Leukemia Inhibitory Factor (LIF), Cardiotrophin-1, and Oncostatin M Share Structural Binding Determinants in the Immunoglobulin-like Domain of LIF Receptor*

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Received for publication, March 27, 2003, and in revised form, April 15, 2003
Published, JBC Papers in Press, April 21, 2003
DOI 10.1074/jbc.M303168200

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 an FXXK motif located at the N terminus of helix D (site III). The immunoglobulin (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, Asp277 and Phe284, 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 Asp277 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 EC50. 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 behaviors 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.

The cytokines of the IL-6 family are multifunctional proteins that 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 seven 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, 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 Ref. 2). The common use of gp130 explains in part the overlapping effects of these cytokines (10). IL-6 and IL-11 binding induces dimerization of gp130 (11–17). LIF, CNTF, CT-1, and CLC induce heterodimerization 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 heterodimerization of gp130 with LIFR or with the specific receptor for OSM, OSMR (23, 24). In addition to transducing receptor chains (gp130, LIFR, and 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) and 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 an 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 that its two FnIII domains have an 1-shaped 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 transmembrane domain (IL-6R and IL-11R) (14, 25) or through a glycosylphosphatidylinositol 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, numbered from I to III by analogy with the growth hormone (32). Cytokines requiring a co-receptor chain
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(e.g., CNTF, CLC, IL-6, and IL-11) binds to this co-receptor (CNTFR, IL-6R, and IL-11R) through binding site I (C-terminal parts of the AB loop and of helix D (8, 20, 26, 33–36). The glycophosphogp130 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 residues from the N-terminal part of the AB loop and from the BC 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 an EXXX 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 Kaposi sarcoma-associated herpesvirus 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 of the LIF site III. Two residues of the LIFR Ig-like domain, Asp214 and Phe284, form a mirror image of LIF Phe156 and Lys159, which constitute the LIFR binding hot spots (41). Single and double LIFR mutants, in which Asp214 or/and Phe284 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, because 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, CA). The structure of human LIF (PDB access number: 1EMR) was modified in three places (Glu57, Val172, and Ser174) to better match the structure of the Ig-like domain of LIFR. The resulting structure was energy-minimized with the DELPHI package (61, 62) under full coulombic. The linear Poisson-Boltzmann equation was then solved iteratively. The Eisenberg charge set was used, with an ionic strength of 0. 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 residues from the N-terminal part of the AB loop and from the BC 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 an EXXX 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 Kaposi sarcoma-associated herpesvirus 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).

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with the second antibody labeled with peroxidase for 60 min. The reaction was visualized on an x-ray film using the ECL reagent (Amersham Biosciences, Les Ullis, France) according to the manufacturer’s instructions.

**Protein Radiolabeling and Binding Experiments**—Because iodination of human CT-1 completely inactivated the biological activity of the cytokine, 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 $10^6$ units/mg. hLIF, hOSM, and mCT-1 were iodinated by the two-phase method as previously described (66). The specific activity of radiolabeled products was $100,000–500,000$ cpm/ng. Cells (5–6 $\times$ $10^6$) were incubated with the indicated concentration of radiolabeled ligand, and the nonspecific binding component was measured by including a 100-fold excess of unlabeled cytokine. After a 90-min 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 frag-
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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 $\beta$ sheet displays a complementary image of the LIF site III (Fig. 2, C and D). The negative core corresponds to Asp$^{114}$, which is part of the deleted helix. Glu$^{159}$ is a residue homologous to Trp$^{144}$ in vIL-6, implying the loss of this H-bond. Glp130 His$^{149}$ is part of the deleted helix and Glu$^{78}$ is mutated to Val in LIFR.

The large structural reorganization at the binding epitope due to the deletion in the $\beta$-strands F and G 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.

**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.

The LIFR 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 (Fig. 2A). This positively charged core is surrounded by two distinct patches of residues forming a rim around it (Fig. 2B). The first patch contains Pro$^{51}$, Phe$^{156}$, Val$^{155}$, Leu$^{104}$, Ile$^{103}$, and Pro$^{106}$ and forms a very hydrophobic area with a horseshoe shape. 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 rotamer state and is held in this orientation by neighbor Pro$^{51}$ and Phe$^{52}$. These three residues form a cluster of interacting residues.

The large structural reorganization at the binding epitope 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 18 residues shorter than that of gp130. A multiple sequence 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 Prediction program (NNSSSP) (57). The positioning of the secondary structure of gp130 and was used for minor manual refinement of the alignment of the Ig-like domains of gp130 and LIFR (Fig. 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 $\beta$-strands B and C of gp130. The second eight-residue long gap was located at the level of the $\beta$-strands F and G and the linking loop. Fig. 1B displays the Ig-like domains of gp130 and of LIFR modeled according to the alignment shown in Fig. 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 $\beta$ sandwich with $\beta$-strands F and G dramatically shortened as compared with gp130.

The deleted regions are part of the structural epitope of vIL-6 binding to gp130 through site III. The $\beta$-strands F and G and the linking loop contain several residues directly involved at the binding interface (Gln$^{78}$, Thr$^{80}$, Asn$^{82}$, Ile$^{83}$, Asn$^{92}$, Val$^{104}$, Tyr$^{94}$, Gly$^{95}$, and Ile$^{96}$). 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$^{4}$ and Cys$^{6}$, located in the N-terminal tail of gp130, with no equivalent residues in LIFR. Three H-bonds (vIL-6 Trp$^{144}$:Ne1 = gp130 Asn$^{92}$:O; vIL-6 Tyr$^{32}$:O = gp130 His$^{149}$:Ne2; and vIL-6 Thr$^{2}$:O = Gln$^{159}$:Ne2) involve side chain-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$^{144}$ is Phe, implying the loss of this H-bond. Gp130 His$^{49}$ is part of the deleted helix and Glu$^{78}$ is mutated to Val in LIFR.

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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 gp130. However, the two sites do not overlap. Equivalent residues of gp130 are Ser and Ile. Only Ser is marginally involved in viral IL-6 site III binding (46).

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Fluorescence intensity

Cell number

Fig. 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 gray histograms correspond to gp130 detection by AN-HH1 anti-gp130 mAb, and the dark gray histograms represent LIFR detection by AN-E1 anti-LIFR mAb.

Phe and Lys 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 as that of the binding site of gp130. However, the two sites do not overlap. Equivalent residues of gp130 are Ser and Ile. Only Ser is marginally involved in viral IL-6 site III binding (46).

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mutation or upon the double mutation prevented measurement of the affinity constant, indicating a $K_d$ of $10$ 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 D214A mutation. The decrease in the affinity upon the F284A and the D214A/F284A mutations did not allow the dissociation constant to be measured ($K_d$ of $10$ nM) (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 by the D214A or the F284A single mutations, with less than 2-fold changes in $K_d$ values found (Table I). However, the D214A/F284A double mutation yielded a decrease in the affinity sufficient to prevent measurable binding, indicating $K_d > 10$ nM.

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Molecular Docking of the LIF-LIFR Complex—The initial assumption of an interaction between LIF Phe$^{156}$ and LIFR Phe$^{284}$ was consistent with the effect of the F284A mutation.
However, the assumption of an interaction between LIF Lys_{159} and LIFR Asp_{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, p rotamer ($x_1 = -165^\circ$, $x_2 = 78^\circ$) and is stabilized by interactions with Pro$_{51}$ and Phe$_{52}$. In this rigidly held 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 g-, p rotameric orientation ($x_1 = -60^\circ$, $x_2 = -88^\circ$), with the phenylalanine ring perpendicular to the protein surface. $x_1 \times x_2$ 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 most stable rotameric orientation. The energy-minimized structure of the best scoring solution is shown in Fig. 6A. In this three-dimensional model, LIFR Phe$_{284}$ is involved in H-bond interactions with LIF Phe$_{156}$ and LIFR Phe$_{284}$ is positioned in g-,$\pi$ interactions with LIF Phe$_{156}$, with an 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$^{150}$. The distance between LIF Lys$^{150}$ N and LIFR Asp$^{214}$ O is 2.6 Å. LIF Glu$^{50}$ interacts with LIF Lys$^{150}$ 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; LIF Ala$^{59}$:O and Gin$^{57}$:O with LIFR Gin$^{213}$:Ne$_2$) (not shown).

When the same procedure was carried out with the D214A LIFR mutant, the best scoring solution was very similar (root mean square deviation $\sim 0.37$ Å). Its energy-minimized structure is shown in Fig. 6B. In this case, LIF Lys$^{150}$ is involved in a H-bond interaction with LIFR Thr$^{281}$:O$_1$. This indicates that an alternative interaction can stabilize the complex. The other interactions are conserved.

It is worth noting 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 ($>600$ Å$^2$) 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 Å$^2$ of buried surface area and electrostatic complementarity (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. However, 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, Asp$^{214}$ and Phe$^{284}$, forming a mirror image of the two known hot spot residues of LIF, Phe$^{156}$ and Lys$^{150}$, 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, because 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\textsuperscript{214} did not contribute to binding affinity or that the interactions disrupted by this mutation were replaced by alternative interactions of similar strength \((|\Delta \Delta G| \leq 0.5 \text{ kcal/mol})\). 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. First, 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 (Fig. 3). Second, 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 with the single mutations. Third, 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 D214A/F284A LIFR mutant was still efficient upon stimulation by OSM (20-fold increase in EC\textsubscript{50}). Moreover, weak STAT3 phosphorylation upon LIF stimulation of Ba/F3 cells transfected with gp130 and the D214A/F284A LIFR mutant could be detected. This indicates that, although 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 is based 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, \textit{i.e.} the effect of multiple mutations is larger than the sum of the individual mutations. A 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 (Fig. 6A) corroborates the initial hypothesis of a salt bridge between LIFR Asp\textsuperscript{214} and LIF Lys\textsuperscript{275} and of \(\pi-\pi\) interactions between LIFR Phe\textsuperscript{286} and LIF Phe\textsuperscript{156}. During the writing of this report, 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. LIFR Phe\textsuperscript{277} should be involved in van der Waals interaction with LIF Leu\textsuperscript{104}, and LIFR Val\textsuperscript{282} should participate with the hydrophobic cluster involving LIFR Phe\textsuperscript{286} and LIF Phe\textsuperscript{156}. 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 (Fig. 6B) indicates that, upon the Asp\textsuperscript{214} to Ala mutation, the salt bridge between LIF Asp\textsuperscript{214} and LIF Lys\textsuperscript{275} can be replaced by a H-bond interaction between LIF Lys\textsuperscript{275}:N\textsuperscript{\textgamma} and LIFR Thr\textsuperscript{281}:O\textgamma.1. The difference in the dissociation constant of LIF to wild type or D214A LIFR corresponds to a change in the binding free energy of \(<0.5 \text{ kcal/mol}\). The strength of a salt bridge at a protein-protein interface is difficult to evaluate because of the entropic cost of desolvation. 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 the 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\textgamma–LIFR Thr\textsuperscript{281}:O\textgamma1-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.

\textbf{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 \((K_d \sim 10^{-8} \text{ m})\) and the high affinity complexes \((K_d \sim 10^{-10} \text{ m})\) 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.

The CT-1 response to LIFR mutations was similar to that of LIF. 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 D214A/F284A 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 very similar sites III of the two cytokines (Fig. 7). In addition to the conserved FXXK motif (Phe\textsuperscript{168} and Lys\textsuperscript{171}), most residues of the AB loop N-terminal part (Leu\textsuperscript{51}, Glu\textsuperscript{52}, Gly\textsuperscript{53}, Asp\textsuperscript{54}, and Pro\textsuperscript{55}) and of the BC loop (Leu\textsuperscript{111}, Asn\textsuperscript{112}, and Pro\textsuperscript{113}) are conserved or type-conserved. Phe\textsuperscript{56} is also conserved. Conservation of Pro\textsuperscript{275} and Phe\textsuperscript{282} allows CT-1 Phe\textsuperscript{168} to be held in the same orientation as LIF Phe\textsuperscript{156}. Non-conserved residues are at the periphery of the interface (Glu\textsuperscript{110}, Arg\textsuperscript{114}, Gly\textsuperscript{166}, and Pro\textsuperscript{169}). This strongly suggests that the formation of the cytokine-LIFR complex with subnanomolar affinity requires very conserved structual properties of the interface. Site-directed mutagenesis of LIF has shown the involvement of some of these residues in the interaction (Pro\textsuperscript{51} and Pro\textsuperscript{106}) (41). The effect of these mutations on the affinity or on the biological activity is, however, reduced (<10-fold), as compared with 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 D214A/F284A 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, because the D214A and F284A single mutations of LIFR had no significant effect on the affinity or the biological activity of OSM and that the mutated receptor was still able to induce efficient BAF proliferation and STAT3 phosphorylation, upon OSM stimulation.

\textbf{Involvement of LIFR Ig-like Domain in Cytokine Binding}
Involvement of LIFR Ig-like Domain in Cytokine Binding

Fig. 7 shows residues conserved in oncostatin M as compared with LIF. In addition to the FXXK motif (Phe160 and Lys163), the few conserved or type-conserved residues are located at the N-terminal part of the AB loop (Gln163 and Gly167), at the C-terminal part of the CD loop (Asp158 and Ala159), and at the N-terminal part of helix D (Gln161 and Arg165). The large insertion in the BC loop yielding an additional helix (Ala169-Glu172) 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 granulocyte-colony stimulating factor 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, OSM Phe284 does not constitute a hot spot for OSM binding, which is consistent with a weak interaction.

On the other hand, for LIF and CT-1, interface where interacting residues have not a geometry optimized for strong interactions. On the other hand, for LIF and CT-1, the Phe284 and Asp214 residues. The behavior of OSM binding epitope in the Ig-like domain of LIFR.

In conclusion, LIF, CT-1, and OSM share an overlapping binding epitope located in the Ig-like domain of LIFR and involving the Phe234 and Asp214 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 Phe234 and Asp214 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-4R 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.

Acknowledgment—We thank Dr. J. Janin (Orsay, France) for stimulating discussion.

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Leukemia Inhibitory Factor (LIF), Cardiotrophin-1, and Oncostatin M Share Structural Binding Determinants in the Immunoglobulin-like Domain of LIF Receptor

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J. Biol. Chem. 2003, 278:27169-27179.
doi: 10.1074/jbc.M303168200 originally published online April 21, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303168200

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