Title:
LEUKEMIA INHIBITORY FACTOR, CARDIOTROPHIN-1 AND ONCOSTATIN M SHARE STRUCTURAL BINDING DETERMINANTS IN THE IG-LIKE DOMAIN OF LIF RECEPTOR

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Running title: Involvement of LIFR Ig-like domain in cytokine binding
SUMMARY

The leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1) and oncostatin M (OSM) are four helix bundle cytokines acting through a common heterodimeric receptor composed of gp130 and LIF receptor (LIFR). Binding to LIFR occurs through a binding site characterized by a FxxK motif located at the N-terminus of helix D (site III). The Ig-like domain of LIFR was modeled and the physico-chemical properties of its Connolly surface were analyzed. This analysis revealed an area displaying properties complementary to those of the LIF site III. Two residues of the Ig-like domain of LIFR, D214 and F284, formed a mirror image of the FxxK motif. Engineered LIFR mutants in which either or both of these two residues were mutated to alanine were transfected in Ba/F3 cells already containing gp130. The F284A mutation impaired the biological response induced by LIF and CT-1 whereas the response to OSM remained unchanged. The D214 mutation did not alter the functional responses. The D214A/F284A double mutation, however, totally impaired cellular proliferation to LIF and CT-1 and partially impaired OSM induced proliferation with a 20-fold increase in EC$_{50}$. These results were corroborated by the analysis of Stat3 phosphorylation and Scatchard analysis of cytokine binding to Ba/F3 cells. Molecular modeling of the complex of LIF with the Ig-like domain of LIFR provides a clue for the superadditivity of the D214A/F284A double mutation. Our results indicate that LIF, CT-1 and OSM share an overlapping binding site located in the Ig-like domain of LIFR. The different behavior of LIF and CT-1, on one side, and of OSM, on the other side, can be related to the different affinity of their site III for LIFR.
INTRODUCTION

The cytokines of IL-6\(^1\) family are multifunctional proteins which regulate cell growth, differentiation and cellular functions and are involved in a variety of biological responses including the immune response, inflammation, neural development and hematopoiesis (1-5). This family of cytokines belongs to the long chain four helix bundle class (6) and is composed of 7 factors: interleukin-6 (IL-6), interleukin-11 (IL-11), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1) and the recently discovered cardiotrophin-like cytokine (CLC) (7,8). A viral form of IL-6 (vIL-6), encoded by the Kaposi sarcoma-associated herpesvirus (KSHV), is also part of this cytokine family (9).

These cytokines act by the formation of a multimeric receptor complex including a common receptor unit, gp130 (for a review, see (2)). The common use of gp130 explains in part the overlapping effects of these cytokines (10). IL-6 and IL-11 binding induces dimerisation of gp130 (11-17). LIF, CNTF, CT-1 and CLC induce heterodimerisation of gp130 and of the leukemia inhibitory factor receptor (LIFR) (7,8,18-22). Human OSM can recruit two kinds of active complexes resulting from the heterodimerisation of gp130 with LIFR or with the specific receptor for OSM, OSMR (23,24). In addition to transducing receptor chains (gp130, LIFR, OSMR), the active complex can contain specific co-receptor chains conferring high affinity. Specific co-receptor chains were observed for IL-6 (IL-6R) (25), IL-11 (IL-11R) (14) and for CNTF and CLC which share the same co-receptor chain (CNTFR) (7,8,26-28).

The receptor transducing chains of the IL-6 family, gp130 (13), LIFR (29) and OSMR (23) have a modular organization, with an extracellular domain, a short transmembrane domain and an intracellular domain. The extracellular domain of gp130 contains a N-terminal Ig-like domain, followed by a cytokine binding domain (CBD) and three fibronectin III domains. The cytokine binding domain is composed of two fibronectin III domains characterized by two conserved disulfide bridges in the N-terminal FnIII domain and a conserved WSXWS motif in the C-terminal FnIII domain (30). This motif is characteristic of class I cytokine receptors and is found for the other class I cytokine receptor family (30). The crystal structure of the gp130 cytokine binding domain indicates
Involvement of LIFR Ig-like domain in cytokine binding that its two FnIII domains have a L-shape quaternary structure (31). In addition to the modules of gp130, LIFR and OSMR possess an additional N-terminal module. For LIFR, this module is a second copy of a cytokine-binding domain (29), whereas for OSMR, it is limited to the C-terminal half of a CBD (23). The co-receptor chains are composed of an Ig-like domain followed by a CBD either linked to the membrane by a trans-membrane domain (IL-6R, IL-11R) (14,25) or through a GPI linker (CNTFR) (26).

Site-directed mutagenesis studies have shown that the cytokines of the IL-6 family interact with the receptor chains by three binding sites, numeredated from I to III by analogy with the growth hormone (32). Cytokines requiring a co-receptor α chain (e.g. CNTF, CLC, IL-6, IL-11) binds to this co-receptor (CNTFRα, IL-6Rα, IL-11Rα) through binding site I (C-terminal parts of loop AB and of helix D (8,20,26,33-36)). The glycoprotein gp130 interacts through binding site II, located on the solvent exposed faces of helices A and C (3,37,38). These sites are similar to binding sites I and II of the growth hormone (32). An additional binding site (site III) is located at the N-terminal part of helix D and may include the N-terminal part of the AB loop and the C-terminal part of the CD loop. It corresponds to an additional gp130 binding site for IL-6 and IL-11 (36,39,40) and to the LIFR binding site for LIF, OSM, CNTF, CT-1 and CLC. These LIFR binding cytokines are characterized by a FxxK motif located at the N-terminus of helix D which is required for LIFR binding and constitutes the signature of this interaction (41-45). The sites III are organized as exchangeable modules (45). The recently determined structure of KSHV IL-6 (vIL-6) complexed with gp130 has shown that IL-6 interacts with the cytokine binding domain of gp130 through site II and with the Ig-like domain of a second gp130 molecule through site III in a complex formed by two vIL-6 and two gp130 molecules (46,47).

Several lines of evidence, based on chimeric receptors, suggest that the Ig-like domain of LIFR is involved in site III binding (48-50). The aim of the present study was to determine the LIF binding site of LIFR. For this purpose, we modeled the Ig-like domain of LIFR and analyzed the properties of its surface, to identify an area with physicochemical properties complementary to those
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of the LIF site III. Two residues of the LIFR Ig-like domain, Asp\textsuperscript{214} and Phe\textsuperscript{284}, form a mirror image of LIF Phe\textsuperscript{156} and Lys\textsuperscript{159}, which constitute the LIFR binding hot spots (41). Single and double LIFR mutants, in which Asp\textsuperscript{214} or/and Phe\textsuperscript{284} were mutated to alanine, were tested for their ability to induce biological effects in response to LIF stimulation. Stimulation by CT-1 or OSM was also studied since these two cytokines share the capability to form an active complex with gp130 and LIFR (19,22,23,51). We show that LIF, CT-1 and OSM share overlapping binding sites located in the Ig-like domain of LIFR.

MATERIALS AND METHODS

Molecular modeling

The figures were drawn with Insight (Accelrys, San Diego, USA). The structure of human LIF (PDB access number: 1EMR) was modified in three places (Glu\textsuperscript{57}, Val\textsuperscript{172}, and Ser\textsuperscript{174}) to better represent the common variant. These three residues were mutated to those found in the common variant (Asp\textsuperscript{57}, Ile\textsuperscript{172}, and Ala\textsuperscript{174}) with the Biopolymer module of Insight. The resulting structure was energy-minimized with CHARMM (52). The structure is similar to that of murine LIF (53). Human cardiotrophin-1 was modeled by homology with LIF and CNTF (PDB access number: 1CNT) (54) by using the molecular modeling program MODELER (55), as implemented in Insight. The missing loop (residues 135-155) of oncostatin M (PDB access number: 1EVS) (44) was also modeled with MODELER.

The Ig-like domain of LIFR was modeled from the crystal structure of the Ig-like domain of gp130 in the complex with viral IL-6 (PDB access number: 1I1R) (46). The Ig-like domains of LIFR and of gp130 were aligned from a multiple alignment of the Ig-like domains of the gp130 receptor family with ClustalW (56). The positions of the \(\beta\)-strands in the Ig-like domain of LIFR were checked with the NNSSP program (57). Twenty models were generated with MODELER and refined by simulated annealing. The quality of the models was checked with Profiles_3D (58) and the Protein Verify module of Insight.
The stability of the mutated Ig-like-domains of LIFR (D214A, F284A and D214A/F284A LIFR) was calculated with the FOLD-X program (59) available at http://fold-x.embl-heidelberg.de. This program predicts the change in the stability of mutated proteins by computing the changes in the free energy of folding upon mutations.

**Surface properties**

A solvent probe radius of 1.4 Å was used to define the protein molecular surfaces (60). Continuum electrostatic calculations were carried out with the DELPHI package (61,62) under Insight. The formal charge set was used, with a ionic strength of 0. The dielectric constants of the proteins and of the surrounding medium were 2 and 80, respectively. The electrostatic potentials were mapped onto cubic grids with a 0.72 Å point spacing. The percentage grid fill was 50% for LIF (159 x 159 x 159 points/slide) and 40 % for the Ig-like domain of LIFR (133 x 133 x 133 points/slide). The boundary potential was full coulombic. The linear Poisson-Boltzmann equation was then solved iteratively. The Eisenberg's hydrophobicity scale (63) was used to display the protein surface hydrophobicity.

**Protein docking**

The lowest energy rotameric orientation of LIFR Phe\textsuperscript{284} was searched by $\chi_1 \times \chi_2$ isomeric mapping with CHARMM. The dihedral angles $\chi_1$ and $\chi_2$ were defined by the bond connectivities N-$\text{C}\alpha$-$\text{C}\beta$-$\text{C}\gamma$ and $\text{C}\alpha$-$\text{C}\beta$-$\text{C}\gamma$-$\text{C}\delta_2$, respectively. A single rotameric orientation of Phe\textsuperscript{284} was stable and corresponded to the g-, perpendicular orientation. This orientation was used in the starting structure for the docking procedure. The docking of the Ig-like domain of LIFR to LIF was carried out with the molecular docking program HEX 2.0 based on spherical polar Fourier correlations (64). This program is available on the Internet at http://www.biochem.abdn.ac.uk/hex/. A filter based on the distance between the rings of LIF Phe\textsuperscript{156} and LIFR Phe\textsuperscript{284} was used to remove false-positive solutions. The best scoring solution was energy minimized using 100 steepest descent steps, followed by ABNR steps.
until a convergence gradient of 0.001 was reached. A similar procedure was carried out with the D214A mutant.

**Cells and reagents**

Transfected Ba/F3 cells were maintained as previously described (45). The medium was supplemented with hygromycin and neomycin for LIFR/gp130 Ba/F3 cell lines. Purified recombinant human LIF and mouse IL-3 were kindly donated by Drs K. Turner and M. Stahl (Genetics Institute, Boston, MA). IL-2 was a kind gift of Dr. G. Zurawski (DNAX Research Institute, CA). Human CT-1, murine CT-1, human IL-6, human OSM and soluble IL-6R were purchased from R&D Systems (Oxon, UK). IgG1 isotype control, AN-HH1 anti-gp130 and anti-LIFR mAbs (AN-B1, AN-C1, AN-D1, AN-E1, AN-F1, ANG1, AN-H1, ANI1, AN-J1) were generated in the laboratory as described elsewhere (45,65). The antibody detecting phospho-STAT3 (Tyr705) was purchased from New England Biolabs (Beverly, MA). Goat anti-mouse peroxidase-labeled immunoglobulins were from Clinisciences (Montrouge, France).

**Site-directed mutagenesis and cell transfection**

The pME18S vector containing the cDNA encoding the human LIFR was subjected to site-directed mutagenesis using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA) following the manufacturer’s instructions. Mutations were performed on the predicted Ig-like domain of LIFR. Mutations were verified by DNA sequencing with an automatic DNA sequencer (Beckman Coulter) using the Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter).

Ba/F3 cell lines stably expressing the gp130 receptor were transfected with cDNA encoding mutated LIFR using electroporation (960 microfarads; 230 V) with 40 μg of the mutated plasmid. Transfected cells were selected for growing in the presence of 300 ng/ml hygromycin and IL-3 (1U/ml).
Flow cytometry analysis

LIFR expression on transfected Ba/F3 cell lines was verified by flow cytometry analysis using nine different monoclonal antibodies (AN-B1, AN-C1, AN-D1, AN-E1, AN-F1, ANG1, AN-H1, ANI1, AN-J1) directed against different conformational epitopes to ensure the correct folding of the protein. Gp130 expression was verified using the AN-HH1 anti-gp130 mAb. Cells were successively incubated for 30 min at 4°C with the appropriate primary antibody or isotype control antibody (10 µg/ml) and a phycoerythrin-conjugated anti-mouse antibody. Fluorescence was subsequently analyzed on a FACScan flow cytometer from Becton Dickinson (Mountain View, CA).

Proliferation assays

Ba/F3 cell lines expressing gp130 and wild type or mutated LIFR were seeded in 96-well plates at a concentration of 5 X 10^3 cells/well in RPMI 1640 medium containing 5% FCS. Serial dilutions of the cytokines tested were performed in triplicate. After a 72-h incubation period, 0.5 µCi of [3H]Tdr was added to each well for the last 4-h of the culture and the incorporated radioactivity determined by scintillation counting.

STAT3 tyrosine phosphorylation analysis

After a 36-h cytokine and serum starvation, cells were stimulated for 10 min in the presence of the indicated cytokine. Then cells were lysed in 10 mM Tris-HCl pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, proteinase inhibitors (1µg/ml pepstatin, 2 µg/ml leupeptin, 5 µg/ml aprotinin, 1mM PMSF). After pelleting insoluble material and protein standardization, the supernatants were submitted to SDS-PAGE and transferred onto an Immobilon membrane (Millipore, Bedford, MA, USA). The membranes were subsequently incubated with the phospho-STAT3 (Tyr^705) polyclonal antibody before being incubated with the second antibody labelled with peroxidase for 60 min. The reaction was visualized on an X-ray
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film using the ECL reagent (Amersham, Les Ullis, France) according to the manufacturer's instructions.

Protein radiolabeling and binding experiments

Since iodination of human CT-1 completely inactivated the biological activity of the cytokine (unpublished results), binding assays were carried out using radioiodinated murine CT-1. Comparison of mCT-1 with hCT-1 on the proliferative response of the gp130/LIFR Ba/F3 cells gave a similar specific activity of $10^6$ U/mg. hLIF, hOSM and mCT-1 were iodinated by the two-phase method as previously described (66). The specific activity of radiolabeled products was 100,000-300,000 cpm/ng. Cells (5-6 X $10^6$) were incubated with the indicated concentration of radiolabeled ligand, and the non-specific binding component was measured by including a 100-fold excess of unlabeled cytokine. After a 90 mn incubation at 4°C, cell-bound radioactivity was separated from the unbound fraction. Determination of affinity binding constants was performed according to Scatchard (67).

RESULTS

Molecular modeling of the Ig-like domain of LIF receptor

The crystallographic structure of the gp130 Ig-like domain was resolved in a complex of viral IL-6 with an extracellular fragment of gp130 bearing the N-terminal three modules (46). The Ig-like domain of gp130 was a seven-stranded $\beta$ sandwich module. This structure was used as template for homology modeling of the LIFR Ig-like domain. The alignment of the two Ig-like domains was not straightforward because of low homology and marked difference in sequence length. The LIFR Ig-like domain was eighteen residue shorter than gp130. A multiple alignment of the Ig-like domains of four receptors belonging to the gp130 family (LIFR, gp130, OSMR and CLF) was carried out with ClustalW (56). This alignment was used as input to the Neural Network Secondary Structure
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Prediction program (NNSSP) (57). The positioning of the β strands predicted by the NNSSP program was consistent with the secondary structure of gp130 and was used for minor manual refinement of the alignment of the Ig-like domains of gp130 and LIFR (Figure 1a). This alignment yielded a 28% identity rate. It was characterized by two large gaps. The first seven residue long gap corresponded to the helix linking the β-strands B and C of gp130. The second eight residue long gap was located at the level of the β-strands F and G and the linking hairpin. Figure 1b displays the Ig-like domains of gp130 and of LIFR modeled according to the alignment shown in Figure 1a. The two deletions could be easily accommodated on a structural background in the modeling procedure. In the resulting three-dimensional model, the LIFR Ig-like domain was a seven-stranded β sandwich with β-strands F and G dramatically shortened as compared to gp130.

The deleted regions are part of the structural epitope of vIL-6 binding to gp130 through site III. The F and G strands and the linking loop contain several residues directly involved at the binding interface (Gln<sup>78</sup>, Thr<sup>80</sup>, Asn<sup>82</sup>, Ile<sup>83</sup>, Asn<sup>92</sup>, Val<sup>93</sup>, Tyr<sup>94</sup>, Gly<sup>95</sup> and Ile<sup>96</sup>). The interaction between vIL-6 and gp130 at site III is stabilized by seven H-bonds (46). Four H-bonds involve backbone-backbone interactions. Two of these H-bonds involve gp130 Asp<sup>4</sup> and Cys<sup>6</sup>, located in the N-terminal tail of gp130, with no equivalent residues in LIFR. Three H-bonds (vIL-6 Trp<sup>144</sup>:Nε1 ≡ gp130 Asn<sup>92</sup>:O; vIL-6 Tyr<sup>32</sup>:O ≡ gp130 His<sup>49</sup>:Nε2; vIL-6 Thr<sup>34</sup>:O ≡ Gln<sup>78</sup>:Nε2) involve side chains-backbone interactions and participate in the specificity of the binding. None of these H-bonds can be conserved in the LIF-LIFR complex. The residue homologous to Trp<sup>144</sup> is Phe, implying the loss of this H-bond. Gp130 His<sup>49</sup> is part of the deleted helix and Gln<sup>78</sup> is mutated to Val in LIFR.

The large structural reorganization at the binding epitope due to the deletion in the F and G strands and the loss of H-bonds prevented straightforward prediction of residues involved in LIF binding by homology with vIL-6. These findings prompted us to perform an analysis of the surface properties of LIF and LIFR.
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Determination of the putative LIF binding site

Protein-protein complexes result from electrostatic, polar (H-bond) and hydrophobic interactions (68). These interactions require complementary shapes and physicochemical properties. To determine the putative complementary site III in the Ig-like domain of LIFR, we computed the molecular surface properties (electrostatic potential and hydrophobicity) of the LIF site III and of the LIFR Ig-like domain, and searched for complementary areas (Figure 2).

The LIF receptor binding site III is located at the N-terminus of helix D, on the 'top' of the cytokine opposite to the N and C terminals. Site-directed mutagenesis has shown that residues from the N-terminal of the AB loop and from the BC loop are also part of the binding epitope (41). The core of the cytokine 'top' corresponds to the protruding Lys^{159} side chain of the FxxK motif and has a very positive potential (Figure 2a). This positively charged core is surrounded by two distinct patches of residues forming a rim around it (Figure 2b). The first patch contains Pro^{51}, Phe^{156}, Val^{155}, Leu^{104}, Ile^{103}, and Pro^{106} and forms a very hydrophobic horse shoe. The second patch is composed of three hydrophilic residues: Glu^{50}, Gln^{48} and Asn^{105}. Among these residues, mutations of Pro^{51} and Pro^{106} to Ala have been shown to alter LIF binding to LIFR (41). Phe^{156} is in the trans rotameric state and is hold in this orientation by neighbor Pro^{51} and Phe^{52}. These three residues form a cluster of interacting residues.

The strategy used to determine the LIFR site III binding epitope was to search an area of the LIFR Ig-like domain with physicochemical properties complementary to those of the LIF site III, i.e., a negative core, surrounded by two patches of hydrophobic and hydrophilic residues. The electrostatic potential and the hydrophobicity pattern of the LIFR molecular surface were computed and carefully analyzed. The side of the upper β sheet displays a complementary image of the LIF site III (Figure 2, c-d). The negative core corresponds to Asp^{214}. This residue is surrounded by a hydrophobic horse shoe composed of Val^{216}, Phe^{284}, Val^{282}, Gly^{280} and Phe^{279} and by a hydrophilic patch composed of Gln^{213}, Thr^{281} and Lys^{215}. LIFR Phe^{284} and Asp^{214} form a mirror image of LIF Phe^{156} and Lys^{159} and are thus putative hot spots of the binding interface. This putative binding site is located on the same sheet of
the Ig-like domain than the binding site of gp130. However, the two sites do not overlap. Equivalent residues of gp130 are Ser\textsuperscript{13} and Ile\textsuperscript{98}. Only Ser\textsuperscript{13} is marginally involved in viral IL-6 site III binding (46).

Site-Directed Mutagenesis of the LIFR Ig-like Domain

Residues 214 and 284 in the Ig-like domain of LIFR were substituted with alanine to experimentally verify the putative binding site of LIFR. Single or double mutations were introduced in the receptor cDNA. cDNAs encoding mutated proteins were stably transfected in Ba/F3 cell lines already transfected with gp130 to generate high affinity LIF receptor. Mutant expression was then analyzed using a set of anti-LIFR monoclonal antibodies which bind to different conformational epitopes. An example using the AN-E1 mAb is given in Figure 3. No significant change in flow cytometry analysis could be detected and the expression of the LIFR mutants was comparable to that of the wild type protein. Similar results were obtained using eight additional mAbs (not shown). These results indicate that the mutations did not alter the folding and did not introduce significant changes in the structure of LIFR.

Ba/F3 proliferation assays

The function of the mutated LIF receptors was assessed by determining proliferation of Ba/F3 cell lines expressing gp130 and LIFR in response to LIF (Figure 4 and Table I). LIF induced a robust proliferation of Ba/F3 cells expressing gp130 and wild type LIFR. Introducing the Phe\textsuperscript{284} to Ala mutation in LIFR impaired the proliferative response of Ba/F3 cells upon stimulation by LIF with a 10-fold increase in EC\textsubscript{50} (Table I). The Asp\textsuperscript{214} to Ala mutation did not impair the proliferation of Ba/F3 cells and did not significantly alter EC\textsubscript{50}. Unlike single mutations which had no or moderate effects, the double mutation had a dramatic effect on BA/F3 cell proliferation with an increase in EC\textsubscript{50} larger than 50 000 fold.

To provide further evidence about the importance of Asp\textsuperscript{214} and Phe\textsuperscript{284} for receptor function, proliferation assays were performed upon stimulation of Ba/F3 cell lines expressing gp130 and wild
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Type or mutated LIFR by cardiotrophin-1 or oncostatin M (Figure 4 and Table I). These cytokines use the same functional receptor as LIF. As previously observed, cardiotrophin-1 was less efficient than LIF for stimulating Ba/F3 cells transfected with wild type LIFR (Table I) (69). However, the effects of the D214A and/or F284A mutations in the LIFR Ig-like domains were similar to those observed with LIF. The Asp\textsuperscript{214} to Ala mutation did not induce any significant change in the proliferative response of the BA/F3 cells induced by CT-1, whereas the Phe\textsuperscript{284} to Ala mutation markedly impaired cellular proliferation (100 fold increase in EC\textsubscript{50}). The double mutation totally abrogated the proliferative response (at least 10 000 fold increase in EC\textsubscript{50}).

The effect of the LIFR mutations on the response of BA/F3 cells was markedly different when these cells were stimulated by oncostatin M. The single Asp\textsuperscript{214} to Ala and Phe\textsuperscript{284} to Ala mutations had no significant effect upon stimulation by OSM. On the other hand, the double mutation impaired cell proliferation with a 20-fold increase in EC\textsubscript{50}, (Table I). This indicates that OSM binds LIFR through the Ig-like domain.

**STAT3 phosphorylation**

The dimerization of gp130 and LIFR upon binding of LIF, CT-1 or OSM induces cell activation via the recruitment of Janus kinases. Tyrosine-phosphorylated gp130 and LIFR in turn serve as docking proteins for signal transduction molecules such as STAT3 (4,18,70,71). To test whether the mutations could influence the levels of STAT3 phosphorylation, we used a Western blot assay to monitor STAT3 activation in transfected Ba/F3 cells (Figure 5). The results corroborated the data obtained with the Ba/F3 cells proliferation assay. The Asp\textsuperscript{214} to Ala mutation did not affect STAT3 phosphorylation in response to LIF, CT-1 or OSM. The Phe\textsuperscript{284} to Ala mutation affected STAT3 phosphorylation in response of CT-1 and LIF, but not to OSM. This is consistent with the larger EC\textsubscript{50} in proliferation required upon stimulation by CT-1 than by LIF (Figure 4). The double mutation altered STAT3 phosphorylation in response of CT-1 and LIF, but did not impair the response to OSM, in agreement with proliferation assays.
Binding affinity

The effect of the mutations in the LIFR Ig-like domain on the biological response induced by LIF, CT-1 or OSM could be due to impaired binding or signal transduction. In order to answer this question, we determined the dissociation constants of these cytokines for the mutated receptors. Binding of radioiodinated LIF, CT-1 and OSM to Ba/F3 cells expressing gp130 and wild type or mutated LIFR was thus carried out, and the results were analyzed according to Scatchard (67) (Table I).

The high affinity dissociation constant of LIF binding to the gp130/LIFR heterocomplex expressed in BA/F3 cells (Kd = 250 pM) was in agreement with previously published data (51). The Asp\textsuperscript{214} to Ala mutation did not induce a significant change in Kd. On the other hand, the decrease in the affinity upon the Phe\textsuperscript{284} to Ala mutation or upon the double mutation prevented measurement of the affinity constant, indicating a Kd > 10 nM. Similar results were observed with CT-1. The high affinity dissociation constant of CT-1 was consistent with previous data (69) and was not significantly altered by the Asp\textsuperscript{214} to Ala mutation. The decrease in the affinity upon the F284 and the D214/F284 mutations did not allow the dissociation constant to be measured (Kd>10nM) (Table I).

The affinity of OSM for the gp130-LIFR heterocomplex expressed in Ba/F3 cells was similar to previously published values (72). The high affinity dissociation constant was not significantly altered upon the D214A or the F284A single mutations, with less than two fold changes in Kd values (Table I). However, the D214A/F284A double mutation yielded a decrease in the affinity sufficient to prevent measurable binding, indicating Kd > 10 nM.

Molecular docking of the LIF-LIFR complex

The initial assumption of an interaction between LIF Phe\textsuperscript{156} and LIFR Phe\textsuperscript{284} was consistent with the effect of the F284A mutation. However, the assumption of an interaction between LIF Lys\textsuperscript{159} and LIFR Asp\textsuperscript{214} was challenged by the experimental results obtained with the D214A LIFR mutant. Molecular docking of LIF to the Ig-like domain of LIFR using the HEX program (64) was thus carried out to get better insight into the molecular details of the interaction.
Energetically favorable Phe-Phe interactions require edge-to-face orientations of the phenylalanine rings (73). The rotameric orientations of the Phe side chains are thus crucial for proper binding. The orientation of LIF Phe$^{156}$ in the crystal structure corresponds to the trans, $\pi$ rotamer ($\chi_1 = -165^\circ,$ $\chi_2 = 78^\circ$) and is stabilized by interactions with Pro$^51$ and Phe$^52$. In this rigidly hold orientation, Phe$^{156}$ lies on the protein surface. In the MODELER built model of the Ig-like domain of LIFR, Phe$^{284}$ is positioned in the $\gamma$, $\pi$ rotameric orientation ($\chi_1 = -60^\circ,$ $\chi_2 = -88^\circ$), with the phenylalanine ring perpendicular to the protein surface. $\chi_1 \times \chi_2$ isomeric mapping of LIFR Phe$^{284}$ indicates that this orientation corresponds to the single rotameric orientation of Phe$^{284}$ possible (not shown). The relative orientations of LIF Phe$^{156}$ and LIFR Phe$^{284}$ are favorable for interacting. Docking of the LIF-LIFR complex with HEX was thus carried out with the side chains of LIF Phe$^{156}$ and LIFR Phe$^{284}$ in their stablest rotameric orientation. The energy minimized structure of the best scoring solution is shown in Figure 6a. In this three-dimensional model, LIFR Phe$^{284}$ is involved in $\pi-\pi$ interactions with LIF Phe$^{156}$, with a edge-to-face geometry. The distance between the Phe ring centroids is equal to 5.0 Å. LIFR Asp$^{214}$ forms a salt bridge with LIF Lys$^{159}$. The distance between LIF Lys$^{159}$ N$\zeta$ and LIFR Asp$^{214}$ O$\delta$1 or O$\delta$2 is 2.6 Å. LIF Glu$^{50}$ interacts with LIFR Lys$^{215}$ to form a second salt bridge. Several H-bonds between the two proteins are also formed at the interface (LIF Leu$^{83}$:O with LIFR Lys$^{209}$: N$\zeta$; LIF Ala$^{26}$:O and Gln$^{27}$:O with LIFR Gln$^{213}$:N$\varepsilon$; (not shown).

When the same procedure was carried out with the D214A LIFR mutant, the best scoring solution was very similar (RMSD = 0.37Å). Its energy-minimized structure is shown in Figure 6b. In this case, LIF Lys$^{159}$:N$\zeta$ is involved in a H-bond interaction with LIFR Thr$^{281}$:O. This indicates that an alternative interaction can stabilize the complex. The other interactions are conserved.

It is worth to note that HEX is a rigid body docking program and that its success for the docking of the Ig-like domain of LIFR to LIF may be related to the fact that the interface does not involve flexible parts, but rigid surfaces requiring only minor side chain rearrangements. In particular, the side chains of LIF Phe$^{156}$ and LIFR Phe$^{284}$ have a single rotameric orientation possible. Their relative orientation is favorable to edge-to-face interaction without any side chain rearrangement.
DISCUSSION

LIF binding site of LIFR

The resolution of an increasing number of protein-protein complexes leads to a better knowledge of rules underlying formation of binding interfaces. Most interfaces (or structural epitopes) of heterocomplexes have usually large surface areas (> 600 Å²) with good shape and physicochemical complementarities (68). Hydrophobic interactions are fundamental for protein-protein interactions (74). Interfaces also have about one hydrogen bond per 100 Å² of buried surface area and electrostatic complementary (75). Analysis of pairing preferences at protein-protein interfaces corroborate that pairing preferentially occur between residues with complementary properties (76). Pairs of large hydrophobic residues or of complementary charged residues are favorable. Analysis of structural interfaces does not give details about the contributions of individual residues to the binding free energy. Despite the large size of binding interfaces, only a few interacting residues actually contribute to the binding free energy. Interface residues contributing the most to the binding energy are known as hot spots (77-79).

These basic principles underlying complex formation were used to search the complementary site III of LIFR, in a two step procedure. First, the putative structural binding epitope was determined by searching a surface area with physicochemical properties complementary to those of the LIF site III. Second, two residues of this area, Asp²¹⁴ and Phe²⁸⁴, forming a mirror image of the two known hot spot residues of LIF, Phe¹⁵⁶ and Lys¹⁵⁹, were the best candidates for the functional binding epitope. They were mutated to alanine to verify this assumption by assaying the biological activity and the binding affinity of the single and double mutants.

The LIFR site-directed mutagenesis study presented here clearly establishes that the F284A mutation in the Ig-like domain of LIFR impairs LIF binding to the gp130-LIFR heterocomplex. The decreased efficiency of LIF for inducing the proliferation of Ba/F3 cells expressing the F284A mutant is consistent with the decreased affinity of the cytokine for the receptor. These results are in agreement with the observation that a LIFR mutant bearing the double mutation F284T and A285S had at least a
30-fold loss in affinity (49). The F284T/A285S double mutation is very disruptive since it replaces two hydrophobic residues, Phe and Ala, by two polar residues, Thr and Ser.

The D214A mutation did not impair binding or biological activity. This indicates either that Asp\(^{214}\) did not contribute to binding affinity or that the interactions disrupted by this mutation were replaced by alternative interactions of similar strength (\(|\Delta G| \leq 0.5 \text{ kcal/mol}\)). The D214A/F284A double mutation had a dramatic effect on the proliferative response of Ba/F3 cells upon stimulation by LIF. This might be due either to a gross disruption of the LIFR structure or to the prevention of alternative interactions possible upon the single D214A mutation.

The former explanation can be ruled out for several reasons. Firstly, the double mutant was correctly expressed and recognized by monoclonal antibodies directed against different conformational epitopes as well as wild type or single mutant proteins (Figure 3). Secondly, we verified the stability of the mutated proteins by computational mutagenesis using FOLD-X (59). The results (Table II) indicate that the mutations do not significantly alter the stability of the Ig-like domain and that the effect of the double mutation is just additive as compared to the single mutations. Thirdly, the double mutation, albeit dramatically impairing the biological activity induced by LIF or CT-1, did not disrupt the biological response induced by OSM. The proliferative response of Ba/F3 cells expressing gp130 and the D214/F284 LIFR mutant was still efficient upon stimulation by OSM (20 fold increase in EC\(_{50}\)). Moreover, weak STAT-3 phosphorylation upon LIF stimulation of Ba/F3 cells transfected with gp130 and the D214/F284 LIFR mutant could be detected. This indicates that, albeit the affinity was strongly reduced by the double mutation, a slight recruitment of the signaling cascade remained. In turn, this corroborates the assumption that the structure of the Ig-like domain was not altered.

The latter explanation lies on the possibility of alternative interactions upon the single D214A mutation. Examples of alternative interactions yielding no apparent effect of a single mutation were reported in the literature (78,80). This phenomenon is usually connected to superadditivity of multiple mutations, i.e. the effect of multiple mutations is larger than the sum of the individual mutations. A
Involvement of LIFR Ig-like domain in cytokine binding

single mutation may be compensated for by neighboring residues making alternative contacts at the interface. However, upon two mutations, these compensatory contacts should no longer be possible, leading to greater conformational perturbation in the complex than single mutations.

Molecular modeling of the complex between LIF and the Ig-like domain of wild type LIFR (Figure 6a) corroborates the initial hypothesis of a salt bridge between LIFR Asp\textsuperscript{214} and LIF Lys\textsuperscript{159} and of $\pi-\pi$ interactions between LIFR Phe\textsuperscript{284} and LIF Phe\textsuperscript{156}. During the manuscript writing, a study reporting mutations in the Ig-like domain of LIFR increasing or decreasing its affinity for LIF was made available (81). The three-dimensional model of the complex between LIF and the Ig-like domain of LIFR that we computed was used to analyze the effect of the reported mutations. F279 should be involved in van der Waals interaction with LIF L104, and V282 should participate to the hydrophobic cluster involving LIFR F284 and LIF F156. The identified residues complete the present work and the definition of LIFR binding site III. Analysis of the interface involving LIF and the D214A LIFR mutant (Figure 6b) indicates that, upon the Asp\textsuperscript{214} to Ala mutation, the salt bridge between LIF Asp\textsuperscript{214} and LIFR Lys\textsuperscript{159} can be replaced by a H-bond interaction between LIF Lys\textsuperscript{159}:N$\zeta$ and LIFR Thr\textsuperscript{281}:O$\gamma$1. The difference in the dissociation constant of LIF to wild type or D214A LIFR corresponds to a change in the binding free energy < 0.5 kcal/mol. The strength of a salt bridge at a protein-protein interface is difficult to evaluate because of the entropic cost of desolvation and salt bridges may be stabilizing or destabilizing. At protein-protein interfaces, the global balance is generally positive with a $\Delta G$ of about 2 kcal/mol for correct geometry (82-84). The strength of H-bond is usually in the 0.5-2 kcal/mol range (84-86). The similar binding free energy of salt bridge or H-bond involving LIF Lys\textsuperscript{159} could be due to a higher desolvation energy of the charged Asp\textsuperscript{214} and Lys\textsuperscript{159} in the salt bridge that would offset the more favorable enthalpy. The interaction of the two Phe rings makes a geometry favorable to the formation of the LIF Lys\textsuperscript{159}:N$\zeta$ - LIFR Thr\textsuperscript{281}:O$\gamma$1 H-bond. Under the double mutation, the absence of $\pi-\pi$ interaction should prevent the correct geometry and thus the formation of this H-bond, yielding a LIFR mutant with strongly reduced binding affinity, which is indeed observed.
Comparison with oncostatin M and cardiotrophin-1

Cardiotrophin-1 and oncostatin M share with LIF the capability to induce biological response through the gp130-LIFR heterocomplex. However, the sequential process yielding complex formation is different. LIF and CT-1 first bind LIFR, forming the so-called “low affinity” complex (22,51). This first event is followed by the recruitment of gp130, which induces the formation of the “high affinity” complex. On the other hand, OSM first binds gp130, forming a “low affinity” complex before recruiting LIFR or OSMR in a “high affinity” complex (19,23). The inability of OSM to directly bind LIFR indicates that its affinity for LIFR is much lower than that of LIF or CT-1. The difference in the dissociation constants of the “low affinity” (Kd \( \approx 10^{-8} \) M) and the “high affinity” complexes (Kd \( \approx 10^{-10} \) M) is about 100. Thus, the free energy of binding is about 10 kcal/mol for the first interacting receptor, but only 3 kcal/mol for the second interacting receptor.

CT-1 response to LIFR mutations was similar to LIF one. The activity and the binding constant were not altered upon the D214A mutation, whereas the biological response was impaired upon the F284A mutation and totally abrogated upon the double mutation. These results clearly indicate that CT-1 binds LIFR through the Ig-like domain and that the binding mechanism is similar to that of LIF. This is consistent with the striking similarities of the sites III of the two cytokines (Figure 7a). In addition to the conserved FxxK motif (Phe\(^{168}\) and Lys\(^{171}\)), most residues of the AB loop N-terminal part (Leu\(^{51}\), Gln\(^{52}\), Gly\(^{53}\), Asp\(^{54}\), Pro\(^{55}\)) and of the BC loop (Leu\(^{111}\), Asn\(^{112}\), Pro\(^{113}\)) are conserved or type-conserved. Phe\(^{56}\) is also conserved. Conservation of Pro\(^{55}\) and Phe\(^{56}\) allows CT-1 Phe\(^{168}\) to be held in the same orientation as LIF Phe\(^{156}\). Non conserved residues are at the periphery of the interface (Glu\(^{110}\), Arg\(^{114}\), Gly\(^{166}\), Pro\(^{169}\)). This strongly suggests that the formation of the cytokine-LIFR complex with subnanomolar affinity requires very conserved structural properties of the interface. Site-directed mutagenesis of LIF has shown the involvement of some of these residues in the interaction (Pro\(^{51}\), Pro\(^{106}\)) (41). The effect of these mutations on the affinity or on the biological activity is however reduced (< 10 fold), as compared to the effect of the F156A and K159A mutations.
This suggests that these residues may have an indirect role for the correct positioning of residues involved in the interaction rather than a direct role in the interaction.

The effect of the D214/F284 double mutation on the biological activity of oncostatin M and on its affinity for its receptor clearly indicates that OSM interacts with the Ig-like domain of LIFR. However, the binding mechanism should be different from that of LIF or CT-1 since the D214 and F284 single mutations of LIFR had no significant effect on the affinity or the biological activity of OSM and that the double mutated receptor was still able to induce efficient BAF proliferation and STAT3 phosphorylation, upon OSM stimulation. Figure 7b shows residues conserved in oncostatin M as compared to LIF. In addition to the FxxK motif (Phe\textsuperscript{160}, Lys\textsuperscript{163}), the few conserved or type-conserved residues are located at the N-terminal part of the AB loop (Gln\textsuperscript{38}, Gly\textsuperscript{39}), at the C-terminal part of the CD loop (Asp\textsuperscript{158}, Ala\textsuperscript{159}) and at the N-terminal part of helix D (Gln\textsuperscript{161}, Arg\textsuperscript{162}). The large insertion in the BC loop yielding an additional helix (Ala\textsuperscript{95}-Gly\textsuperscript{102}) should make steric hindrance to binding. This is consistent with the much smaller free energy of binding observed for the second interacting receptor in the “high affinity” complex (LIFR binding to the OSM-gp130 complex) than for the first interacting receptor (LIFR binding to LIF or CT-1). In a study aimed to find the binding epitope of the Ig-like domain of G-CSF receptor, Layton et al (37) observed that single mutations in the Ig-like domain of the G-CSF receptor did not impair binding or biological activity, but that double mutations were required to observe an effect. They explained this behavior by the low additional change in free energy upon the second receptor binding, which should be shared by several residues at the interface, corresponding to weak interactions.

The interaction of OSM with the Ig-like domain of LIFR corresponds to a weak affinity binding interface and the molecular details must be different from those observed for CT-1 or LIF, corresponding to a much stronger affinity interface. Both the F284A and the D214A mutations do not impair the interaction of OSM with LIFR. Contrary to LIF or CT-1, LIFR Phe\textsuperscript{284} does not constitute a hot spot for OSM binding, which is consistent with weaker interactions (19). OSM Phe\textsuperscript{160} is not held in an efficient geometry for interacting with LIFR Phe\textsuperscript{284} by a cluster of aromatic residues, which may
weaken the free energy of binding due to entropic cost. The additional helix in the BC loop of OSM (44) should also be destabilizing. The effect of the double mutation, however, indicates that Asp$^{214}$ and Phe$^{284}$ participate to the binding and are part of the OSM binding epitope on the Ig-like domain of LIFR.

In conclusion, LIF, CT-1 and OSM share an overlapping binding epitope on the Ig-like domain of LIFR, involving the Phe$^{284}$ and Asp$^{214}$ residues. The behavior of OSM can be related to its lower affinity for LIFR, implying a binding interface where interacting residues have not a geometry optimized for strong interactions. On the other hand, for LIF and CT-1, the Phe$^{284}$ and Asp$^{214}$ pair corresponds to the binding hot spot. These residues should be involved in salt bridge and π-π interactions with the site III FxxK motif. Such interactions are frequently observed in cytokine-receptor complexes and, more generally, in protein-protein complexes. Examples are given by the IL-4/IL-4Rα complex (87,88) and by the p35/p40 complex (89). These interactions yield high free energy of binding when their geometry is optimized by favorable environment of neighbor residues.

ACKNOWLEDGMENTS: We thank Dr J. Janin (Orsay, France) for stimulating discussion.

FOOTNOTES

1Abbreviations: IL-6: interleukin-6; IL-11: interleukin-11; LIF: leukemia inhibitory factor; CT-1: cardiotrophin-1; OSM: oncostatin M; CNTF: ciliary neurotrophic factor; CLC: cardiotrophin-like cytokine; LIFR: LIF receptor; OSMR: OSM receptor; Ig-like: immunoglobulin-like; CBD: cytokine binding domain; FnIII: fibronectin type III.
Involvement of LIFR Ig-like domain in cytokine binding

LEGENDES

Figure 1: (A) Alignment of the Ig-like domains of LIFR and gp130 and (B) ribbon drawings of the Ig-like domains of gp130 (left) (46) and modeled LIFR (right). The positions of gp130 corresponding to gaps in the alignment are shown in green in the ribbon model. The side chains shown in red correspond to the residues in the Ig-like domain of gp130 involved in the interface with vIL-6 (indicated with a star in the sequence of gp130) and to those of these side chains conserved in LIFR. Asp<sup>214</sup> and Phe<sup>284</sup> of LIFR are shown in cyan.

Figure 2: Connolly surfaces of the LIF site III (a, b) and of the LIFR Ig-like domain (c, d). The surfaces are colored with either the electrostatic potential (a, c) or with the hydrophobicity index of the exposed residues (b, d) as described in Materials and Methods. LIF is viewed from the “top”. The orientation of the LIFR Ig-like domain has been manually adjusted to match the hydrophobic patches of the two proteins.

Figure 3: Receptor expression on Ba/F3 cell lines transfected with gp130 and wild type or mutated LIFR. The expression of gp130 and LIFR was monitored by flow cytometry analysis. The black histograms correspond to the isotype controls, the light grey histograms correspond to gp130 detection by AN-HH1 anti-gp130 mAb and dark grey histograms by LIFR detection by AN-E1 anti-LIFR mAb.

Figure 4: Proliferative response of Ba/F3 cell lines transfected with gp130 and wild type or mutated LIFR upon stimulation by LIF, CT-1, OSM. Cells were cultured in triplicate using 3-fold dilutions of LIF (filled squares), CT-1 (filled triangles), OSM (filled circles), hIL-6 plus 500 ng/ml of soluble IL-6 receptor (open squares), or IL-2 (open diamonds), used as an irrelevant cytokine (150 ng/ml at highest concentration). Vertical bars indicate the SEM.
Figure 5: Tyrosine phosphorylation of STAT3 in Ba/F3 cells transfected with gp130 and wild type or mutated LIFR. The cells were exposed to 20 ng/ml of IL-2, hIL-6 + 500 ng/ml of soluble IL-6 receptor, LIF, CT-1 or OSM, then lysed. The lysates were subjected to immunoblot analysis with antibodies specific for activated forms of STAT3 (STAT3-P) as described in Materials and Methods.

Figure 6: Three-dimensional model of the complex of LIF with the Ig-like domain of wild type LIFR (A) or of the D214A LIFR mutant (B) after molecular docking with HEX and energy minimization, with a zoom on interacting side chains. Phenylalanine and proline residues are shown in green, acid residues in red, basic residues in blue, Thr<sup>281</sup> in orange and Ala<sup>214</sup> in the D214A mutant in cyan.

Figure 7: (A) Sequence alignment of human LIF, cardiotrophin-1 and oncostatin M and (B) Connolly surfaces of modeled CT-1 (left) and of oncostatin M (44) (right) with a color code indicating the site III residues which are conserved relative to LIF. Conserved residues are shown in red, type-conserved residues in yellow, no conserved residues in green. Residues that do not belong to site III are in grey. Oncostatin M residues in cyan correspond to the Ala<sup>95</sup>-Gly<sup>102</sup> α-helix.
TABLE I

Proliferation and binding affinity of cytokines on Ba/F3 cells expressing gp130 and wild type or mutated LIFR

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mutation</th>
<th>EC$_{50}$ (pg/ml)$^a$</th>
<th>EC$<em>{50}$ / EC$</em>{50}$ (wt)</th>
<th>Kd (nM)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIF</td>
<td>WT</td>
<td>22</td>
<td>1</td>
<td>0.25</td>
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<tr>
<td></td>
<td>D214A</td>
<td>9</td>
<td>0.4</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>F284A</td>
<td>200</td>
<td>9</td>
<td>&gt; 10</td>
</tr>
<tr>
<td></td>
<td>D214A,F284A</td>
<td>&gt;10$^6$</td>
<td>&gt;50 000</td>
<td>&gt; 10</td>
</tr>
<tr>
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<td>1</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>D214A</td>
<td>1500</td>
<td>1.2</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>F284A</td>
<td>120 000</td>
<td>100</td>
<td>&gt; 10</td>
</tr>
<tr>
<td></td>
<td>D214A,F284A</td>
<td>&gt;10$^6$</td>
<td>&gt;10 000</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>OSM</td>
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<td>0.58</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>F284A</td>
<td>70</td>
<td>0.5</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>D214A,F284A</td>
<td>2800</td>
<td>20</td>
<td>&gt; 10</td>
</tr>
</tbody>
</table>

$^a$ EC$_{50}$ refers to the cytokine concentration required to active the proliferation of the Ba/F3 by 50% of maximum stimulation.

$^b$ Kd corresponds to the high affinity dissociation constant of cytokine binding to Ba/F3 cells transfected with gp130 and wild type or mutated LIFR as determined by Scatchard analysis.
TABLE II

Stability of LIFR mutants

<table>
<thead>
<tr>
<th>Mutation</th>
<th>(\Delta\Delta G) (kcal/mol)</th>
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</tr>
<tr>
<td>F284A</td>
<td>0.20</td>
</tr>
<tr>
<td>D214A, F284A</td>
<td>0.64</td>
</tr>
</tbody>
</table>

1 The changes in the free energy of folding upon mutations have been calculated using the FOLD-X program (59).
REFERENCES

Involvement of LIFR Ig-like domain in cytokine binding


Figure 1

A

LIFR: 207 ----QTKVPFCDKVILVGSLLTFGCCVSQEX----VLSALIGHTNCPPL 245
GP130: 2 LLDPCCGYIPSPEPVQLSNFTAVCVLKEKCMYFHVNANYITWKTTHFT 51

LIFR: IHLDGENVAKIR-NISVSASS-G-TNWVFTT------EDNIFGTIFPACY 292
GP130: TP--KEQTHTINFTASSVTFTDIASLNIQLTCNILTFQLENYGITTISGL 101

B

![Image of protein structures with labeled amino acids and secondary structures.]

H49
Q78
N92
N82
E90
T80
Q91
F36
E12
S13
Y94
P5
C6
D4
L2
F284
T273
E275
N277
I278
F279
D214
Figure 2

(a) K159
(b) I103, L104, V155, K159, F156, Q48, P51, E50
(c) D214
(d) G280, F279, N213, D214, K215, V216, V282, F284, T281

Color scales:
- For (a): kT/e
- For (b-d): Eisenberg scale
Figure 3

Fluorescence intensity vs. Cell number

- gp130
- Isotype control
- LIFR
- gp130
- gp130 - WT LIFR
- gp130 - F284A LIFR
- gp130 - D214A LIFR
- gp130 - F284A/D214A LIFR
Figure 5

P-STAT3 → gp130
STAT3 →

P-STAT3 → gp130 - WT LIFR
STAT3 →

P-STAT3 → gp130 - F284A LIFR
STAT3 →

P-STAT3 → gp130 - D214A LIFR
STAT3 →

P-STAT3 → gp130 - F284A/ D214A LIFR
STAT3 →
Figure 6
Figure 7

A

HLIF: 22 LMNLQIRSLQLNLSNALFILYTAQGEPPNLPLEKLCGP-NVTDFP-PFHANGTEKAKLVELRYIVVLGTSLGNITRDQK
hCT1: 25 KIRQTNSLAILLTKYAEQLQYEYQLQCTDFGL-PSFSPPRPLFVAGLSAPAPSHAGLPYHERLRLDAAALAALEPPLLDAVCRRAQ
hOSM: 13 LGQLKQKTDLMQDTS-RLDPYIRIQLQRLVPK-LREHCQ-PRGAPSE-ETLRLGRRGFLQTLNATLGVCLHRHLEQRLPFAQDLE

B

K163
K171
F168

F160
Leukemia inhibitory factor, cardiotrophin-1 and oncostatin M share structural binding determinants in the Ig-like domain of LIF receptor

Hélène Plun-Favreau, David Perret, Caroline Diveu, Josy Froger, Sylvie Chevalier, Eric Lelievre, Hugues Gascian and Marie Chabbert

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