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# Characterization of the Receptor Binding Sites of Human Leukemia Inhibitory Factor and Creation of Antagonists*

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**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 LIF[1] is essential for embryonic implantation (6, 7). In contrast to the mild phenotype in mice lacking the LIF gene, the targeted deficiency of the specific LIF receptor (LIF-R), results in mice that have multiple placental, skeletal, neural, and metabolic disorders, which cause perinatal death (8). The 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 $\alpha$-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 $\beta$ 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 theses complexes. Such complexes use a specific cytokine binding receptor for each ligand and often share a common signaling $\beta$ 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 $\beta$ receptor together with a ligand-specific chain (28). Similarly, the interleukins: 4, 7, 9, 15, and possibly 13 use the interleukin-2 (IL-2) $\gamma$ chain together with a specific interleukin receptor binding component for signal transduction (29). Heterotrimeric 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-

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[1] The abbreviations used are: LIF, leukemia inhibitory factor; m, murine; h, human; LIF-R, LIF receptor; CNTF, ciliary neurotrophic factor; CT, cardiotrophin; IL, interleukin; CSF, colony-stimulating factor; hGH, human growth hormone; GHR, growth hormone receptor; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CPK, Corey-Pauling-Koltun; Fc, constant region domain of human $\gamma$-immunoglobulin; IgG, $\gamma$-immunoglobulin.

americ complex, consisting of two molecules each of IL-6, IL-6-R, and gp130 (31, 32).

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 ($K_D \approx 10^{-9}$ 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-*X*-Trp-Ser (20). The structure of this domain has been solved for both the prolactin receptor (35) and GHR (17), these structures are composed of two $\beta$-barrels each consisting of seven anti-parallel $\beta$-strands.

The high affinity signaling complex ($K_D \approx 10^{-11}$ M) for LIF is formed by the association of the LIF-R and a related member of the hematopoietin receptor family, gp130 (36, 37). However, like many other receptor complexes. the stoichiometry of components in the activated complex is unknown. The gp130 receptor differs to the LIF-R in that this receptor is predicted to have only a single hematopoietin domain (33, 38). Both LIF-R and gp130 are involved in the high affinity receptor complex for cytokines related in structure to LIF, these include oncostatin M (37, 39), CT-1 (14, 40), and CNTF (41–43). The high affinity CNTF receptor complex also requires the presence of a third component, the CNTF receptor (44), whereas, oncostatin M also uses an alternative receptor to the LIF-R (45). The gp130 receptor and not the LIF-R is also involved as a co-receptor and signal transducer for the cytokines, IL-6 (46, 47) and IL-11 (48, 49). The common usage of gp130 and in some cases also LIF-R for signal transduction by LIF, oncostatin M, CNTF, CT-1, IL-6, and IL-11 explains their overlapping functional characteristics. Both LIF-R and gp130 mediate intracellular signal transduction via activation of cytoplasmic tyrosine kinases belonging to the JAK and Src families (50–52).

A clear understanding of how a particular cytokine interacts with its receptors is a crucial requirement for both comprehension of receptor activation and also the design of specific agonists and antagonists of cytokine action. Previous mutagenesis studies of LIF have utilized the observation that both murine and human LIF (hLIF) bind to the mLIF-R, but only hLIF will bind to the hLIF-R with high affinity. These studies suggested that residues in the C-D loop were important for this species specificity (15, 53).

This study extended the previous work by evaluating the role of individual residues of hLIF to the binding to both hLIF-R and gp130. Human LIF mutants were analyzed both by direct binding assays to recombinant LIF-R-Fc and gp130-Fc and also for activity in a bioassay responsive to hLIF. Apart from revealing residues of hLIF critical for binding hLIF-R and gp130, abrogation of the gp130 binding site created antagonists of wild type hLIF.

## MATERIALS AND METHODS

*Construction of Mutant Human LIF Expression Plasmids*—The construction of pGeX-hLIF and the characterization of the expressed LIF has been described previously (15, 54). All mutant DNA sequences were created by PCR overlap (55) using pGeX-hLIF and specific oligonucleotides for each individual mutant, the sequence of the primers used are available on request. The mutant hLIF sequences were cloned into the pGeX plasmid, and the nucleotide sequence of all constructs was confirmed by DNA sequencing with Sequenase (Amersham).

*Expression and Purification of Human LIF Mutants and Oncostatin M*—All mutants were expressed as glutathione *S*-transferase fusion proteins in *Escherichia coli* JM109. The expression, purification, and cleavage of mutant fusion hLIF proteins was performed as described previously (15, 56). Purity of individual mutants was determined by inspection of silver-stained SDS-PAGE gels run in a Pharmacia Biotech Inc. Phast gel system. All mutants had a purity of greater than 80% by this method. The protein concentration of each mutant protein was determined by the Coomassie Plus Protein Assay (Pierce) and verified by comparative SDS-PAGE analysis.

The mature form of oncostatin M (57) was a kind gift from Dr. D Staunton; this protein was also expressed as a glutathione *S*-transferase fusion protein and purified as above.

*Construction of Human LIF-R-Fc and gp130-Fc Receptor Expression Plasmids*—PCR was used to amplify the region coding for amino acids 2–538 of the human LIF receptor. This fragment was cloned into the pIG plasmid (58) using a *Hin*dIII site 5′ to the first ATG codon and a *Bam*HI site 3′ to the codon for amino acid 528. Both sites were introduced by the PCR primers: amino terminus, TAGAAGCTTCCACCATGGATATTTACGTATGTTTGA; carboxyl terminus, ACGGATCCACTTACCTGTCCCCTTTGAAGGACTGGCTTC.

The PCR primer matching the amino-terminal coding sequence also introduced an optimized Kozak sequence (59), while the other primer created a splice donor sequence between codon 528 and the *Bam*HI site. An identical approach was used to clone the human gp130 into the pIG plasmid, except the region amplified coded for residues 1–328. The PCR primers used were: amino terminus, TAGAAGCTTCCACCATGTTGACGTTGCAGACTTGGG; carboxyl terminus, ACGGATCCACTTACCTGTTGCTTTAGATGGTCTATCTT.

The nucleotide sequences of both constructs were confirmed by DNA sequencing with Sequenase (Amersham).

*Expression and Purification of Human LIF-R-Fc and gp130-Fc*—Both the human LIF receptor and the gp130 receptor were expressed as fusion proteins with the Fc region (hinge-CH2-CH3) of human IgG1. The pIG expression plasmids were transfected by the calcium-phosphate technique (60) into a human epithelial kidney cell line 293T (61), which expresses the large T antigen of SV40. After transfection of 293T cells, the medium was changed to a serum-free medium (Ultra-Cho, BioWhittaker), and the Fc-fusion proteins were left to accumulate in the medium for 6 days. Receptor-Fc proteins were purified from filtered supernatants by chromatography on protein A-Sepharose (Pharmacia). Elution of the receptor was achieved with 0.1 M sodium citrate (pH 3.0). The purity of the receptor-Fc proteins was assessed as greater than 90% as determined by inspection of silver-stained SDS-PAGE gels run in a Pharmacia Phast gel system.

*Iodination of and Biotinylation of Ligands*—Recombinant human LIF produced as described above was iodinated using IODOBEADS (Pierce) and [$^{125}$I]iodine (Amersham) as recommended by the manufacturers. Iodinated LIF had a specific activity of $1–4 \times 10^3$ cpm/fmol and was equally active in the bioassay as wild type hLIF. Recombinant oncostatin M was biotinylated with biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma) following a published procedure (62).

*Ligand Bioassays*—All mutant hLIF proteins were tested for biological activity on the Ba/F3-hLIF-R/hgp130 cell line, which expresses both hLIF-R and hgp130 (15). The assay was performed exactly as described previously (15). All assays were performed in triplicate for at least three independent experiments. It was noted that different batches of cells exhibited quantitative differences in their response to hLIF although the relative potencies of hLIF and mutants were preserved. Antagonism assays were also performed with the Ba/F3-hLIF-R/hgp130 cell line. Briefly, the antagonists were titrated in the presence of a 50% maximum stimulation concentration of hLIF; otherwise, the cells were treated exactly as for the standard bioassay.

*Ligand Binding Assays*—All binding assays were performed in Nunc Maxisorp 96-well plates. For LIF-R-Fc binding, plates were first coated with 100 $\mu$l of protein A (Sigma) at 1 $\mu$g/ml, blocked with PBS-1% BSA, and then incubated with 100 $\mu$l of LIF-R-Fc also at 1 $\mu$g/ml. Initial experiments indicated that the supernatant from 293T cells transfected with LIF-R-Fc was equivalent to purified LIF-R-Fc; therefore, all subsequent experiments used the LIF-R-Fc-containing supernatant. After LIF-R-Fc binding, the plates were washed with PBS-0.05% Tween 20 and then used for the binding assay. Competition binding assays between $^{125}$I-hLIF and wild type hLIF or mutant hLIF were performed in a volume of 60 $\mu$l in a buffer of RPMI (Life Technologies, Inc.), 20 mM Hepes, pH 7.2, 25 mg/ml BSA, 2 mg/ml azide, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA overnight at 4 °C. After washing twice with 200 $\mu$l of PBS-0.05% Tween 20, bound $^{125}$I-hLIF was released by incubation in 80 $\mu$l of 1 M NaOH and counted in a $\gamma$ counter (LKB). All LIF-R-Fc binding studies were performed in triplicate for at least two independent experiments.

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 $^{125}$I-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 $\mu$l 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 II (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 $^{125}$I-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 $^{125}$I-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).

Of the mutants with significantly reduced LIF-R-Fc binding, all but three mutants had unchanged gp130-Fc affinity. The three mutants with altered gp130-Fc affinity all had less than 4-fold decreases in affinity. These mutants were hLIF P106A, hLIF K153A, and hLIF D154A (Table I). The reduced affinity for gp130-Fc in the hLIF P106A mutant may be due to a change in the conformation of the B-C loop and thus may affect the packing of the C-helix, a contributor to gp130 binding (see below). For the hLIF K153A mutant, an additional mutation randomly introduced by PCR is present in the D-helix (G162D, Table I), this extra change may alter the packing of the A-B loop against the D-helix and thus disrupt the structure. The presence of this second mutation confounds the contribution of Lys-153 to LIF-R binding.

Overall, the residues in hLIF that contribute the majority of free energy for binding to the LIF-R are located at the beginning of the D-helix (Phe-156 and Lys-159); these residues are spatially close together and have their side chains prominently exposed to the solvent in the hLIF structure (Fig. 5). Other residues that influence LIF-R binding map to the beginning of the D-helix (Lys-158), B-helix (Lys-102), and C-D loop (Thr-150); these amino acids are proximal to Phe-156 and Lys-159. Residues in the A-B loop (Asp-57, Lys-58) and the carboxyl terminus of the D-helix (Lys-170, Ala-174, Val-175) may form a second site of interaction with the LIF-R.

*Identification of the gp130 Binding Site on Human LIF*—Mutant hLIF molecules were also assayed in two different systems for binding to the gp130 receptor. First, the relative affinity for binding to gp130-Fc was assessed by the ability of LIF mutants to inhibit biotinylated oncostatin M binding to immobilized recombinant human gp130-Fc (Table I and Fig. 3). Oncostatin M binds gp130-Fc with a significantly higher affinity than LIF and thus provides a convenient tracer for this type of binding assay. Second, the LIF mutants were also examined in the Ba/F3-LIF-R/gp130 proliferation assay (Table I and Fig. 2). All mutants with alterations in the beginning of both the A-helix and C-helix exhibited undetectable gp130-Fc binding and varied from a 250-fold reduction to undetectable stimulation in the bioassay (Table I and Figs. 2 and 3). In contrast to the gp130-Fc binding, the A- and C-helix mutants showed a similar affinity to wild-type LIF in the LIF-R-Fc binding assay (Table I and Fig. 1). The results from the bioassay using mutants with multiple alanine substitutions in the A- or the C-helix (hLIF-O4 and hLIF-O2) strongly suggest that residues in both the A- and C-helices of hLIF contribute to binding the gp130 receptor (Fig. 2). The greater reduction in bioactivity seen in the hLIF-O4 mutant compared to the hLIF-O2 mutant (Fig. 2*B*) indicates that, for LIF, the majority of binding energy for gp130 resides in the A-helix residues. Identification of the individual contribution of each hLIF residue to gp130 binding will require further mutagenesis of hLIF.

*Antagonist Activity of Human LIF Mutants Impaired in gp130 Binding*—LIF mutants shown to have large decreases in gp130-Fc binding were assayed for their ability to antagonize hLIF stimulation of the cell line Ba/F3-hLIF-R/hgp130. The results presented in Fig. 4 demonstrate that several of these mutants show antagonistic activity. In this assay, the inhibition was specific to hLIF since murine IL-3 stimulation of the Ba/F3-hLIF-R/hgp130 cell line was unaffected by the various antagonists (data not shown). Only mutants with the greatest reduction in the proliferation assay showed antagonism activity (hLIF-O1, -O3, -O4, and -O5; Table I and Fig. 4). Mutants with significant residual proliferation in the bioassay such as hLIF-O2 and -O6 exhibited no antagonistic characteristics (Table I and Fig. 4). Of the four mutants that exhibited antago-

TABLE I
*Summary of receptor binding and LIF bioassay data for LIF mutants*
Data represents the mean values of the various experiments.

| wild type or hLIF mutant | Location of mutation on hLIF structure[a] | Ratio LIF-R binding: $IC_{50}$[b] mutant/$IC_{50}$ hLif | Ratio gp130 binding: $IC_{50}$ mutant/$IC_{50}$ hLif | Ratio Baf stimulation: $EC_{50}$[c] mutant/$EC_{50}$ hLif |
|---|---|---|---|---|
| hLIF | | 1 | 1 | 1 |
| 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 |
| K58A | 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 | 1.3 | 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[d] | C-D loop | 45 | 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 | 3.0 | 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 |
| Q171A | 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 |
| hLIF-O1[e] | A- and C-helix | 1.0 | >10 | >100,000 |
| hLIF-O2[f] | C-helix | 1.0 | >10 | 250 |
| hLIF-O3[g] | A-helix | 1.8 | >10 | >100,000 |
| hLIF-O4[h] | A-helix | 1.2 | >10 | >100,000 |
| hLIF-O5[i] | A- and C-helix | 2.0 | >10 | >100,000 |
| hLIF-06[j] | A- C-helix | 1.0 | >10 | >1,000 |
| Oncostatin-M | | >100 | 0.01 | 10 |

[a] Residues were assigned structural types according to the designations in the crystal structure of mLIF (15).
[b] $IC_{50}$ refers to the concentration of mutant or wild type protein required to inhibit 50% of the binding of the labeled species.
[c] $EC_{50}$ 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.
[e] Mutations in hLIF-O1: Q25A, S28A, A117E, D120R, I121K, G124N, S127L.
[f] Mutations in hLIF-O2: D120A, I121A, G124A, S127A.
[g] Mutations in hLIF-O3: Q25L, S28E, Q32A, S36K.
[h] Mutations in hLIF-O4: Q25A, S28A, Q32A.
[i] Mutations in hLIF-O5: A117E, D120R, I121K, G124N, S127L, Q25L, S28E, Q32A, S36K.
[j] Mutations in hLIF-O6: Q25A, S28A, S36A, D120A, I121A, G124A, S127A.

nism of hLIF-dependent Ba/F3-hLIF-R/hgp130 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).[2] 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

[2] R. C. Robinson *et al.*, submitted for publication.

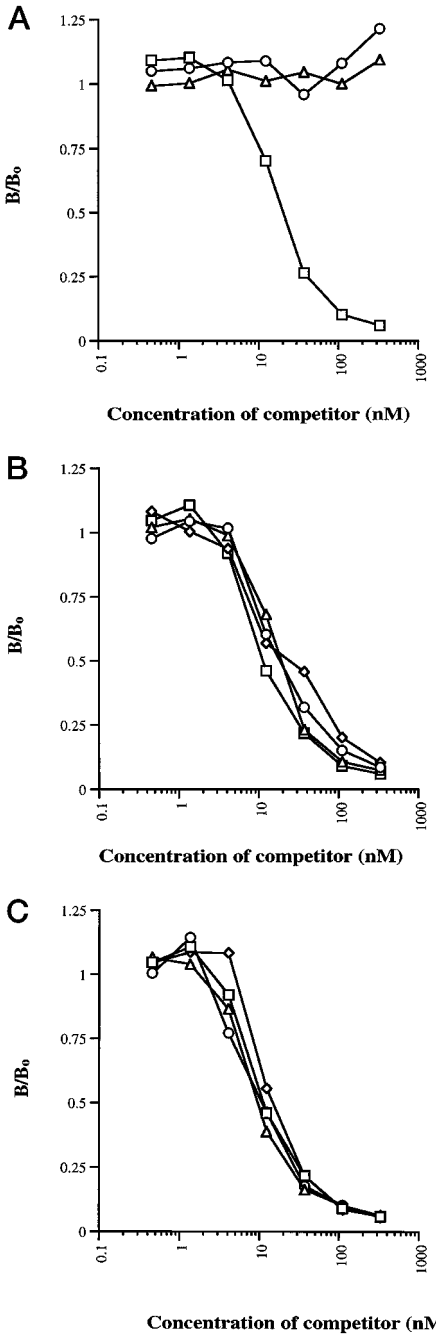


FIG. 1. **Competitive inhibition of $^{125}$I-hLIF (0.165 nM) binding to LIF-R-Fc by hLIF or hLIF mutants.** Results are expressed as a ratio of counts bound at a particular concentration of competitor [*B*] divided by counts bound in the absence of competitor [$B_0$]. Values represent the mean of triplicate 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.

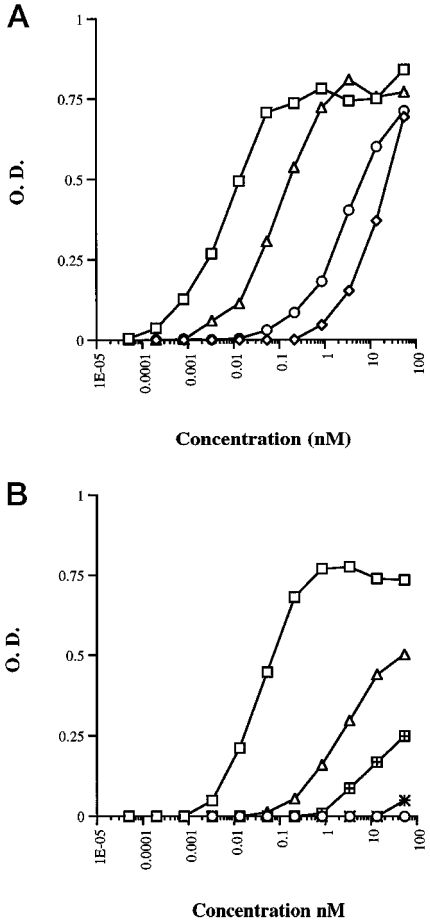


FIG. 2. **Biological activity of hLIF or hLIF mutants in the Ba/F3-hLIF-R/hgp130 assay.** Results are expressed as the $A_{570}$ value of cells assayed for proliferation by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (79). Values represent the mean of triplicate samples, the S.E. for all points was less than 10% of the mean. *A*: □, hLIF; ○, hLIF F156A; ◇, hLIF K159A; △, oncostatin M. *B*: □, hLIF; ○, hLIF-O1; △, hLIF-O2; ◇, hLIF-O3; *, hLIF-O4; ⊕, hLIF-O5; ⊞, hLIF-O6.

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. That these residues were not identified in this investigation as significant contributors of hLIF binding to 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

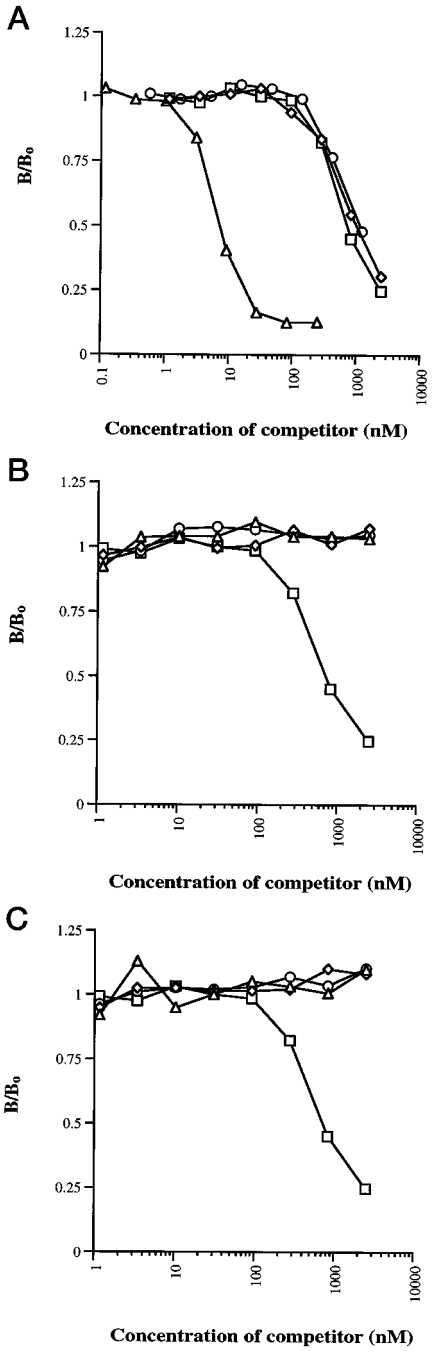


FIG. 3. **Competitive inhibition of biotinylated oncostatin M (0.8 nM) binding to gp130-Fc by hLIF or hLIF mutants.** Results are expressed as a ratio of biotinylated oncostatin M bound att a particular concentration of competitor [*B*] divided by biotinylated oncostatin M bound in the absence of competitor [$B_0$]. Values represent the mean of duplicate samples. *A*: □, hLIF; ◇, hLIF F156A; ○, hLIF K159A; △, oncostatin M. *B*: □, LIF; △, hLIF-O1; ◇, hLIF-O3; ○, hLIF-O5. *C*: □, hLIF; △, hLIF-O2; ◇, hLIF-O4; ○, hLIF-O6.

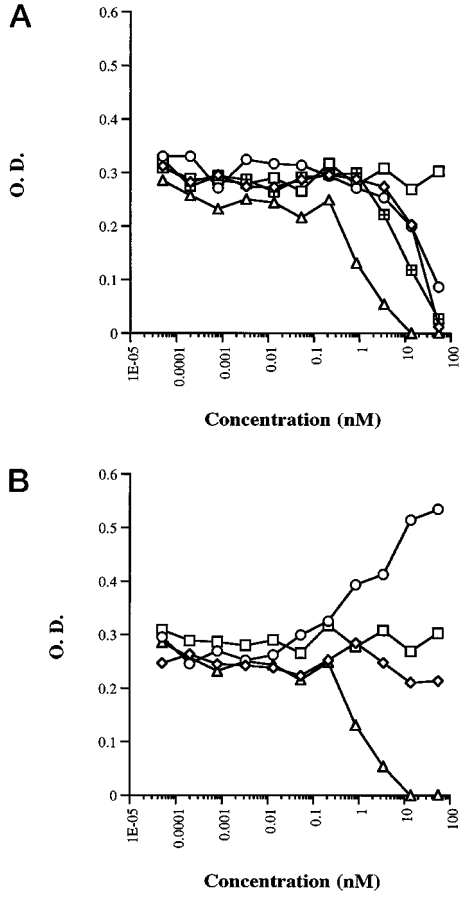


FIG. 4. **Antagonism of hLIF stimulation of the Ba/F3-hLIF-R/hgp130 cell line by hLIF mutants impaired in gp130 binding.** Human LIF mutants were titrated in the presence of a constant concentration of hLIF (0.015 nM). Results are expressed as the $A_{570}$ value of cells assayed for proliferation by 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*: □, no antagonist; ○, hLIF-O1; ◫, hLIF-O3; △, hLIF-O4; ◇, hLIF-O5. *B*: □, no antagonist; ○, hLIF-O2; ◇, hLIF-O6; △, hLIF-O4.

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

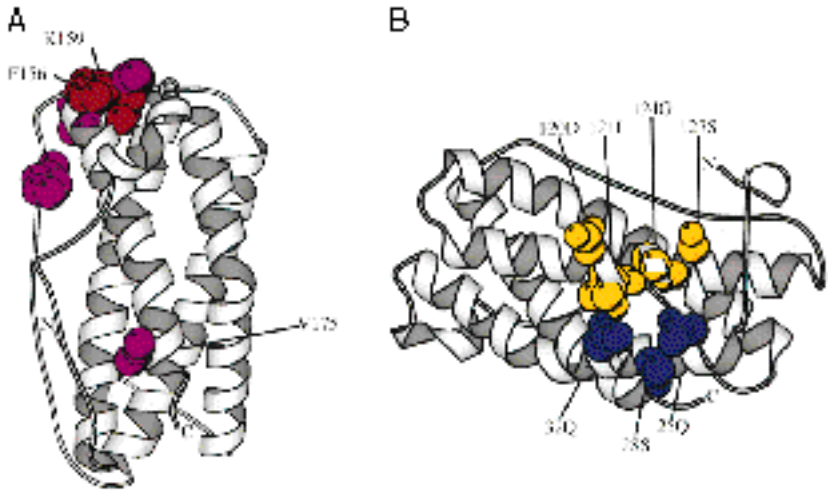


FIG. 5. **Diagrammatic representation (Molscript; Ref. 80) of the hLIF structure[2] depicting residues important for binding to its receptors.** The hLIF structure is very similar to the mLIF molecule apart from a shift in which the A-B loop crosses the D-helix. *A*, residues of hLIF with a major impact on hLIF-R binding are displayed in *red* CPK and labeled. Residues with a moderate impact on binding are displayed in *magenta* CPK. These residues are: Pro-51, Thr-150, Lys-158, and Val-175. *N* = amino terminus and *C* = carboxyl terminus. The putative site I in hLIF would be centered around residue Val-175, site III is centered on residues Phe-156 and Lys-159. *B*, residues of hLIF that when mutated collectively on either the A- or C-helix caused reduced binding to gp130. Residues on the A-helix are displayed in *blue* CPK, those on the C-helix are displayed in *yellow* CPK. The residues in these two helices compose the equivalent of site II in hLIF. *N* = amino terminus and *C* = carboxyl terminus.

TABLE II
*Summary of receptor binding locations in the long chain hematopoietin cytokines*

Locations of receptor binding sites were assigned as noted under "Discussion" and the references noted below.

| Ligand | Site I | Site II | Site III |
|---|---|---|---|
| hGH | GHR[a] | GHR[b] | |
| LIF | LIF-R? | gp130 | LIF-R |
| CNTF | CNTF-R[c] | gp130[d] | LIF-R? |
| IL-6 | IL-6-R[e] | gp130[f] | gp130[g] |

[a] Ref. 22.
[b] Ref. 25.
[c] Refs. 18 and 74.
[d] Ref. 74.
[e] Refs. 75–78.
[f] Ref. 63.
[g] Ref. 32.

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-R binding ligands will be published elsewhere)[3] 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·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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[3] A. B. Vernallis, K. R. Hudson, and J. K. Heath, manuscript in preparation.

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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# Characterization of the Receptor Binding Sites of Human Leukemia Inhibitory Factor and Creation of Antagonists

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