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The interaction of G-CSF with its receptor

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1. ABSTRACT

The function of the G-CSF receptor has been of considerable interest, particularly because of the clinical usefulness of G-CSF. The first step in receptor activation, which is the interaction of G-CSF with its receptor, has been studied by mapping the binding sites of neutralizing antibodies, by studying the complexes formed between G-CSF and various receptor fragments in solution and by mutagenesis of the receptor and ligand. In addition, the structure of G-CSF has been determined. Part of the ligand-binding domain of the receptor in complex with G-CSF has been crystallized and its structure described. Consideration of all these studies has allowed us to make a model of the complete ligand-binding domain in complex with G-CSF that accounts for the published data. The complex has a 2:2 stoichiometry, with two binding sites on both the ligand and receptor that are equivalent to site II and site III of the IL-6 receptor complex. This model was based on the published structure of gp130 in complex with viral IL-6, which we believe to be very similar.

2. INTRODUCTION

The G-CSF receptor (G-CSF-R) is expressed predominantly on blood cells of the neutrophilic granulocytic lineage, in which it is required for proliferation, differentiation and survival (1, 2). Activation of the receptor by G-CSF initiates downstream signaling cascades that include the Jak-STAT and MAP kinase pathways (2). Deletion of either the G-CSF gene or the G-CSF-R gene in mice results in neutropenia and a compromised response to some types of infection (3-5).

The G-CSF-R gene was first cloned in 1990 and analysis of its predicted amino acid sequence showed that it comprised six structural domains of approximately 100 amino acid residues each in the extracellular region, a single transmembrane domain and an intracellular domain without intrinsic kinase activity (6-8). The extracellular domains are commonly called D1–D6, with the N-terminal D1 being an immunoglobulin-like (Ig) domain and D2-D6 being fibronectin type III (FN III) domains (Figure 1A). FN

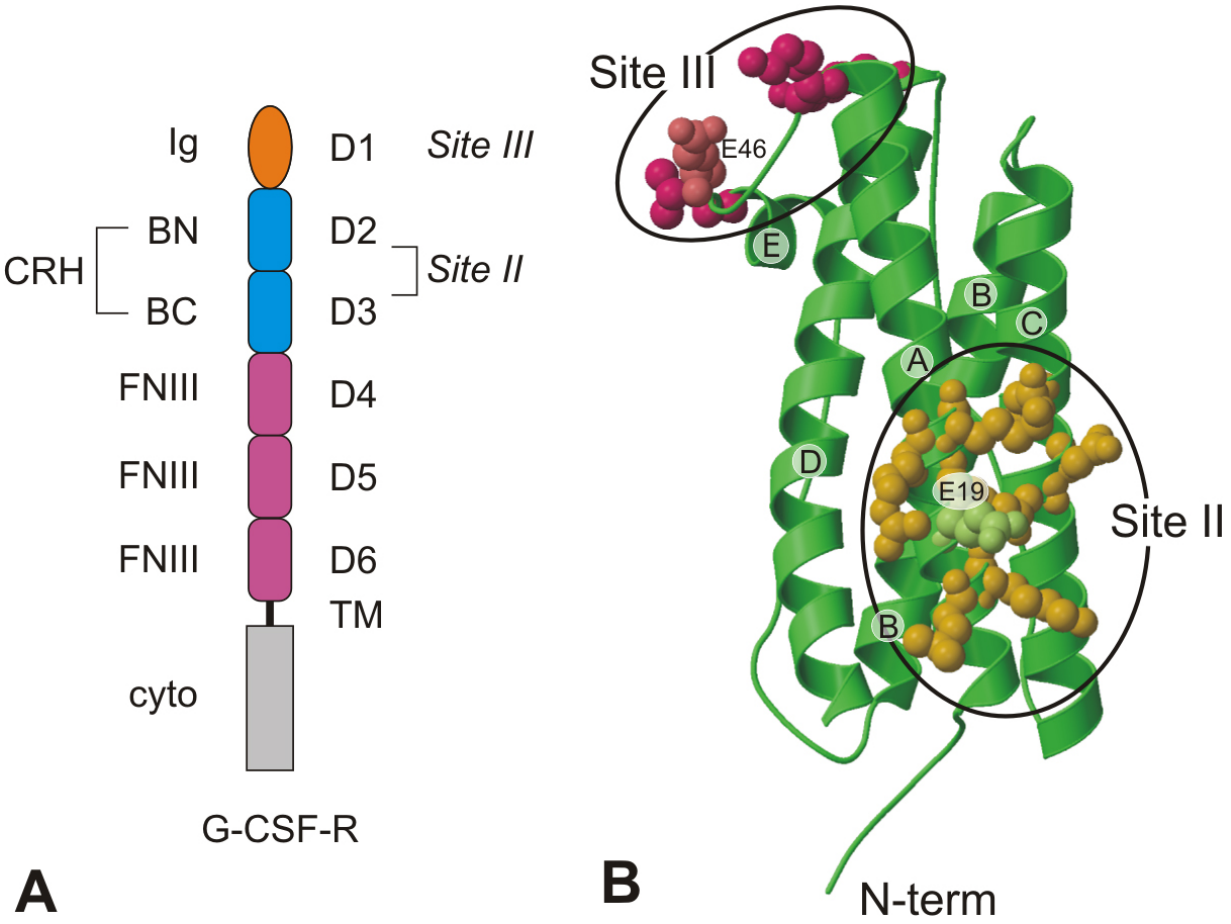

Figure 1. Representations of the G-CSF receptor and ligand. A, domain structure of G-CSF-R showing the six extracellular domains (labeled D1-D6), the transmembrane domain and the cytoplasmic domain (cyto). D1 is an Ig-like domain, and together D2-D3 (sometimes referred to as BN and BC domains) are called the CRH module. Receptor binding site II is on the CRH module and binding site III is on the Ig domain. B, ribbon diagram of G-CSF showing the main helices labeled A-D and the additional E-helix in the A-B loop. The residues involved in receptor binding (Site II: L15, K16, E19, Q20, R22, K23, D109 & D112; site III: K40, E46, F144 & L49) (34) are shown in space filling representation. The central residues of sites II and III are E19 and E46 respectively and are shaded differently.

III domains contain 7 β-strands (A-G) arranged in 2 β-sheets with flexible loops connecting the strands. Ig domains are similar, but the fourth strand is switched from the second sheet to the first (9). This domain structure is most similar to that of gp130, the signal-transducing receptor chain of the IL-6 receptor family, with which it shares 46% amino acid sequence similarity in the extracellular region (10). Domains D2 and D3 form the cytokine-receptor homologous (CRH) module, which contains 4 cysteine residues and a WSXWS motif conserved in the type I cytokine receptor family (11). In total, there are 8 disulphide bonds in the extracellular region: 2 in D1, 3 in D2, 2 in D3 and 1 in D4 (12). The human G-CSF-R contains 3 free cysteine residues in addition to those forming disulphide bonds, but these are not conserved in the murine receptor. There are 9 potential N-linked glycosylation sites (7). Eight of these were fully or partially glycosylated in the extracellular region expressed in CHO cells and appear to be required to protect the protein from aggregation in solution (12). Little is known about the function of the D4-D6 domains, which do not appear to be involved in ligand binding (13).

The structure of human G-CSF has been determined by both X-ray crystallography (14) and NMR spectroscopy (15, 16), revealing a 4-alpha-helical bundle protein, as predicted by Bazan (Figure 1B) (17). In addition to helices A-D, there is a short E-helix in the A-B loop. The 4-alpha-helical structure is shared by all the ligands of class I cytokine receptors, although they have little sequence similarity. Prior to the structure determination, a region of G-CSF important for function was identified by mapping the binding site of neutralizing antibodies (18). This region formed part of the A helix and the beginning of the A-B loop. Later mutagenesis studies of G-CSF have established that residues in the A and C helices form a binding site for the receptor (19, 20). In addition, residues near the N-terminal end of the D helix that could form a second binding site were identified (20).

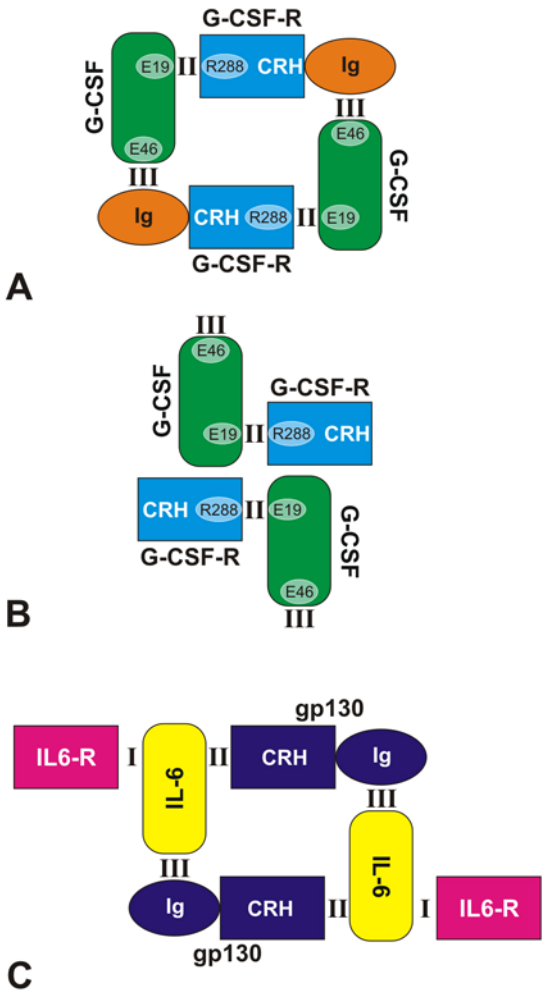

Figure 2. Schematic representation of G-CSF/G-CSF-R and IL-6/IL-6R/gp130 complexes. A, the 2:2 signaling complex of G-CSF (green) with G-CSF-R with binding sites II and III labeled. The most significant residues of the site II interaction are E19 on the ligand with R288 on the receptor (CRH module, blue) and for site III E46 on the ligand interacts with the Ig domain of the receptor (orange). B, the Aritomi crystal structure showing an improbable dimer interface of two 1:1 G-CSF:G-CSF-R complexes, where the site III of G-CSF remains exposed and not in contact with the receptor. C, the hexameric complex of IL-6 (yellow) with its alpha receptor (IL-6R, magenta) and gp130 (navy blue). The core of the complex looks equivalent to that of G-CSF and G-CSF-R, but in addition, IL-6 binds to IL-6R through a site I interaction.

3. STOICHIOMETRY OF THE G-CSF-R-LIGAND COMPLEX

Members of the cytokine receptor type I family that signal as homodimers, such as the growth hormone receptor and the erythropoietin receptor, form receptor:ligand complexes with 2:1 stoichiometry (21, 22). In contrast, the cytokine receptors that form heterodimers or multimers appear to contain receptor A, receptor B and ligand in a 2:2:2 ratio. An example of this stoichiometry is the IL-6R-gp130-IL-6 complex (Figure 2C) (23, 24). The G-CSF-R appears to signal as a homodimer but is closely related to gp130, therefore it was not clear whether it would form a 2:1 or 2:2 complex with G-CSF. In order to determine which of these alternatives was correct, various domains from the extracellular region of the receptor were expressed as soluble proteins and their interaction with G-CSF was analyzed. The CRH module (D2-D3) formed a 1:1 complex with G-CSF in solution, suggesting that it was insufficient to allow dimerization (25). Receptor fragments D1-D2 and D2-D3 were both able to bind a single G-CSF molecule in a 1:1:1 complex, suggesting that G-CSF had 2 binding sites, one of which bound D1 or D1-D2 and the other which bound D2-D3 (26). Hiraoka et al. suggested that these results were evidence for a 2:1 receptor:ligand complex. The complete extracellular region was prone to oligomerize in solution (27), nevertheless, Horan et al described 2:2 complexes with G-CSF (28). They suggested that each G-CSF had only a single receptor-binding site and that receptor-receptor interactions were responsible for dimerization. Similarly, they found that the Ig-CRH region without D4-D6 formed 2:2 complexes with G-CSF and appeared to be the minimum region of the receptor required for high affinity ligand binding (29). A more recent study has confirmed the 2:2 stoichiometry of the extracellular receptor-ligand complex in solution (30).

We hypothesized that these apparently contradictory versions of the complex proposed by Hiraoka and Horan could be reconciled in a model (Figure 2A) based on the proposed IL-6-receptor complex (Figure 2C) (31). The sequence and structural domain similarity of the G-CSF-R with gp130 indicated that the G-CSF-R complex might be a 2:2 complex containing the equivalent of site II and site III of the IL-6-receptor complex, but not site I, because there is no equivalent to the IL-6 receptor chain required to form site I (Figure 2A). Hiraoka's data is consistent with this 2:2 complex if each complete receptor (D1-D3) has 2 ligand binding sites and if receptor dimerization is the result of each ligand molecule binding 2 receptors at 2 different sites, rather than the receptor-receptor interaction proposed by Horan and colleagues.

Support for this model was provided by the determination of the crystal structure of the complex of gp130 with a viral IL-6 homolog (32). Viral IL-6 forms a functional 2:2 complex with gp130, without an α receptor, thus could be similar to the G-CSF-R complex. Each vIL-6 molecule interacts with 2 receptors, one via site II on D2-D3 and one via site III on the Ig domain, thus resulting in a cross-linked complex. Recently, the crystallization of a 2:2 complex of D1-D3 with G-CSF was reported, thus the structure of this complex should be available in the near future (33). Until then, we have developed a model of the G-CSF-R complex, based on the viral IL-6 complex, which can explain most of the published data as described below in section 6 (34).

4. EVIDENCE FOR THE SITE II INTERACTION.

4.1. Mutagenesis studies

The first structure of a type I cytokine receptor complex to be determined was that of the growth hormone

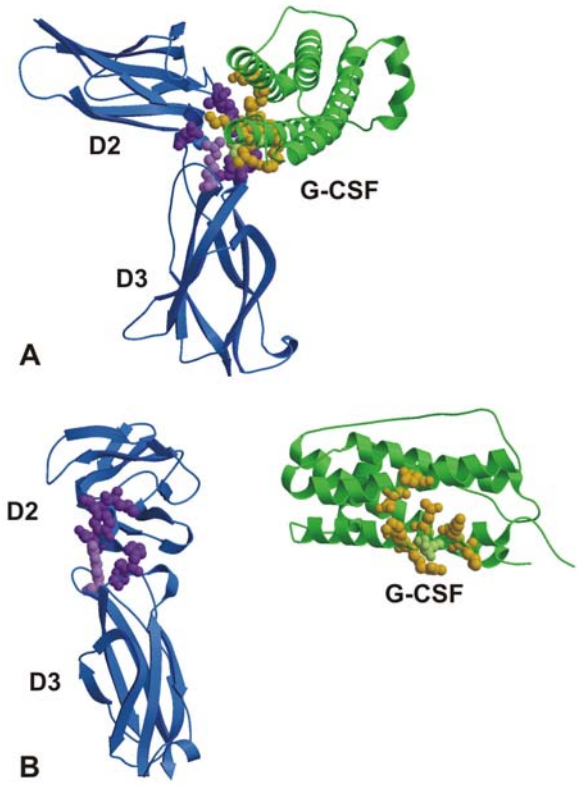

Figure 3. Ribbon diagram of the site II complex between G-CSF and D2-D3 of G-CSF-R. A, Model of human G-CSF (green) binding to human G-CSF-R CRH module (D2 and D3, blue) through the site II interaction. The model is based on the Aritomi human G-CSF:mouse G-CSF-R 1:1 complex (34, 37). Residues important to binding G-CSF: L15, K16, E19, Q20, R22, K23, D109 & D112; G-CSF-R L172, Y173, D200, H238, I239 & R288 (19, 20, 35) are shown in space fill representation. G-CSF residues are colored yellow and receptor residues purple, with the central E19-R288 interaction shaded differently. B, "Open book" representation of the G-CSF/G-CSF-R site II complex, where receptor and ligand have been rotated by 90 degrees in opposite directions from their orientations in panel A.

receptor (GH-R) (21). This receptor contains two extracellular domains, equivalent to D2 and D3, which form an angle of about 90^{o} and bind ligand at the 'elbow' mainly through residues on four loops. We thought it likely that the G-CSF-R would have a similar binding site, therefore we made a model of D2-D3 based on the GH-R structure. Although the sequence identity was only 13%, the core regions of FN III domains with known structure at the time were highly conserved, enabling us to use an alignment of G-CSF-R and GH-R with the gp130 receptor family to predict beta-strands. Several residues in the regions predicted to be equivalent to the four loops of GH-R involved in ligand binding were mutated to alanine. The function of the mutated receptors was determined by measuring G-CSF binding and proliferation responses in transfected Ba/F3 cells. The structural integrity of the mutant receptors was confirmed by determining binding of a panel of conformation-dependent monoclonal antibodies. Six residues in the predicted loop regions were thus identified as being important for ligand binding and receptor function (35). The loops most strongly involved were the E-F loop in D2 and the B-C and F-G loops in D3. The six residues formed a plausible binding site on the model comprised of charged and hydrophobic residues that could make both electrostatic and hydrophobic interactions with G-CSF (Figure. 3).

The residue that appeared to be the most important for G-CSF binding was R288 in the F-G loop of the D3 domain. To determine which G-CSF residue(s) R288 interacted with, the effect of various charged residue mutations in G-CSF was tested. We reasoned that mutation of residues that were involved in binding but did not interact with R288 would cause reduced activity when the G-CSF mutants were tested on both WT and R288A mutant receptors. In contrast, mutation of residues that interacted with R288 would cause reduced activity on the WT receptor but not on the R288A mutant receptor. Thus, comparison of the activity of WT and mutant G-CSFs on wild-type and R288A mutant receptors, showed that E19 of G-CSF interacted with R288 of the receptor (36). This G-CSF residue is in the A helix and forms part of a charged binding site previously identified by mutagenesis (19, 20) (Figure 1B, 3).

4.2. Crystal structure of a 2:2 G-CSF:D2-D3 G-CSF-R complex.

The crystal structure of the murine D2-D3 with human G-CSF was recently reported (37). Given that D2-D3 and G-CSF formed a 1:1 complex in solution (26), it was a little surprising that the crystallized complex was a 2:2 structure. Nevertheless, the site II interaction that was determined in this complex was consistent with other receptor–ligand interactions and the mutagenesis data. A schematic illustration of this complex is shown in Figure 2B for comparison with our model (Figure 2A). It seems likely that the dimerization interface in this complex is an artifact of crystallization because it is not consistent with other data about the complex (see section 6). Of the six residues identified by mutagenesis in the human D2-D3 (Figure 3), four are conserved in the murine receptor used for crystallization. Three of these are in the major interface of the crystal structure (L172, L173 and R288), while the fourth (D200) is not, although the neighboring residue, M199, is involved in ligand binding. Interestingly, mutation of M199 had a two-fold effect on receptor function but this did not reach statistical significance (35). Although H238 and I239 are not conserved in the murine receptor, the residues in the equivalent positions (Y237, M238) were in the major binding interface. The critical G-CSF residue E19 was hydrogen-bonded to Y173 and R288.

5. EVIDENCE FOR THE SITE III BINDING SITE

5.1. Effect of removing the Ig domain

Deletion of most of the Ig domain resulted in a 20-fold loss of binding affinity for G-CSF and a very reduced proliferation response in cells expressing this Ig-

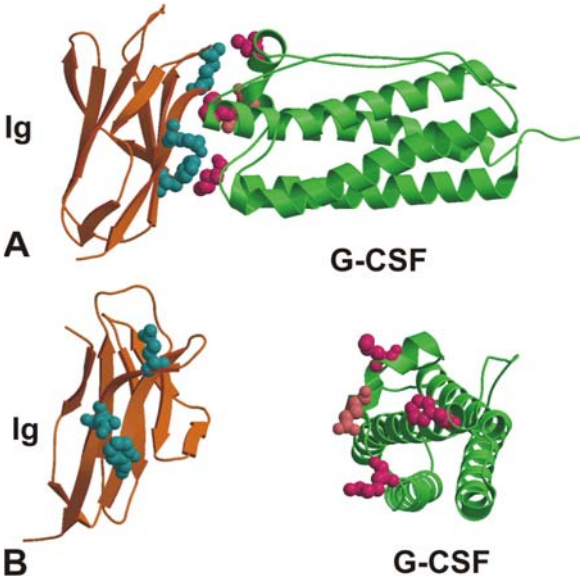

Figure 4. Ribbon diagram of the site III complex between G-CSF and the Ig domain of G-CSF-R. A, Model of human G-CSF (green) binding to human G-CSF-R Ig domain (D1, orange) through the site III interaction. The model is based on the structure of viral IL-6 with gp130 (34). Residues important for binding in G-CSF: K40, E46, F144, L49 and G-CSF-R: F75, Q87 and Q91 (20, 34, 36) are shown in space fill representation. G-CSF residues are colored pink and receptor residues cyan. B, "Open book" representation of the G-CSF/G-CSF-R site III complex, where receptor and ligand have been rotated by 90 degrees in opposite directions from their orientations in panel A.

deleted receptor (13). Similarly, if the Ig domain was swapped with that of gp130, the resulting chimeric receptor bound G-CSF with reduced affinity and did not transduce a detectable proliferation response (36). This chimeric receptor was also used to show that E46 of G-CSF appeared to interact with the Ig domain. Previously, E46 was implicated in receptor binding by mutagenesis (19). As mentioned in section 3, the Ig domain was required to cause receptor dimerization in the presence of G-CSF in solution (26). Taken together, these data strongly suggest that the Ig domain is involved in receptor dimerization via a direct interaction with G-CSF.

5.2. Effect of antibodies to the Ig domain

Additional evidence for the presence of a G-CSF binding site on the Ig domain was provided by the mapping of binding sites of neutralizing anti-receptor antibodies (34, 38). Three monoclonal antibodies that blocked G-CSF binding were shown to bind to the Ig domain and one of these was particularly strongly neutralizing. Other blocking antibodies bound to the D2 domain, whereas those binding D4-D6 did not block G-CSF binding. While it is possible that the blocking antibodies had an indirect effect on ligand binding, this observation is consistent with a binding site on the Ig domain.

5.3. Mutagenesis of the Ig domain

An alignment of the Ig-like domains of the G-CSF-R and gp130 from several species was used to predict secondary structural features and a model of the domain was constructed to aid the prediction of exposed residues for mutagenesis. The alignment revealed that there were four conserved cysteine residues as well as other conserved hydrophobic and structurally important residues. The disulphide bonds formed in the gp130 family Ig domain are different from those determined experimentally for the G-CSF-R by Haniu et al. (12). The latter disulphide linkages could not be used to produce a model of a traditional Ig-domain fold, therefore, based on the conservation with gp130, the gp130 linkages were used. Single or multiple residue mutations were produced and tested for effect on receptor function. Although no single residue mutation had a large effect on receptor function, several smaller effects were observed and these residues on the predicted F and G strands formed a credible binding surface on the Ig domain model (34). Larger reductions in receptor function were observed with combinations of mutations, confirming the identification of this binding site, in which the most important residues were F75, Q87 and Q91 (Figure 4). When the viral IL-6-gp130 structure was published, it was apparent that the site III interface in this structure was remarkably similar to the surface defined by mutagenesis on the G-CSF-R Ig domain (32). It also enabled us to refine our model and predict that other residues we had not mutated would be involved in binding (I88 and N90).

6. MODEL OF THE G-CSF COMPLEX WITH G-CSF-R

The crystal structure of D1-D3 of gp130 in complex with viral IL-6 showed a tetramer of two viral IL-6 and two gp130 subunits (32). There is a site II interaction of viral IL-6 with D2 and D3 and a site III interaction between viral IL-6 and D1 as we proposed for the G-CSF-R complex (Figure 2A). There is no ligand-ligand or receptor-receptor interaction. We used this gp130 structure together with the murine G-CSF-R structure of D2-D3 (37) to provide templates for our model of the G-CSF-R complex. The resulting structure is illustrated in Figure 5, with further detail of site II in Figure 3 and site III in Figure 4. The site II interaction in this model is essentially the same as that in the crystal structure described by Aritomi et al. (37). In contrast, the site III interaction with D1 results in a different dimerization interface from that in the crystal structure. We believe that the absence of the Ig domain in the crystal structure may have resulted in dimerisation that was an artifact of crystallization. This dimerization does not seem likely because it would not allow any interaction between the Ig domain and G-CSF (Figure 2B) (37). In our model, most of the residues in G-CSF and the receptor that were identified by mutagenesis as important, are found in the site II and site III interfaces (discussed in more detail in ref. (34)). The critical E19-R288 interaction is present in the site II interface. The G-CSF residues L49, F144 and E46, identified by mutagenesis as possible site III residues, are in the site III interface, along with F75, Q87 and Q91 of G-CSF-R. The model can also explain the various complexes that were described in solution with partial

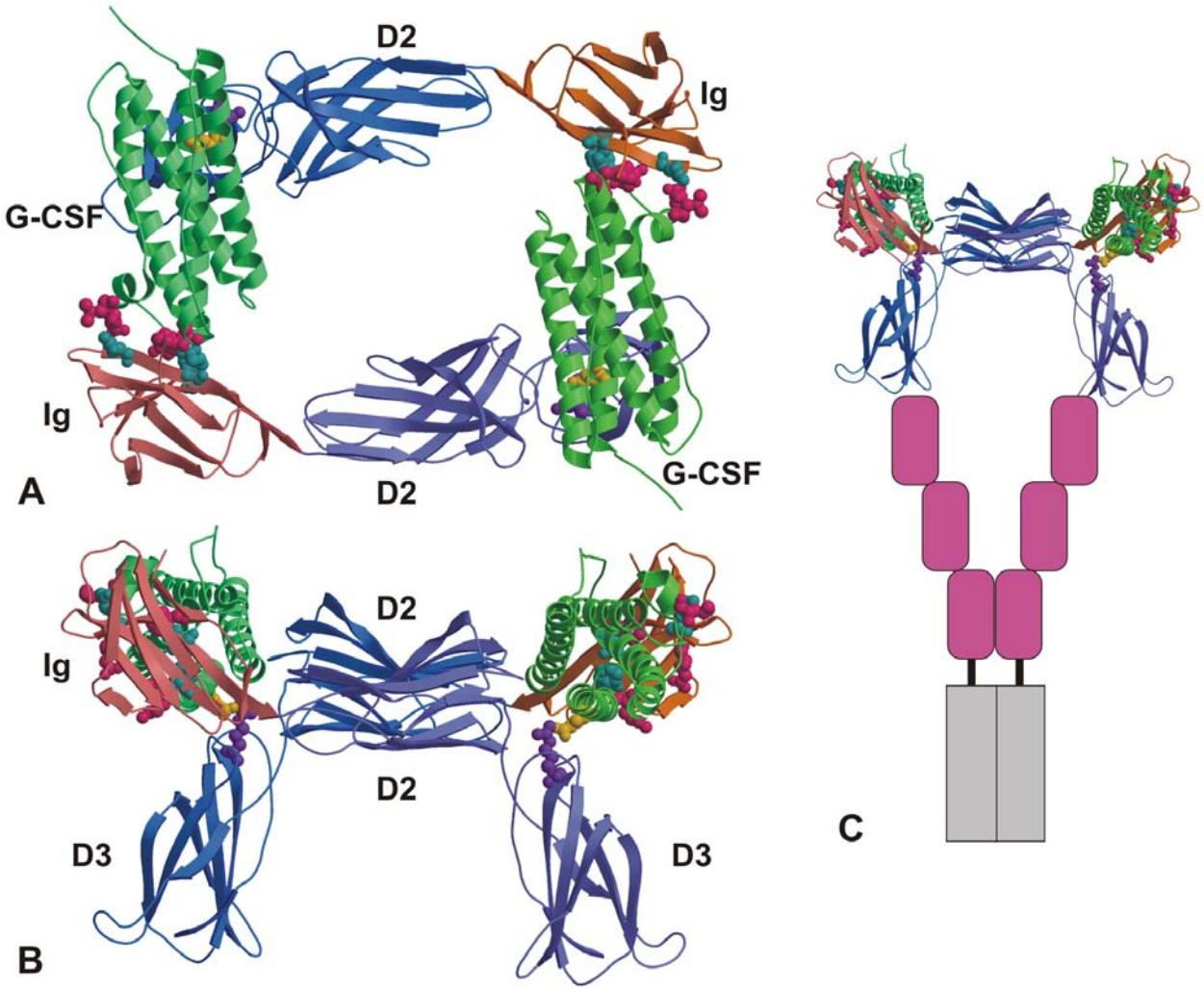

Figure 5. Model of the 2:2 signaling complex of G-CSF with G-CSF-R. A, Top view of the ribbon representation of the two-fold symmetrical tetramer complex. Each G-CSF molecule is involved in binding one receptor though a site II interaction with the CRH module (blue) and a second receptor though a site III interaction with the Ig domain (orange). For simplicity, only residue E19 on G-CSF and R288 on G-CSF-R are shown for the site II interaction. The coloring schemes are consistent with figures 3 and 4. B, side view of the G-CSF:G-CSF-R tetramer where the complex has been rotated by 90 degrees from the view in panel A. C, schematic representation of the whole signaling complex where domains D4-D6 of G-CSF-R facilitate dimerization of the cytoplasmic regions. D4-D6 the transmembrane and cytoplasmic domains are represented in the same way as in figure 1.

receptor constructs and is consistent with the neutralizing antibody binding data.

Mapping of the glycosylation sites revealed that they were compatible with the model. The O-linked glycosylation site of G-CSF at T133 (39) is in the C-D loop and far away from binding sites II and III. The four N-linked glycosylation sites in D1-D3 of G-CSF-R (12) are all exposed and would not interfere with G-CSF binding.

7. FUNCTION OF D4-D6 G-CSF-R

Little is known about the role of the three membrane-proximal domains. Deletion of most of D4-D6 produced a receptor that had severely reduced signaling capacity but essentially normal ligand binding ability, showing that these domains are not involved directly in ligand binding (13). This result was confirmed when D4-D6 of the G-CSF-R was swapped with the equivalent region from gp130, and the chimeric receptor showed normal G-CSF binding and signal transduction (10). Thus, it seems likely that D4-D6 are required to bring about dimerization of the cytoplasmic domain in the correct orientation for signaling to occur (Figure 5C). Several antibodies that bind to D4-D6 have been shown to partially inhibit signaling without affecting ligand binding, consistent with this view (38). In addition, a non-protein compound has been identified that activates the murine receptor by interacting with D4-D6, showing that dimerization of this region is sufficient to allow

downstream signaling (40). It is clear from the viral IL-6/gp130 complex and the IL-6/IL-6R/gp130 complex that the C-termini of the gp130 D3 domains are a considerable distance apart (~ 100 Å). One function of the D4-D6 domains is therefore likely to be bridging this gap to enable the juxtaposition of the transmembrane and cytoplasmic domains for downstream signaling. A recent study of the IL-6 complex, containing the complete extracellular region of gp130, by single particle electron microscopy, showed that the membrane proximal domains were indeed close together at the surface of the grid (41).

8. PERSPECTIVE

The mutagenesis studies together with some structural and other information have enabled us to prepare a model of the G-CSF-R complex. This model, based on a substantial amount of data, provides the first detailed representation of the signaling complex. Ultimately, the crystal structure will provide with more certainty the finer details of the interactions of G-CSF with its receptor. This will provide the necessary atomic resolution information for rational design of agonists or antagonists in the future. The complex that has been crystallized contains D1-D3 of the receptor, thus the structure of D4-D6 will remain to be determined.

There are several cases now described of mutations in the G-CSF-R found in patients with severe congenital neutropenia. These naturally occurring mutations can sometimes provide useful data about receptor function. The P206H mutation described by Ward et al. (42) is in the conserved proline-rich linker region between D2 and D3. This mutation resulted in reduced responses to G-CSF in myeloid cell lines transduced with the mutant receptor, although binding affinity for G-CSF was relatively normal. On our model, this residue is directed away from the binding interface with G-CSF and we would suggest a possible destabilizing effect on the receptor structure. Two deletions have been described that result in truncation of the receptor shortly after the conserved WSXWS sequence in D3. In one of these, this sequence was mutated to WSDWG followed by 28 missense amino acids and a stop codon. This leaves domain 3 essentially intact, only missing the last few correct amino acids of the domain. It is unknown whether the mutant receptor could still bind G-CSF. This receptor was expressed on the cell surface in association with wild type receptor and inhibited the response to G-CSF (43). The second deletion altered the WSXWS sequence to WGHPA, followed by 24 missense amino acids and a stop codon. This receptor was not expressed on the cell surface and also altered the intracellular trafficking of the wild type receptor so that the response of cells expressing both receptors was reduced (44). Domain 3, with the WGHPA mutation is unlikely to fold up correctly (see EPO receptor mutation analysis (45)). It is likely that domains 1 and 2 would still be able to fold correctly, thus allowing the interaction with the wild-type receptor. Finally, a mutation in the transmembrane domain has been described (T617N), which resulted in a constitutively active receptor, presumably because it altered receptor dimerisation and/or orientation in the absence of ligand (46).

There are still questions to be asked about the mechanism of receptor activation. Is the function of G-CSF solely to bring the two receptor subunits into close enough proximity, and in the correct orientation, to allow Jak activation and cross-phosphorylation of the cytoplasmic domains, thus initiating the cytoplasmic signaling cascade? Is there any conformational change upon ligand binding? There have been two studies that have detected conformational changes (30, 47) but it is not clear whether these changes are necessary for signaling. The fact that a compound that binds to the D4-D6 region is able to activate the receptor in the absence of G-CSF (40) suggests that conformational changes in D1-D3 may not be required, but could possibly enhance the efficiency of activation. How does the receptor exist in the cell membrane? Studies with soluble receptors have found that dimers tend to form in solution in the absence of ligand (28) but there is no evidence yet of preformed dimers in the cell membrane. We await further biophysical studies to answer these and other questions.

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