Affinity Maturation of Leukemia Inhibitory Factor and Conversion to Potent Antagonists of Signaling

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Leukemia inhibitory factor (LIF)-induced cell signaling occurs following sequential binding to the LIF receptor α-chain (LIFR), then to the gp130 co-receptor used by all members of the interleukin-6 family of cytokines. By monovalently displaying human LIF on the surface of M13 phage and randomizing clusters of residues in regions predicted to be important for human LIF binding, we have identified mutations, which lead to significant increases in affinity for binding to LIFR. Six libraries were constructed in which regions of 4–6 amino acids were randomized then panned against LIFR. Mutations identified in three distinct clusters, residues 53–57, 102–103, and 150–155, gave rise to proteins with significantly increased affinity for binding to both human and mouse LIFR. Combining the mutations for each of these regions further increased the affinity, such that the best mutants bound to human LIF with >1000-fold higher affinity than wild-type human LIF. NMR analysis indicated that the mutations did not alter the overall structure of the molecule relative to the native protein, although some local changes occurred in the vicinity of the substituted residues. Despite increases in LIFR binding affinity, these mutants did not show any increase in activity as agonists of LIF-induced proliferation of Ba/F3 cells expressing human LIFR and gp130 compared with wild-type LIF. Incorporation of two additional mutations (G29A and G124R), which were found to abrogate cell signaling, led to the generation of highly potent antagonists of both human and murine LIF-induced bioactivity.

Leukemia inhibitory factor (LIF)$^1$ is a member of the family of cytokines that includes interleukin (IL)-6, IL-11, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), oncostatin M (OSM), and cardiotrophin-like cytokine/cytokine-like factor-1 (CLC/CLF) $^1$. As with most of these proteins, LIF displays a wide variety of biological actions on numerous cell types and tissues, including neurons, embryonic stem cells, hepatocytes, megakaryocytes, adipocytes, and osteoblasts $^2$. All are four α-helical bundle type cytokines that exert their biological effects following the formation of high affinity receptor complexes involving at least one gp130 molecule. In the case of LIF, formation of the signaling complex is a two-step process: LIF initially binds to the LIF receptor α-chain (LIFR) with low (nm) affinity, then LIF-LIFR binds to gp130 to form the high (pm) affinity LIF-LIFR-gp130 signaling complex $^1, 3, 4$. Using alanine scanning mutagenesis, the sites on human LIF important for binding to both LIFR and gp130 have been characterized $^5$. In particular, a Phe and Lys near the start of the D-helix were found to be critical for LIFR binding, while a number of residues in both the A- and C-helices were important for interaction with gp130. These receptor binding sites are referred to as Site III and Site II, respectively, following the convention established for IL-6 $^6$.

While many of the in vitro functions of LIF appear to overlap with those of other cytokines in the family, it has been shown through gene deletion studies in mice that LIF has a critical role in embryo implantation that cannot be compensated for by the other family members $^7$. It therefore follows that antagonists of LIF may be able to act as novel contraceptives. Indeed, in several studies in which anti-LIF antibodies have been injected into both mice and monkeys, a decrease in the pregnancy rate of both animals was observed $^8–10$. Further studies of the contraceptive potential of anti-LIF agents would benefit from the development of novel antagonists of LIF signaling.

One non-antibody approach to the development of LIF antagonists is through the design of mutant LIF molecules which inhibit signaling by binding to only one of the two receptor chains. Antagonists of LIF $^5$, or other related cytokines (11, 12), have previously been made by mutation of residues important for interaction with gp130. Such antagonists, however, are not particularly potent as they only bind the cytokine-specific receptor, the affinity of which is considerably lower than that of the wild-type cytokine forming the higher affinity gp130-containing complex. To improve the potency of such molecules, and hence convert them into so-called “superantagonists,” additional mutations are introduced that improve the affinity for binding the cytokine-specific receptor. This affinity maturation process is usually achieved using phage display technology.
Affinity Maturation of LIF

(13). Of the gp130-signaling cytokines, superantigenic forms of IL-6 (14–16) andCNTF (17) have been reported, but not of LIF. In the present report, we describe the affinity maturation of LIF through the use of phage display, and the subsequent conversion of these mutants into potent superantagonists.

EXPERIMENTAL PROCEDURES

Construction of Mutant LIF Libraries—To enable monovalent display of human LIF as a fusion to the gene III coat protein of M13 phage (geneIIIp), LIF cDNA was subcloned into an in-house phage display vector. Libraries were constructed essentially as described by Sidhu et al. (18). Prior to construction, the codons for each residue to be randomized were changed to amber codons. Library B: 53 random residues (positions 89–141 and 171–223) were mutagenized with the degenerate oligonucleotides in which an NNS codon was incorporated at each randomized position. Following transformation of electrocompetent SBS20 E. coli (18) with the mutated phagemids, M13K07 helper phage was added and the culture incubated overnight in 500 ml of 2YT medium containing 100 mg/ml ampicillin. Phage particles were then isolated from the supernatants by polyethylene glycol-NaCl precipitation as described previously (18).

Panning of Libraries—Each library was subjected to four rounds of panning against recombinant human LIFR. Five wells of a 96-well microtiter plate were coated with LIF at 2.5 μg/ml (rounds 1 and 2) or 1 μg/ml (rounds 3 and 4) in phosphate-buffered saline (PBS) overnight at 4 °C. After blocking with PBS containing either 1% (v/v) bovine serum albumin (rounds 1 and 3) or 0.5% (v/v) casein (rounds 2 and 4), the initial phage stocks, or phage from the previous round of panning, were diluted 10-fold with PBS containing 0.1% (v/v) Tween-20 and 1% (v/v) bovine serum albumin. Five wells of culture supernatant from individual colonies grown in the presence of helper phage was used with 100 μl of 107 PFU of M13KO7 helper phage. Phage particles were propagated from individual colonies following incubation on Luria agar plates for 16 h.

After the fourth round of panning, eluted phage were serially diluted and used to infect XLI-Blue cells for 1 h prior to plating onto agar plates containing 100 μg/ml ampicillin. To determine the sequences of the mutants selected from the NNS codons, DNA sequencing was performed as described previously (20).

For production of LIF proteins, the cDNA for either human or murine LIF, or the human/murine chimera MH35 (21) were subcloned into a modified pGEX-2T vector (Amersham Biosciences, Uppsala, Sweden) in which an ϕ15M origin of replication had been inserted to enable single-stranded DNA preparation for mutagenesis. All mutant forms were made using the method of Kunkel et al. (19). Recombinant proteins were expressed as c-terminal fusions to glutathione S-transferase (GST) fusion proteins in BL-21 DE3 pLys E. coli. Phage proteins were propagated from individual colonies following incubation on Luria agar plates for 16 h.

Expression of LIF Mutants on M13 Phage—Mutants of LIF were prepared according to the method of Kunkel et al. (19) in a phagemid vector which enabled monovalent display on phage as fusions to geneIIIp. These constructs were used to transform XL-1 Blue E. coli, and phage were propagated from individual colonies following incubation on Luria agar plates for 16 h.

Kinetic Analysis by Surface Plasmon Resonance (SPR)—Kinetic analysis of wild-type or mutant cytokines was performed by SPR using a BIAcore 3000 instrument (Biacore AB, Uppsala, Sweden). Proteins were immobilized onto a CMS biosensor chip at 1000 RU per channel. Sensorgrams were obtained following injection of various concentrations of recombinant human LIF at a flow rate of 40 μl/min for 4 min. Bound receptor was then allowed to dissociate for times up to 10 h. Binding profiles were analyzed by using BIACore evaluation software Ver. 3.2 (Biacore AB, Uppsala, Sweden).

NMR Spectroscopy—Lyophilized uniformly 15N-enriched MH35-LIF mutant MH35-BD was dissolved in 0.5 ml of 90% H2O/10% 2H2O without the addition of buffer. The concentration was ∼0.7 m at pH 7.5 and the pH was adjusted to 4.4 without correcting for the deuterium isotope effect. Spectra were recorded at 37 °C on a Bruker DRX-600 spectrometer using triple-resonance probes equipped with triple-axis gradients; these included two-dimensional 1H-15N HSQC, three-dimensional 1H-15N-NOESY-HSQC with a 150-ms mixing time, and three-dimensional HNHA spectra. Measurements of steady-state 1H-15N NOE values were carried out as described previously (27). All spectra were processed using XWINNMR, version 3.5 (Bruker AG, Karlsruhe, Germany), and analyze was used for analysis of spectra. 1H chemical shifts were referenced to 4,4-dimethyl-4-silapentane-1-sulfonate at 0 ppm via the H2O signal, and the 15N chemical shifts were referenced indirectly using the 15N/1H γ-ratios (29). The standard deviations of NOE values were determined from the background noise level of the spectra as described by Farrow et al. (30).

Bo/F3 Cell Proliferation Assay—Ba/F3, an IL-3-dependent cell line, is derived from murine pro B lymphocytes and does not normally express LIFR or gp130 (31). Ba/F3 cells stably transfected with either murine LIFR/gp130 or human LIFR/gp130 (32) were seeded at 3×104 cells/well in 10 μl of Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum. For agonist assays, the various wild-type or mutant forms of LIF were added at increasing concentrations in a total volume of 100 μl/well. After incubation for 48 h, proliferation was measured colorimetrically at 570 nm using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich). In the antagonist assays, cells were incubated with increasing concentrations of LIF antagonist in the presence of a fixed, submaximal concentration of either human LIF (60 pg), murine LIF (40 pg), or IL-3 (4 pg) in a total volume of 100 μl/well. Proliferation was again measured as described for the agonist assays. Agonist and antagonist assays were always performed in triplicate, and mean values for each assay point were plotted.

M Cell Colonization Assay—M1 differentiation assays were performed as described previously (33). Cells were stimulated with a fixed, submaximal concentration of recombinant murine LIF (8 pg) in the presence of increasing concentrations of the various LIF antagonists.

RESULTS

Design and Construction of Libraries—Six mutant libraries of LIF were designed in which residues that contribute to LIFR binding were randomly mutated. The selection of residues to be randomized was based primarily on the alanine-scanning mutagenesis study of Hudson et al. (5), although data from other LIF structure-function studies (21) were also considered. Residues which had been shown to contribute to LIFR binding were mapped onto the three-dimensional structure of human LIF, then this and other nearby residues were incorporated into the random library designs.
into the libraries (Fig. 1A). Each library contained a total of six randomized residues. Libraries A–E formed a near contiguous surface in vicinity of Phe156 and Lys159, two residues on LIF that were found to be critical for binding to LIFR (Fig. 1B). Library F, which included three residues that had been shown to result in a 2–3-fold decrease in binding to receptor when substituted with Ala, was located at the C-terminal end of the D-helix (Fig. 1A).

Following electroporation into E. coli, each library contained between 10⁸ and 10⁹ transformants, with mutation rates of 40–80%. While this is below the level required for representation of all possible protein sequences within a library containing six randomized positions (2.5 × 10⁹ transformants required for a complete library at the 90% confidence level, Ref. 18), it was expected to provide a high enough level of diversity for the purposes of the study.

Panning of Libraries—Each of the libraries was panned against human LIFR. After four rounds of panning, individual clones were picked and the sequences in the randomized region were determined. Panning of library A (residues 48–53) failed to identify any specifically binding mutants, as many of the clones sequenced after four rounds of panning contained the template sequence, or contaminating wild-type LIF or high affinity clones from other libraries. It has been suggested that vulnerability to contamination in panning phage display libraries indicates an absence of clones within the library that exhibit high affinity for the target protein (34). We therefore decided to prepare a new library with reduced diversity by limiting the randomized segment to four residues located near the beginning of the AB loop (amino acids 49–52). Following four rounds of panning with this library, 20/24 clones analyzed contained the wild-type sequence. Only three clones contained a sequence, which differed from wild type, and this variation only occurred at residue 50.

No single dominant sequence emerged from library B in which residues 53–58 in the A-B loop were randomized, but certain amino acids were selected preferentially at several of the randomized positions. In particular, Gly was strongly selected at residue 53 in place of the wild-type Pro (10/17 clones), and a large hydrophobic residue (Leu/Met/Phe/Trp) in place of Asn at 54 and 55 (11/17 and 14/17 clones, respectively). There was also a preference for Gly or Pro (8/17 clones) at residue 57 in place of Asp, and retention of a positively charged residue (7/17 clones), at residue 58.

![Design of mutant LIF libraries. A, residues that were randomly mutated in each of the libraries were mapped onto the backbone of the human LIF structure (PDB accession number: 1EMR) and colored as indicated. B, molecular surface of human LIF is shown at the top of the 4-helix bundle in the region of Phe156 and Lys159 (shown in red); two residues that are critical for LIFR binding (5). Residues randomly mutated in libraries A–E are colored as indicated.](image-url)
Clones from each library with sequences similar to the consensus were characterized in a competitive binding ELISA. Mutants were expressed monovalently as fusions to gene IIIp and tested for their ability to bind immobilized LIFR. The IC\textsubscript{50} was the concentration of soluble LIFR required to inhibit 50\% of the maximal binding response. The wild type sequence within each of the randomized segments is shown in column 1, and the modified sequences within the clones analyzed are shown in column 3.

### Table I

| Library (wt sequence) | Clone | Sequence | IC\textsubscript{50} | IC\textsubscript{50} wt/IC\textsubscript{50} mutant |
|-----------------------|-------|----------|-----------------------|-----------------------------------------------|
| wt LIF\textsuperscript{a} | NA\textsuperscript{b} | NA | 3.6 | NA |
| B (53/54NLNLDK\textsuperscript{58}) | B-11 | 53GLLGLGK\textsuperscript{58} | 0.036 | 100 |
| C (100/KILNIPS\textsuperscript{107}) | C-2 | 100AMLNPE\textsuperscript{107} | 0.82 | 4.4 |
| D (150/TSGDVV\textsuperscript{155}) | D-8 | 150WAQEXTT\textsuperscript{155} | 0.10 | 36 |
| E (150/VFQKKK\textsuperscript{160}) | E-2 | 150YFSRKM\textsuperscript{160} | 1.2 | 3.0 |
| F (170/KQHIAV\textsuperscript{175}) | F-10 | 170WSYSEE\textsuperscript{175} | 2.6 | 1.4 |

\textsuperscript{a} wt, wild type.

\textsuperscript{b} NA, not applicable.

The results of these experiments (Table I) indicate that clones selected from most of the libraries showed increased affinity for binding LIFR relative to wild-type LIF. In particular, clone B-11 from library B had the highest affinity and bound to LIFR with an IC\textsubscript{50} that was 100-fold lower than that of wild-type LIF. Clones from libraries C, D, and E showed improvements in LIFR binding affinities between 3- and 36-fold. Where two clones were tested from any one library, the clone that was more highly represented in the final pool tended to have the higher affinity. Only clones from library F did not show any significant improvement in binding affinity compared with wild-type LIF. As the sequences of clones analyzed from this library did not show any obvious consensus, it is possible that they were selected on the basis of improved display or expression on the phage surface, and not enhanced LIFR binding affinity.

#### Affinities of Combination Mutants—According to additivity principles (35), mutations in non-interacting parts of a protein should combine to give simple additive changes in the free energy of binding. As the LIF mutants selected from library B gave the greatest improvement in affinity for binding to LIFR, mutations derived from the remaining libraries were grafted onto this template in an effort to further improve its affinity. A pentamutant of LIF (P53G/N54L/N55L/L56Q/D57G), designed from the consensus sequence of library B selectants, was used as a template for combining additional mutations derived from the consensus sequences of libraries C (K102W/I103E), D (T150W/G152Q/K153E/D154Y/V155T), or E (V155Y/Q157S/K158Q/K160M). Binding affinities were again measured by competition ELISA, where each of the LIF mutants was expressed monovalently on M13 phage. However, no change in binding affinity was observed for any of these combination mutants (B+C, B+D, B+E, B+C+D, B+C+E) relative to the affinity of the parent library B pentamutant (data not shown).

Given the apparent lack of improvement in binding affinity for any of the combination LIF mutants, or conversely of any decrease in affinity, we suspected that these results were a limitation of the phage ELISA assay to measure IC\textsubscript{50} values below ~50 pM, rather than the combination mutants not possessing additive improvements in LIFR binding affinity. To overcome this potential limitation and better assess the relative changes in LIFR binding affinity, the consensus mutations, and combinations thereof, were introduced into the mouse/human chimeric LIF known as MH35 (21). The sequence of MH35 is 85\% identical to that of human LIF and its three-dimensional structure is quite similar to that of the human protein (36). Relative to human LIF, this protein has a reduced affinity (~20-fold) for binding human LIFR, hence it
was expected that a greater range in MH35 affinity improvement would be measurable by phage ELISA, assuming a similar ~50 pM lower limit of IC₅₀ determination.

Analysis of the binding affinities of wild-type and mutant MH35 proteins demonstrated a number of important points (Table II). First, the affinity-enhancing mutations identified by screening on a human LIF backbone provided comparable improvements in binding when placed onto the MH35 backbone. Second, by using MH35 as a template, we were able to observe a greater range in binding affinity improvement, as predicted, and more importantly, now demonstrated that the combinations of mutations led to additive enhancements of binding affinity. The order in binding affinity for each consensus mutant followed that observed for the human LIF backbone (MH35-B > MH35-D > MH35-C > MH35-E > wild-type MH35). Further improvements in affinity were observed for combinations of the consensus mutations. For example, the combination of consensus mutations from libraries B and D (MH35-BD) gave a 1300-fold improvement in binding affinity over wild-type MH35, as opposed to improvements of 180- and 34-fold for MH35-B and MH35-D. As with the phage ELISA assays on a human LIF backbone, the lowest IC₅₀ value obtained was on the order of ~50 pM. Again, this probably represented the lower limit of IC₅₀ that could be determined in this assay and may explain why the MH35-BCD mutant had a similar IC₅₀ to MH35-BD (70 pM and 54 pM, respectively).

The MH35 mutants were also tested in a competition phage ELISA to determine their binding affinity for human LIFR (Table II). Murine LIFR binds to wild-type MH35 and human LIF with unusually high affinity that is significantly higher than for human LIFR (21). Despite the subnanomolar affinity of wild-type MH35 for binding murine LIFR, each of the affinity matured MH35 mutants MH35-B, -C, -D, and -BD exhibited higher affinity binding. However, the apparent improvements in affinity appeared to be modest, perhaps due to the limitation of the phage ELISA assay to measure IC₅₀ values below ~50 pM. Consequently, the relative enhancements in murine LIFR binding affinities may have been greater than suggested by these data.

**Binding Analysis of Recombinant MH35 Mutants—**The binding studies described thus far made use of LIF mutants displayed on phage. To compare these data to those obtained for soluble protein, various LIF mutants were recombinantly expressed. For these studies, we chose to use MH35 LIF as a template rather than human LIF. This had two important advantages. First, the weaker affinity of MH35 for binding human LIFR, relative to human LIF, would make it easier to determine the range of affinity changes displayed by the panel of mutants. Second, MH35 LIF, and mutants thereof, express at a much higher level than the corresponding human LIF proteins. In fact, we were unable to produce most of the human LIF mutants in quantities suitable for these or other studies.

Each MH35-based protein was expressed and purified, and its human LIFR binding affinity determined by SPR analysis on a BLAcore instrument. For reference, we also included wild-type human LIF in this analysis as its LIFR binding affinity was recently determined by another group using BLAcore (37). Due to the very slow off-rates exhibited by the highest affinity mutants, long dissociation times of up to 10 h were used to calculate dissociation rate constants (kₒff). The association rate constants (kₐff) were calculated for each LIF mutant based on data for injection of human LIFR at several different concentrations (typically four different concentrations for each kₒff determination). The kₐff and kₒff values, and equilibrium binding constants (Kₒ) calculated from this data, are summarized in Table III. The kinetic constants obtained for human LIF were almost identical to values recently published by Bitard et al. (37). All of the proteins analyzed had very similar association rates, with less than 3-fold difference between the slowest (MH35) and fastest (MH35-D) kₒff values. By contrast, very large differences were observed in the dissociation rates of the proteins. The values obtained for kₒff extend over five orders of magnitude between the fastest (MH35) and the slowest (MH35-BCD) rates. Thus, the large differences in binding affinity between wild-type MH35 and the affinity-matured mutants arise almost entirely from reductions in the rate of dissociation of the LIFR complexes. The most potent mutant, MH35-BCD, had a calculated Kₒ of 1.1 pm, that is, 150,000-fold lower than that of wild-type MH35. While this is a greater difference than that observed by phage ELISA assay, this is likely to be due to the ability of the BLAcore analysis to determine Kₒ values down to the very low picomolar range.

**Structural Analysis of MH35-BD by NMR—**To determine whether there were any significant structural differences between affinity matured MH35 and wild-type, mutant MH35-BD was examined by NMR and compared with data for the native protein (36). Essentially complete ¹H and ¹³N NMR resonance assignments for MH35-BD were made from three-dimensional NOE-SPY-HQSC and HNHA spectra. The assignment process was assisted by the close similarity between two-dimensional ¹³N-¹H HSQC spectra of the mutant and native proteins (see Supplementary Data). The weighted average deviations of ¹H and ¹³N chemical shifts for MH35-BD from
Kinetic constants were determined by injection of soluble human LIFR over biosensor chips onto which the various LIF mutant were immobilized. The association ($k_{\text{on}}$) and dissociation ($k_{\text{off}}$) rate constants were calculated using the BIAEquation software (Biacore). The equilibrium binding constant ($K_d$) was calculated from the ratio of rate constants ($k_{\text{on}}/k_{\text{off}}$).

| Construct | $k_{\text{on}}$ (M$^{-1}$ s$^{-1}$) | $k_{\text{off}}$ (s$^{-1}$) | $K_d$ (nM) |
|-----------|-----------------|-----------------|------------|
| MH35      | $3.7 \times 10^7$ | $6.4 \times 10^{-2}$ | 180 nM     |
| human LIF | $4.3 \times 10^7$ | $6.8 \times 10^{-3}$ | 16 nM      |
| MH35-BD   | $6.9 \times 10^7$ | $1.2 \times 10^{-2}$ | 170 pM     |
| MH35-D    | $9.1 \times 10^7$ | $5.0 \times 10^{-4}$ | 560 pm     |
| MH35-BD   | $6.3 \times 10^7$ | $2.4 \times 10^{-6}$ | 3.7 pm     |
| MH35-BCD  | $5.3 \times 10^7$ | $5.7 \times 10^{-7}$ | 1.1 pm     |

These differences in local structure are emphasized by a detailed analysis of the NOEs from the backbone amide resonances of residues in the two sites to conserved regions of the structure (Supplementary Data). The observed NOE differences for residues 53–58 (site B) can be accounted for by a movement of the polypeptide backbone in this region, which encompasses the A’ mini-helix at the start of the AB loop, toward the N-terminal region of the D-helix (Fig. 1A). The NOE differences involving residues 150–155 (site D) are mostly in medium-range connectivities (i.e. between residues three or four apart in the amino acid sequence), but new contacts between Trp150 and Leu161 and Gln152 and Lys159 in MH35-BD imply that site D, near the end of the CD loop, has moved slightly closer to the N-terminal region of the D-helix than in native MH35.

**Bioactivities of Affinity-matured LIF Mutants—**To determine whether improvements in LIFR binding affinity translated into enhanced bioactivity, we examined the ability of wild-type and mutant MH35 proteins to stimulate proliferation of a Ba/F3 cell line, which stably expressed human LIFR and gp130. Relative to wild-type MH35, MH35-BD, and MH35-BCD had similar potencies in acting as agonists of cell proliferation. The concentration of each protein required to achieve half-maximal stimulation ($EC_{50}$) was very similar, with less than 2-fold difference between the highest and lowest value obtained (i.e. MH35: 16 pm; MH35-BD: 12 pm; MH35-BCD: 22 pm). This result demonstrated that the improvement in LIFR binding affinity did not result in a corresponding increase in bioactivity.

**Identification of the gp130 Binding Site and Construction of LIF Superagonists—**One of the aims of this study was to produce superagonists of both human and murine LIF signaling by combining mutations which enhance LIFR binding affinity with those that disrupt binding to the gp130 coreceptor. Because of the difficulties in recombinantly producing the human LIF mutants, we again used the MH35 backbone for conversion of the high affinity mutants into superagonists. The ability to make milligram quantities of the MH35-based proteins was particularly advantageous, as a long term aim of this project is to examine the effect of such antagonists in vivo, which would likely require relatively large amounts of recombinant protein.

According to Hudson et al. (5), human LIF can be converted to an antagonist by making Ala substitutions of three residues (Gln$^{25}$, Ser$^{28}$, and Gln$^{32}$) on the A-helix which the authors had identified as being important for interaction with gp130. We therefore made Ala substitutions of the equivalent residues (Gln$^{29}$, Asn$^{28}$, and Gln$^{32}$) on the MH35 protein. To determine whether this recombinant protein possessed any residual LIF agonist activity, its ability to induce proliferation of Ba/F3 cell lines stably expressing either human LIFR and gp130, or murine LIFR and gp130 was tested. Different results were obtained according to the species of receptor (Fig. 2A). For human LIFR/gp130-transfected cells, Q29A/G124R MH35 showed negligible agonist activity up to a concentration of 4 nM; however, it did show activity in stimulating proliferation of murine LIFR/gp130 transfected Ba/F3 cells. Thus, while this combination of three mutations was sufficient to prevent activation of the human LIFR/gp130 signaling complex, it did not prevent signaling through the corresponding murine receptor complex.

As we had aimed to produce antagonists of human and murine LIF signaling, alternate mutations were sought that would abrogate binding to both human and murine gp130. In a review article by Bravo and Heath (1), the authors allude to unpublished data, which demonstrate that the triad of residues Gln$^{29}$, Gly$^{124}$, and Asn$^{128}$ on human LIF are important for the interaction with gp130. We therefore prepared new mutants of MH35 in which Gly$^{124}$ was mutated to Arg, either alone, or in combination with an Ala substitution of Gln$^{29}$. These mutants were again tested for their ability to stimulate proliferation of the engineered Ba/F3 cell lines. The Q29A/G124R MH35 mutant had a significantly decreased ability to induce proliferation of murine LIFR/gp130-transfected Ba/F3 cells, but still acted as a partial agonist of signaling. The Q29A/G124R double mutant was found to be completely inactive as an agonist (up to 4 nM), importantly, both of human or murine LIFR/gp130-transfected Ba/F3 cells (Fig. 2B). The inability to stimulate cell proliferation was presumably due to a failure of this protein to engage gp130, as the affinity of Q29A/G124R MH35 for binding to human or murine LIFR was similar to that of wild-type MH35 (data not shown). The Q29A/G124R double mutation was therefore used to convert the affinity-matured MH35 mutants into antagonists.

**Analysis of MH35-based Superagonists—**The Q29A/G124R double mutation was introduced into several of the affinity-matured MH35 variants and these proteins were prepared recombinantly. For reference, we also prepared the corresponding double mutants of human and murine LIF (Q29A/G124R hLIF; Q29A/G124R mLIF). These proteins were tested for their ability to inhibit LIF-induced proliferation of Ba/F3 cells expressing human LIFR/gp130 (Fig. 3A). As expected, these mutants of LIF exhibited dose dependent inhibition of proliferation; moreover, differences in the potency of inhibition correlated well with their anticipated affinities for binding human LIFR. Affinity maturation had a dramatic effect on the ability of these mutants of LIF to act as antagonists. The parent MH35 molecule (Q29A/G124R MH35) had weak activity, showing partial inhibition of LIF-induced proliferation only at the highest concentration tested (2500 nM). However, the Q29A/G124R-containing forms of the highest affinity MH35 mutants (MH35-BD, MH35-BCD) were potent antagonists of signaling. The concentration of the MH35-BD antagonist required to inhibit 50% of LIF-induced proliferation was just 2-fold higher ($IC_{50} = 140$ pM) than the amount of LIF used to stimulate the cells. By comparison, the Q29A/G124R mutant of human LIF was a relatively weak antagonist of cell proliferation ($IC_{50} = 120$ nM), as expected based on its weaker LIFR binding affinity (Table II). The action of these antagonists was specific to LIFR-mediated signaling, as none was able to inhibit...
IL-3-induced cell proliferation (data not shown).

The Q29A/G124R-containing mutants of MH35 were also potent antagonists of LIF-induced proliferation of Ba/F3 cells expressing murine LIFR/gp130 (Fig. 3B). MH35 LIF has ~50-fold higher affinity for binding murine LIFR compared with murine LIF, and thus represents an affinity-matured LIF variant with respect to murine LIFR binding. Accordingly, Q29A/G124R MH35 was considerably more potent as an antagonist of signaling than Q29A/G124R mLIF. Further improvements in the superantagonist activity of Q29A/G124R MH35 were achieved by incorporating mutations identified from the phage-display screen. In the Ba/F3 cell proliferation assay, we observed further increases (4–25-fold) in the potencies of Q29A/G124R MH35 and Q29A/G124R-B, -D, -BD, and -BCD antagonists compared with Q29A/G124R MH35. The most potent molecule (Q29A/G124R MH35-BD) inhibited 50% of proliferation at a concentration that was similar to the amount of murine LIF used to stimulate the cells (IC50 = 36 pm).

We also tested a number of these antagonists against unmanipulated M1 murine myeloid leukemic cells, a cell line which differentiates into macrophages in response to LIF (Fig. 4). As expected, the superantagonists were considerably more potent than Q29A/G124R mLIF in inhibiting LIF-induced differentiation of this cell line. Relative to the murine LIFR/gp130 Ba/F3 cell assay, however, the differences in the potency of the various superantagonists was minor, with only a 5-fold difference in the concentrations required to inhibit 50% of cell differentiation.

DISCUSSION

Creation of High Affinity Mutants—In this report, we have identified mutations of LIF which result in significant enhancements for binding the LIFR. These mutations were located at the ends of helices -B and -D, or within the A-B and C-D connecting loops proximal to two residues, Phe156 and Lys159, which were previously shown to be critical for LIFR binding (5). Interestingly, we observed retention of the wild-type sequence at two clusters of residues, 49–52 and 104–107, following randomization and panning against LIFR. Hence it is likely that the sequence of LIF is intolerant to substitution within these regions. These two clusters form part of the connecting loops that join either the A-B or C-D helices, and are highly conserved in another LIFR ligand, CT-1. An adjoining LIF residue, Ile103, was commonly replaced by Asp or Glu, and these correspond to the amino acids found in the equivalent position of three of the four other LIFR-binding cytokines.
Both of the 49–52 and 104–107 clusters include a Pro residue, suggesting that some conformational constraint is important for connection of the helices. Indeed, Phe$^{156}$ is held in a trans rotameric state by Pro$^{51}$ and Phe$^{52}$, and this rotameric conformation has been predicted to be crucial for interaction with LIFR (38).

**Fig. 3.** Inhibition of Ba/F3-LIFR/gp130 cell proliferation by LIF mutants. LIF mutants were titrated in the presence of a constant amount of wild-type LIF, and proliferation after 48 h incubation was measured colorimetrically by MTT assay (42). Values represent the mean of triplicate samples. Closed circles, Q29A/G124R hLIF; open circles, Q29A/G124R mLIF; diamonds, Q29A/G124R MH35; closed triangles, Q29A/G124R MH35-B; closed squares, Q29A/G124R MH35-D; open squares, Q29A/G124R MH35-BD; open triangles, Q29A/G124R MH35-BCD.

**Fig. 4.** Inhibition of M1 cell differentiation by LIF mutants. LIF mutants were titrated in the presence of a constant amount of murine LIF (8 pM). Results are expressed as the percentage of M1 colonies, grown in semi-solid agar, that showed a differentiated (disperse) phenotype. Circles, Q29A/G124R mLIF; diamonds, Q29A/G124R MH35; triangles, Q29A/G124R MH35-B; closed squares, Q29A/G124R MH35-D; open squares, Q29A/G124R MH35-BD.
The results from the present study, in addition to those of Hudson et al. (5), indicate that LIF residues in the vicinity of Phe^156 and Lys^159, which lie across one end of the four-helix bundle, can make contact with LIFR, and that the affinity of this interaction can be increased significantly by mutations in this region. Some of the affinity-enhancing substitutions were quite dramatic, including D57G, K102W, T150W, K153E, and D154Y, and indicated that the incorporation of additional hydrophobic contacts, as well as the elimination or reversal of charges, must provide a more complementary LIFR binding surface. Comparison of NMR data for MH35-BD with that of the native protein showed that the affinity enhancing mutations did not alter the overall structure of the molecule, but that some local changes occurred in the vicinity of the mutations, more so in the B site than D. Thus, the observed enhancement in receptor binding affinity appears to be a consequence not only of the altered side chain chemistry associated with substitutions at sites B and D but also local structural changes in the vicinity of these regions. The improvements in affinity were almost entirely due to reductions in the rates of dissociation of the LIFR complex, consistent with what has been observed previously for a variety of other affinity matured proteins (13, 34).

**Effect of Affinity Maturation on LIF Bioactivity**—No improvements in biological activity were observed for the high affinity LIF mutants when tested for signaling through the human LIFR. This is not entirely surprising, as an analogous situation occurs with naturally occurring ligands for the murine LIFR: human LIF binds to the murine LIFR with significantly higher affinity (100–1000-fold) than murine LIF (39), but the biological activities of both molecules are very similar when tested on cells expressing murine LIFR (21, 40). A similar result was obtained with growth hormone and heregulin in which affinity matured forms with 50–400-fold improvements in affinity for their respective receptors were no more active than the wild-type proteins (34, 41). In a model proposed by Pearce et al. (41), the dissociation rate for the 1:1 growth hormone receptor complex was suggested to be slow enough for it to laterally diffuse across the membrane surface in search of an unoccupied second receptor. Hence, a reduction in the dissociation rate of the 1:1 complex will not necessarily enhance the rate of formation of the higher order signaling complex. As was shown experimentally for growth hormone, only mutants in which the dissociation rate was significantly decreased, rather than to wild-type growth hormone, showed an effect (reduced bioactivity) on downstream signaling events. A similar situation may exist for LIF, whereby the rate of dissociation of wild-type LIF from the LIF-LIFR complex is below the threshold level, over which no further enhancement in bioactivity results from a reduction in the dissociation rate.

**Conversion of High Affinity Mutants to Superantagonists**—Using information about the gp130 binding epitope described in a review article by Bravo and Heath (1), we were able to generate LIF mutants that maintained binding to LIFR, but had no biological activity, presumably because of a loss of gp130 binding activity. Initial attempts to create an antagonistic variant of LIF by introducing mutations previously described by Hudson et al. (5), gave rise to a protein that maintained significant agonist activity on cells expressing murine, but not human LIFR/gp130. Given that the Hudson et al. data was based on human LIF mutants acting on cell lines expressing human LIFR/gp130, our results suggested that there may be subtle species-specific differences in the key residues on LIF involved in the interaction with gp130. However, as the Q29A/G124R double mutation was sufficient to abrogate signaling of cells expressing either mouse or human receptors, these residues on the ligand must be involved in binding gp130 from either species.

By incorporating mutations which disrupted gp130 binding into the high affinity LIF mutants, we were able to generate highly potent antagonists of LIF bioactivity. The most active of these antagonists were able to inhibit LIF-induced proliferation of cells at concentrations similar to that of the wild-type LIF. This is consistent with the results from a reduction in the order of 1000-fold compared with the non-affinity matured forms of these antagonists. As shown with previous cytokine superantagonists (16), the potency varied depending on the cell line tested (e.g. Ba/F3 versus M1 cells) and these differences can probably be explained by the different receptor numbers on the cells and the types of biological responses used as read-outs for the assays. While we predict that the high affinity mutations on a human LIF backbone would be more potent antagonists than their MH35 counterparts on cell lines expressing human receptors (due to the higher affinity of human LIF compared with MH35 for binding human LIFR), we were unable to characterize such proteins due to problems experienced in the recombinant expression of many of these proteins. We are currently attempting alternate expression strategies to overcome this obstacle.

The LIFR is shared by a number of related proteins including CT-1, CNTF, and CLC/CLF, thus, it is likely that the LIF superantagonists generated in this study will also be potent antagonists of these other cytokines, as was previously the case for a non-affinity matured LIF antagonist (11). These superantagonists may therefore be useful in defining the in vivo function of cytokines, which signal through the LIFR, and we are currently using them to further investigate the role of LIF in fertility.

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