Identification of a Ligand-biding Site on the Granulocyte Colony-stimulating Factor Receptor by Molecular Modeling and Mutagenesis*

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Granulocyte colony-stimulating factor (G-CSF) initiates its effects on cells of the neutrophil lineage by inducing formation of a homodimeric receptor complex. The structure of the G-CSF receptor has not yet been determined, therefore we used molecular modeling to identify regions of the receptor that were likely to be involved in ligand binding. The G-CSF receptor sequence was aligned with all the available sequences of the gp130 and growth hormone receptor families and a model of the cytokine receptor homologous domain was constructed based on the growth hormone receptor structure. Alanine substitution mutagenesis was performed on loops and individual residues that were predicted to bind ligand. Mutant receptors were expressed in factor-dependent Ba/F3 cells and assessed for proliferation response and ligand binding. Six residues were identified that significantly reduced receptor function, with Arg288 in the F’-G’ loop having the greatest effect. These residues formed a binding face on the receptor model resembling the growth hormone receptor site, which suggests that the model is reasonable. However, electrostatic analysis of the model provided further evidence that the mechanism of receptor dimerization is different from that of the growth hormone receptor.

The granulocyte colony-stimulating factor receptor (G-CSF-R) is a member of the class 1 cytokine receptor family which is defined by the presence of a cytokine receptor homology (CRH) domain that is characterized by four conserved Cys residues and a conserved WSXWS sequence (Trp-Ser-X-Trp-Ser, where X is any amino acid) sequence (1). The receptor is expressed predominantly on cells of the neutrophil lineage (2), hence interaction with G-CSF stimulates proliferation and differentiation of neutrophils from precursor cells (3). G-CSF is required for normal neutrophil production in vitro (4). The molecular mechanisms of receptor activation and signal transduction are at present poorly understood. The receptor is most probably homodimerized by ligand binding (5–7), resulting in activation of cytoplasmic signaling pathways including the Jak-STAT pathway and the MAP kinase pathway (reviewed by Avalos (8)).

Bazan (1) proposed that the conserved CRH domain is comprised of two modules of fibronectin type III (FnIII)-like structure, each containing seven β-strands which form two β-sheets. This model has been confirmed by determination of the crystal structures of several receptors including the growth hormone receptor (GH-R) (9), prolactin receptor (10), erythropoietin receptor (EPO-R) (11), and related class 2 cytokine receptors: tissue factor (12) and interferon-γ receptor α (13). The highly conserved Cys residues and WSXWS sequence are likely to be required for maintenance of the structure of the CRH domain. In the G-CSF-R, each of the CRH domain modules (termed BN and BC) have been expressed as soluble proteins, both of which bind G-CSF with low affinity (14, 15). In both the BN and BC domains, the disulfide bonds were critical for maintaining a stably folded protein and the WSXWS sequence in the BC domain was also important for maintaining structure rather than ligand binding (14, 15).

In addition to the CRH domain, the extracellular region of the receptor contains an N-terminal Ig-like domain and three FnIII domains that are C-terminal to the CRH domain (16, 17). Deletion analysis of the receptor established that the CRH BN domain was essential for ligand binding and signal transduction, while the Ig-like domain also contributed to receptor function (18). The soluble form of the extracellular region of the receptor forms a complex with G-CSF that has a 2:2 stoichiometry (7) and the Ig-like domain is required for receptor dimerization in solution (6). In addition, neutralizing monoclonal antibodies to the receptor that blocked G-CSF binding mapped to the Ig and CRH domains, further implicating these domains in ligand binding (19).

The results of Hiraoka et al. (6) suggest that there are two binding sites for G-CSF on the receptor, one of which is in the CRH domain. Their model is compatible with a recent mutagenesis study which identified two regions of G-CSF apparently required for interaction with the receptor (20). However, Horan et al. (7) have suggested that there is one binding site on the receptor and that dimerization involves a conformational change that follows ligand binding (7).

Although the mechanism of receptor dimerization is likely to be different from that of the GH-R, it is possible that one binding site on the G-CSF-R is similar to the ligand-binding site on the GH-R. We have therefore modeled the structure of the G-CSF-R CRH domain on the GH-R structure (9) and predicted regions likely to be involved in ligand binding. These regions have been substituted with alanine residues to determine their contribution to ligand binding and receptor function. This approach has enabled us to define a likely ligand-binding site.
EXPERIMENTAL PROCEDURES

Sequence Alignment—The sequences of CRH domains were aligned by primarily manual means. Special care was taken to align conserved sequence patterns like the "WSXWS" motif, conserved disulfide bonds between β-strands, tryrosine corners (21), the proline-rich linker between the two FnIII domains, and the alternating pattern of hydrophobic/hydrophilic residues observed in β-sandwich proteins. To find an optimal alignment between the CRH domains of G-CSF-R and the human GH-R, the only structure of a CRH domain available at that time, multiple alignments were performed using CRH domains of different species of the growth hormone receptor, G-CSF-R, the prolactin receptor, IL-6 receptor, gp130, and leukemia inhibitory factor receptor.α A β-bulge was introduced into the F-strand of the BC domain to maintain the pattern of positively charged residues forming part of the "WSXWS" structural motif. This was also required for the alignment of gp130.

Modeling of the G-CSF-R CRH Domain—The model of the CRH domain of the G-CSF receptor is based on the sequence alignment described in the preceding paragraph and the coordinates of the chain B of protein data bank entry 3HHR (9). The growth hormone receptor coordinates were used as a template for the β-sheet regions of the receptor and some of the loops. Other loops were taken from available structures of other FnIII domains or from a search of the protein data bank (22).

The model building and refinement procedure followed in general the procedure described in detail by Smith et al.22 (23). Similar procedures were used to construct models of IL-6 (24) and the epidermal growth factor receptor kinase (25). An initial model was built using the Homology module of InsightII (Molecular Simulations Inc., San Diego, CA). This structure was refined using the X-PLOR program (26) in conjunction with the OPLS force field (27). The two conserved disulfide bridges of the BN domain formed naturally in the model building. The third disulfide bridge (14) between the C-C' loop and the F-strand was created using Xplor and refined separately to have reasonable stereochemical parameters before proceeding with the molecular dynamics stages. In the BC domain the modeling suggested the two disulfide bridges subsequently confirmed by Anaguchi et al. (15).

The quality of the model was examined as described elsewhere (23). All irregularities observed during our modeling procedures were analyzed in detail and subjected to further refinement with additional or modified restraints, or the initial sequence alignment was modified and the model was rebuilt.

Electrostatic Calculations—As one method to find possible dimerization sites of the G-CSF-R, we performed an analysis of the electrostatic potential generated by the CRH of the G-CSF-R. The isopotential surfaces of the model were calculated by following, in general, the method described elsewhere (23, 28) using the program DelPhi (29, 30) with the following parameters: a probe radius of 1.4 Å, an ion exclusion layer of 2.0 Å, a salt concentration of 0.145 M, standard van de Waals radii, partial charges from the OPLS force field, the solution dielectric of 80, and a solute dielectric of 2. Histidine residues were treated as having a net charge of ±0.5 e.

Mutagenesis of the G-CSF-R—Site-directed mutagenesis (31) was used to introduce mutations into the receptor cDNA in most cases. A few mutants were made with a mutagenesis kit (Quickchange site-directed mutagenesis kit, Stratagene, La Jolla, CA). Oligonucleotides were synthesized incorporating the desired mutation(s) and usually a silent restriction enzyme site for identification of mutant cDNAs. Mutations were confirmed by nucleotide sequencing and the mutant cDNAs were subcloned into pEFBOS expression vector (32) for transfection.

Expression of Receptor Mutants and Cell Culture—Ba/F3 murine myeloid cells (33) were transfected with wild type or mutant cDNAs by electroporation. Cells (4 × 10⁶) in 0.8 ml of phosphate-buffered saline with 20–30 µg of G-CSF-R cDNA and 1.5–2 µg of pGKneo (34) were treated to obtain 200 microfarads and 270 volts. Cells were cultured in 24-well plates in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 10% WEHI-3B D cell conditioned medium (9).

Flow Cytometry—G-CSF-R expression in transfected cell lines was determined by fluorescence analysis. Cells were stained with 10 µg/ml LMM741, an antireceptor monoclonal antibody (mAb) that recognizes the FnIII domains (19), followed by fluorescein isothiocyanate-conjugated sheep anti-mouse IgG antibody (Silenus, Amrad, Melbourne, Australia) and 2 µg/ml propidium iodide to exclude dead cells from analysis. Cells were analyzed in a FACScan flow cytometer with Lysis II software (Becton Dickinson, San Jose, CA). Transfected cell lines were selected on the basis of apparent homogeneity of receptor expression and were not further cloned.

To probe the structural integrity of the mutant receptors, binding of a panel of anti-G-CSF-R mAbs (19) was assessed. The panel contained 5 conformation-dependent, neutralizing mAbs that recognize 3 epitopes and 3 mAbs that are not conformation-dependent or neutralizing and recognize 3 different epitopes on the FnIII domains. A semiquantitative assay was established by titrating the mAbs and selecting a concentration for staining that was slightly suboptimal. Binding of each antibody (median fluorescence channel) was compared with that of LMM741 (used as standard) and with binding of the same pair to the wild type receptor.

G-CSF Binding Assay—YPY-G-CSF (Tyr 1,3-G-CSF, Kirin, Japan) was iodinated using IODO-GEN (Pierce, Rockford, IL) as described previously (19). The bindability of each batch was greater than 60% and the specific activity was 4–10 × 10⁶ cpm/µg as determined by self-displacement analysis (35). Data were corrected for bindability of ligand. In most cases, binding affinity was determined by titration of [125I]-G-CSF up to saturating concentration, but the lower affinity mutant receptors were assessed by cold competition as described previously (19). Data were analyzed with the EBDA (96) and LIGAND (37) programs (Biosoft, Cambridge, UK).

Proliferation Assay—Ba/F3 cells expressing wild type or mutant G-CSF-R were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum/well with serial 3-fold dilutions of rh-G-CSF (Amgen, Thousand Oaks, CA) in triplicate. The 96-well plates were incubated for 48 h at 37 °C and pulsed with 0.5 µCi/well [methyl-³²H]thymidine (ICN Pharmaceuticals, Irvine, CA) for the last 4 h before harvest. Cells were harvested onto glass fiber filters and counted in a liquid scintillation counter (Betaplate, LKB-Wallac, Turku, Finland). At least three independent cell lines were tested for each mutant and each assay was repeated at least once.

RESULTS

Modeling of the G-CSF-R CRH Domain—We created a model of the CRH domain of G-CSF-R based on its homology to the human growth hormone receptor and conserved sequence patterns found for 9 known members of closely related cytokine receptors, the so called "gp130" family of cytokine receptors. Between the sequences of the CRH domains of G-CSF receptor and growth hormone receptor we found a 34% sequence similarity and 13% sequence identity, which is in a range where homology modeling is difficult, but still feasible. The final alignment of the CRH domains of G-CSF-R with human growth hormone receptor and the gp130 family members is shown in Fig. 1. The known β-strands of GH-R and the prolactin receptor are underlined, together with the predicted β-strands of the G-CSF-R.

In addition to a reasonable sequence similarity, we observed a highly conserved core region in the known structures of FnIII domains: pairwise structural alignments of the β-strands B, C, E, and F among eight known FnIII domain structures show values of the root mean square deviation of atomic positions of about 1.2 Å, although all eight FnIII domains show only very low levels of pairwise sequence identities. This observed close structural similarity for the core region of FnIII domains considerably improves the chances of creating a reliable homology model of G-CSF-R. The primary aim of our modeling was to assist in site-directed mutagenesis in identifying G-CSF-binding sites on G-CSF-R, and further to investigate the possibility of G-CSF and G-CSF-R forming a receptor complex analogous to the growth hormone receptor complex (9).

The binding site on GH-R consists mainly of four loops in the hinge region between the BN and BC domains. Based on the sequence alignment and our three-dimensional model, we identified the main regions on the CRH domain homologous to the binding site observed on GH-R. The 4 loop regions are high-

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lighted in Fig. 1. Because there was little sequence similarity in these regions, charged, aromatic, or large hydrophobic residues were chosen for alanine substitutions as indicated in Fig. 1 and Table I.

Effect of Mutation of the Predicted Binding Site in the G-CSF-R

The residues indicated in Fig. 1 and Table I were substituted with alanine by site-directed mutagenesis of the G-CSF-R cDNA. Mutant receptor DNAs were transfected into Ba/F3 cells and transfected cell lines were selected after analysis of receptor expression by flow cytometry. The binding of a panel of conformation-dependent anti-receptor mAbs was also tested in comparison with conformation-independent mAbs to determine whether the mutation affected the conformation of the receptor. Binding of all mAbs except LMM774 was not affected by the mutations (data not shown). Three of the mutations had little effect on LMM774 binding but mutant hGR2 showed a reduction in binding (Fig. 2A). Since this antibody is one of a pair binding to an epitope on the CRH BN domain, and the binding of the other mAb was not affected, it is likely that the residues mutated form part of the epitope recognized by this mAb.

The transfectants were tested for proliferation in response to G-CSF and binding affinity for G-CSF. A representative assay of each mutant is shown in Fig. 3 and a summary of assays of at least three independent cell lines expressing each mutant or the wild-type receptor is given in Table II. All the mutant receptors were expressed at similar levels to the wild type receptor as determined by Scatchard analysis of binding data and flow cytometry (Fig. 2A). Since this antibody is one of a pair binding to an epitope on the CRH BN domain, and the binding of the other mAb was not affected, it is likely that the residues mutated form part of the epitope recognized by this mAb.

Additional Mutations in the CRH Domain—The residues mutated in hGR2 (E-F loop) and hGR3 (G-A\text{9}\text{loop}) were mutated singly to alanine to determine the contribution of each residue to receptor function. In addition, we used the G-CSF-R

### Table I

| Mutant | Mutation position | Residues mutated | Ba/F3 transfectants |
|--------|-------------------|------------------|--------------------|
| hGR 1 A-B loop | Thr115, Thr114, Ser113 | 6,000 ± 2,800 All positive |
| hGR 2 P-F loop | Lys192, Tyr173, Gln174 | 8,800 ± 2,800 All positive, 774 red. |
| hGR 3 G-A loop | Asp197, Met198, Asp200 | 8,900 ± 2,100 All positive |
| hGR 4 B-C loop | His236 | 7,100 ± 1,600 All positive |

*Number of receptors per cell determined by Scatchard analysis of binding data. Mean ± S.E. of three independent cell lines. Wild type cells expressed 12,600 ± 2,200 receptors.*

*Five neutralizing, conformation-dependent antireceptor mAbs that recognize 3 different epitopes were tested by flow cytometry.*

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Fig. 1. Alignment of the human G-CSF-R, GH-R, and prolactin receptor (PLR) sequences with members of the gp130 family. The \( \beta \)-strands of the GH-R and PLR are underlined as well as the G-CSF-R \( \beta \)-strands determined from our modeling. The GH-R residues important for ligand binding are highlighted in bold. Also in bold are the residues initially mutated in the G-CSF-R (mutants hGR1-hGR4). Every tenth residue in the sequences is marked and the conserved WSXS\( \text{W} \) region is boxed.
model to identify new residues with side chains near those already identified. Five such residues in the G-A loop (Val201), B-C loop (Ile239, Asn240), and the F-G loop (Arg288, Trp289) were also mutated to alanine. All these single residue mutants were expressed in Ba/F3 cells at levels similar to the wild type receptor (2,000–16,000 receptors/cell) and were able to bind the conformation-dependent mAbs, indicating that the mutations did not affect the structure of the receptor (data not shown). Of the residues from the hGR2 mutant, which showed reduced binding of LMM774, only the L172A mutation resulted in reduced binding of this antibody (Fig. 2B).

The effect of these mutations was tested in proliferation and binding assays (Table III). Of the residues from the hGR2 mutant (E-F loop), substitution of Tyr173 had the greatest effect, with a 30-fold reduction in proliferative response to G-CSF and a 5-fold loss of binding affinity. Mutation of Leu172 also reduced activity but had no detectable effect on binding affinity, whereas mutation of Gln174 had no effect. Mutation of the residues from hGR3 (G-A loop) identified one (Asp200) that significantly reduced activity, but the effects of the individual mutations did not appear to account for the loss of activity of hGR3, suggesting a synergistic effect of multiple substitutions. Mutation of the additional neighboring residues identified two that had significant effects: Ile239 from the B-C loop and Arg288 from the F-G loop. The R288A mutation had the greatest effect of any of the single residues, causing greater than 100-fold loss of activity and 8-fold loss of binding affinity.

Model of the CRH Domain Binding Site—The position of the residues that significantly affected receptor function is shown on our model of the G-CSF-R CRH domain in Fig. 4. The residues form a binding site containing both charged and hydrophobic residues that could be involved in both electrostatic and hydrophobic interactions with G-CSF.

Electrostatic Analysis of the Model—The electrostatic potentials of growth hormone and its receptor were found to be complementary in those areas of the molecules that are involved in binding, including the region of receptor-receptor interaction (28). In addition, electrostatic complementarity has been found between the binding interfaces of antigen-antibody and other protein-protein complexes (38). It is therefore tempting to assume that electrostatic complementarity will play a significant role in definition of important interaction sites between other members of the helical cytokine family.2

Fig. 5 shows our results for the electrostatic analysis of our model of the CRH domain of G-CSF-R in comparison with the GH-R. The electrostatic isopotential surfaces of GH-R1 and GH-R2 (Fig. 5, C and D) show regions of opposite charge at the sites of receptor interaction (numbered 1–5). In contrast, the analysis of the G-CSF-R reveals a large uniformly distributed negative electrostatic potential in the area where dimerization...
activity and are labeled NT and by residue type and number. Important for receptor function are shown as CPK models and labeled complex.

forms a homodimer similar to the growth hormone receptor shown). We, therefore, consider it unlikely that the G-CSF-R receptor. Small alterations in alignment and side chain conformation of four regions chosen in this way gave a reduction in prediction likely binding residues for testing by mutagenesis. Molecular modeling based on the GH-R structure was used to select residues are replaced to evaluate the contribution of 300 amino acid residues that have been implicated in ligand binding, has provided of single residues resulted in the identification of three loops containing ligand binding residues, the E-F loop in the BN domain and the B’-C’ and F’-G’ loops in the BC domain. In addition, the G-A’ linker region may contribute to ligand binding. The greatest effect was obtained by mutation of Arg288 in the F’-G’ loop. Residues implicated in ligand binding in these three loop regions of other cytokine receptors are shown in Fig. 6, where the loop sequences are aligned with those of G-CSF-R.

The E-F loop in the GH-R contains Trp104 which is critical for interaction with GH (39). Similarly, in the EPO-R, Phe63 was found to be important for EPO binding (40). Thus our finding of involvement of Tyr173 in the G-CSF-R suggests that use of an aromatic residue in the E-F loop for ligand binding may be a common feature of class 1 cytokine receptors. However, in the IL-5-R α-chain, a charged residue (Arg188) in the predicted E-F loop was important for IL-5 binding (42), in the IL-6R α-chain Val199 was implicated and to a lesser extent, Tyr188 (43). In the human common β-subunit (hβc), there was no effect of mutation of the putative E-F loop (46). Thus, an equivalent residue is not universally used by all receptors for ligand binding (Fig. 6).

The second residue that contributed a large proportion of the binding energy to the GH-RGH complex was Trp169 in the B’-C’ loop (39). In the G-CSF-R B’-C’ loop, we found a hydrophobic, non-aromatic residue, Ile239, was the most important of the residues tested, with a lesser contribution from His238. Other receptor chains that contain a ligand-binding aromatic hydrophobic residue in predicted B’-C’ loops include the IL-6-Rα (43), IL-2-Rβ (44), AIC2A (47), and hβc (41). However, polar residues in the B’-C’ loop also appear to be important in ligand binding of the EPO-R (40), IL-2-Rβ (44), and hβc (41, 48).

Although the F’-G’ loop in the GH-R was not important for ligand binding, it was involved in contact with GH (9). The residue apparently contributing the most to ligand binding by the G-CSF-R, Arg288, is in the predicted F’-G’ loop. Surprisingly, the neighboring aromatic Trp289 was not important. This region contained important ligand-binding residues also in hβc (Tyr421) (49), the EPO-R (a peptide ligand) (11), the IL-6Rα (44), and hβc (47), and GM-CSF-β (Arg286) (45). A recent NMR study of the interaction of the soluble BC domain with G-CSF also implicated the F’-G’ loop in ligand binding (50).

Mutation of three residues in the predicted G-A’ loop of the G-CSF-R (hGR3) reduced receptor activity by 40-fold but mutation of the individual residues did not account for this effect, suggesting that there may be a synergistic effect of the multiple mutation. Residues in this region that connects the BN and BC domains may form interactions that are important for maintaining the correct orientation of the domains and thus may have indirect effects on ligand binding.

Overall, there are many similarities in the sites of ligand interaction among the cytokine receptors, as has been discussed by others (40, 46), although the type of residue involved differs between receptors. It has been suggested that hydrophobic interactions may be the most important (39), but this is not universally the case. Interestingly, receptor interaction of IL-4 appears to require predominantly electrostatic interactions (51), whereas the residues we have identified in the G-CSF-R CRH domain have a mixture of charged, polar, and hydrophobic side chains.

G-CSF does not show any species specificity among the species tested, therefore residues important for function would be expected to be conserved between the species. In the CRH domain, there is 66% sequence identity between the murine and human receptors. Of the residues identified as important in this study, four are conserved and two are not (human His238, Ile239, murine Tyr237, Met238), reflecting the overall sequence conservation. Evolutionary sequence changes in receptor and ligand within a species may produce complementary

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### Table III

Effect of additional mutations on G-CSF-R function

| Mutant position | Proliferation EC_{50} ratio | Binding EC_{50} ratio | Binding K_d ratio |
|----------------|-----------------------------|-----------------------|-------------------|
| Wild-type      | 20 ± 5                      | 20 ± 5                | 20 ± 5            |
| L172A E-F loop | 110 ± 22                    | 5.6 ± 2               | 5.6 ± 2           |
| Y173A          | 660 ± 130                   | 33 ± 2               | 33 ± 2           |
| Q174A          | 24 ± 2                      | 1.3 ± 2              | 1.3 ± 2          |
| D197A G-A'     | 12 ± 5                      | 0.63 ± 1             | 0.63 ± 1         |
| M199A          | 39 ± 6                      | 2.0 ± 9              | 2.0 ± 9          |
| D200A          | 97 ± 19                     | 5.0 ± 2              | 5.0 ± 2          |
| V201A          | 24 ± 4                      | 1.2 ± 1              | 1.2 ± 1          |
| I239A B’-C’   | 1100 ± 360                  | 59 ± 10             | 59 ± 10         |
| N240A          | 23 ± 3                      | 1.3 ± 0.9            | 1.3 ± 0.9        |
| R288A F’-G’   | 5500 ± 1600                 | 280 ± 10             | 280 ± 10         |
| W289A          | 12 ± 2                      | 0.63 ± 4             | 0.63 ± 4         |

* a Significantly different from wild-type (p < 0.01), determined by Student’s t test.

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### Figure 4

Ribbon diagram of the three-dimensional model of the CRH domain of G-CSF-R. Loops are represented as thin gray coils and β-strands as wide green ribbons. Residues that were found to be important for receptor function are shown as CPK models and labeled by residue type and number. Red residues had >30-fold effect on activity. The N and C termini are labeled NT and CT, respectively. The figure was prepared using the programs MOLSCRIPT (55) and Raster3D (56, 57).

of the CRH domains could be expected, if the receptors were to form a contact dimer similar to that of the growth hormone receptor. Small alterations in alignment and side chain conformation did not affect the electrostatic potential (data not shown). We, therefore, consider it unlikely that the G-CSF-R forms a homodimer similar to the growth hormone receptor complex.

**DISCUSSION**

Site-directed mutagenesis of cytokine receptors, in which selected residues are replaced to evaluate the contribution of the amino acid side chains to ligand binding, has provided information about the binding sites of many receptors in this family (39–45). The G-CSF-R contains three domains or over 300 amino acid residues that have been implicated in ligand binding. Alanine scanning was therefore impractical and molecular modeling based on the GH-R structure was used to predict likely binding residues for testing by mutagenesis. Mutation of four regions chosen in this way gave a reduction in receptor activity and binding affinity in three cases, suggesting that the model was substantially correct. Further mutagenesis of single residues resulted in the identification of three loops containing ligand binding residues, the E-F loop in the BN domain and the B’-C’ and F’-G’ loops in the BC domain.
FIG. 5. Surface representation of the modeled CRH domain of G-CSF-R (A and B) compared with the GH-R (C and D). A, the area which corresponds to the dimerization interface of the two growth hormone receptor CRH domains is colored purple. The green surface patch indicates the area homologous to the GH-binding site on GH-R. B, electrostatic isopotential surfaces of the CRH domain of G-CSF-R. The isopotential surface at +0.5 kT/e is shown in blue, whereas the one at −0.5 kT/e is rendered in red. C and D show the electrostatic isopotential surfaces of two GH receptor molecules rotated to reveal the dimerization surfaces (areas 1–5). The figure was created using the GRASP program (58).

FIG. 6. Alignment of the loop regions containing ligand binding residues from G-CSF-R with those of other cytokine receptors. Residues implicated in ligand binding (see “Discussion”) are in bold and known β-strands are underlined. Alignments are adapted from Bazan (1) and Goodall et al. (59).

mutations resulting in no overall loss of affinity. It is more difficult to understand why the affinity of a xenogeneic interaction would be preserved during sequence divergence, except perhaps by chance. It would be interesting to determine whether the non-identical murine residues are functional in the human receptor, especially the Tyr residue, which is a non-conservative substitution for His.

None of the residues mutated in this study had an effect that was equivalent to the two Trp residues in GH-R that contribute most of the binding energy to the complex (39). While the number of residues that we mutated in this study was relatively small, so that we could have missed a residue of major importance, this is less likely to be true in the case of G-CSF, where extensive mutagenesis failed to reveal any residues having a large effect (20). Therefore the high affinity of the G-CSF ligand-receptor complex may be due to a substantially larger number of interactions than the 11 that were important in the GH-R complex (39), or there may be a secondary interaction involved. The binding affinity of monomeric G-CSF-R is apparently about 2 nm, or 20-fold less than the dimeric affinity (52, 53), in contrast with the GH-R, where monomeric binding is only 2–3-fold lower affinity than dimeric receptor binding (54). This difference suggests that the second binding site and/or secondary interactions following ligand binding are more important in the formation of a high affinity complex with the G-CSF-R than the GH-R. The apparent effects of mutations in either G-CSF or its receptor in one binding site may be reduced by the presence of an intact second binding site allowing dimerization.

Previously published data indicate that the G-CSF receptor complex involves only binding site 2 (comprising residues on helices A and C) and site 3 (residues on the N-terminal region of helix D) on G-CSF but not site 1 (comprising residues on helices A and C-terminal residues of helix D) (20), in contrast to growth hormone which employs sites 1 and 2. In addition, the Ig-like domain present in IgG-R is necessary for high affinity ligand binding (6, 19), again in contrast to the growth hormone receptor which does not include an Ig-like domain. The soluble extracellular domain of the G-CSF receptor forms a tetrameric 2:2 G-CSF-G-CSF-R complex with ligand (7), rather than 2:1. Together, these reports indicate that it is unlikely that the G-CSF-G-CSF-R complex interacts according to the well known growth hormone binding paradigm.

Our analysis of the electrostatic potential generated by G-CSF-R confirms the above mentioned studies (6, 7, 20) by showing that the formation of a (CRH)2 contact dimer is unlikely to follow the binding pattern observed in the growth hormone receptor complex. The recently published NMR structure of the BC domain (50) is very similar to our model, establishing that our calculations for this domain are likely to be correct. Because the BC domain is unlikely to be involved in dimerization, it may be that additional contacts between the receptor monomers are formed utilizing the three membrane proximal FnIII domains. The contact between the FnIII domains would form a secondary interaction following ligand binding, and could significantly contribute to the binding affinity of the complex.

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