of hemopoietic and vascular endothelial cells. Moreover, GM-CSF suppresses apoptosis in hemopoietic precursors (2–4) while enhancing the response of neutrophils to bacterial antigens and the phagocytic activity of macrophages/macrophages (reviewed in Ref. 5). The GM-CSF receptor is composed of two chains that belong to the superfamily of cytokine receptors typified by the growth hormone receptor (GHR) (6–8). The α-chain confers low affinity binding only ($K_d = 1–5$ nM) (9, 10), whereas the β-chain (βc) (11) does not bind GM-CSF by itself but confers high affinity binding when associated with the α-chain ($K_d = 40–100$ pM) (12–15). We have previously shown that the high affinity GM-CSF binding site results from the stoichiometric association of GMα with βc, resulting in a stabilization of the GM-GMR complex by 3 orders of magnitude (16). Previous studies also suggest that the low affinity binding of GMα to the ligand may be attributed to a major difference in the off rate when compared with that of the GMαβc complex, whereas the on rates are not significantly different (17, 18).

Through sequence alignment, Bazan (6) first predicted that cytokine receptors are made up of two fibronectin folds, each containing seven antiparallel β-strands, similar to the immunoglobulin fold. These strands are labeled A–G for the first fibronectin fold and A′–G′ for the membrane proximal fibronectin fold. Crystallization of the GHR-GHR complex (19), of erythropoietin receptor (EpoR) complexed with an Epo agonist (20), as well as the solution structure of the granulocyte colony-stimulating factor receptor (21) confirmed the predicted structure. Interestingly, the RGD integrin binding site of human fibronectin is located at the F-G loop of the four-domain segment (22). In addition, the major ligand binding site for the granulocyte colony-stimulating factor receptor was also identified at the F-G’ loop just upstream of the WSXWS box. The three-dimensional structure of GMR is not available, but that of the ligand was solved by x-ray crystallography and NMR analysis (23). We have previously constructed a three-dimensional model of the GMR complex on the basis of the GH-GHR complex in which the GH crystal was replaced by that of GM-CSF, while the α- and β-chains of GMR were modeled according to the R1 and R2 chains of GHR (24). Through site-directed mutagenesis guided by homology modeling with the growth hormone receptor complex, we identified a single residue, Arg368, located at the tip of a β-turn of the F-G’ loop of GMα that drives the ligand recognition process (24). In addition, alanine scanning mutagenesis of the β-chain identified two tyrosine residues, Tyr365 at the B′-C’ and F’-G’ loops of GMα, and of βc, thus establishing a functional symmetry within an apparently asymmetrical heterodimeric structure.

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GM-CSF Receptor Complex

Epo binding (28), consistent with their locating at the hydrophobic interface between the Epo mimetic peptide and EpoR in the structure of the crystal (20). Together, these studies identified three loops within the two fibronectin folds of the superfamily of cytokine receptors that are potentially involved in ligand binding, the E-F, B'-C', and F'-G' loops. The present study is designed to define the functional determinants of the GM-CSF receptor assembly, through a convergence of binding studies performed at equilibrium and kinetic studies of ligand-receptor interaction. In addition, molecular dynamics simulations were performed on a model of the GM-CSF receptor assembly in order to determine amino acids potentially located at contact points between the ligand and receptor and to guide our mutagenesis aimed at defining the functional interface of the complex.

MATERIALS AND METHODS

Cells and Plasmids—Jurkat cells (ATCC) were passaged three times weekly, at 5 × 10^6/ml in Iscove’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies). The human GMRα and β open reading frame, cloned in pME18S, were a generous gift of Dr. Toshio Kitamura (Tokyo, Japan) (11). Site-directed mutagenesis was performed through polymerase chain reaction overlap, as described previously (24). All polymerase chain reaction-generated fragments were completely sequenced in both orientations after cloning into pME18S (Pharmingen). 

Electroporation of Jurkat Cells for Transient Expression of GMRα and β—Exponentially growing Jurkat cells were resuspended at 2.5 × 10^7 cells in a volume of 600 μl of medium and electroporated at 960 microfarads, 320 V with 5 μF of GMRα and 5 μF of β, all buffer. Transfected plasmids were linearized with restriction enzymes. 

Electroporation was performed through a transformer chain reaction overlap, as described previously (24). All polymerase chain reaction-generated fragments were completely sequenced in both orientations after cloning into pME18S (Pharmingen). Mutant GM-CSFs were produced as described previously (30).

Electroporation of Jurkat Cells for Transient Expression of GMRα and β—Exponentially growing Jurkat cells were resuspended at 2.5 × 10^7 cells in a volume of 600 μl of medium and electroporated at 960 microfarads, 320 V with 5 μg of GMRα and 5 μg of β, all buffer. Transfected plasmids were linearized with restriction enzymes. All polymerase chain reaction-generated fragments were completely sequenced in both orientations after cloning into pME18S (Pharmingen). 

RESULTS

Role of the B'-C' and F'-G' Loops of the α-Chain of the GM-CSF Receptor in Ligand Binding—In our three-dimensional model of the GM-CSF receptor complex, several loops appear to be oriented toward the ligand: the E-F loop of the first cytokine receptor domain as well as the B'-C' and F'-G' loops of the second cytokine domain. In order to directly address the importance of these loops in ligand binding, we chose to generate chimeras in which the three loops of GMRα were substituted by the corresponding loops of IL-3Rα (15). Our rationale for choosing IL-3Rα is based on the evidence that it is the closest to GMRα in primary sequence, in predicted three-dimensional structure, as well as in functional and biological properties (18, 33, 34), yet IL-3Rα demonstrates exquisite binding specificity for its ligand. The primary sequences of the three loops as well as their flanking β-strands are illustrated in Fig. 1. The alignment shows that the primary sequences of the loops are highly variable, except for the arginine at the F'-G' loop identified in our previous study (24) and for the WS box as shown by Bazan (6). In addition, the F'-G' loops are conserved in length, whereas the E-F and B'-C' loops are more variable. In order to preserve the overall structure of the chimeric molecule, we have chosen to maintain the length of the loops as in GMRα, such that amino acid residues of the interleukin-3 receptor were removed (E-F chimera) or amino acid residues of GMRα were added (B'-C' chimera) to those of the interleukin-3 receptor loops as shown in Fig. 1. NIH 3T3 cells were transiently transfected with the different GM-CSF chimeras alone and submitted to GM-CSF binding assays (Fig. 2). Substitution of the B'-C' loop as well as the F'-G' loop of GM-CSF with those of IL-3Rα completely abrogated GM-CSF binding, while the E-F loop of the first fibronectin domain showed only a 2-fold decrease in ligand binding.

Since our previous results indicate that a mutant α, which is unable to bind GM-CSF, can still associate with wild type β to favor ligand binding (24), we co-transfected the different chimeric GMRα with wild type β. Results shown in Fig. 2C and Table I indicate that the substitution of interleukin-3 receptor sequences at the B'-C' loop of GM-CSF converted ligand binding so that a single low affinity binding site of 1–4 nM, whereas the E-F chimera can direct both high and low affinity GM-CSF binding when co-expressed with wild type β in NIH 3T3 cells (Fig. 2B), much in the same way as wild type GMRα (data not shown). These results confirm the overall importance of the F'-G' loop in ligand binding as observed previously (24) and reveal the importance of the B'-C' loop in this process. In contrast, the
Fig. 1. Alignment of the E-F, B'-C', and F'-G' loops of the GM-CSF receptor α-chain, the IL-3Rα chain, and βc. The seven β-strands of the first fibronectin-like domain are labeled A–G, and those of the membrane-proximal fibronectin-like domain are labeled A'–G'. For the chimeric αF'-G' (residues 277–289) and for the chimeric α-E-F (residues 160–166).

Fig. 2. Determination of GM-CSF binding to chimeric GMR. The chimeras as shown were transfected into exponentially growing NIH-3T3 cells, in the presence or in the absence of βc (α only). 125I-GM-CSF was added at a concentration of 6.7 nM for the α-chain only (A). Results shown are the average ± S.D. of duplicate determinations corrected for nonspecific binding, which was determined with 100-fold excess of unlabeled GM-CSF. Results are calculated as percentage of 6

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Functional Importance of the Aromatic Ring at Position 226 of GMRα—Since Tyr\textsuperscript{226} of GMRα, located at the B’-C’ loop, moved into a strategic position after the simulations, we addressed its importance as well as that of another aromatic residue of the same loop, Tyr\textsuperscript{221}. Two mutations were introduced at these positions, a tyrosine to phenylalanine mutation aimed at preserving hydrophobic interactions conferred by the aromatic ring as well as a tyrosine to glutamine mutation, which removes the possibility of hydrophobic interaction. When binding was performed in the absence of β\textsubscript{c} (Fig. 6A and Table I), Y226F was able to associate with GM-CSF to the same extent as wild type GMRα. In contrast, the Y226Q mutation showed a complete loss of GM-CSF binding as illustrated in Fig. 6A and determined through saturation analyses (data not shown). In comparison, mutation of Tyr\textsuperscript{221}, which is also located at the B’-C’ loop to either glutamine or phenylalanine did not affect GM-CSF binding under the same conditions (Fig. 6A and Table I). The loss of low affinity binding caused by the Tyr\textsuperscript{226} mutation was not due to structural alterations of the molecule, as assessed by reactivity with a monoclonal anti-GMRα (Fig. 7). Our observations are therefore consistent with an important function for the aromatic ring at position 226 in the ααα configuration.

The impact of the Y226Q mutation was further evaluated in the presence of β\textsubscript{c}. Data shown in Fig. 6C indicate that the Y226Q mutant exhibits a single GM-CSF binding site of high to intermediate affinity, whereas the low affinity binding site was lost. Thus, the Y226Q mutation resulted in a loss of binding free energy of 0.5–0.8 kcal/mol when co-transfected with wild type β\textsubscript{c}, which accounts for almost half of the low observed with the B’-C’ chimera (Table I). Together, our results indicate an important role for the aromatic ring in establishing hydrophobic interactions or π-charge interaction at position 226 of GMRα in the absence of β\textsubscript{c} and, to a lesser extent, in the presence of β\textsubscript{c}.

Binding studies performed at equilibrium allow for an estimation of the energetic contribution of individual residues but do not discriminate between a role in the association process itself (K\textsubscript{on}) or in the dissociation rate (K\textsubscript{off}). The contribution of Tyr\textsuperscript{226} to the GMR complex was therefore investigated through determination of the kinetic constants for the GMRα Y226Q mutant and wild type GM-CSF (Fig. 8).

In the absence of β\textsubscript{c}, there was a 3.5-fold increase in the dissociation rate for the Y226Q mutant, consistent with the observed shift in the equilibrium constant determined by saturation analysis (data not shown). In the presence of β\textsubscript{c}, there was a 5-fold increase in the off rate when the aromatic residue at position 226 of the α-chain was mutated to a hydrophilic residue, comparable with that observed with the α-B’-C’ chimera or with the αR280M mutant. This mild increase in K\textsubscript{off} is not comparable with those produced by mutations of tyrosine residues of β\textsubscript{α}, as discussed underneath. There was a significant decrease in the on rate, resulting in an overall 4–8-fold increase in the dissociation constant. In comparison, mutation of Arg\textsuperscript{280} produced a 10–12-fold decrease in K\textsubscript{on} as reported previously (24). Together these decreases in K\textsubscript{on} suggest that α-Tyr\textsuperscript{226} contributes to the ligand recognition process, albeit not to the same extent as α-Arg\textsuperscript{280}.

Comparative Contribution of Tyrosine Residues on the β-Chain to the Stability of the GM-GMR Complex—We first compared the kinetics of binding of the α-chain alone (low affinity) to that of the αβ complex (high affinity). Data shown in Table I indicate a 100-fold difference in the dissociation rate between α and αβ complex, whereas the difference in the association rate was 100-fold resulting in a 1000-fold difference in affinity for the ligand. These results are in agreement with the difference in K\textsubscript{on} observed between α and the αβ complex as determined through saturation analysis. Our observations are therefore consistent with the view that the recruitment of β\textsubscript{c} results in stabilization of the GM-GMR complex. The difference in the on rates between α and the αβ complex is, however, higher than anticipated (18) and suggests that β\textsubscript{c} can contribute to ligand binding as described elsewhere (24, 25, 27) albeit to a lesser extent when compared with the α-chain.

Previous studies identified two residues of the β-chain that are important for high affinity GM-CSF binding, Tyr\textsuperscript{365} at the B’-C’ loop and Tyr\textsuperscript{421} at the F’-G’ loop. These studies, however, did not discriminate between a direct contribution to the ligand recognition process or a structural function involved in complex stability. We therefore mutated these two residues into Gln and verified that the mutations did not induce gross structural changes in the molecule through staining with a monoclonal antibody against β\textsubscript{c} (Fig. 7). The binding kinetics of mutant GMR βY365Q and βY421Q were then compared with those of wild type β\textsubscript{c} in association with wild type ααα-chains. The association rate (K\textsubscript{on}) for βY365Q was 4-fold lower than that of wild type β\textsubscript{c}, whereas the K\textsubscript{off} of βY421Q was unaffected by the mutation (Fig. 8), suggesting that Tyr\textsuperscript{365} but not Tyr\textsuperscript{421} is important for ligand association. The dissociation rate constant (K\textsubscript{off}) for αβY365Q and αβY421Q were, however, 20- and 30-fold higher than that of wild type αβ\textsubscript{c}. These results indicate that the difference in binding affinities observed for the GMR βY365Q and βY421Q mutants compared with wild type GMR is

### Table I

| Transfected genes | Kinetic analyses (K\textsubscript{on}/K\textsubscript{off}) | Analyses at equilibrium | Location |
|-------------------|--------------------------|-------------------------|---------|
|                   | Binding sites             | K\textsubscript{d} (high affinity) | K\textsubscript{d} (low affinity) |
| α                 | 18 nM                    | 22 ± 6                  | B’-C’   |
| aY226F            | ND                       | 47 ± 5                  | B’-C’   |
| aY226Q            | ND                       | No binding              | B’-C’   |
| aY221Q            | ND                       | No binding              | B’-C’   |
| a + β             | 21 pm                    | 80 ± 27                 | E-F     |
| a + β + β         | 320 pm                   | 108 ± 61                | E-F     |
| aB’-C’ + β       | 1 nM                     | 2.6 ± 0.9               | B’-C’   |
| aY226Q + β       | 320 pm                   | 217 ± 60                | B’-C’   |
| a + βY365Q       | 1.8 nM                   | 4 ± 1                   | B’-C’   |
| a + βY421Q       | 0.73 nM                  | 3 ± 0.3                 | F’-G’   |

a Kinetic analyses were performed as described in the legend to Fig. 7.
b Binding constants were determined at equilibrium through analysis of saturation curves.
c ND, not detected.
mainly due to an increase in the dissociation rate constant. The extrapolated cumulative effect of the two mutations on the dissociation rate constant was 50-fold, which recapitulated the difference between the αβ complex and α alone. Consistent with these kinetic properties, our three-dimensional model also positions β-Tyr365 in the vicinity of the ligand, whereas β-Tyr421 was remote from the ligand, either in our original model (24) or after molecular dynamics (Fig. 4). Together, our observations indicate that the aromatic rings at positions 365 and 421 of βc are critical to the stability of the liganded complex, consistent with the role assigned to βc, and that Tyr 365 also fulfills a ligand association function for βc.

Despite the locating of Tyr365 in the vicinity of helix A, the closest residue on the ligand was Arg23, which was still at a distance of 9 Å. In contrast, we found on closer examination of this interface that the neighboring residue Arg23 was located within 5 Å of β-Asp369, suggesting the possibility of electrostatic interaction at this position. We therefore determined the importance of the charge at position 23 of the ligand, through analysis of the capacity of mutant GM-CSF to compete for the binding of wild type radiiodinated GM-CSF. Binding characteristics were determined at equilibrium. As illustrated in Fig. 9, the dissociation constant for wild type GM-CSF is 58 pM, which is comparable with values estimated through saturation analysis (Table I) and comparable with published results (12, 13, 17, 37, 38). Removal of the charge (R23N) results in a 2-fold shift in the dissociation constant. In contrast, reversal of the charge at position 23 (R23D) induces a 5-fold increase in $K_d$ (Fig. 7), indicating that Arg 23 has an apparent contribution of 0.9 kcal/mol. Furthermore, mutations of Asp 369 of βc affected the binding equilibrium to an extent that was comparable with that observed with Arg23 mutations on the ligand (data not shown). Together, our observations indicate the importance of the charge at position 23 of the ligand, which is required for proper association between the ligand and βc. Since Glu23 is also critical for high affinity binding (39), with an apparent contribution of 2.5 kcal/mol, the observations are consistent with a hot spot of binding energy on helix D (Glu23 and Arg23) of GM-CSF, which establishes a functional interface with βc.
through several residues, including His\textsuperscript{367}, Tyr\textsuperscript{365}, and, potentially, Asp\textsuperscript{369}.

**DISCUSSION**

The proper assembly of a high affinity complex involving GM-CSF and the α- and β-chains of the receptor is essential for signal transduction. In the present study, we define the molecular determinants required for ligand recognition and for stabilization of the complex through a convergence of several approaches, the construction of chimeric receptors, the molecular dynamics of our original three-dimensional model of the GM-GMR complex, and site-directed mutagenesis. Critical to this tripartite complex is the establishment of four non covalent bonds, three that determine the nature of the ligand recognition process involving residues Arg\textsuperscript{280} and Tyr\textsuperscript{365} of the α-chain and residue Tyr\textsuperscript{365} of the β-chain. Interestingly, the latter serves a dual function in that it cooperates with another residue of β, Tyr\textsuperscript{226}, to stabilize the complex.

**Charge Interactions and π-Charge Interactions Determine GM-CSF Binding**—The specificity of protein-protein interactions is often conferred by charge interactions or π-charge interactions, as in enzyme-substrate or major histocompatibility complex-antigen recognition processes. In the GM-GMR complex, we have previously identified two salt bridges that are critical for high affinity binding, one between the α-chain and the ligand α-Arg\textsuperscript{280}-(GM)Asp\textsuperscript{112} (24) and the other one between β, and the ligand β-His\textsuperscript{367}-(GM)Glu\textsuperscript{21} (25, 39). In the present study, we provide evidence for the importance of two aromatic rings in this process, Tyr\textsuperscript{366} of GMα and Tyr\textsuperscript{365} of β. Interestingly, both are located at the B'-C' loops and contribute to the ligand recognition process, as evidenced by a significant decrease in the on rate when these residues were mutated to hydrophilic residues. Molecular dynamic simulations and functional binding studies concur to indicate that α-Tyr\textsuperscript{226} serves a critical ligand binding function in the absence of β. Hence, our results suggest that the low affinity complex is established through the interactions of Arg\textsuperscript{280} on the first α-chain with site 1 on helix D of the ligand and of Tyr\textsuperscript{226} on the second α-chain with site 2 on helix A of the ligand. In contrast, in the high affinity complex formed by the αβ heterodimer, Our model also predicts that the contact points between the ligand and β may be multiple and involve at least three residues at the B'-C' loop of β, Tyr\textsuperscript{365}, His\textsuperscript{367}, and Asp\textsuperscript{369}, and three residues on helix A of the ligand, Leu\textsuperscript{28} or Arg\textsuperscript{24}, Glu\textsuperscript{21}, and Arg\textsuperscript{23}, respectively.

In the present study, we have identified the role of aromatic residues within the B'-C' loop of both chains of the GM-CSF receptor that are important for ligand binding. Interestingly, such interactions for the residues reported here and elsewhere contribute to more than 60% of the free energy of the whole complex. It is possible that the remaining free energy may be

**FIG. 4. Movement of Tyr\textsuperscript{226} during the simulation.** A, interaction on site 1 between α\textsubscript{1}-Tyr\textsuperscript{226} (α\textsubscript{1}Y226) and (GM)Leu\textsuperscript{115} (GML115); B, interaction on site 2 between α\textsubscript{2}-Tyr\textsuperscript{226} (α\textsubscript{2}Y226) and (GM)Arg\textsuperscript{23}.

**FIG. 5. Structure of the low affinity GM-CSF receptor complex.** Three-dimensional model of the low affinity GM-CSF receptor complex after molecular dynamics simulation for 20 ps. Site 1 is identical to that shown in Fig. 3A, whereas site 2 binding is established with a second molecule of GMα. Color codes are the same as in Fig. 3.
contributed by hydrophobic interactions between residues that are buried at the ligand-receptor interface. Two aromatic residues that are important for interleukin-6 binding are also found at the B'9-C'9 loop of interleukin-6 receptor α, Phe230, and Tyr231 (40). In addition, another aromatic residue, Trp169, also located at the B'9-C'9 loop of GHR is essential to the growth hormone receptor complex (32). Thus, the conservation of one or more aromatic residues within the B'9-C'9 loop suggests a nonredundant function for π-π interactions or π-charge interactions at this position in ligand binding. Not surprisingly, in the EpoR that lacks an aromatic residue at the B'9-C'9 loop, this function is fulfilled by another residue at the E-F loop that is important for ligand binding, Phe239 (28). Interestingly, the E-F loop of the growth hormone receptor also presents Trp104 at the hormone-receptor interface, which together with Trp169 contributes to 60% of the free energy of the complex (32). Since both EpoR and GHR are homodimers, one may speculate that they constitute a subfamily in which the ligand binding function is established at the E-F loop. Although the latter appears to be dispensable for the GMR complex, both chains of GMR have retained two functional interfaces, one at the F'-G' loop, comparable with EpoR and granulocyte colony-stimulating factor receptor, while the other interface is established at the B'-C' loop, in the same way as the growth hormone receptor.

Hence, within the superfamily of cytokine receptors, each receptor chain, be it part of a homodimeric or a heterodimeric complex, appears to establish two major functional interfaces with the ligand through two of the three loops, E-F of the first fibronectin fold and B'-C' or F'-G' of the second fibronectin fold.

**Structural Determinants of GMR That Define the Ligand Binding Function**—The F-G loop of fibronectin harbors the RGD sequence that defines the integrin binding site (22). Similarly, crucial binding determinants for both GMR and EpoR are also located at the F'-G' loop. Thus, the conservation of a
functional interface at the F'-G' loop suggests that structural elements have evolved to maintain this interface. The crystal structure of EpoR revealed an important structural element that holds the F'-G' loop in a configuration, which is optimal for presentation of the functional residue Phe282, i.e. the WS box region also found in all members of the superfamily of cytokine receptors (20). In our three-dimensional model, we have previously shown that the two Trp residues of the WS box of GMα are in the proximity of Arg276 of the F' strand, thus establishing a strong π-charge interaction that forces a β-turn at the location of Arg276, required for ligand association (24). On the α-chain, another interaction reinforces this structural requirement, the salt bridge between Asp278 at the F'-G' loop, just upstream of the WS box, and Lys191 of the linker domain (Ref. 24 and data not shown). Since the linker domain holds the two fibronectin-like modules together, we anticipate that interactions may be found at this location in other cytokine receptors.

Finally, an important residue was previously found at the F'-G' loop of βc, Tyr421 (26), which was thought to be involved in ligand binding. In contrast, our results indicate that Tyrr421 does not serve a ligand binding function. First, kinetic studies performed here indicate that Tyr421 is primarily involved in stabilizing the complex. Second, in our three-dimensional model of the complex, Tyr421 is not oriented toward the ligand. Even when the residue is flipped in trans, we did not detect any residue on the ligand that might be within a reasonable distance of Tyr421. Consistent with these observations, solving the structure of domain 4 of βc complexed with an antagonistic antibody2 shows that Tyr421 indeed points to a different face than the cytokine binding site located on the B-C loop. Since we have previously shown that a functional GMα complex is at least a hexamer of two GMα, two βc, and two molecules of GM-CSF (35), it is tempting to speculate that Tyr421 is important in establishing a higher order of receptor assembly required for signal transduction. Future studies will be required to resolve this issue.

Symmetry within an Asymmetrical Structure—The α-chain of the GMα complex comprises 378 amino acid residues, most of these constituting the extracellular domain (9), while βc has 881 amino acid residues, half of which are located in the intracellular domain (11). Despite its short cytoplasmic tail, GMα cooperates with βc in signal transduction (35, 41, 42), and residues essential for cellular activation have been located within its 40-amino acid tail. Thus, despite a variability in length, both chains of GMα appear to fulfill specific functions in signal transduction. In addition, the present study identifies a symmetrical disposition of the functional loops involved in GMα complex assembly. Evidence presented here and elsewhere indicates that both B'-C' and F'-G' loops of GMα and βc are critical for the assembly of the GM-CSF receptor and its ligand into a high affinity complex. In our original model, the F'-G' loop of GMα is the mirror image of the B'-C' loop of βc, both determining the ligand association process, whereas the B'-C' loop of GMα appears to be the counterpart of the F'-G' loop of βc, both more remote from the ligand. After molecular dynamics, the B'-C' loop of GMα moves closer to helix D of the ligand to establish the second bridge with GM-CSF. As a result, GMα has two loops in the vicinity of the ligand, F'-G' and B'-C', both contributing to the ligand recognition process, whereas βc is predicted to have a single loop (albeit with several contact points), consistent with a ligand binding function which is secondary to that of GMα. Hence, after molecular dynamics, the two functional loops of GMα are disposed as mirror images of the corresponding loops of βc, establishing a symmetrical structure within the extracellular domains of the GMα complex. In summary, residues located at the turn of the

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B'-C' and F'-G' loops of both GMRα and β, specify the nature of the functional interface of the GM-GMR complex.

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