Solution Structure of Leukemia Inhibitory Factor*

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The solution structure of a murine-human chimera of leukemia inhibitory factor (LIF), a 180-residue cytokine with a molecular mass of 20 kDa, has been determined using multidimensional heteronuclear NMR techniques. The protein contains four α-helices, the relative orientations of which are well defined on the basis of long-range interhelical nuclear Overhauser effects. The helices are arranged in an up-up-down-down orientation, as found in other four-helix bundle cytokines, and the overall topology of the chimera is similar to that of the crystal structure of murine LIF (Robinson, R. C., Grey, L. M., Staunton, D., Vankelecom, H., Vernallis, A. B., Moreau, J. F., Stuart, D. I., Heath, J. K., and Jones, E. Y. (1994) Cell 77, 1101–1116). Differences between the structures are evident in the N-terminal region, where the peptide bond preceding Pro17 has a trans-conformation in solution but a cis-conformation in the crystal, and in the small antiparallel β-sheet encompassing residues in the N terminus and the CD loop in the crystal structure, which is not apparent in solution. There are also minor differences in the extent of the helices. Other than at the N terminus, the main difference between the two structures occurs at the C-terminal end of the CD loop. As this loop is close to a receptor-binding site on LIF that makes a major contribution to high affinity binding to the LIF receptor α-chain, these differences between the solution and crystal structures should be taken into account in structural models of LIF receptor interactions.

Leukemia inhibitory factor (LIF) is a glycoprotein identified and purified on the basis of its ability to induce differentiation in murine myeloid leukemic M1 cells (1). LIF is produced, however, in a diverse array of cell types and is a highly pleiotropic cytokine, exerting a range of actions on a number of cell types, including hepatocytes; adipocytes; megakaryocytes; neuronal, muscle, and embryonic stem cells; and osteoblasts (2).

These actions include the induction of cell proliferation (in myoblasts and megakaryocytes), the induction (in M1 cells) or suppression (in embryonic stem cells) of differentiation, and the induction of mature cell function (in neurons, adipocytes, and hepatocytes) (2, 3).

Although it has multiple activities, LIF appears to be absolutely necessary only for embryo implantation. Male mice lacking the gene for LIF are apparently normal, whereas the corresponding female mice are infertile, and the deficiency can be corrected by injection of purified LIF (4). In contrast, mice lacking the LIF receptor have multiple placental, skeletal, neural, and metabolic disorders, which lead to perinatal death (5). The explanation for these observations, at least in part, is that several other cytokines share components of the LIF receptor, the functional form of which consists of the LIFR α-chain and a common β-chain, gp130 (3). The β-chain is shared with interleukin (IL)-6 and IL-11, and both chains form part of the receptor complexes for oncostatin M, ciliary neurotrophic factor (CNTF), and cardiotrophin-1.

With its range of biological functions and activity against a variety of target cells, LIF also has potential therapeutic applications. Its ability to induce the growth and differentiation of peripheral and central nerve cells indicates a potential use in the treatment of peripheral nerve damage (6) and motor neuron disease. Furthermore, the receptor subunit gp130, which is shared among the IL-6 family of cytokines, is a potential target for antagonists that could be used in treating certain cancers and autoimmune diseases associated with overproduction of these cytokines (7). A knowledge of the structures of the cytokines and their receptor complexes will open the way for the design of cytokine antagonists or agonists directed toward specific tissues.

Despite the low sequence identity among the four-helix bundle cytokines (17–24%) (8), there are common features of their three-dimensional structures. On the basis of their sequences and tertiary structures, they have been divided into two classes (9–11), which share a common motif consisting of four helices (referred to as the A, B, C, and D helices) arranged in an antiparallel up-up-down-down fashion, with long loops between the A and B helices and the C and D helices. The short-chain molecules are distinguished from their long-chain counterparts by the lengths of their helices, which span only 15 or so residues compared with ~25 in the long-chain class, and by the secondary structure elements in the long AB and CD loops, with the short-chain molecules typically having a small β-sheet and the long-chain molecules having α-helices.

We have undertaken an investigation by NMR spectroscopy of the solution structure of LIF. Mature LIF has three disulfide bonds and is heavily N-glycosylated, with an apparent molecular mass range of 32–62 kDa in the glycosylated state, but only 20 kDa in the non-glycosylated state (12). Samples for NMR were obtained from Escherichia coli, where the protein is
not glycosylated, but it has been shown that glycosylation is not important for activity (2, 13). The solution structure was determined for MH35-LIF, a murine-human chimera of LIF consisting of residues 1–47 and 83–180 of mLIF and residues 48–82 of hLIF, except for residues 107, 112, 113, 155, and 158, which are from hLIF (Fig. 1). hLIF binds both the human and murine receptors with high affinity, whereas mLIF binds strongly only to the murine receptor (14, 15). Although MH35-LIF has a greater sequence similarity to murine LIF than to human LIF, it has essentially the same biological activity as hLIF (15), and thus, it was of interest to determine its structure to characterize changes in the receptor-binding domains of the protein. MH35-LIF also expressed at high levels in E. coli grown on minimal medium and could be labeled efficiently (16).

**EXPERIMENTAL PROCEDURES**

**NMR Spectroscopy**—The preparation of [13C]- and [15N]-labeled MH35-LIF and the chemical shift assignments and secondary structure of MH35-LIF have been reported previously (17). NMR data were acquired on samples at pH 4.4 and 40 °C.

A two-dimensional NOESY spectrum was acquired on unlabeled MH35-LIF with a 150-ms mixing time, and the following spectra were acquired on uniformly [15N]-labeled MH35:LIF: three-dimensional [13C]- and [15N]-edited NOESY-HSQC (18) with a 150-ms mixing time to obtain distance constraints, HNHA (19) or HMQC-J (20) to determine NH–C\(^\alpha\) coupling constants, and HNHB (21) to obtain stereospecific assignments. The triple-resonance experiments [13C]-edited NOESY-HSQC (22), [13C]-edited ROESY-HSQC (23), HACAHB-COSY (24), and HCACO (25) were acquired on the [13C,15N]-labeled sample to obtain further distance constraints, further stereospecific assignments, and limits on the \(\phi\) and \(\psi\) angles. Pulsed-field gradients were used to suppress unwanted coherences and the water resonance (26).

**Structural Constraints**—Approximate interproton distances were derived from two-dimensional NOESY and three-dimensional [13C] and [15N]-edited NOESY spectra. Peak intensities were classified as strong, medium, weak, or very weak on the basis of contour levels and transverse relaxation times. Pulsed-field gradients were used to suppress uninformative NOE cross-peaks. This process was repeated until all the distance and angle restraints produced a set of structures that had no NOE distance violations >0.3 Å or dihedral angle violations >5°. Once the final set of restraints had been obtained, a new family of structures was calculated using HYSS (13) and the torsion angle dynamics program DYANA (29). Several rounds of structure calculation were carried out using DYANA to resolve violated distance constraints and to determine possible assignments for ambiguous NOE cross-peaks. This process was repeated until all the distance and angle restraints produced a set of structures that had no NOE distance violations >0.3 Å or dihedral angle violations >5°. Once the final set of restraints had been obtained, a new family of structures was

**Fig. 1.** Amino acid sequences of hLIF, mLIF, and MH35-LIF. Glycosylated residues are indicated in **boldface.** Only differences from human LIF are shown. The helical regions in MH35-LIF are **underlined.**

**Fig. 2.** Strip plots at C\(^{\alpha}\) chemical shifts for the C-terminal residues Lys\(^{170}\)-Phe\(^{180}\) of [13C,15N]-labeled MH35-LIF, taken from a 600-MHz three-dimensional [13C]-HSQC spectrum. C\(^{\alpha}\)–C\(^{\beta}\) NOE pairs typical of an \(\alpha\)-helix are indicated by **horizontal lines.** An NOE between the C-terminal Phe\(^{180}\) aromatic protons and the C\(^{\beta}\) of Val\(^{172}\) is also indicated.
generated using DYANA; the 50 structures with the lowest penalty functions were selected from a calculation of 1000 structures, and these were refined in X-PLOR (30) using dynamical simulated annealing (31) and energy minimization, as described previously (28), but without neutralization of charged side chains. The 20 best structures, on the basis of their stereochemical energies, were chosen for structural analysis.

Structures were analyzed using MOLMOL (32) and PROCHECK-NMR (33). Hydrogen bonds were identified in MOLMOL using a maximum C–N distance of 2.4 Å and a maximum angular deviation of 35° from linearity. Structural figures were generated using MOLMOL.

RESULTS

Structure Determination—Structural restraints were determined from a combination of two- and three-dimensional double- and triple-resonance experiments using both 15N- and 13C,15N-labeled samples as well as unlabeled protein. A representative region of a three-dimensional 13C NOESY-HSQC spectrum, from which distance restraints were obtained, is shown in Fig. 2. There were very few constraints for the first nine residues, which are apparently disordered in solution.

Structures were determined initially using DYANA, then refined by simulated annealing in X-PLOR, and finally energy-minimized. Parameters characterizing the final 20 structures are plotted in Fig. 3; structural statistics are summarized in Table I; and a superposition of the final 20 structures is shown in Fig. 4. The best defined regions of the structure, residues 25–48, 75–133, and 153–180, correspond to the A helix, the B and C helices and the short BC loop, and the D helix including the C terminus, respectively. The structures shown in Fig. 4 were superimposed over the backbone atoms nitrogen, α-carbon, and carbon of the four helices that define the α-helical bundle.

The data in Table I show that the ensemble of NMR structures is energetically reasonable and has acceptable covalent geometry. Indeed, 90% of the residues have φ-ψ values in the allowed regions of a Ramachandran plot, and there is good clustering of residues in the most favored α-helical region (φ = −60°, ψ = −60°), as would be expected for a four-helix bundle protein. The mean pairwise RMSD for the backbone heavy atoms is 0.65 Å over the four-helix bundle and 3.06 Å for the entire molecule (including the disordered N terminus). The corresponding values for all heavy atoms are 1.44 and 3.50 Å, respectively.

A measure of the quality of the three-dimensional structure is the consistency of the RMSDs determined by alignment over alternative combinations of individual secondary structure elements. If the tertiary structure is well defined, then a superposition over the major elements of secondary structure should give consistent RMSDs. In MH35-LIF, alignment over one of the major helices gave low RMSD values over the whole bundle, with the mean being 0.71 Å for the backbone atoms and 1.46 Å for all heavy atoms.

Structure of MH35-LIF—The overall topology is a four-helix bundle, with the helices arranged in an up-up-down-down left-handed fashion. The N-terminal region is distinguished by its lack of long-range order, with the first nine residues being unstructured and residues 10–22 being poorly ordered. The A helix begins at residue 24 and contains a marked kink in the vicinity of residues 34–36. This kink, which was also observed in the crystal structure of mLIF (34), is reflected in the backbone amide exchange behavior of the amides (27) and the chemical shifts for residues in this region (17). The first of the two long interhelical loops, the AB loop, connects the C terminus of the A helix with the N terminus of the B helix. The AB loop contains some elements of secondary structure near its N terminus, with two irregular turns preceding a short helix between residues 55 and 60. This loop is well packed against the helical bundle, with long-range NOEs observed between residues in the loop (Leu56, Leu59, and Cys60) and residues in the A and D helices. There is a very short, well ordered loop
linking the B and C helices. The C and D helices are connected by the second long loop. In contrast to the AB loop, the CD loop has no regular secondary structure and is less ordered overall, as judged by the angular order parameters and RMSD values over these residues compared with the AB loop (Fig. 3).

Comparison with Crystal Structure of mLIF—Fig. 3 shows the residue by residue RMSD from the crystal structure of mLIF (34). The overall RMSD when the two structures are aligned over the backbone of the common helical bundle residues is 1.55 Å, indicating good agreement. Comparison of the uncertainty in the positions of the atoms as measured by crystallographic B factors in mLIF (Fig. 3G) with the backbone RMSDs of the solution structure (Fig. 3D) shows that they follow a similar pattern, emphasizing the similarity between the two structures. The lengths of the four main helices in the solution structure differ slightly from those of the x-ray structure, with the limits for the helices (according to PROCHECK-NMR) being 24–45, 76–104, 108–133, and 154–176 for the A, B, C, and D helices, respectively, compared with 22–48, 76–104, 109–135, and 155–177 in the crystal structure. The short helix involving residues 55–60 is also present in the crystal structure. Analysis of the crystal structure using PROCHECK shows that there is a short antiparallel β-sheet involving residues 10–12 in the N-terminal tail and residues 140–142 in the backbone heavy atoms superimposed structures of MH35-LIF showing near the N terminus, Pro17 adopts a CD loop, but this is not present in solution. The other region where differences were evident is the CD loop, especially at its C-terminal end (Fig. 3F). These differences are close to one of the receptor-binding sites, as discussed below. In mLIF, the AB loop crosses the D helix roughly one-third of the way down its length (34), rather than at its N terminus as in growth hormone. In MH35-LIF, the crossing point is only slightly closer to the N terminus of the D helix than in mLIF.

**DISCUSSION**

Comparison with Proteins of Related Folds—A number of structures have been determined for four-helix bundle cytokines or related molecules by either NMR or x-ray crystallography, with the limits for the helices (according to PROCHECK-NMR) being 24–45, 76–104, 108–133, and 154–176 (34). The overall RMSD when the two structures are aligned over the backbone of the common helical bundle residues is 1.55 Å, indicating good agreement. Comparison of the uncertainty in the positions of the atoms as measured by crystallographic B factors in mLIF (Fig. 3G) with the backbone RMSDs of the solution structure (Fig. 3D) shows that they follow a similar pattern, emphasizing the similarity between the two structures. The lengths of the four main helices in the solution structure differ slightly from those of the x-ray structure, with the limits for the helices (according to PROCHECK-NMR) being 24–45, 76–104, 108–133, and 154–176 for the A, B, C, and D helices, respectively, compared with 22–48, 76–104, 109–135, and 155–177 in the crystal structure. The short helix involving residues 55–60 is also present in the crystal structure. Analysis of the crystal structure using PROCHECK shows that there is a short antiparallel β-sheet involving residues 10–12 in the N-terminal tail and residues 140–142 in the backbone heavy atoms superimposed structures of MH35-LIF showing near the N terminus, Pro17 adopts a CD loop, but this is not present in solution. The main differences occur, as expected, in the loop regions. Near the N terminus, Pro17 adopts a trans-conformation in the solution structure of MH35-LIF (17), in contrast to the cis-conformation in the crystal structure. The N-terminal eight residues were not observed in the crystal structure due to the lack of order in this region, which is consistent with the lack of long-range NOEs in this region and the sharper NH resonances from the N-terminal residues in two-dimensional HSQC spectra. The other region where differences were evident is the CD loop, especially at its C-terminal end (Fig. 3F). These differences are close to one of the receptor-binding sites, as discussed below. In mLIF, the AB loop crosses the D helix roughly one-third of the way down its length (34), rather than at its N terminus as in growth hormone. In MH35-LIF, the crossing point is only slightly closer to the N terminus of the D helix than in mLIF.

**TABLE I**

| Residues superimposed (Å) | Mean pairwise RMSD | Backbone | All heavy atoms |
|---------------------------|---------------------|----------|-----------------|
| 1–180                     | 3.06 ± 0.86         | 3.50 ± 0.72 |
| 9–180                     | 1.57 ± 0.25         | 2.34 ± 0.27 |
| ABCD                      | 0.65 ± 0.08         | 1.44 ± 0.09 |
| A                         | 0.56 ± 0.12         | 1.30 ± 1.20 |
| B                         | 0.46 ± 0.09         | 1.42 ± 0.12 |
| C                         | 0.42 ± 0.09         | 1.32 ± 0.17 |
| D                         | 0.45 ± 0.12         | 1.38 ± 0.20 |
| AB loop                   | 1.50 ± 0.47         | 2.16 ± 0.47 |
| CD loop                   | 2.14 ± 0.84         | 3.32 ± 1.03 |

* Approximately half of these intraresidue constraints are redundant with the covalent geometry.
FIG. 5. Solution structure of MH35-LIF showing receptor binding and glycosylation sites. A, receptor-binding sites identified by alanine scanning mutagenesis (41). Solvent-accessible surfaces are shown for the following residues: site I, Lys170, Ser174, and Val177; site II, Gln25, Ser28, Gln32, Asp120, Ile121, Gly124, and Ser127; and site III, Pro51, Pro106, Thr150, Lys153, Asp154, Phe156, Lys158, and Lys159 (41). Phe156 and Lys159 are shown in red, and Pro106 in pink. Two residues that may also be associated with site III, Asp57 and Lys58, are shown in white (largely hidden in this view). B, six residues capable of conferring high affinity binding to the human LIFR α-chain on mLIF (15): Asp57, Ser107, His112, Ser113.
graphy. Six of these, G-CSF, growth hormone, CNTF, IL-6, leptin, and LIF itself, are in the "long-chain" class of cytokine. The sequence identity among these molecules is low, but they share common structural motifs and similar receptor systems. In particular, LIF, IL-6, and CNTF all share the signal-transducing protein gp130, and this is reflected in at least one common receptor-binding site among this group of cytokines.

The solution structure of MH35-LIF is in good agreement with that of mLIF determined by x-ray crystallography (34). As well as sharing the overall antiparallel four-helix bundle motif, structural details similar to those determined for MH35-LIF have been observed for G-CSF (35), CNTF (36), IL-6 (37, 38), and human leptin (39). The A helix has a kink approximately half way along its length, as also found for CNTF, IL-6, and leptin in a similar although not identical position. Although there is no three-dimensional structure available for oncostatin M, differences from chemical shift patterns expected for a purely α-helical conformation have been observed in its A helix (40), making it likely that this molecule also has a kink here. This structural feature serves to maximize the close contact between the A and C helices (39), and it has been suggested that it may also be important in receptor interactions (36). CNTF, G-CSF, leptin, and mLIF have kinks or bends in their D helices, whereas growth hormone and IL-6 have kinks in their B helices.

The AB loop in LIF differs from those in IL-6 and CNTF in that it is relatively well defined in both the solution and crystal structures and it contains two irregular turns preceding a short helix. The AB loop in IL-6 contains only two β-turns, and these occur at the C-terminal end of the loop, whereas the AB loop is poorly ordered in CNTF. In comparison with the AB loop, the CD loop in LIF is not as well defined in the NMR and crystal structures and does not contain any ordered secondary structure. Leptin (39) and IL-6 (37, 38) both have short helices in this loop, of 10 and 12 residues, respectively.

Receptor Interactions—The receptor complex for LIF is believed to involve a single ligand molecule in association with gp130 and LIFR, and this ternary complex is responsible for signal transduction. A partial alanine scan of hLIF (41) suggested that there were three distinct receptor-binding sites on the surface of LIF, designated sites I–III. Similar sites have been identified on the surfaces of CNTF (36, 42) and IL-6 (37, 38). Site I, at the C terminus of the D helix, confers specificity of the ligands IL-6, CNTF, and LIF for their respective receptor α-chains, whereas site II, at the A-C helical interface, interacts with the signal-transducing protein gp130. Site III, at the N terminus of the D helix and the N-terminal end of the C helix, interacts either with LIFR in the cases of CNTF and LIF or with a second gp130 molecule in the case of IL-6. The locations of these sites on the structure of MH35-LIF are shown in Fig. 5A.

The residues responsible for high affinity binding of hLIF to the LIFR α-chain contribute to sites I and II (34, 41). Site I, centered on Lys158, Ala174, and Val175, is located at the C-terminal end of the D helix (Fig. 5A). Lys170 and Val175 are conserved between murine and human LIFs, and their orientations in the solution structure of MH35-LIF and the crystal structure of mLIF are essentially the same, although the Lys170 side chain is disordered in solution. Ala174 is replaced by Ser in both MH35-LIF and mLIF, and once again, their orientations are similar in the two structures. Two additional residues in hLIF, Asp57 and Lys58, were nominated as making weak contributions to site I (41), but inspection of the structure shows that they are located between sites I and III, although closer to the latter (Fig. 5).

Hudson et al. (41) found that the dominant contributors to the affinity of hLIF for its receptor α-chain were the site III residues Phe156 and Lys159, located in the "D1 motif" (8) at the N-terminal end of the D helix. Both Phe156 and Lys159 are conserved among hLIF, mLIF, and MH35-LIF. Whereas Lys159 is in essentially the same orientation in the structures of mLIF and MH35-LIF, Phe156 shows a small displacement from its position in the crystal structure. The displacement of Phe156 is more significant at the backbone than the side chain and reflects the divergence between the C-terminal ends of the CD loops in the two structures, evident in the RMSDs between the solution and crystal structures in this region (Fig. 3F). As the CD loop is surface-exposed, it is less likely to adopt a rigid conformation than the helical bundle, but the solution structure of MH35-LIF is reasonably well defined at its C-terminal end (Fig. 3, D and E), and the structure of mLIF lies well outside the family of solution structures in this region (Fig. 4). Differences in amino acid sequence are unlikely to be responsible for the structural differences, as the sequence of MH35-LIF is identical to that of mLIF in the CD loop. More important, there are NOEs observed in the NMR spectra, reflecting distances of <5 Å, that are not consistent with the crystal structure in the C-terminal part of the CD loop. Furthermore, several NOEs predicted by short distances in the crystal structure are not observed in our spectra, although not all NOEs predicted from the NMR structures are observed in this region. Taken together, these observations suggest that the significant differences in the region of residues 150–155 (Figs. 3F and 4) reflect a genuine difference between the solution and crystal structures close to site III.

Other contributors to site III are Pro51 (which adopts a cis-configuration in both the solution and crystal structures), from the N-terminal section of the AB loop; Pro106, from the short BC loop; and Thr150, Lys153, and Lys158, from the end of the CD loop and the start of the D helix. The only one of these residues altered in MH35-LIF is Thr150, which is replaced by His. The imidazolium pKₐ of His150 is 5.4 (16); therefore, at physiological pH, this side chain would be neutral, but its larger size may contribute to slightly weaker binding of MH35-LIF to the LIFR α-chain. As Pro106 is separated from the other residues in site III (Fig. 5) and located in the tight turn linking the B and C helices, it is possible that its replacement by Ala may have affected receptor binding indirectly via a local conformational change and therefore that it may not be a direct contributor to site III (41). The same may also be true for Pro51, as the cis-configuration of the peptide bond may not be maintained in the P51A mutant, but this residue is in a more flexible region of the structure (the AB loop), and any conformational changes are likely to be less extensive.

As noted previously (41), Phe156 and Lys159 are conserved in oncostatin M, CNTF, and cardiotrophin-1, all of which bind to the LIFR α-chain in their signal transduction complexes. By contrast, in related long-chain cytokines that do not bind to the LIFR α-chain, such as IL-6 and IL-11, these residues are not conserved. The importance of the Phe and Lys residues has been confirmed by mutagenesis studies in the case of CNTF (42, 43). In IL-6, this site is thought to interact with a second gp130 molecule in the hexameric signaling complex (37, 38).

A study of murine-human chimeras of LIF (14, 15) identified

Lys159 and Val175. C, Asn side chains that are glycosylated in naturally occurring LIF (44). Asnδ, Asnδ, and Asnδ are colored orange, and Asnδ, Asnδ, and Asnδ cyan. The individual solution structure shown is the closest to the mean (calculated by averaging over the backbone heavy atoms (nitrogen, α-carbon, and carbon) of all residues of the 20 final structures). Helices and loops are colored as described in the legend to Fig. 4.
a group of six residues from hLIF that created high affinity for the hLIF receptor α-chain when introduced into mLIF (which normally binds only very weakly). These residues, shown in Fig. 5B, are located in the same region of the structure as site III (Fig. 5A), but the only residue in common is Asp97. The explanation for the differences between the residues identified by alanine mutagenesis and those identified using chimeras is not clear, but local structural changes associated with alanine substitutions may be one factor, and structural studies on some of the mutants involving site III would be informative. Steric hindrance associated with the six key residues on mLIF may also contribute to its low affinity for the human receptor. It is likely that this receptor-binding surface includes contributions from both sets of residues.

The remaining receptor-binding site on LIF, site II, is responsible for interaction with gp130. This site has been localized to residues in the A and C helices, but because the interaction of LIF with gp130 is significantly weaker than that with the LIFR α-chain, it was more difficult to identify all of the residues that contribute to site II (41). Nevertheless, Gln26, Ser28, and Gln32 in the A helix and Asp126, Ile121, Gly124, and Ser127 in the C helix were shown to be involved (Fig. 5). Of these residues in hLIF, only Ser28 and Ile121 are different in MH35-LIF, being substituted conservatively by Asn and Val, respectively. The solution and crystal structures are in good agreement in this region.

In CNTF and IL-6, the number of residues identified as contributing to the common gp130-binding site is lower than for hLIF. Thus, in CNTF, only Lys91 and Asp126, which are on the exposed face of the A helix and interact via a salt bridge (42), participate, whereas in IL-6, the two corresponding residues (Tyr31 and Gly35) are involved, together with Ser118 and Val121, on the exposed face of the C helix. Thus, it appears that many different combinations of side chains can satisfy the requirements of interaction with gp130, probably reflecting the fact that this is, in most cases, a comparatively weak interaction. It would be interesting to know which residues contribute to site II on oncostatin M, which is able to bind to gp130 before binding to the LIFR α-chain and has an affinity for gp130 at least 2 orders of magnitude higher than that of hLIF (41).

Post-translational Modifications—LIF is N-glycosylated on Asn residues 9, 34, 63, 73, 96, and 116 when expressed in Chinese hamster ovary cells (44). Structurally, these sites on LIF do not form a single contiguous surface, but are distributed over the surface (Fig. 5C). The sites are located in the unstructured N-terminal tail (Asn9), the middle of the A helix near the kink (Asn34), the AB loop (Asn63 and Asn73), the C-terminal end of the B helix (Asn96), and the N-terminal end of the C helix (Asn116). As the degree of glycosylation does not appear to affect the interaction of LIF with its receptor, the carbohydrate moieties must not interfere with receptor interaction. Three of the sites (Asn residues 63, 96, and 116) encircle site III, but are directed away from it; Asn96 and Asn33 are ~9 Å away from site III. Asn34 lies between sites I and II, but the location of these sites on opposite faces of LIF accounts for the lack of effect of glycosylation on binding to either gp130 or the LIFR α-chain.

Glycosylation probably modifies the solution properties of LIF and protects it from proteolytic degradation (44). However, in a recent NMR investigation of glycosylated G-CSF, it has been suggested that the carbohydrate may also increase the stability of the protein by decreasing the mobility of these residues near the site of glycosylation (45). Given that LIF is already more rigid than other cytokines for which structures are available,2 it would be interesting to know if the glycosylated form was even less flexible. One possible implication of this is that many of the surface residues involved in receptor binding might undergo little conformational change upon receptor binding, thus reducing the conformational entropy penalty associated with binding. Such a case has been observed for a mutant of G-CSF, in which enhanced biological activity was attributed to a decrease in the mobility of the long loops connecting the helices (46), probably stabilizing the biologically active conformation.

Histidine Environments—Previously, we determined the imidazolium pKα values for the six His residues of MH35-LIF using two-dimensional 1H NMR (16). Three of these are in the range 6.0–6.4, but the other three are perturbed, and the structure accounts satisfactorily for these observed effects. His71 is close to Glu82, which is likely to be responsible for the low pKα inflection in the titration curve for His71 and the elevated imidazolium pKα (>7.5). His112 is very close to the side chain of Lys102 and is partially buried, which both would contribute to its low pKα (4.1), and His150, with a pKα of 5.4, is also partially buried.

Small Molecule Design—One approach to the development of small molecule agonists or antagonists of cytokines is the screening of oligopeptide libraries displayed on bacteriophage surface proteins. An alternative strategy is the de novo design of mimetics of individual receptor sites on the cytokine surface, although this approach is probably more suited to the generation of an antagonist than an agonist. The IL-6 family of cytokines has similar three-dimensional structures and three topologically conserved binding sites, and there are even greater similarities among those members of the family that bind to the LIFR α-chain, e.g., the common D1 motif in site III. A small molecule that mimicked site III of the LIF family of cytokines by binding tightly to the LIFR α-chain might have a wider spectrum of activities than desirable therapeutically, as it would inhibit the actions of all members of the LIF family (41). An antagonist that bound tightly to LIF itself would overcome this problem. The detailed knowledge now available of the structure of LIF and the residues contributing to the three receptor-binding sites on its surface also opens the way for a structure-based approach to the development of low molecular mass analogues.

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