Residues in human leukemia inhibitory factor (hLIF) crucial for binding to both the human LIF receptor (R) and gp130 were identified by analysis of alanine scanning mutants of hLIF in assays for both receptor binding and bioactivity. The region of hLIF most important for binding to the hLIF-R is composed of residues from the amino terminus of the D-helix, carboxyl terminus of the B-helix, and C-D loop. This site forms a distinct surface at the end of the four-helix bundle in the tertiary structure of the closely related murine LIF. The two residues of hLIF that contribute the majority of free energy for hLIF-R binding, Phe-156 and Lys-159, are surrounded by other residues which have only a moderate impact. This arrangement of a few key residues surrounded by less important ones is analogous to the functional binding epitope of human growth hormone for its receptor. A second region of hLIF that includes residues from the carboxyl terminus of the D-helix and A-B loop also had a weak influence on hLIF-R binding. Residues in hLIF from both the A- and C-helices are involved in binding the gp130 co-receptor. Abolition of the gp130 binding site in hLIF created antagonists of LIF action.

Leukemia inhibitory factor (1–3) is a secreted cytokine that elicits pleiotropic effects on a diverse range of cell types, these include embryonic stem cells, primordial germ cells, neurons, adipocytes, hepatocytes, and osteoblasts (4, 5). In mice, gene knockout experiments have demonstrated that LIF1 is essential for embryonic implantation (6, 7). In contrast to the mild biological differences between these two genetic deficiencies is an outcome of the use of the LIF-R for signal transduction by several ligands. The cytokines known to bind to the LIF-R are included in a group that share some biological properties with LIF: oncostatin M, ciliary neurotrophic factor (CNTF), cardiotrophin (CT-1), interleukin-6 (IL-6), and interleukin-11 (9–14).

The crystal structure of recombinant murine LIF (mLIF) has been solved (15). LIF has a four-α-helical bundle topology with up-up-down-down helix orientation that has long crossover loops between the first two and last two helices. The structure of LIF shows greatest homology to granulocyte-colony-stimulating factor (G-CSF; Ref. 16), human growth hormone (hGH; Ref. 17) and the recently determined CNTF (18). These proteins all belong to the hematopoietin cytokine family, which is characterized by the four-helix bundle structure, but limited sequence homology between family members (19–21). The hematopoietin cytokine family is divided into the short and long chain subfamilies (21). The long chain group of which both LIF and hGH are members is characterized by helices of approximately 25 residues, the presence of short helical regions in the long loops, and the complete absence of β strands. The canonical member of the hematopoietin cytokine family and most characterized is hGH.

The co-crystal structure of hGH and its receptor (17), together with extensive mutagenesis studies of both the ligand (22, 23) and receptor (24), have defined both the signaling complex and binding surfaces of these molecules. The activated GHR is formed by homodimerization of two identical receptor subunits and a single hGH molecule (17, 25). hGH uses two distinct sites to bind sequentially to the two growth hormone receptors, first via site I and secondarily to site II. The higher affinity GHR binding site on hGH, site I, involves residues in the carboxyl terminus of both the A- and D-helices and the A-B loop, whereas site II includes residues in both the A- and C-helices. In contrast to the ligand, almost identical residues on the GHR are used to bind both sites I and II on hGH.

Receptor homodimerization leading to signal transduction as used by the GHR, erythropoietin R (26), and G-CSF-R (27) represents the simplest form of receptor assembly. A more complex form of receptor association involves heterodimerization, although the exact stoichiometry of receptor components has not been determined for the majority of these complexes. Such complexes use a specific cytokine binding receptor for each ligand and often share a common signaling β chain among several cytokines. This arrangement is demonstrated by interleukin 3, granulocyte macrophage-colony stimulating factor (GM-CSF), and interleukin-5 (IL-5), all of which use a common β receptor together with a ligand-specific chain (28). Similarly, the interleukins: 4, 7, 9, 15, and possibly 13 use the interleukin-2 (IL-2) γ chain together with a specific interleukin receptor binding component for signal transduction (29). Heterotrimetric receptor formation of three different subunits is utilized for the association of the high affinity IL-2 receptor (30). The most complicated receptor assembly so far characterized is that of the signaling unit of the interleukin 6 receptor (IL-6-R). This complex involves receptor heterodimerization, but forms a hex-
LIF transduces its biological signal via transmembrane receptors, of which both low and high affinity forms have been characterized. The low affinity species of the receptor (K<sub>D</sub> \approx 10^{-9} \mu M) is the single chain 190-kDa LIF receptor (33). Primary sequence comparison indicates that the LIF-R is part of the hematopoietin receptor family (20, 34). The extracellular region of the LIF-R is predicted to be composed of two hematopoietin domains separated by an immunoglobulin module and three fibronectin type III repeats proximal to the membrane (33). Hematopoietin cytokine binding domains have several primary sequence elements conserved in nearly all members of the family, including several cysteine residues and the motif Trp-Ser-.
Binding studies to gp130-Fc were performed in manner similar to that for LIF-R-Fc. These assays differed in that for gp130-Fc competition binding, biotinylated oncostatin M was used instead of 125I-hLIF and the bound oncostatin M was detected by incubation with a streptavidin-horseradish peroxidase conjugate (Amersham). Specifically, after washing plates with PBS-0.05% Tween 20, the wells were rinsed with PBS and then incubated with 100 μl of streptavidin-horseradish peroxidase (1/1000 dilution) in PBS-1% BSA. After again washing with PBS, the horseradish peroxidase was detected by incubation with the chromagen OPD (orthophenylenediamine; Dako) according to the manufacturer’s instructions. Absorbance was read at 492 nm in a 96-well plate reader (Anthos). All gp130-Fc binding studies were performed in duplicate for at least two independent experiments.

RESULTS

Rationale for Human LIF Mutagenesis—The solvent accessibility of residues in the structure of the closely related mLIF (15; 79% sequence identity with hLIF) was used in conjunction with previous mutagenesis data to target amino acids of hLIF for mutagenesis. Alanine scanning mutagenesis (22) was used to identify hLIF residues involved in LIF-R binding by mutagenesis of amino acids in the D-helix, C-D loop, the carboxyl-terminal end of the A-helix, and A-B loop of hLIF. The choice of these locations was based on the results of previous LIF mutagenesis (15, 53), and the site I hGH paradigm (22, 23). Subsequently, residues in the vicinity of the B-C loop were also selected for mutagenesis because of their proximity to Phe-156 (see below). The location of both the hGH site I (25) and also the residues important for gp130 binding in the predicted IL-6 structure (63) were used as a guide for mutagenesis of the putative gp130 binding site in hLIF. Instead of alanine-scanning mutagenesis, both multiple alanine and also multiple nonconservative substitutions were engineered to identify the hLIF gp130 binding site.

Identification of the LIF-R Binding Site on Human LIF—Mutant LIF molecules were assayed in two different systems for binding to the LIF-R. First, the ability of LIF mutants to inhibit 125I-hLIF binding to immobilized recombinant human LIF-R-Fc is presented in Table I and Fig. 1. Second, the LIF mutants were tested for their biological activity in a proliferation assay of the LIF-dependent cell line Ba/F3-LIF-R-gp130 (Table I and Fig. 2).

Two mutants exhibited dramatic reductions in the LIF-R-Fc binding assay, hLIF F156A and hLIF K159A (Table I and Fig. 1), the change in LIF-R-Fc affinity for these two mutants was also paralleled by a large decrease in activity in the proliferation assay (Table I and Fig. 2). A similar reduction in LIF-R-Fc binding was also observed for mutants hLIF F156A and hLIF K159A, when assayed for competition binding with 125I-hLIF on 293T cells transfected with the entire LIF-R reading frame (data not shown). Despite the reduction in LIF-R-Fc affinity in these mutants, no alteration in gp130-Fc affinity was observed (Table I and Fig. 3), thus arguing that these mutations were specific for LIF-R binding. Several other mutants demonstrated significantly reduced binding to the LIF-R-Fc. These included hLIF P51A, hLIF K153A, hLIF P106A, hLIF T150A, hLIF K158A, and hLIF V175A (Table I). Finally, several mutants showed only weak (2-fold) reduction in LIF-R-Fc binding. These mutants included hLIF D57A, hLIF K58A, hLIF D66A, hLIF K102A, hLIF D154A, hLIF K170A, and hLIF A174Q (Table I).

In general, the reduced LIF-R-Fc binding by the various mutants was also accompanied by a decrease in activity in the proliferation assay. However, the reduction in the LIF-R-Fc binding was usually greater than the reduction in the proliferation assay, even though the proliferation assay was able to detect lower LIF-R affinities. A lowered affinity for the LIF-R-Fc did not necessarily translate into a parallel reduction in biological activity, such as in mutant K153A. An analogous observation has been reported for mutants of IL-5 (64).
nism of hLIF-dependent Ba/F3-hLIF-R/gp130 stimulation, the mutant hLIF-O4 was clearly the most effective (Fig. 4).

**DISCUSSION**

Activation of hematopoietin receptors requires at least the dimerization of two transmembrane receptors with cytoplasmic domains capable of signal transduction. The role of the ligand is to facilitate the oligomerization process. In the simplest example, signal transduction results from receptor homodimerization like the classical GHR. A more complex interaction involves receptor activation through heterodimerization; the activated human LIF receptor belongs in this category.

This study has identified residues of hLIF crucial for binding to both the LIF-R and gp130 by analysis of hLIF mutants in solid phase binding studies to both the LIF-R-Fc and gp130-Fc and also a bioassay responsive to LIF. The detectable binding of two different receptors to hLIF provided a convenient control for protein folding in mutant molecules. Thus, mutants that had decreased binding in one receptor but not the other strongly supported the idea that individual mutations caused local rather than global changes in structure.

The hLIF residues Lys-159 and Phe-156 provide the majority of free energy for binding to the LIF-R. These two residues are located at the beginning of the D-helix, with their side chains adjacent and prominently exposed to the solvent in both the mLIF molecule and the recently determined hLIF structure (Fig. 5). Surrounding Lys-159 and Phe-156 are several other residues in close proximity that influence the binding of hLIF to the hLIF-R (Table I); collectively, these amino acids form a

**TABLE I**

| Wild type or hLIF mutant | Location of mutation on hLIF structure | Ratio LIF-R binding: IC50 mutant/IC50 hLIF | Ratio gp130 binding: IC50 mutant/IC50 hLIF | Ratio BaF stimulation: EC50 mutant/EC50 hLIF |
|--------------------------|---------------------------------------|---------------------------------------------|--------------------------------------------|---------------------------------------------|
| hLIF                     | Y45A A-helix                          | 1.0                                         | 1.0                                        | 1.0                                         |
|                          | T46A A-helix                          | 0.8                                         | 1.0                                        | 0.5                                         |
|                          | E50A A-B loop                         | 1.0                                         | 0.75                                       | 1.0                                         |
|                          | P51A A-B loop                         | 8.0                                         | 1.0                                        | 5.0                                         |
|                          | P53A A-B loop                         | 1.1                                         | 0.9                                        | 1.6                                         |
|                          | N54A A-B loop                         | 1.0                                         | 1.0                                        | 0.8                                         |
|                          | N55A A-B loop                         | 1.0                                         | 1.0                                        | 1.0                                         |
|                          | D57A A-B loop                         | 2.2                                         | 1.2                                        | 1.1                                         |
|                          | K59A A-B loop                         | 2.0                                         | 0.8                                        | 2.0                                         |
|                          | G61A A-B loop                         | 1.5                                         | 1.0                                        | 1.0                                         |
|                          | P62A A-B loop                         | 1.8                                         | 1.0                                        | 1.5                                         |
|                          | N63A A-B loop                         | 1.0                                         | 1.0                                        | 1.0                                         |
|                          | V64A A-B loop                         | 1.0                                         | 1.0                                        | 1.0                                         |
|                          | T65A A-B loop                         | 1.0                                         | 1.0                                        | 1.0                                         |
|                          | D66A A-B loop                         | 2.0                                         | 0.8                                        | 1.0                                         |
|                          | K102A B-helix                         | 2.0                                         | 1.0                                        | 2.0                                         |
|                          | I103A B-helix                         | 0.5                                         | 1.0                                        | 1.0                                         |
|                          | P106A B-C loop                        | 4.0                                         | 3.3                                        | 2.0                                         |
|                          | S107A B-C loop                        | 1.0                                         | 0.9                                        | 0.8                                         |
|                          | D149A C-D loop                        | 1.6                                         | 1.0                                        | 1.0                                         |
|                          | T150A C-D loop                        | 4.0                                         | 1.0                                        | 1.2                                         |
|                          | S151A C-D loop                        | 1.6                                         | 0.4                                        | 1.0                                         |
|                          | G152A C-D loop                        | 1.0                                         | 1.0                                        | 1.0                                         |
|                          | K153A C-D loop                        | 4.0                                         | 3.0                                        | 4.0                                         |
|                          | D154A C-D loop                        | 2.0                                         | 2.5                                        | 2.0                                         |
|                          | V155A D-helix                         | 1.0                                         | 0.7                                        | 1.0                                         |
|                          | F156A D-helix                         | >100                                       | 1.1                                        | 700                                         |
|                          | Q157A D-helix                         | 0.8                                         | 1.0                                        | 1.0                                         |
|                          | K158A D-helix                         | 1.0                                         | 1.0                                        | 1.0                                         |
|                          | K159A D-helix                         | >100                                       | 1.0                                        | 3,000                                       |
|                          | K168A D-helix                         | 1.0                                         | 1.0                                        | 1.0                                         |
|                          | K170A D-helix                         | 2.0                                         | 0.7                                        | 1.1                                         |
|                          | Q173A D-helix                         | 1.4                                         | 1.0                                        | 1.5                                         |
|                          | A174Q D-helix                         | 2.0                                         | 0.9                                        | 2.0                                         |
|                          | V175A D-helix                         | 3.0                                         | 1.1                                        | 1.1                                         |
|                          | A177Q D-helix                         | 1.0                                         | 1.0                                        | 2.0                                         |
|                          | Q178A D-helix                         | 1.0                                         | 1.0                                        | 1.0                                         |
|                          | H1F-O1 A- and C-helix                 | 1.0                                         | >10                                        | >100,000                                    |
|                          | H1F-O2 A-helix                        | 1.0                                         | >10                                        | 250                                         |
|                          | H1F-O3 A-helix                        | 1.8                                         | >10                                        | >100,000                                    |
|                          | H1F-O4 A-helix                        | 1.2                                         | >10                                        | >100,000                                    |
|                          | H1F-O5 A- and C-helix                 | 2.0                                         | >10                                        | >100,000                                    |
|                          | Oncostatin-M                          | >100                                       | 0.01                                       | 10                                          |

a Residues were assigned structural types according to the designations in the crystal structure of mLIF (15).

b IC50 refers to the concentration of mutant or wild type protein required to inhibit 50% of the binding of the labeled species.

c EC50 refers to the concentration of mutant or wild type protein required to activate the LIF bioassay by 50% of maximum stimulation.

d The mutant hLIF-K153A has the additional mutation G162D introduced randomly by PCR.

The hLIF residues Lys-159 and Phe-156 provide a convenient control for protein folding in mutant molecules. Thus, mutants that had decreased binding in one receptor but not the other strongly supported the idea that individual mutations caused local rather than global changes in structure.

The hLIF residues Lys-159 and Phe-156 provide the majority of free energy for binding to the LIF-R. These two residues are located at the beginning of the D-helix, with their side chains adjacent and prominently exposed to the solvent in both the mLIF molecule and the recently determined hLIF structure (Fig. 5). Surrounding Lys-159 and Phe-156 are several other residues in close proximity that influence the binding of hLIF to the hLIF-R (Table I); collectively, these amino acids form a
distinct region at the end of the four-helix bundle in the tertiary structure of hLIF (Fig. 5). The arrangement of the LIF-R binding site of hLIF into a few pivotal residues surrounded by ones of lesser importance was also observed in the interaction of the hGH site I with the GHR (23).

The C-D loop and surrounding residues of hLIF have been identified previously by chimera studies as being responsible for the difference in binding affinity of murine and hLIF to the hLIF-R (15, 53, 65). In the most refined form of this analysis, six mLIF residues were mutated to the equivalent hLIF residues (E57D, T107S, Q112H, V113S, A155V, and R158K; Ref. 65) to give the mutated mLIF molecule high affinity binding to the hLIF-R. These residues were not identified in this investigation as significant contributors of hLIF binding to the hLIF-R may suggest these residues prevent mLIF binding to the hLIF-R with high affinity by either structural or chemical interference. This hypothesis is supported by the observation that the human and mLIF structures show no major shifts in the peptide backbone in the vicinity important for LIF-R binding.2

Other cytokines related to hLIF in primary sequence: mLIF, oncostatin M, CNTF, and CT-1 that can involve the hLIF-R in the activated receptor complex all have the hLIF residues Phe-156 and Lys-159 conserved (15, 40), whereas IL-6 and IL-11 which do not bind the LIF-R lack these residues. No other residues of hLIF that influence binding to the LIF-R are conserved in all of these hLIF-R binding ligands. Thus, the equivalents of hLIF Phe-156 and Lys-159 in these other ligands may represent a common LIF-R binding motif. However, like mLIF, all of these molecules (excluding CT-1, which has yet to be examined) bind hLIF-R with significantly lower affinity than hLIF (15, 39, 41, 43, 53, 66). Therefore, a LIF-R binding epitope involving homologues of Phe-156 and Lys-159 must be modulated by other residues in each individual cytokine.

The majority of hLIF residues identified in this study that were important for hLIF-R binding cluster at the end of the four-helix bundle. However, five residues with a weak influence

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**Figure 1.** Competitive inhibition of 125I-hLIF (0.165 nM) binding to LIF-R-Fc by hLIF or hLIF mutants. Results are expressed as a ratio of counts bound at a particular concentration of competitor (B) divided by counts bound in the absence of competitor (B0). Values represent the mean of triplicate samples, the S.E. for all points was less than 10% of the mean. A: □, hLIF; ○, hLIF F156A; △, hLIF K159A; B: □, hLIF; △, hLIF-O1; ○, hLIF-O3; ○, hLIF-O5; C: □, hLIF; △, hLIF-O2; ○, hLIF-O4; ○, hLIF-O6.

**Figure 2.** Biological activity of hLIF or hLIF mutants in the Ba/F3-hLIF-R/hgp130 assay. Results are expressed as the A570 value of cells assayed for proliferation by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (79). Values represent the mean of triplicate samples, the S.E. for all points was less than 10% of the mean. A: □, hLIF; ○, hLIF F156A; △, hLIF K159A; △, oncostatin M. B: □, hLIF; ○, hLIF-O1; Δ, hLIF-O2; ○, hLIF-O3; *, hLIF-O4; ○, hLIF-O5; △, hLIF-O6.
on hLIF-R binding map to the carboxyl-terminal end of the D-helix and the A-B loop (Asp-57, Lys-58, Lys-170, Ala-174, Val-175). These residues may represent a second site of contact between hLIF and the hLIF-R. This second site could represent interaction with either another LIF-R molecule or another part of the LIF-R. Both these possibilities would be compatible with the use of a similar site on other long chain hematopoietin cytokines (see below and Table II). The latter possibility is also in accordance with models proposed for LIF binding based on both the predicted two-hematopoietin domain structure of the LIF-R and competition binding characteristics of murine and hLIF (15, 67). In these models, one LIF molecule is able to bind simultaneously to two distinct sites on a single LIF-R. However, the existence of a second site on LIF for LIF-R binding site requires further verification.

Residues of hLIF important for binding to gp130 were identified in a manner analogous to those involved in the LIF-R binding site, except that multiple simultaneous substitutions were used to locate the gp130 binding site. The interaction of LIF with gp130 is significantly weaker than the interaction with LIF-R which limits the ability to detect the influence of individual mutations with weak effects on the interaction with gp130. This analysis indicated that residues at the amino terminus of the A-helix contributed the majority of free energy for binding to gp130. In particular, all or a subset of the A-helix residues Gln-25, Ser-28, and Gln-32, participate directly in gp130 binding. The mutation of the C-helix residues: Asp-120, Ile-121, Gly-124, and Ser-127 also reduced gp130 binding, suggesting that all or some of these residues also interact with gp130. However, the role of individual hLIF amino acids in binding to gp130 cannot be determined for either the A- or C-helices without further mutagenesis investigations.

Antagonists for several different four-helical bundle cytokines have been created previously by mutations in ligand receptor binding surfaces. These engineered antagonists include those for ligands which homodimerize their receptors, for
example hGH (68) and also cytokines which involve heterodimerization in receptor activation such as interleukin 4 (69), IL-5 (64), and GM-CSF (70, 71). Similarly, in hLIF, abolition of the gp130 binding site generates specific antagonists of hLIF in the hLIF-responsive bioassay. The most active of these antagonists, hLIF-O4, required 50–100-fold molar excess to inhibit 50% activity of hLIF (Fig. 4). The requirement for an excess of hLIF-O4 for significant inhibition is most likely a result of the LIF-R-gp130 complex having approximately a 100-fold greater affinity than the LIF-R for hLIF.

Generally, the antagonistic activity of the mutants impaired in gp130 binding was negatively correlated with the residual stimulation activity in the bioassay (Table I and Figs. 2 and 4). However, the four mutants with essentially no bioactivity, yet nearly identical LIF-R-Fc binding, also showed distinct differences in antagonism. For example, hLIF-O4 (Q25A, S28A, and Q32A) was more effective than hLIF-O3 (Q25L, S28E, Q32A, S36A; Fig. 4). These differences in the antagonist activity of the hLIF mutants may be due to affinity differences for gp130, undetectable in the binding assay. Alternatively, a more complex interaction between the mutants and gp130 may explain these antagonism differences.

The pleiotropic nature of LIF and the number of ligands that utilize the LIF-R will probably mean that LIF-R-specific antagonists (the ability of these mutants to antagonize other LIF-Rbinding ligands will be published elsewhere) will not be of use therapeutically. However, the presence of soluble forms of gp130 in human serum that are able in vitro to inhibit gp130-dependent cell stimulation may indicate that general antagonists operate in vivo (72). Moreover, the antagonists will be of use in dissecting the complex and overlapping actions of cytokines that use LIF-R and gp130 as receptors.

Finally, the results presented here strongly reinforce a pattern of receptor site usage among the long chain hematopoietin cytokines (73). In this pattern, topologically conserved epitopes on different cytokines are used to bind cytokine receptors. To the initial paradigm of site I and II on hGH for GHR binding, a third receptor binding location has been definitively added by the mutational analysis of the known LIF structure presented here (site III; Fig. 5). Site III has also been recently predicted to bind a second molecule of gp130 on the modelled structure of IL-6 (32). The known receptor binding sites for other long chain cytokines are listed in Table II. The common usage of receptor binding sites in this cytokine family suggests that other members such as CT-1, oncostatin M, and IL-11 will also use topologically similar epitopes.

The distribution of receptor binding epitopes in non-overlapping regions of four helical bundle cytokines provides a spatial explanation for multiple receptor engagement. However, whether all complexes that involve heterotrimeric or even heterodimeric receptor engagement use a single ligand in the activated receptor or also involve higher order associations of ligands and receptors such as in the IL-6 complex (31, 32) remains to be resolved. This issue represents an important future goal for understanding the LIF-R-gp130 signaling complex.

In summary, residues in hLIF that are important for binding both the LIF-R and gp130 were identified in this study, the disruption of the gp130 binding site resulted in the creation of LIF-R antagonists.

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\[\text{Table II}\]

| Ligand   | Site I | Site II | Site III |
|----------|--------|---------|----------|
| hGH      | GHR\(^a\) | GHR\(^b\) |            |
| LIF      | LIF-R\(^c\) | gp130\(^d\) | LIF-R    |
| CNTF     | CNTF-R\(^e\) | gp130\(^d\) | LIF-R?    |
| IL-6     | IL-6-R\(^f\) | gp130\(^d\) | gp130\(^d\) |

\(^{a}\) Ref. 22.  
\(^{b}\) Ref. 25.  
\(^{c}\) Ref. 18 and 74.  
\(^{d}\) Ref. 74.  
\(^{e}\) Refs. 75–78.  
\(^{f}\) Ref. 63.  
\(^{g}\) Ref. 32.
293T cells. We are also grateful to C. Moores for assistance in creating the hLIF mutants, J. Bond for aiding the production of the LIF-R-Fc and gp130-Fc, and E. Y. Jones and R. C. Robinson for useful discussions.

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