Interaction of Granulocyte Colony-stimulating Factor (G-CSF) 
with Its Receptor

EVIDENCE THAT Glu19 of G-CSF INTERACTS WITH Arg288 OF THE RECEPTOR

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Granulocyte colony-stimulating factor (G-CSF)1 is a member of a family of cytokines that have a four-α-helical bundle structure, with the four helices conventionally labeled A–D from the N terminus (1). The structures of human, bovine, and canine G-CSF have been determined by x-ray crystallography (2, 3) and NMR spectroscopy (4, 5). In addition to the four main α helices, there is a short 3₁₀ helix in the A–B loop. The main biological activities of G-CSF are the proliferation, differentiation, and survival of cells of the neutrophil lineage (6, 7). These responses are initiated by interaction with a specific receptor (G-CSF-R) which is expressed on neutrophils, their precursors, and some leukemic cell lines (6, 7). Binding of G-CSF causes receptor dimerization and activation of signaling cascades such as the Jak-STAT and mitogen-activated protein kinase pathways (8).

The receptor extracellular region comprises six structural domains as follows: an N-terminal immunoglobulin-like (Ig) domain, followed by a cytokine receptor homologous module (CRHM) (containing two fibronectin type III-like (FNIII) domains as follows: an N-terminal “BN” and a C-terminal “BC” domain) and three further FNIII domains (9, 10). This domain structure is closely related to that of gp130, the shared signal transducer of the IL-6 family of cytokines (11), with which the G-CSF-R shares 46% sequence similarity in the extracellular region (12). The CRHM and Ig domain of the G-CSF-R have been implicated in ligand binding by deletion analysis (10) and mapping of neutralizing mAbs (13), whereas the three membrane-proximal FNIII domains are not required for ligand binding but may be important for receptor stability and/or signal transduction (10, 14). Studies of a soluble form of the extracellular region of the receptor show that a 2:2 complex of ligand and receptor form in solution (15), but the structure of the complex is currently unknown. The BN and BC domains of the G-CSF-R have been expressed individually, and each has been shown to bind G-CSF with low affinity (about 10⁻⁷ M) in a 1:1 complex (16, 17). The structure of BC has been determined recently by NMR spectroscopy, and the data indicated that the F–G loop of this domain is likely to be involved in ligand recognition (18). However, it is not yet clear whether there are one or two ligand-binding sites on the G-CSF-R. Horan et al. (14, 15) concluded that there is probably one ligand-binding site with dimerization caused by receptor-receptor interaction. On the other hand, studies on the complexes formed with G-CSF and two soluble receptor fragments (Ig-BN and the CRHM) indicated that each G-CSF-R in the complex has two binding sites for G-CSF, one in the CRHM and one requiring the Ig domain (19). In addition, this study established that the Ig domain of the G-CSF-R is required for receptor dimerization (19). Similarly, the Ig domain of gp130 is required for receptor complex formation and signaling by interleukin-6 (IL-6) (12).

We recently identified several residues in the CRHM that interact with G-CSF (20), forming a binding site similar to the growth hormone receptor-binding site (21). Of these residues, Arg288 in the F–G loop of the BC domain appeared to be the
most important for G-CSF binding, in agreement with the study of Yamasaki et al. (18). This large, positively charged residue might interact with a negatively charged Glu or Asp residue in G-CSF. Mutagenesis studies of G-CSF have identified several charged residues that are important for receptor binding, including Glu19, Lys33, Glu46, and Asp112 (22, 23). In both these studies, Glu19 in the A helix of G-CSF was identified as particularly important and appeared to be a possible candidate for interaction with Arg288. In the present study, to identify which G-CSF residues interact with Arg288 in the receptor, we have mutated Glu19, Lys33, Glu46, and Asp112 and compared the activity of these G-CSF mutants on cells expressing wild-type (WT) and mutant (R288A) receptors. In addition, to clarify further whether there are one or two binding sites on G-CSF, a chimeric receptor was constructed in which the Ig domain of gp130 replaced the G-CSF-R Ig domain. This chimera was used to determine whether the above G-CSF residues interacted with the CRHM-binding site or the putative Ig domain-binding site.

**Experimental Procedures**

**Preparation of G-CSF Mutants**—Human G-CSF mutants were prepared by oligonucleotide-directed mutagenesis (24), and the mutations were confirmed by sequencing the complete G-CSF cDNA. The G-CSF mutant proteins were expressed in Escherichia coli and purified essentially as described previously (25). Samples of mutant and WT rG-CSF were analyzed by reverse phase high pressure liquid chromatography to confirm homogeneity.

**G-CSF-R Mutants and Chimeric Receptor**—The human G-CSF-R mutants were prepared as described previously (20). To generate the Ig domain chimeric receptor (gp130-Ig-GR), EcoRV restriction enzyme sites were introduced in both the G-CSF-R and gp130 between the Ig and CRH domains as described for the G-CSF-R (12, 13) [oligonucleotide for gp130, 5'-GGAAATCTAATATAGGGATATCCCTGAAA- AA-3']. A BstXI-EcoRV fragment of gp130 was ligated with an EcoRV-XbaI fragment of G-CSF-R into BstXI- and EcoRV-digested pcDNA1Amp (Invitrogen, The Netherlands). The chimeric receptor was excised with HindIII and XbaI and ligated with linkers that contained XbaI and HindIII sites. The receptor was then subcloned into the XbaI site of pEFBOS expression vector (26). The chimera encoded residues Met1-Gly129 of gp130 followed by residues Tyr29-Asp312 of the G-CSF-R (residues numbered as in Ref. 27). The receptors were transfected into the murine pro-B cell line, Ba/F3 (28), as described previously (20).

**Flow Cytometry**—Analysis of receptor expression by flow cytometry was performed as described previously (13) using the following mAbs: LMM741, which binds to the G-CSF-R FNIII domains (13), LMM775, which binds to the G-CSF-R Ig domain (13), and GPZ35, which binds to gp130 (29).

**G-CSF Binding Assay**—Tyr1,3-Ig-CSF (provided by Kirin, Japan) was iodinated using IODO-GEN (Pierce) as described previously (13). The ability of each batch to bind was at least 60%, and the specific activity was 4–10 × 10⁶ cpm/μg as determined by self-displacement analysis (30). Binding affinity of G-CSF mutants was determined by titration of mutants in the presence of 125I-Tyr1,3-G-CSF (~100 pM) to WT-GR and 200 pM for (R288A)GR, (R288E)GR, and (gp130-Ig-GR). Data were analyzed with the "drug" section of the LIGAND program (31) ( Biosoft, Cambridge, UK).

**G-CSF Proliferation Assay**—Proliferation of Ba/F3 cells expressing WT and mutant G-CSF-R was determined by measuring incorporation of [methyl-3H]thymidine (ICN Pharmaceuticals, Irvine, CA) as described previously (20).

**Results**

**Preparation and Characterization of gp130-Ig-GR**—Because we believed that the evidence for two separate Ig domain-binding sites in the G-CSF-R was stronger than the evidence for a single site, we first wanted to determine which of the G-CSF residues Glu19, Lys33, Glu46, or Asp112 were required for binding to each site. Initially we deleted the Ig domain, to leave only the CRHM site, but found that this receptor was poorly expressed and apparently not folded correctly as determined by mAb binding (13) (data not shown). Therefore a chimeric receptor containing the Ig domain of the related receptor chain gp130 (gp130-Ig-GR) was constructed and expressed in Ba/F3 cells. This construct expressed well and was tested for binding of anti-gp130 and anti-G-CSF-R mAbs (Fig. 1A). As expected, cells expressing (gp130-Ig-GR) bound the mAb against the G-CSF-R FNIII domains (LMM741) but not the mAb against the G-CSF-R Ig domain (LMM775). The recognition site of GPZ35 on gp130 was not previously known. Staining of (gp130-Ig-GR) with this mAb establishes that it recognizes the Ig domain. The chimera bound WT G-CSF with reduced affinity (see below) but had no detectable activity in a proliferation assay (Fig. 1B), suggesting that the receptor cannot dimerize correctly.

**Binding of G-CSF Mutants to the CRHM Site**—To determine which of the mutated amino acid residues of G-CSF interacted with the CRHM site, binding of mutant G-CSFs to (gp130-Ig-GR) was determined by cold competition with radiolabeled WT G-CSF (Fig. 2). (E46A)G-CSF bound to (gp130-Ig-GR) with a similar affinity (Kd = 0.50 nM) to WT G-CSF (Kd = 0.42 nM), indicating that this residue was not required for binding to the CRHM site and probably interacts with the Ig domain. No competition was detected with (E19A)G-CSF and only very weak inhibition with (K23A)G-CSF (Kd = 11 nM) and (D112A)G-CSF (Kd = 72 nM), showing that these residues were required for interaction with (gp130-Ig-GR), presumably with the CRHM site. Binding of WT G-CSF to the complementary chimaera (GR-Ig-gp130 (12) was not detected (data not shown).

**Proliferation Response of (R288A)GR to the Mutant G-CSFs**—To determine whether any of the G-CSF residues selected for mutation interacted with Arg288 in the CRHM of the receptor, we compared the proliferation of Ba/F3 cells expressing the WT-GR with cells expressing (R288A)GR in response to the mutant G-CSFs. Typical results are shown in Fig. 3, and the mean EC₅₀ values from two independent experiments are summarized in Table I. The mutant G-CSFs have been compared with WT G-CSF by calculating the mutant/WT EC₅₀ ratios (Table I). In the case of the WT-GR, the mutations resulted in a reduced proliferation response of 10–70-fold, with the exception of (D112A)G-CSF. As shown by the ranges given by the data, the two experiments gave somewhat different EC₅₀ values; however, the mutant/WT G-CSF ratios from each experiment were very similar (range <10%, excluding D112A which was 50%). With (R288A)GR, we were unable to add sufficient mutant G-CSF to reach plateau responses in some cases, because of the large effects of some mutant combinations; thus some EC₅₀ estimates are approximate. Nevertheless, it is clear that (E19A)G-CSF gave a similar response to WT G-CSF in cells expressing (R288A)GR, whereas the other G-CSF mutants gave a substantially reduced response. The effect of combining each of the K23A, E46A, and D112A G-CSF mutations with the (R288A)GR mutation was greater than would be predicted from the individual effects of the G-CSF- and G-CSF-R mutations. If the G-CSF and receptor mutations behaved independently, the mutant/WT ratios (Table I) should have been similar for the WT and R288A receptors.

To determine whether any of the G-CSF mutations affected the stability of the G-CSF mutants in culture, recovery of WT G-CSF and the mutants from the culture supernatant after 48 h incubation was determined by ELISA (modified from Ref. 32). Recovery of WT G-CSF was 40% and recoveries of (E19A)G-CSF and (D112A)G-CSF were greater than WT (Table I). Recovery of (K23A)G-CSF was 7.5% and (E46A)G-CSF was 15%; therefore, only a small fraction of the reduced activity of these mutants could be explained by reduced stability. Binding of the mAb used in the ELISA was not affected by the G-CSF mutations (data not shown).

**Binding of G-CSF Mutants to (R288A)GR**—To determine whether the effects of the mutations on proliferation could be
explained by changes in binding affinity, binding of the G-CSF mutants to Ba/F3 cells expressing WT-GR or (R288A)GR was compared. Typical inhibition curves for the binding of 125I-Tyr1,3-G-CSF in the presence of increasing concentrations of mutant or WT G-CSF are shown in Fig. 4. The mean $K_d$ values from two independent experiments with each receptor and G-CSF combination is given in Table II. Fig. 4A shows that the G-CSF mutations had only slight effects on the affinity of binding to the WT-GR. The K23A, E46A, and D112A G-CSFs bound less well to (R288A)GR than did WT G-CSF, showing that these G-CSF residues interacted with residues other than Arg288 on the receptor CRHM or Ig domain (Fig. 4B). In contrast, (E19A)G-CSF bound as well as WT G-CSF to (R288A)GR, indicating that Glu19 interacts with Arg288 of the receptor. As observed with the proliferation data (Table I), the mutant/WT ratios of the binding affinity of K23A, E46A, and D112A G-CSFs to (R288A)GR were greater than the corresponding ratios with WT-GR, suggesting a synergistic effect of receptor and
G-CSF mutations.

*The Effect of Charge Reversal Mutations*—If the interaction of Glu19 of G-CSF with Arg288 of the receptor were predominantly electrostatic, we would predict that charge reversal mutations would have a greater effect than charge-to-alanine mutations and that simultaneous charge reversal of both residues might restore the interaction. The proliferation of Ba/F3 cells expressing WT-GR, (R288A)GR, or (R288E)GR in response to WT, E19A, and E19R G-CSFs was determined by analysis of the data with the LIGAND program (31) and are mean values of two independent experiments.

**Fig. 2.** Binding of G-CSF mutants to Ba/F3 cells expressing the receptor chimera (gp130-Ig)GR. Unlabeled mutant or WT G-CSF was titrated in the presence of about 200 pM 125I-Tyr1,3-G-CSF and allowed to bind for 4 h at 4°C. Binding was calculated as a percentage of binding in the absence of inhibitor after subtraction of nonspecific binding. Kd values for the mutant G-CSFs were determined by analysis of the data with the LIGAND program (31) and are mean values of two independent experiments.

**Fig. 3.** Proliferation of Ba/F3 cells expressing WT-GR or (R288A)GR in response to G-CSF mutants. Uptake of [methyl-3H]thymidine was measured after 48 h culture.

**Table I**

| G-CSF Mutant | Receptor       | Recoverya |
|--------------|----------------|-----------|
| WT           | WT-GR          | 1.0       |
| E19A         | (R288A)GR      | 1.7       |
| K23A         | (R288A)GR      | 1.7       |
| E46A         | (R288A)GR      | 1.7       |
| D112A        | (R288A)GR      | 1.7       |

a Recovery of G-CSF at the end of culture determined by ELISA.

**Fig. 4.** Binding of G-CSF mutants to Ba/F3 cells expressing WT-GR or (R288A)GR. Method as in legend for Fig. 2. 125I-Tyr1,3-G-CSF was used at about 100 pM for WT-GR and about 200 pM for (R288A)GR.

**Table II**

| G-CSF Mutant | Receptor       | Kd (nM) | Mut/WTb |
|--------------|----------------|---------|---------|
| WT           | WT-GR          | 0.045 ± 0.008 | 1.0     |
| E19A         | (R288A)GR      | 0.050 ± 0.004 | 1.1     |
| K23A         | (R288A)GR      | 0.077 ± 0.015 | 1.7     |
| E46A         | (R288A)GR      | 0.076 ± 0.003 | 1.7     |
| D112A        | (R288A)GR      | 0.060 ± 0.003 | 1.3     |

b Ratio of Kd for mutant G-CSF/WT G-CSF.

c Data are mean ± S.D. of three assays, including data shown in Fig. 4.

| Recoverya |
|-----------|
| 1.0       |

a Data are means ± range of two assays, including data shown in Fig. 3.

b Data are means ± range of two assays, including data shown in Fig. 3.

c Data are means ± range of two assays, including data shown in Fig. 3.

d No response detected.

explain the low activity. However, with (R288A)GR, this effect disappeared and all three G-CSFs behaved similarly, as would be expected when the charge interaction was removed. This result also shows that the E19R mutation did not have a detectable effect on the folding of the mutant G-CSF. The response of (R288E)GR to WT G-CSF was only slightly reduced in comparison with the response of (R288A)GR, suggesting that
that is equivalent to growth hormone site II (21). Of the residues interacting with the CRHM, we conclude that only Glu19 in the A helix interacts with Arg288 of the receptor. The large loss of activity of (E19RG-CSF in comparison with (E19AG-CSF shows that Glu19 probably makes an electrostatic interaction with Arg288. Glu19 is sandwiched between Lys16 and Lys23 in the A helix (2), creating a net single positive charge in this region that the charge reversal (E19R) converts to a triple positive charge. This large change in charge could explain the substantial loss of activity of (E19RG-CSF with the WT-GR. It is interesting that the binding affinity for this combination was not proportionately reduced (Table III), suggesting that the mechanism responsible for the loss of activity in this case is different from that with (R288A)GR, which had a similar binding affinity but not as great a loss of activity with any of the G-CSF mutants. The charge reversal mutation of the receptor, (R288E)GR, did not have as great an effect as the charge reversal mutation of G-CSF. It seems likely that the receptor has folded slightly differently to accommodate the substitution, thus reducing its effect. Arg288 is in the F–G loop of the BC domain, which was observed to be highly flexible in an NMR study (18). In addition, Arg is a smaller residue than Lys, thus rearrangement of the F–G loop may be possible when two Glu residues are involved in the complex (WT-G-CSF and (R288E)GR) but not when there are two Arg residues ((E19RG-CSF and WT-GR).

Similar electrostatic interactions have been described in the human GM-CSF-receptor complex (33, 34) and the IL-4-IL-4 receptor α chain complex (35). Arg280 in the α-chain of the GM-CSF-R makes an electrostatic interaction with Asp112 in the D helix of GM-CSF (33), whereas Glu23 in the A helix probably interacts with Tyr365, His367, and Ile368 of the β-common receptor chain (34). IL-4 binding to the IL-4 receptor α chain involves a patch of charged residues on the A and C helices that is likely to interact with charged residues on the receptor (35). In other cases such as the growth hormone-receptor complex, the most important interactions are hydrophobic (36). The binding of (E46AG-CSF and (D112A)G-CSF to (R288A)GR revealed synergy or cooperativity between the receptor and G-CSF mutations, because the combined effect was substantially greater than predicted from the effect of each individual mutation. This observation suggests that a single mutation may be partially compensated for by the neighboring residues making alternative contacts in the receptor-ligand complex. However, when there are two mutations, compensatory contacts may no longer be possible, leading to greater conformational perturbation in the complex than was seen with single mutations.

The two recently reported mutagenesis studies of G-CSF differ in their conclusions about whether there are one or two receptor-binding sites (22, 23). Both agree that there is a binding site involving charged residues on the A and C helices of G-CSF, and our data on the effects of mutating Glu19 and Lys23 in helix A and Asp112 in helix C further confirm this. Reidhaar-Olson et al. (22) proposed a second binding site in G-CSF involving Lys40, Phe144, Val149, and Leu49. These residues, other than Lys40, are hydrophobic, suggesting that this site may interact with a hydrophobic region of the receptor. Like Lys40, Val144, and Leu49, Glu46 is in the 310 helix in the A-B loop of G-CSF (2) and may thus form part of this second binding site. Although Young et al. (23) found no evidence that Lys40 was important, they did not test the other residues. We also have found no biological effect of a K40A mutation (data not shown), but the effects of (E46AG-CSF provide support for the existence of a second binding site involving the 310 helix of G-CSF.
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**Fig. 6. Model of the G-CSF receptor-ligand complex.** G-CSF interacts with the CRHM of one receptor (site II) and the Ig domain of the second receptor (site III). Interacting amino acid residues are shown.

and the Ig domain of the receptor (site III in Fig. 6). Alternatively, the residues in site III may not interact directly with the receptor but may be important structurally for stabilizing the complex with the G-CSF-R.

The chimeric receptor (gp130-Ig)GR was not able to transduce a proliferative signal in response to G-CSF, although it appeared to be folded correctly and bound G-CSF, albeit with reduced affinity. The reduction in binding affinity in itself did not account for the lack of response because (R288A)GR had a similarly reduced binding affinity and was able to transduce a response. We conclude that exchange of the Ig domain has reduced binding affinity, creating a receptor that cannot dimerize and contains a single ligand-binding site in the CRHM. This is consistent with previous studies of soluble G-CSF-R domains showing that the Ig domain is necessary for receptor dimerization (19).

In addition, our data on (E46A)G-CSF suggest that the Ig domain interacts specifically with site III on G-CSF, rather than with the Ig domain of the second G-CSF-R, to cause dimerization (Fig. 6). A recent study using Fourier-transformed infrared spectroscopy detected a conformational change in G-CSF following receptor binding which likely reflects change of the $\alpha$ helix to an $\alpha$ helix, consistent with the suggestion that this region is important in the G-CSF-G-CSF-R complex (37).

Our proposed G-CSF-R complex has many similarities with the complex proposed for IL-6 with the IL-6 receptor (IL-6R) and gp130 (38). In this hexameric complex (consisting of two molecules each of IL-6, IL-6R, and gp130), IL-6 has three sites that are important for complex formation as follows: site I that binds to the IL-6R (39) and sites II and III that contact gp130 (40, 41). Site II comprises residues in helices A and C, and site III includes residues at the beginning of the D helix and residues in the AB loop of IL-6. G-CSF has a binding site equivalent to site II in IL-6 (22, 23) and may have a site equivalent to site III (this study and Ref. 22). Thus the topology of the G-CSF-G-CSF-R complex may resemble that of the IL-6-IL-6R-gp130 complex, without the IL-6R and the interaction with site I. In both cases, the Ig domain is required for formation of a signaling complex (10, 12).

We propose that G-CSF first binds through site II to the CRHM of one receptor. This binding induces a conformational change in G-CSF and the G-CSF-R that allows interaction of the Ig domain of the second receptor with the $\beta$ helix region and possibly the N terminus of the D helix of G-CSF (site III), resulting in receptor dimerization and formation of a 2:2 (G-CSF-G-CSF-R) complex. There is evidence from other studies that both G-CSF and the G-CSF-R undergo conformational changes in the receptor complex (15, 37). Moreover, we have not been able to detect binding of G-CSF to the Ig domain in a chimeric receptor, (GR-Ig)gp130 (12), which is consistent with the proposed requirement for a conformational change in G-CSF and/or the G-CSF-R for binding to occur. In addition, it suggests that the CRHM is the main ligand-binding site. There is some evidence that the growth hormone receptor also undergoes conformational changes after ligand binding that are required for biological responsiveness (42, 43). Thus, receptor activation by cytokines may require more than simply achieving physical proximity of signal transducing receptor components. Our model of G-CSF-G-CSF-R complex formation accounts for the published data pertaining to the structure of this complex (15, 19, 20, 37) and suggests that maturation of the Ig domain of the G-CSF-R will be informative.

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**TABLE III**

| Mutant/WT ratio | WT | 0.019 ± 0.009 | 0.051 ± 0.013 | 5.6 ± 1.3 | 0.39 ± 0.03 | 19 ± 3 | 0.66 ± 0.09 |
|-----------------|----|---------------|---------------|----------|-----------|-------|------------|
|                 | E19A | 0.14 ± 0.01 | 0.059 ± 0.016 | 9.8 ± 0.79 | 0.31 ± 0.05 | 40 ± 4 | 0.62 ± 0.05 |
|                 | E19R | 270 ± 135 | 0.35 ± 0.01 | 9.0 ± 0 | 0.30 ± 0.01 | 15 ± 2 | 0.32 ± 0.003 |
|                 | Mutant/WT ratio | WT | 1.0 | 0.01 | 0.059 | 0.009 | 0.051 | 0.79 |
|                 | E19A | 7.4 | 1.2 | 0.01 | 0.059 | 0.009 | 0.051 | 0.79 |
|                 | E19R | 14,000 | 6.9 | 0.01 | 0.059 | 0.009 | 0.051 | 0.79 |

$^{a}$ Mean ± S.D. of three assays using one cell line expressing each receptor, including data from Fig. 5.

$^{b}$ Ratio with respect to WT G-CSF and WT-GR.
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