Molecular Modeling-guided Mutagenesis of the Extracellular Part of gp130 Leads to the Identification of Contact Sites in the Interleukin-6 (IL-6)-IL-6 receptor-gp130 Complex*

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The transmembrane protein gp130 is involved in many cytokine-mediated cellular responses and acts therein as the signal-transducing subunit. In the case of interleukin-6 (IL-6), the signal-transducing complex is composed of the ligand IL-6, the IL-6 receptor (IL-6R, gp80, CD120), and at least two gp130 (CD130) molecules. The extracellular part of the signal transducer gp130 consists of six fibronectin type III-like domains. It has recently been shown that the three membrane distal domains bind to the IL-6-IL-6R complex. A structural model of the IL-6-IL-6R-gp130 complex enabled us to propose amino acid residues in these domains of gp130 interacting with IL-6 bound to its receptor. The proposed amino acid residues located in the B'C loop (Val552) and in the F'G loop (Gly306, Lys307) of domain 3 and in the hinge region (Tyr218) connecting domains 2 and 3 of gp130 were mutated to disturb ternary complex formation. Binding of wild type and mutants of the extracellular region of gp130 was studied by use of a co-precipitation assay and Scatchard analysis. All mutants showed decreased binding to the IL-6-IL-6R complex. Biological function of the membrane-bound gp130 mutants was studied by STAT (signal transducer and activator of transcription) activation in COS-7 cells and by proliferation of stably transfected Ba/F3 cells. Reduced binding of the mutants was accompanied by decreased biological activity. The combined approach of molecular modeling and site-directed mutagenesis has led to the identification of amino acid residues in gp130 required for complex formation with IL-6 and its receptor.

Interleukin-6 (IL-6)1 is a multifunctional cytokine that induces various biological responses on different target cells. The most prominent IL-6 effects are the terminal differentiation of B cells, growth promotion of B and T lymphocytes, and induction of acute phase protein synthesis in hepatocytes (1, 2). IL-6 exerts its action via a surface receptor composed of an IL-6 binding subunit (gp80 or IL-6 receptor) (3) and a signal-transducing subunit (gp130) (4, 5). First, IL-6 forms a low affinity complex with the IL-6 receptor (IL-6R). Subsequently, this binary complex interacts with two molecules of gp130, resulting in a high affinity receptor complex (6). Both receptor components are members of the hematopoietic cytokine receptor superfamily (7).

The dimerization of the cytoplasmic parts of gp130 initiated by receptor complex formation is believed to be the decisive step for the onset of the signal transduction cascade, which leads to phosphorylation and thereby activation of associated tyrosine kinases of the Jak family (8, 9). The activated Jak kinases in turn phosphorylate distinct cytoplasmic tyrosine residues of gp130. In the context of their surrounding sequence, these phosphotyrosine side chains serve as docking sites for two transcription factors of the STAT family, STAT1 and STAT3 (10, 11). The bound transcription factors also become tyrosine-phosphorylated by the Jak kinases. Via their SH2-domains, the tyrosine-phosphorylated STAT factors homodimerize or heterodimerize. Finally, after an additional serine phosphorylation and translocation into the nucleus (12, 13), the dimerized STAT factors bind to enhancer sequences of IL-6 target genes and induce their expression (14).

In contrast to the intensively studied molecular events of the Jak/STAT signaling pathway emerging from the cytoplasmic part of gp130, the molecular determinants of the extracellular part of gp130 required for the interaction with the IL-6-IL-6R complex are less well characterized. By using deletion mutants of the cytoplasmic and transmembrane parts of gp130 the region required for ternary complex formation was recently confined to the membrane distal half of gp130 (15). This region consists of three fibronectin type III-like domains. The second and third ones are designated the cytokine receptor homology domains and are characterized by four conserved cysteine residues and the WSEWS motif, respectively.

To map the contact site of gp130 to IL-6 in the IL-6-IL-6R complex in more detail we have introduced single amino acid substitutions in the cytokine binding domain and studied their influence on ternary complex formation. Also, the signaling capabilities of gp130 wild type and point mutants were analyzed. The selection of residues as targets for mutagenesis was guided by a molecular model consisting of IL-6 and the cytokine receptor domains of the IL-6R and gp130 (16). The model was built using the x-ray structure of the related human growth hormone-growth hormone receptor complex as a template (17).

Whereas growth hormone exhibits two binding sites (sites 1 and 2) for its receptor, extensive mutational analysis of IL-6 led to the identification of three binding sites. The interactions of site 1 with the IL-6R (18, 19) and of site 2 with gp130 (20) correspond to those in the growth hormone receptor complex and are represented in the model. Due to lack of structural
information, the second gp130 molecule, which contacts site 3 of IL-6 (21, 22), cannot be integrated in this model.

The crucial residues involved in the interaction of IL-6 with the IL-6R from this model were confirmed by mutagenesis studies of both molecules (16, 23–25). The defined contact sites of IL-6R in the IL-6/IL-6R interface are located in the predicted loop regions of the third fibronectin type III-like domain. We assumed that the interaction of gp130 with IL-6 is mediated by residues in analogous positions as in the respective cytokine receptor homology domain of the human growth hormone receptor.

This approach led to the identification of four amino acid residues of gp130 that, when exchanged, resulted in a reduced ternary complex formation and a decreased signaling capability. These data suggest that our model of the IL-6

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and T4-DNA ligase were obtained from Boehringer Mannheim (Mannheim, Germany). Tran35S-label was purchased from ICN (Meckenheim, Germany). Dulbecco's modified Eagle's medium was purchased from Life Technologies, Inc. (Eggenstein, Germany), and fetal calf serum was purchased from ICN (Meckenheim, Germany). Dulbecco's modified Eagle's medium and RPMI 1640 were purchased from Life Technologies, Inc. (Eggenstein, Germany), and fetal calf serum was purchased from ICN (Meckenheim, Germany). Dulbecco's modified Eagle's medium and RPMI 1640 were purchased from Life Technologies, Inc. (Eggenstein, Germany), and fetal calf serum was purchased from ICN (Meckenheim, Germany). Dulbecco's modified Eagle's medium and RPMI 1640 were purchased from Life Technologies, Inc. (Eggenstein, Germany), and fetal calf serum was purchased from ICN (Meckenheim, Germany). Dulbecco's modified Eagle's medium and RPMI 1640 were purchased from Life Technologies, Inc. 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PhosphorImager is presented in the form of bar graphs for wild type gp130 or the mutants Y218K and V252D were co-electrotransfected with 2 μg of plasmid DNA by electroporation. After electroporation, the cells were stimulated with 4 ng/ml IL-6 and 1 μg/ml of the shgp130K307E-Flag fusion protein expression vectors and metabolically labeled with [35S]cysteine/methionine. Subsequently, the soluble proteins were precipitated with 0.5 μg/ml of the monoclonal anti-Flag antibody M2 (Kodak, Germany) at 4 °C and immobilized using 1.25 mg/ml protein A-Sepharose. The Sepharose-bound [35S]shgp130-Flag fusion proteins were then incubated with 250 μl of COS-7 cell supernatant containing [35S]shIL-6R and 400 ng of IL-6 in a total volume of 1 ml. After overnight incubation at 4 °C, the IL-6/[35S]shIL-6R/[35S]shgp130-Flag complexes were sedimented by centrifugation via protein A-Sepharose. Expression of the [35S]shgp130-Flag fusion proteins and ternary complex formation were visualized by detection of 100-kDa (shgp130-Flag) and 66-kDa (shIL-6R) proteins after SDS-polyacrylamide gel electrophoresis and fluorography. The expression levels of the shgp130-Flag fusion proteins and level of shIL-6R co-precipitated in the ternary complex were quantified by use of a PhosphorImager (STORM 840, Molecular Dynamics, Inc.).

Electrophoretic Mobility Shift Assay (EMSA)—COS-7 cells were transiently transfected with wild type gp130-Y440, gp130Y218K-Y440, gp130V252D-Y440, gp130V252D6W-Y440, or gp130K307E-Y440 expression plasmids. 48 h after transfection, the cells were stimulated with 4 ng/ml IL-6 and 1 μg/ml of the indicated concentrations of IL-6 in a total volume of 1 ml. After overnight incubation at 4 °C, the IL-6/[35S]shIL-6R/[35S]shgp130-Flag complexes were sedimented by centrifugation via protein A-Sepharose. Expression of the [35S]shgp130-Flag fusion proteins and ternary complex formation were visualized by detection of 100-kDa (shgp130-Flag) and 66-kDa (shIL-6R) proteins after SDS-polyacrylamide gel electrophoresis and fluorography. The expression levels of the shgp130-Flag fusion proteins and level of shIL-6R co-precipitated in the ternary complex were quantified by use of a PhosphorImager (STORM 840, Molecular Dynamics, Inc.).

Immunofluorescence Staining—Approximately 1 × 10⁴ transfected COS-7 cells were seeded on glass coverslips. 48 h after transfection, the cells were fixed with 2% paraformaldehyde for 20 min as described (31), and unspecific binding sites were blocked with 1% bovine serum albumin for 30 min. Subsequently, the cells were incubated with 50 ng/ml of the monoclonal gp130-antibodies B-T12 or B-S12 for 20 min in darkness. The coverslips were then mounted on slides with Mowiol containing 7.5% glycerol. The electrophoresis was run in 0.25-fold TBE buffer at 200 V/cm for 4 h.

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Binding of IL-6/shIL-6R Complexes to Stably Transfected Ba/F3 Cells—5 × 10⁵ Ba/F3 cells, BAF-gp130, BAF-Y218K, or BAF-V252D cells were incubated with the indicated concentrations of [125I]IL-6 (specific activity, 2,500 cpm/ml) and 3.3 μg/ml shIL-6R in 100 μl of medium for 16 h at 4 °C. After sedimentation of the cells through dibutyryl-cAMP-thalidomide solution (1.020 g/ml), cell-associated and free radioactivity were measured in a γ-counter. Specific binding was obtained by subtracting the non-specific binding from that total binding to the Ba/F3 transfecants (total binding).

Cell Proliferation Assay—2 × 10⁵ Ba/F3 cells stably transfected with gp130, gp130Y218K, or gp130V252D were cultured in RPMI 1640 containing the indicated concentrations of IL-6 and shIL-6R or of the antibodies B-S12 and B-P8. B-S12 is an agonistic gp130-antibody, while B-P8 is non-specific.
B-P8 acts synergistically (32). After 60 h of incubation, viable and metabolically active cells were quantified by using the colorimetric Cell Proliferation Kit II (XTT) (Boehringer Mannheim).

RESULTS

Selection of gp130 Amino Acid Residues for Site-directed Mutagenesis Guided by a Molecular Model of the IL-6/IL-6R/gp130 Complex—Based on the x-ray structure of the human growth hormone-human growth hormone receptor complex, a model of the IL-6/IL-6R/gp130 complex was built and used successfully for the rational design of site-directed mutagenesis experiments with IL-6 and the IL-6R (24). We have taken this model to guide mutagenesis experiments also with gp130 and focused on residues predicted to be in contact with site 2 of IL-6. Like in the human growth hormone-growth hormone receptor complex this area is characterized by several hydrophobic interactions covered by a hydrophilic, charged layer. Besides others, the residues involved and therefore mutated in this study are tyrosine 218 (Y218), glycine 306 (G306), lysine 307 (K307), and valine 252 (V252) (Fig. 1). The mutations were chosen for the following reasons: 1) Y218K and V252D are expected to destroy the central hydrophobic interaction between gp130 and IL-6, 2) G306W should introduce steric constraints in the contact region, and 3) K307E should destabilize the hydrophobic core of the interaction by making it more accessible for water molecules. All of these mutations were expected to impair the interaction between gp130 and the ligand-receptor complex.

Binding of Soluble gp130 Mutants to the IL-6-shIL-6R Complex—The point mutations described above were introduced by site-directed mutagenesis in addition, the transmembrane and cytoplasmic parts of gp130 were replaced by a Flag epitope to allow immunoprecipitation with a specific monoclonal anti-Flag-antibody. Formation of ternary complexes was analyzed by co-precipitation of [35S]shIL-6R in the presence of IL-6.

The mutant proteins were transiently expressed in COS-7 cells. After metabolic labeling with [35S]cysteine/methionine, the soluble proteins were immunoprecipitated from supernatants, with the anti-Flag antibody M2 and immobilized using protein A-Sepharose. The Sepharose-bound [35S]shgp130-Flag proteins were subsequently incubated with IL-6 and the shed form of metabolically labeled IL-6R ([35S]shIL-6R). The ternary complexes were precipitated and subjected to SDS-polyacrylamide gel electrophoresis. Both the [35S]shgp130 proteins and the [35S]shIL-6R were visualized by fluorography (Fig. 2).

In the presence of IL-6, the shIL-6R was immunoprecipitated with a monospecific anti-IL-6R-antiserum (lane 1). As expected, shIL-6R appeared as a protein of about 66 kDa. The shIL-6R was not precipitated in the absence of a specific antiserum, regardless whether IL-6 was present (lane 2) or not (lane 3), indicating that the shIL-6R does not bind unspecifically to protein A-Sepharose. When shIL-6R was incubated with wild type shgp130-Flag and IL-6 (lane 5), it was co-precipitated, indicating that the ternary complex had been formed.

The results obtained with the gp130 point mutants are shown in lanes 6–9. The IL-6R band corresponding to the mutant

TABLE I

| Protein             | Binding % |
|---------------------|-----------|
| shgp130-Flag        | 100       |
| shgp130 Y218K-Flag  | 76.7 ± 5.1|
| shgp130 V252D-Flag  | 45.3 ± 4.0|
| shgp130 G306W-Flag  | 63.6 ± 12.7|
| shgp130 K307E-Flag  | 73.7 ± 17.7|

FIG. 3. Immunofluorescence staining of wild type and mutant gp130 proteins. About 2 × 10⁵ COS-7 cells expressing the proteins indicated were grown on glass coverslips. 48 h after transfection, the cells were incubated with either the monoclonal gp130-antibody B-T12 (left panel) or with the monoclonal gp130-antibody B-S12 (right panel). The antibodies bound to the cell surface were visualized by subsequent incubation with a rhodamine-conjugated anti-mouse-IgG. The stained cells were analyzed by fluorescence microscopy and photographed at a × 500 magnification.
Y218K-Flag (lane 6) exhibited a similar intensity as the wild type gp130, whereas only faint bands were visualized for the mutants V252D (lane 7), G306W (lane 8), and K307E (lane 9).

The 100-kDa shgp130-Flag wild type and mutant proteins available for complex formation can be seen in addition to the shIL-6R protein in lanes 4–9. Using a PhosphorImager, we quantified the relative amounts of [35S]shgp130-Flag fusion proteins and [35S]shIL-6R as shown in Fig. 2B. This enabled us to compare the amounts of shgp130-Flag proteins with their capabilities to form complexes with IL-6 and the shIL-6R.

A comparison of the binding data of wild type and mutant gp130-Flag proteins calculated from four independent experiments is shown in Table I. The mutant V252D-Flag showed 45.3 ± 4.0% binding relative to wild type gp130, which was
defined as 100%. Y218K-Flag and K307E-Flag exhibited 76.7 ± 5.1% and 73.7 ± 17.7% binding, respectively. The mutant G306W showed 63.6 ± 12.7% binding.

Evaluation of the Structural Integrity of gp130 Mutants by Immunofluorescence—To ensure that the reduced binding of the gp130 mutants was not due to misfolded proteins, we performed immunofluorescence staining experiments. COS-7 cells transfected with membrane-bound wild type and mutant gp130 proteins were incubated with the monoclonal human gp130 antibody B-T12 or B-S12. The B-T12 antibody recognizes an epitope within the gp130 fibronectin type III domains 4–6, and the B-S12 antibody binds to the third domain of gp130, comprising the mutated residues (32). Both antibodies do not recognize linear peptide sequences of gp130 and are therefore suitable for the detection of properly folded proteins. The bright staining of the cells shows that the B-T12 and the B-S12 antibodies were able to bind to all of the gp130 point mutants (Fig. 3). Thus, the reduced binding of the gp130 mutants to the IL-6-IL6R complex does not seem to be due to changes either in their overall structure or in the fold of the third domain.

Biological Activity of gp130 Mutants Determined by STAT1 Activation—Since ternary complex formation is essential for signal transduction, we supposed that changes in binding of the gp130 point mutants to IL-6-IL6R complexes should also lead to alterations in signaling. In human hepatoma cells (HepG2), the transfection factors STAT1 and STAT3 are efficiently activated by tyrosine phosphorylation after IL-6 stimulation (8). In COS-7 cells, however, the concentration of STAT3 is too low to study its activation. Therefore, we examined only STAT1 activation. To obtain a selective and strong activation of STAT1, we have used constructs encoding the extracellular domains of gp130 or of the respective mutants, the transmembrane and the membrane-proximal cytoplasmic domains (box 1 and box 2) of gp130 fused to the tyrosine 440 module of the interferon-γ receptor (YDKPH; Y440) (Fig. 4A). Similar constructs using the extracellular domain of the erythropoietin receptor have recently been shown to efficiently activate STAT1 in COS-7 cells (11).

COS-7 cells were transfected with the expression plasmids coding for the wild type gp130-Y440 and the mutants Y218K-Y440, V252D-Y440, G306W-Y440, and K307E-Y440. 48 h after transfection, the cells were stimulated with IL-6 and shIL-6R for 15 min. Subsequently, the cells were divided into two fractions to investigate STAT1 activation and cell surface expression of the gp130 proteins.

Nuclear extracts were prepared from the first fraction, and STAT1 activation was investigated in an EMSA using a radioactively labeled m67SIE probe. Wild type gp130-Y440 protein as well as the point mutants were able to recruit STAT1 and to promote the formation of STAT1 homodimers (Fig. 4B) after stimulation with IL-6 and shIL-6R but to different extents (lanes 4, 6, 8, 10, and 12). No STAT1 activation was detectable without stimulation (lanes 1, 3, 5, 7, 9, and 11). When cells were transfected with the pSVL control plasmid, STAT1 activation induced by endogenous gp130 upon stimulation was not observed (lane 2). The relative intensities of the STAT1 bands were quantified using a PhosphorImager.

To compare cell surface expression levels of wild type and mutant gp130-Y440 proteins and to quantify the numbers of cells that are able to respond to IL-6 stimulation, we performed FACS analyses with the second fraction of the transfected COS-7 cells using the monoclonal gp130-antibody B-T12. Since B-T12 recognizes an epitope within the membrane-proximal half of the gp130 extracellular region, the single amino acid exchanges should not lead to alterations in the affinity of this antibody. To examine autofluorescence of the COS-7 cells and unspecified binding of the secondary antibody, these cells were incubated with the R-phycocerythrin-conjugated anti-mouse IgG only (Fig. 4C, histogram a). The shift toward higher fluorescence intensities measured after incubation with the B-T12 antibody (histogram b) is due to endogenous gp130 expressed by COS-7 cells. In histograms c to g, the marker M1 defines cells expressing wild type or mutant gp130-Y440 proteins in addition to endogenous gp130. About 10–15% of the cells showed cell surface expression of the gp130-Y440 proteins.

The distribution of the fluorescence intensities (i.e. gp130-Y440 cell surface expression) as a function of the number of cells is similar for wild type and mutant gp130-Y440 proteins. Therefore, the STAT1 activation induced by each mutant after stimulation with IL-6-shIL-6R can be compared with STAT1 activation induced by wild type gp130-Y440. To account for the variations in numbers of cells expressing wild type or mutant gp130-Y440 due to different transfection efficiencies, we calculated the relative biological activities by comparing the IL-6-IL6R-induced STAT1 activation with the numbers of cells expressing gp130-Y440 wild type or mutant proteins as determined by FACS analysis. The biological activities of the gp130-Y440 point mutants averaged from three independent experiments are summarized in Table II. Wild type gp130-Y440 activity was set to 100% and compared with the mutants. The STAT1 activation of the mutant proteins were 60.7 ± 10.2% for Y218K-Y440, 17.9 ± 5.7% for V252D-Y440, and 19.6 ± 6.2% for the mutant G306W-Y440. K307E-Y440 exhibited a biological activity of 54.8 ± 23.3%.

Binding of the IL-6-shIL-6R Complexes to Ba/F3 Cells Stably Transfected with gp130 or gp130 Mutants—To study binding of the IL-6-shIL-6R complexes to membrane-bound gp130 proteins, we have chosen gp130-deficient Ba/F3 cells for transfection with either wild type gp130 or gp130Y218K and gp130V252D, respectively. After transfection of Ba/F3 cells with pSVL containing the cDNA of wild type or mutant gp130 proteins together with a plasmid carrying the neomycin resistance gene, we selected for G418 resistance. To isolate neomycin-resistant Ba/F3 subclones expressing the expected proteins, the selected cells were analyzed for binding of the anti-gp130-antibodies B-T12 (Fig. 5B) and B-S12 (not shown) by flow cytometry.

For equilibrium binding studies, Ba/Fgp130, BaF-Y218K, and BaF-V252D subclones were incubated with increasing amounts of [125I]IL-6 (0.5–10 nM) and an excess of soluble IL-6R (Fig. 5A). Based on the amount of IL-6 bound at the saturating concentration, the number of cell surface gp130 molecules per cell was calculated by Scatchard analysis to be 670 for BaF-gp130 (Kd = 1.7 nM) and 790 for BaF-Y218K (Kd = 2.9 nM), assuming an IL-6-gp130 ratio of 1:2. No binding of [125I]IL-6 was detectable for BaF-V252D cells.

| Protein | Biological activity |
|---------|---------------------|
| gp130-Y440 | 100 |
| gp130 Y218K-Y440 | 60.7 ± 10.2 |
| gp130 V252D-Y440 | 17.9 ± 5.7 |
| gp130 G306W-Y440 | 19.6 ± 6.2 |
| gp130 K307E-Y440 | 54.8 ± 23.3 |
FIG. 5. Binding of the IL-6/IL-6R complex to Ba/F3 cells stably transfected with gp130 proteins. A, 5 × 10^6 Ba/F3 cells stably transfected with either wild type gp130 or the mutants gp130Y218K and gp130V252D, respectively, were incubated with increasing amounts of radiolabeled IL-6 and an at least 6-fold molar excess of shIL-6R for 16 h at 4 °C. After sedimentation through dibutyl/dinonyl phthalate oil, cell-associated and free radioactivity were measured in a γ-counter. Specific binding as the difference of total and nonspecific binding (i.e. IL-6 binding to untransfected BaF/3 cells) is plotted. Scatchard plots are presented as insets. Three independent experiments were performed. The
Growth Characteristics of Ba/F3 Transfectants—We next measured the proliferation of the Ba/F3 subclones BAF-gp130, BAF-Y218K, and BAF-V252D. The cells were grown in the presence of either IL-6/shIL-6R or the agonistic antibodies B-S12/B-P8. The proliferation curve observed for BAF-Y218K was almost identical to BAF-gp130, whereas the BAF-V252D subclone showed a drastically decreased sensitivity to stimulation with IL-6/shIL-6R (Fig. 6A). The reduced sensitivity of the BAF-V252D cells is not due to a lower expression level of this mutant, since stimulation of all three Ba/F3 subclones with the agonistic gp130 antibodies B-S12 and B-P8 led to comparable proliferative responses (Fig. 6B). These observations are in good agreement with the impaired binding of radioiodinated IL-6 to BAF-V252D, indicating that V252 of gp130 exerts a central role in ternary complex formation with IL-6 and IL-6R.

DISCUSSION

In the present study, site-directed mutagenesis and molecular modeling were combined to examine structural determinants of the interaction of IL-6 and gp130. Experimental structures of IL-6, IL-6R, gp130, and their complexes do not exist. Instead, our model (16) served as the basis for selecting residues of gp130 that are presumed to be in contact with site 2 of IL-6 (Fig. 1) and, therefore, candidates for mutation. The residues we mutated are located in the B'C' loop (Val252), in the F'G' loop (Gly306, Lys307) of domain 3 and in the hinge region (Tyr218) connecting domains 2 and 3 according to our model. Val252 and Tyr218 are part of a hydrophobic cluster formed by IL-6 and gp130, including Val121 of IL-6, known to be involved in this interaction (20). The role of arginine and lysine residues in contact regions of such complexes is to participate in shielding the central hydrophobic core from access by water (33, 34). Such a role can also be assigned to Lys307. The important aspect of Gly306 seems to be that the absence of a side chain provides space occupied by Phe125 of IL-6 in our model of the complex. The nature of the residues we introduced by site-directed mutagenesis was intended to be incompatible with the presumable role of the side chains in the wild type complex.

averages of one triplicate experiment are shown. B, the cell surface expression of the gp130 proteins in stably transfected Ba/F3 cells is demonstrated by binding of the gp130 antibody B-T12 as analyzed by flow cytometry. The closed peaks represent Ba/F3 subclones incubated with only the secondary R-phycocerythrin conjugated antibody. The shift (open peaks) toward higher fluorescence intensities indicate the cells after binding of B-T12.
Therefore, the mutants Y218K and V252D were produced to destroy the hydrophobic cluster. G306W was chosen to cause sterical constraints in the interface by the most bulky side chain available. In case of K307E, charge inversion and shortening of the side chain was expected to weaken the shielding and, hence, the stability of the central hydrophobic cluster.

Our results of binding as well as of functional assays suggest that gp130 interacts with the IL-6-IL-6R complex in the manner derived from the model. All four mutants, Y218K, V252D, G306W, and K307E behaved as expected; a reduction of complex formation capability of the soluble gp130 mutants (wild type > Y218K ~ K307E > G306W > V252D; Table I) is accompanied by a corresponding reduced ability of the membrane-bound form to induce STAT1 activation in transiently transfected COS-7 cells (wild type > Y218K ~ K307E > G306W ~ V252D; Table II).

Since COS-7 cells express low levels of endogenous gp130, an exact quantification of surface expression of the mutants in these cells is not possible. Therefore, we decided to stably transfect Ba/F3 cells, which are known to lack endogenous gp130. The V252D mutant exerted the most prominent effects in both the co-precipitation assay and EMSA and thus was selected for stable transfection. In addition, as a representative for the mutants showing modest effects, the Y218K mutant was chosen.

The reduced binding of the Y218K mutant observed in the co-precipitation assay with soluble receptor proteins (76.7 ± 5.1% of wild type) was confirmed by Scatchard analysis of the binding data obtained from the BAF-Y218K subclone. Here, this mutant exhibited a 1.7-fold reduced association constant compared with wild type gp130 (Fig. 5). In the proliferation assay, the Y218K mutant was rather indistinguishable from wild type gp130. Possibly, this results from a compensation of the reduced binding by an increased surface expression of Y218K (790 sites/cell) compared with wild type (670 sites/cell) as revealed by Scatchard analysis.

Surprisingly, the shgp130V252D-Flag mutant, which showed 45% of wild type binding in the co-precipitation assay, exhibited only a residual binding activity when expressed in Ba/F3 cells and a strongly reduced ability to induce proliferation in these cells. This cannot be due to misfolded protein, since (a) the V252D mutant can be activated by an agonistic monoclonal antibody recognizing an epitope located in the domain where the mutation resides and (b) FACS analysis revealed surface expression comparable with wild type as well as with the Y218K mutant. The low activity of this mutant in both signaling assays, STAT activation in transiently transfected COS-7 cells and induction of proliferation in stably transfected Ba/F3 cells, reflects its incapability of being efficiently dimerized by the IL-6-shIL-6R complex.

The discrepancy between binding observed with the soluble receptor protein compared with the membrane-bound V252D mutant can be explained by assuming that two different sites on gp130 are involved in the interaction with the two well characterized gp130 binding sites on IL-6. In the case of the co-precipitation binding assay with immobilized soluble gp130 mutants, the IL-6-IL-6R complex may still bind with low affinity via site 3 of IL-6 to the IL-6 binding site of gp130, which should not be affected by the mutation. This interaction can be detected in this assay due to the high local concentration of the immobilized shgp130 and the large excess of IL-6 and shIL-6R. However, two intact IL-6 binding sites on gp130 are required for high affinity binding on the cell surface and thus for initiation of the signal transduction cascade. As derived from the model, the V252D mutation is supposed to destroy the interaction of gp130 with site 2 of IL-6 leading to a strongly reduced affinity and to a severely impaired ligand induced dimerization as observed in the cell proliferation and STAT activation assays.

Two models of the functional IL-6-IL-6R-gp130 complex are discussed in the literature, which are contradictory with respect to their stoichiometry. The hexameric complex (35, 36) consists of two molecules each of IL-6, IL-6R, and gp130. In this model, each gp130 is in contact with both sites (sites 2 and 3) of IL-6. In the tetrameric complex (16) consisting of one IL-6, one IL-6R, and two gp130 molecules, one gp130 contacts site 2, and the second gp130 contacts site 3 of IL-6. Thus, since site 2 of IL-6 is involved in either model, reduced binding of our gp130 mutants explains impairment of ternary complex formation regardless of the actual complex stoichiometry.

The central role of Val252 for the interaction of gp130 with site 2 of IL-6 proposed by the model and supported by our findings strengthens a common mechanism emerging from different studies on the interaction of four-helical bundle cytokines with class I cytokine receptors. According to our model, the valine residue described in this study is located in the B’C’ loop of the third domain of gp130 and is superimposable with Trp149 in the corresponding domains of the human growth hormone receptor as well as Phe248 in the IL-6R. By site-directed mutagenesis, these two residues have been shown to be essential for receptor/ligand interaction (25, 34). Furthermore, in the human growth hormone-human growth hormone receptor complex other residues in the receptor/ligand interface contribute only marginally to the free energy of binding (34). The modest reduction of binding observed for the gp130 mutants Y218K, G306W, and K307E suggests that these residues are part of the gp130/IL-6 interface but do not participate in the central interaction. The rational approach of combining site-directed mutagenesis and molecular modeling has led to a first characterization of a region in the ectodomain of gp130 that is involved in IL-6-IL-6R-gp130 ternary complex formation.

Gp130 is a central signal-transducing receptor subunit utilized by a whole set of structurally related cytokines: leukemia inhibitory factor, ciliary neurotrophic factor, interleukin-11, oncostatin M, cardiotrophin-1, and IL-6. Whereas one gp130/IL-6R contact site is described in detail by this study, the other cytokine/gp130 interaction sites are still unknown. The mutants presented here are promising molecular tools to examine the specificity and promiscuity of gp130 in those various cytokine-receptor complexes.

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Molecular Modeling-guided Mutagenesis of the Extracellular Part of gp130 Leads to the Identification of Contact Sites in the Interleukin-6 (IL-6)-IL-6 receptor-gp130 Complex
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