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Dimerization of Granulocyte-Colony Stimulating Factor Receptor: The Ig Plus CRH Construct of Granulocyte-Colony Stimulating Factor Receptor Forms a 2:2 Complex with a Ligand

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We have previously shown that the extracellular domain of granulocyte-colony stimulating factor receptor (soluble G-CSFR), prepared from CHO cell conditioned media, dimerizes upon binding its ligand, G-CSF. The most stable ligand-receptor complex occurs at a 2:2 stoichiometry, unlike the growth hormone and erythropoietin systems. In the latter cases, each ligand uses two binding sites to bring two receptors together. In this study, we have generated a truncated human G-CSF receptor, known to be sufficient for high affinity ligand binding, which consists of an Ig-like domain and a cytokine receptor homology module. With an affinity purified receptor, sedimentation equilibrium experiments clearly demonstrated that this truncated form of the receptor behaves very similarly to the entire extracellular domain. The sedimentation equilibrium data are consistent with the model that the truncated receptor has a weak tendency to self-associate into a dimer in the absence of a ligand, this receptor-receptor interaction is enhanced by ligand binding, and the most stable complex occurs at a 2:2 stoichiometry. These results are very different from those described by others for various murine G-CSF receptor constructs from either *Escherichia coli* or insect expression systems.

Key words: G-CSF receptor, sedimentation equilibrium, dimerization, receptor purification.

Cytokines play a major role in mediating information flow between cells. They regulate cell growth, differentiation, and programmed death by binding to their cognate receptors. Ligand-induced dimerization of cell surface receptors leads to interactions of their cytoplasmic domains. Therefore, understanding how ligand binding causes a receptor to dimerize is a first step for studying the process of ligand-induced signaling. We have been studying this process for a variety of ligand-receptor pairs.

Recently, we purified a Chinese hamster ovary (CHO) cell-derived soluble human G-CSF receptor, which consists of an Ig domain, a CRH module, and three FNIII domains (*1*). The CRH module can be divided into two subdomains, BN and BC (*2-4*). Because of 9 potential *N*-glycosylation sites in the human sequence, this recombinant CHO cell-derived receptor is heavily glycosylated (*1, 5*). When mixed with G-CSF, the receptor forms a stable complex with a stoichiometry of 2 G-CSF: 2 receptor; *i.e.*, the ligand dimerizes the receptor, as expected from the cases of other cytokine receptors, but unexpectedly with a stoichiometry of 2:2 (*1*). Using a different expression system, Ohta and his associates generated different forms of the murine receptor, *i.e.*, Ig-CRH, and Ig-BN, BN-BC, BN, and BC, all of which showed a stoichiometry of ligand complexes different from that observed by us (*6-9*). In order to determine if the three FNIII domains present in the sG-CSFR used in our study played any role in the observed 2:2 stoichiometry, we have now generated a truncated sG-CSFR, consisting of the Ig domain and the CRH module, using a CHO cell expression system. As reported in this paper, we confirmed that the truncated human sG-CSFR forms a 2:2 receptor-ligand complex in a similar fashion to the full-length sG-CSFR. Hence the three FNIII domains play no apparent role in complex formation, which is consistent with the conclusion of Fukunaga *et al.* (*10*) on cell-bound receptor deletion analysis.

MATERIALS AND METHODS

Cloning and Expression of Truncated sG-CSFR—PCR primers for the expression of codons 1-328 of the human G-CSF receptor were synthesized: 5′-GTCTCTAGACCA-CCATGGCAAGGCTGGGAAACT-3′ and 5′-GGAGTCGA-CTTATCTCAGCTCCAGGCTGGGGCT-3′. PCR amplification was performed with Pfu DNA polymerase as described by the manufacturer (Stratgene, La Jolla, CA) for 32-step cycles (30 s at 94°C, 1.3 min at 74°C) in a volume of 50 μl with 1 ng of a plasmid containing the full length sG-CSFR coding region. The product was digested with *Xba*I and *Sal*I, cloned into mammalian expression vector pDSRα

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Abbreviations: G-CSF, granulocyte-colony stimulating factor; CHO, Chinese hamster ovary; Ig, immunoglobulin; CRH, cytokine receptor homology; BN, amino-terminal domain of CRH module; BC, carboxy terminal domain of CRH module; FNIII, fibronectin type III; sG-CSFR, soluble G-CSF receptor; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HEPES, (*H*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid]).

(*11*), and verified by DNA sequencing.

The expression plasmid (3 μg), which contains a dihydrofolate reductase expression cassette, was introduced into CHO-D$^-$ cells by calcium phosphate-mediated transfection (*12*). Colonies appearing after selection were assayed for truncated sG-CSFR expression by Western blotting with G-CSFR antibodies (see below). One positive colony was selected for further expansion. Roller bottles were inoculated with 2×10^7 cells in 200 ml of growth medium (DMEM:Ham's F12 [1:1] supplemented with nonessential amino acids, and 5% fetal bovine serum [reagents from Gibco, Grand Island, NY]). After the cells had reached confluence in 3–4 days, the medium was replaced with 200 ml of growth medium with no serum. Conditioned medium was harvested after 4 days.

Antisera Preparation—New Zealand White rabbits were injected subcutaneously on day 1 with 0.2 mg full-length sG-CSFR and an equal volume of Freund's complete adjuvant. Further boosters (Days 7, 21, 35, and 56) were given with the substitution of Freund's incomplete adjuvant. Antibody titers were monitored by enzyme-linked immunoassaying. After the third booster, 20 ml of blood was obtained from each animal.

Purification—CHO cell-conditioned medium containing truncated sG-CSFR was concentrated 3-fold with a Pellicon tangential flow ultrafiltration device (Amicon, Danvers, MA) fitted with a 10K molecular weight cutoff filter (Filtron, Bearfoot, MA) after adding phenylmethylsulfonyl fluoride to 40 μg/ml. The concentrate was diafiltered with 4 volumes of 20 mM Bis-Tris propane, 40 mM NaCl, pH 7.45, and then mixed with Q-Sepharose Fast Flow (Pharmacia) equilibrated in the same buffer (1.5 ml to 100 ml conditioned media). The column was eluted with a linear gradient of 40–450 mM NaCl in 20 mM Bis-Tris propane, pH 7.45. Aliquots of column fractions were applied to 4–20% polyacrylamide gels (Novex) and run under reducing SDS-PAGE conditions, followed by electroblotting onto nitrocellulose membranes at 10 V for 18 h at room temperature in 25 mM Tris-glycine, 20% methanol. After blotting, each membrane was treated with polyclonal rabbit antisera to the sG-CSFR (1:1,500 dilution) and then donkey anti-rabbit Ig horseradish peroxidase conjugate (1:6,000 dilution), followed by enhanced chemiluminescence detection reagents (Amersham Life Science). The fractions which contained the truncated receptor were pooled and batch-bound to G-CSF Sepharose at 4°C for 18 h with gentle mixing. The G-CSF Sepharose was prepared by mixing 2.5 mg recombinant human G-CSF per ml CNBr-activated Sepharose (Pharmacia) and blocking unreacted sites with 1 M ethanolamine HCl at pH 8.1 (Aldrich). The truncated receptor bound to G-CSF Sepharose was eluted with Immunopure neutral elution buffer (Pierce), and immediately dialyzed against 20 mM HEPES, 100 mM NaCl, pH 7.2, at 4°C. The dialyzed material was concentrated by stirred cell ultrafiltration and then applied to a Superose 12 gel filtration column (Pharmacia) equilibrated in 20 mM HEPES, 300 mM NaCl, pH 7.2. Fractions corresponding to the major peak were pooled and diafiltered in a stirred cell to reduce the concentration of NaCl to 150 mM.

Sedimentation Equilibrium—Sedimentation equilibrium data were acquired with a Beckman Optima XL-A centrifuge at 25°C in 0.1 M potassium phosphate buffer, pH 6.95, using absorbance scans at either 280 or 230 nm. The experimental protocols and data analysis followed the methods used for the full-length sG-CSFR, as described in Horan *et al.* (*1*) and references therein.

RESULTS

The purification of the truncated form of sG-CSFR is analogous to that we reported for the entire extracellular domain. Both receptors display relatively weak binding and similar elution from Q-Sepharose at pH 7.45. Ligand affinity chromatography on G-CSF Sepharose was also found to be useful for purifying the truncated soluble receptor. As before with the full-length sG-CSFR (*1*), nearly all of the receptor, which was eluted from the ligand affinity column using Immunopure elution buffer, was active. When subjected to gel filtration, a small percentage of the affinity purified material formed a shoulder preceding the major peak (see Fig. 1), and was removed from the sample used for studying interactions with G-CSF. The major peak was eluted at a position corresponding to an apparent molecular weight of 44,000, which is higher than the monomeric molecular weight calculated from the amino acid sequence, *i.e.*, 33,500. When the purified truncated receptor was run on an SDS-PAGE gel, an apparent molecular weight of 47,000 was observed (Fig. 2). These data are consistent with the receptor being highly glycosylated.

In accord with the molecular weight estimated on gel filtration, sedimentation equilibrium data for the truncated sG-CSFR clearly indicate that it is predominantly monomeric at concentrations of <1 mg/ml. Our earlier studies on the full-length soluble receptor established that it dimerizes through self-association even in the absence of a

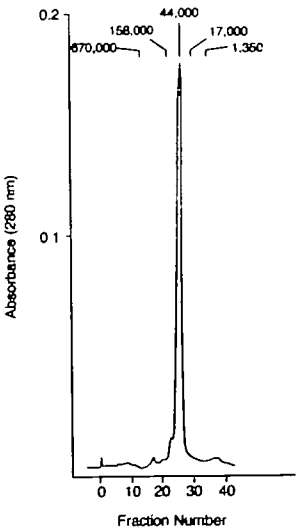

Fig. 1. **Superose 12 gel filtration chromatography of affinity purified truncated sG-CSFR.** Approximately 0.5 mg was loaded onto the column and eluted as described under "MATERIALS AND METHODS." The column was monitored at 280 nm using 1.0 AUFS and calibrated with gel filtration standards from BioRad (Hercules, CA). The standards consisted of thyroglobulin (670,000), bovine gamma globulin (158,000), chicken ovalbumin (44,000), equine myoglobin (17,000), and vitamin B-12 (1,350). The elution position of each standard is shown above and the fractions collected from the column are shown below.

ligand, but that this self-association is very weak ($K_d = 200$ μM), so significant amounts of the dimer are only formed with high protein concentrations. The data for the truncated sG-CSFR are also consistent with a weak tendency to dimerize. Because of the limited availability of this protein, we were unable to carry out sedimentation equilibrium at protein concentrations high enough to allow highly accurate assessment of its monomer-dimer equilibrium. The data obtained are best fitted with $K_d = 120$ μM, but the 95% confidence interval for this parameter extends from 50 to 390 μM. Thus, within this uncertainty, the self-association of the truncated form is indistinguishable from that of the full-length soluble receptor. The data are consistent with a monomer molecular weight of 47,200 [45,300 to 48,700],[2] implying 28.3% carbohydrate [25.2 to 30.4%].

Before discussing results for mixtures of the truncated receptor with G-CSF, it is worthwhile to review the results we obtained previously for the full-length form. We found that the full-length form has a very weak tendency to dimerize in the absence of G-CSF, and that this weak receptor-receptor interaction is very strongly enhanced as a consequence of G-CSF binding, leading to the formation of 2:2 receptor-ligand complexes. The thermodynamic binding model used to explain these results is shown in Fig. 3 along with the dissociation constants determined from sedimentation equilibrium data. These results showed that the association of the two receptors is enhanced ~2,000-fold when both receptors have bound G-CSF. The binding to G-CSF itself is too tight to be well determined directly, but thermodynamic linkage implies that G-CSF binding to preformed receptor dimers is 20-80-fold stronger than that to receptor monomers.

The qualitative sedimentation equilibrium data for the truncated receptor plus G-CSF closely resemble those for the full-length sG-CSFR, with G-CSF clearly producing extensive dimerization of the receptor over wide ranges of mixing ratios and total protein concentrations, and with a 2:2 complex as the highest molecular weight species. More quantitative analysis of the interactions of these proteins was performed by globally fitting data obtained in 18 experiments, encompassing several different molar ratios of the two proteins, total protein concentrations, and rotor speeds, to the binding model shown in Fig. 3. These 18 data sets can be well fitted by this model, but the fitted parameters indicate that the association of a G-CSF-liganded receptor with an unliganded one ($K_{dim,2}$) is indistinguishable from the association of two liganded receptors ($K_{dim,3}$). Therefore, these results are well fitted by a simpler model in which these two associations are required to be equivalent, a model which corresponds to "model 7" of Horan *et al.* (*1*). Figure 4 shows all 18 data sets overlaid by the fitted curves with such a model, as well as the residuals from the fit. While not perfect, this model gives a good overall representation of the data. In contrast, if we try to fit these data with the same model and values as those used for the full-length soluble receptor, the fit is unacceptably poor and gives 34% greater variance. This binding model and the best-fit dissociation constants are summarized in Fig. 5. As before, the binding of G-CSF to receptor monomers or dimers exhibits such high affinity that we cannot accurately measure this K_d, but the interactions between the receptors

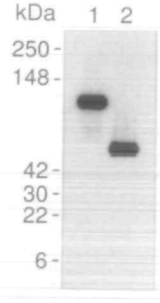

Fig. 2. **Western blot of affinity purified G-CSF receptors.** Molecular weight standards (Novex), 10 ng of full-length extracellular G-CSF receptor (lane 1), and 20 ng of truncated receptor (lane 2) were electrophoresed on a 4-20% gel, electroblotted, and detected as described under "MATERIALS AND METHODS." The relative mobilities of the molecular weight standards are shown to the left of the receptors and are as follows: myosin (250,000), phosphorylase B (140,000), carbonic anhydrase (42,000), myoglobin (30,000 and 22,000), and aprotinin (6,000).

[2] Values within square brackets indicate 95% confidence intervals for fitted parameters.

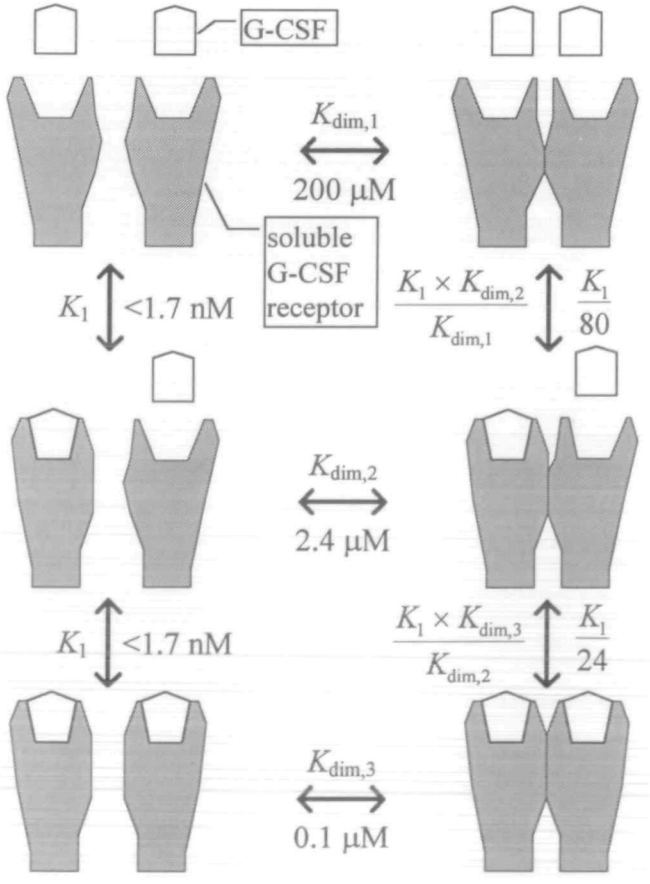

Fig. 3. **Binding model and dissociation constants derived on global analysis of 18 sedimentation equilibrium data sets for mixtures of G-CSF and the full-length sG-CSFR.** The binding model is actually defined by the indicated reaction pathways and binding constants. The pictures of the receptor and ligand are merely schematic, and any changes in their structures upon ligand binding and/or receptor self-association are meant only to illustrate different affinity states, *i.e.* not specific structural features or mechanisms.

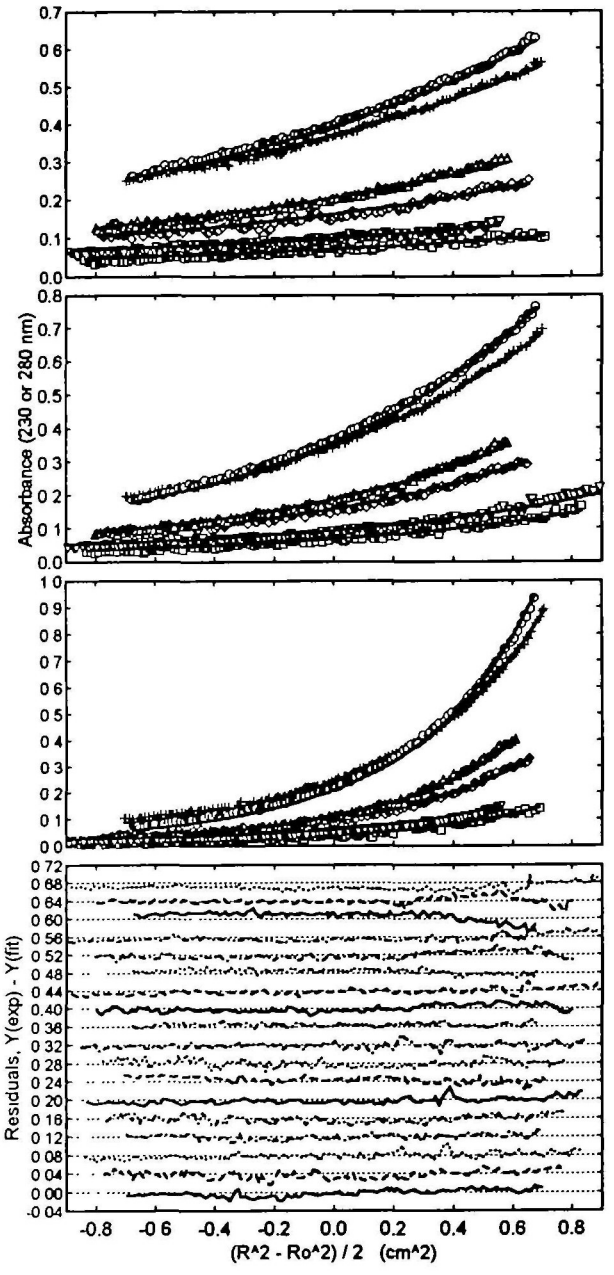

Fig. 4. **Sedimentation equilibrium data and fitted curves for mixtures of the truncated receptor and G-CSF are shown in the upper three panels, which correspond to experiments at 7,000, 9,000, and 13,000 rpm (from top to bottom).** All 18 sets of experimental data were globally fitted to the same binding model, giving the results summarized in Fig. 5. The bottom panel shows an expanded view of the residuals for each of the 18 experiments.

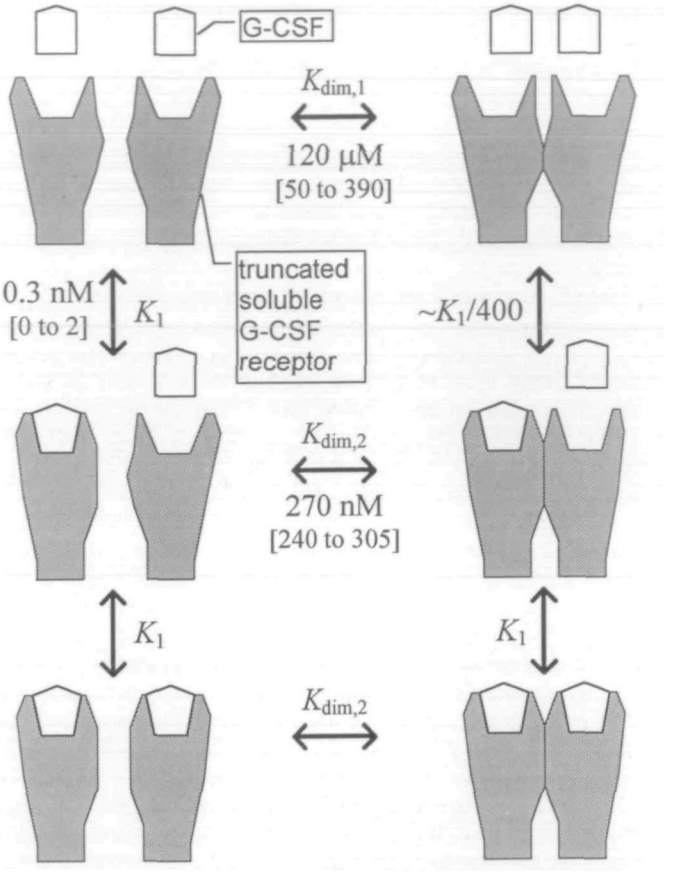

Fig. 5. **Binding model and dissociation constants for the truncated receptor derived from sedimentation equilibrium data shown in Fig. 4.** The 95% confidence interval for each of the fitted dissociation constants is shown within square brackets.

are well determined. Also, during this analysis we fixed the strength of the association between unliganded receptors at the value determined when the truncated receptor was run alone; the fairly large uncertainty of that value does not influence the results, since even an order of magnitude change has almost no impact on the values of the fitted parameters.

These results show in more detail that the overall interactions and dimerization mechanism of the truncated sG-CSFR are qualitatively similar to those of the full-length form. When G-CSF is bound, the receptor-receptor interaction is strongly enhanced (~400-fold), and thus receptor dimerization occurs *via* the enhanced receptor-receptor bonds. This ~400-fold enhancement of the receptor-receptor interaction also implies (through thermodynamic linkage) that an unliganded sG-CSFR dimer has an ~400-fold higher affinity for binding G-CSF than the unliganded monomer. The receptor-receptor dissociation constant of 270 nM, when both receptors are liganded, is quite similar to the 100 nM value for the equivalent reaction with the full-length form. The principal difference between the full-length and truncated receptors arises for the association of a liganded receptor with an unliganded one, which gives rise to a 2:1 receptor:ligand complex. For the full-length receptor, this association is significantly (~20-fold) weaker than that when both receptors are liganded. For the truncated receptor, it appears that only one receptor needs to be liganded to promote a strong receptor association, and the simpler model shown in Fig. 5 (which has one fewer parameter to be fitted) appears to be sufficient to explain the data.

DISCUSSION

Sedimentation, electrophoretic and gel filtration analyses of the truncated human sG-CSFR indicated that the recep-

tor is highly glycosylated. Peptide mapping together with sequence analysis of the full-length human sG-CSFR showed that of 9 potential *N*-linked glycosylation sites, 8 are glycosylated, 4 of which are present in the Ig and CRH domains (*5*). From the carbohydrate content of the truncated receptor determined on sedimentation analysis, *i.e.*, 13,500, each site contains an average molecular weight of ~3,300. This would correspond to tri- to tetraantennary structures on *N*-linked glycosylation for the truncated form.

Detailed binding analysis involving sedimentation equilibrium data showed quantitative differences in the behavior of the truncated and full-length soluble receptors, particularly with regard to the association of a liganded receptor with an unliganded one. This suggests that the FNIII domains influence ligand binding and receptor dimerization. However, while we believe the differences in the behavior of the truncated form are real, in our view what is more significant is the overall similarity of the two receptor forms. These data clearly establish that the truncated form is sufficient to produce all the fundamental behavior of this system: ligand binding with nanomolar affinity, weak receptor dimerization in the absence of a ligand, formation of 2:2 complexes, and a strongly enhanced receptor-receptor interaction when a ligand is bound.

With regard to the differences between the truncated and full-length forms, given that the G-CSF receptor exhibits conformationally-regulated self-association, it seems quite possible that deletion of the FNIII domains might influence the transitions between receptor conformations that strongly and weakly self-associate, and thereby change the detailed energetics of the interactions, even if the FNIII domains are themselves not directly involved in either ligand binding or receptor-receptor interactions. It is also important to remember that the conformations of all soluble forms of the receptor might differ significantly from that of the holoreceptor due to relaxation of constraints imposed by the transmembrane domain. For example, it is possible that in the full-length sG-CSFR the lack of restraints at the carboxyl terminus allows the FNIII domains to adopt a conformation which causes some steric interference with receptor dimerization (and indeed our data cannot rule out a 6-fold difference in the dimerization of the unliganded receptors), and thus removal of the FNIII domains may give a form that more accurately reflects the interactions of the holoreceptor.

Hiraoka *et al.* (*7*) observed a much greater tendency for the unliganded receptor to dimerize when their murine Ig-CRH construct was expressed in insect cells. After affinity-purification with low pH elution, it was essentially all dimeric, as determined by gel filtration. Although we have no clear explanation for this discrepancy in dimerization behavior between this preparation and our CHO cell-derived material, it is possible that there are significant differences between the murine and human receptors. It is also possible that the different degrees and types of glycosylation resulting from the different expression systems may also be significant. Insect cells typically synthesize *N*-linked high mannose type sugar linkages, while CHO cells form complex carbohydrates with varying amounts of sialic acids. We also note that Hiraoka *et al.* (*7*) eluted the receptor from their ligand affinity column under low pH conditions (which causes significant loss of G-CSF binding activity for our human receptor), whereas we used a neutral pH elution step as described under "MATERIALS AND METHODS." An even more surprising difference in the results is their observation that the insect cell-derived material was converted from a dimer to a tetramer upon mixing with G-CSF at a 1:1 molar ratio. Although the observed molar ratio is the same as that obtained in the present study, we have been unable to detect the formation of any 4 G-CSF:4 receptor complexes by our CHO cell-derived material. Again, this difference may be due at least partly to the different species, expression systems, or elution conditions.

However, it is possible to easily reconcile these results if the dimerization of their construct occurs through a site different from that responsible for ligand-induced dimerization. That is, we believe G-CSF binding to their receptor dimer promotes new receptor-receptor interactions, and a second receptor dimer binds, giving rise to the 4:4 complex.

Ota and his associates have also studied various truncated forms of murine sG-CSF receptor, including Ig-BN, BN-BC, BN, and BC (*6, 8, 9, 13*), obtained using an *Escherichia coli*-expression system. Although all of these forms bound G-CSF with high affinity, none of them showed dimerization in the presence of G-CSF. However, they did observe dimerization when Ig-BN and BN-BC were mixed together in the presence of G-CSF, with a molar ratio of 1:1:1 (*13*). This result, which had not appeared at the time our results for the full-length receptor were submitted, seems to be in conflict with our model and is worthy of further discussion. They explain this result by assuming that G-CSF has two binding sites, one for the Ig or Ig-BN interface and the other for the BC or BN-BC interface, and hence that G-CSF is bivalent, like growth hormone or erythropoietin, with two different receptor molecules binding to these two different sites.

Recent alanine-scanning mutagenesis studies provided strong support for the existence of two distinct binding sites on G-CSF (*14*). However, it is important to remember that the existence of two binding sites does not necessarily imply that these sites interact with two different receptors; in receptor constructs containing Ig, BN, and BC domains, a single receptor may span both sites. Furthermore, the suggestion that G-CSF dimerizes through a mechanism similar to that in the case of growth hormone is inconsistent with the preferred 1:1 receptor:ligand ratio, which was observed in both our studies and those by Ohta's group for all forms containing Ig, BN, and BC domains. In our view, the 1:1:1 complex of Ig-BN, BN-BC, and G-CSF may represent an abnormal state, possibly resulting from the splitting of the cytokine receptor homology molecule into two fragments. That is, G-CSF binds Ig-BN and BN-BC constructs simultaneously through its two binding sites, but not necessarily in a way that bridges these two fragments, as in growth hormone or erythropoietin-induced receptor dimerization, and perhaps binds these fragments in a way that would not be possible for the holoreceptor.

Overall, we do not believe there is any fundamental disagreement between our results and assembly model, and the results of Ota and coworkers, or between our model and the existence of two binding sites on G-CSF. We both agree that Ig-BN-BC is the minimal construct that possesses all the normal behavior of the G-CSF receptor, and that such

constructs prefer to bind G-CSF with a 1:1 stoichiometry, not the 2:1 stoichiometry characteristic of the growth hormone paradigm. Conceptually, our views differ in that we believe the two binding sites of G-CSF are each normally engaged by a single receptor, inducing a conformation with stronger receptor-receptor interactions, and leading to receptor dimerization, and not that the two different sites bring the receptors together by simultaneously binding two different receptor molecules.

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