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Inter-species chimeras of leukaemia inhibitory factor define a major human receptor-binding determinant

Catherine M.Owczarek, Meredith J.Layton, Donald Metcalf, Peter Lock, Tracy A.Willson, Nicholas M.Gough and Nicos A.Nicola[1]

The Walter and Eliza Hall Institute of Medical Research and The Cooperative Research Centre for Cellular Growth Factors, PO Royal Melbourne Hospital, Victoria 3050, Australia

[1]Corresponding author

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Human leukaemia inhibitory factor (hLIF) binds to both human and mouse LIF receptors (LIF-R), while mouse LIF (mLIF) binds only to mouse LIF-R. Moreover, hLIF binds with higher affinity to the mLIF-R than does mLIF. In order to define the regions of the hLIF molecule responsible for species-specific interaction with the hLIF-R and for the unusual high-affinity binding to the mLIF-R, a series of 15 mouse/human LIF hybrids has been generated. Perhaps surprisingly, both of these properties mapped to the same region of the hLIF molecule. The predominant contribution was from residues in the loop linking the third and fourth helices, with lesser contributions from residues in the third helix and the loop connecting the second and third helices in the predicted three-dimensional structure. Since all chimeras retained full biological activity and receptor-binding activity on mouse cells, and there was little variation in the specific biological activity of the purified proteins, it can be concluded that the overall secondary and tertiary structures of each chimera were intact. This observation also implied that the primary binding sites on mLIF and hLIF for the mLIF-R were unaltered by inter-species domain swapping. Consequently, the site on the hLIF molecule that confers species-specific binding to the hLIF-R and higher affinity binding to the mLIF-R, must constitute an additional interaction site to that used by both mLIF and hLIF to bind to the mLIF-R. These studies define a maximum of 15 amino acid differences between hLIF and mLIF that are responsible for the different properties of these proteins.

Key words: cytokine receptor/haemopoietin receptor/ligand-receptor interaction/structure-function relationship

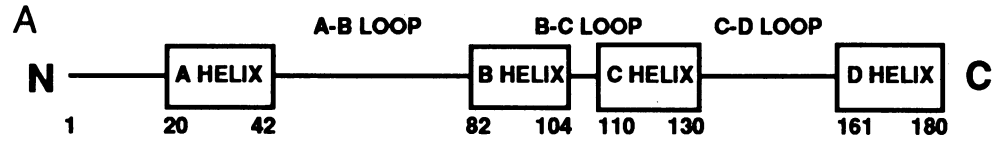
Introduction

Leukaemia inhibitory factor (LIF) is a glycoprotein that has varied actions on a wide range of cell types including megakaryocytes, osteoblasts, hepatocytes, adipocytes, neurons, embryonic stem cells and primordial germ cells (Metcalf, 1991). Many of its activities are also displayed by other cytokines, including interleukin-6 (IL-6), oncostatin-M (OSM) and granulocyte colony-stimulating factor (G-CSF) (Rose and Bruce, 1991).

LIF transduces its biological signal via a transmembrane receptor, of which both high- and low-affinity forms have been described (Hilton and Nicola, 1992). Sequence analysis of cDNA clones for low-affinity LIF-binding α-chains of the human and mouse LIF receptors (Gearing *et al.*, 1991) revealed these proteins to be members of the haemopoietin family of receptors (Bazan, 1991). Both receptors have duplicated haemopoietin domains, and weak homology to both the G-CSF receptor (Fukunaga *et al.*, 1990) and gp130, the signal transducing β-subunit of the IL-6 receptor (Hibi *et al.*, 1991). High-affinity receptors for LIF are generated by heterodimerization of the LIF-R α-chain with gp130, and gp130 has also been proposed to be a component of the OSM receptor (Gearing and Bruce, 1992; Gearing *et al.*, 1992) and the ciliary neurotrophic factor (CNTF) receptor (Ip *et al.*, 1992). Thus, the high-affinity receptor for each of these cytokines comprises at least an $\alpha-\beta$ heterodimer in which the β-subunit is shared, a situation that is reminiscent of the GM-CSF, IL-3 and IL-5 receptor system (Miyajima *et al.*, 1992).

Whilst mouse LIF (mLIF) is unable to bind to the human LIF receptor (hLIF-R), human LIF (hLIF) is able to bind to both high- and low-affinity mouse LIF receptors (mLIF-R), and is fully biologically active on mouse cells. Intriguingly, hLIF binds to both the naturally occurring, soluble form of the mLIF-R α-chain, mLIF-binding protein (mLBP) (Layton *et al.*, 1992), and the high-affinity mLIF-R on PC.13 cells with a higher affinity than mLIF, due to markedly different dissociation kinetics (M.Layton, P.Lock, D.Metcalf and N.Nicola, in preparation). Competitive displacement curves showed that unlabelled mLIF and hLIF had a similar ability to compete with [^{125}I]mLIF for binding to mLBP, while unlabelled hLIF was consistently 1000- to 5000-fold more effective than mLIF in competing with [^{125}I]hLIF for binding to mLBP (Layton *et al.*, 1992). Mouse LBP is also able to act as a competitive inhibitor of LIF binding to its cellular receptor, leading to inhibition of biological responses to LIF. Again, mLBP was an ~1000-fold more potent inhibitor of hLIF than mLIF in this system (Layton *et al.*, in preparation).

Thus, at least two features of hLIF distinguish it from mLIF: first, its capacity to bind to the hLIF-R where mLIF cannot and second, its capacity to bind to the mLIF-R with higher affinity than does mLIF. Establishing the amino acid residues that determine the structural basis for these two features of hLIF was simplified by studying the binding of LIF to the low-affinity LIF-R α-chain, rather than the high-affinity receptor complex. Mouse LBP, a normal component of mouse serum, was used as a source of soluble mLIF-R α-chain, whilst COS cells transfected with a plasmid encoding the hLIF-R were the source of the α-chain of the hLIF receptor. Acquisition of the ability to bind to the hLIF-R α-chain would map the first property of hLIF, while the unusual cross-species binding of hLIF to mLBP provides an ideal system with which to map quantitatively



B

HYBRID	SPECIFIC ACTIVITY (UNITS/mg) x10^{-8}	SCORE (% HUMAN) MEAN ± S.E.M. (%)	RECIPROCAL DILUTION OF MOUSE SERUM TO BLOCK 50% M1 COLONY DIFFERENTIATION	ID$_{50}$ FOR INHIBITION OF ^{125}I-hLIF BINDING TO COS hLIF-R CELLS (μg/ml)
M	1.8	0	8	>100
H	2.1	100	8192	0.04
MH1	1.4	23 ± 3	16	>20
MH2	1.8	87 ± 4	4096	0.15
MH3	0.82	0 ± 0	N.D.	>200
MH4	2.4	26 ± 1	32	25
MH5	1.6	52 ± 1	256	2
MH6	1.4	78 ± 2	1024	0.25
MH7	1.9	54 ± 4	512	1.2
MH8	1.8	77 ± 3	1024	0.3
MH9	1.7	20	64	35
MH10 *a*	1.5	80 ± 4	2048	0.25
MH11 *b*	1.4	67 ± 1	N.D.	0.6
MH12 *c*	1.5	73 ± 3	1024	0.4
MH13 *d*	1.4	73 ± 3	1024	0.4
MH14 *e*	2.9	71 ± 2	2048	0.3
MH15	1.1	92	N.D.	0.15

Fig. 1. (**A**) Schematic linear representation of the secondary structure of the LIF protein based on the predictions of Bazan (1991). The amino and carboxy termini, and the positions of the predicted α-helical regions (boxes) and connecting loops (lines) are shown relative to the amino acid sequence of both mLIF and hLIF (see Figure 2 for numbering). (**B**) Summary of data from biological assays and competitive binding assays of mLIF, hLIF and chimeric LIF proteins. Mouse LIF sequence is represented by open boxes and hLIF sequence is represented by black boxes. The hybrid proteins are numbered from 1 to 15 and are prefixed by 'MH' to denote that they contain both mLIF and hLIF amino acid sequence. Additional single amino acid substitutions are indicated as follows: *a*, K168T; *b*, H112Q; *c*, S113V; *d*, Q112H, V113S, T168K; *e*, Q112H, V113S. The notation Q112H indicates that the glutamine at position 112 in the mLIF amino acid sequence has been substituted with histidine, which is at position 112 in hLIF. The relative positions of the predicted α-helices and connecting loops in the hybrid proteins can be determined by visual alignment of the schematic representations with the diagram immediately below. The percentage human score of mLIF, hLIF and hybrid proteins is calculated from the ID_{50} for inhibition of [^{125}I]hLIF binding to mLIF-binding protein. Most assays were performed two or more times with essentially identical results. N.D., not determined.

the determinants on the hLIF molecule that confer the second feature of hLIF-specific binding.

We have shown that the amino acid residues that confer both of these attributes map to the same region of the hLIF molecule and that these residues are located in the carboxy-terminal half of the molecule. More specifically, a region comprising the predicted connecting loop located between the two carboxy-terminal α-helices contains a major receptor binding determinant, with additional contributions from the loop between the second and third α-helices, and two amino acid residues in the amino-terminal region of the third α-helix.

Results

Molecular modelling

Secondary and tertiary structural predictions have suggested that the LIF molecule is an anti-parallel, four α-helical bundle, a topology common to a number of growth factors and cytokines (Diederichs *et al.*, 1991; Parry *et al.*, 1991; de Vos *et al.*, 1992). The three-dimensional structure of LIF has not yet been determined, so we utilized the model of Bazan (1991) to divide the LIF amino acid sequence into a series of modules of predicted α-helices and connecting loops (Figure 1A). A series of plasmids was then designed to encode mouse–human chimeric LIF (m-hLIF) molecules (Figure 1B) in which regions of hLIF sequence were incorporated into a mLIF molecular framework. As the sequences of mLIF and hLIF are ~80% identical (Gough *et al.*, 1988) (Figure 2), swapping even large domains of mLIF and hLIF sequence resulted in changes of relatively few amino acid residues.

Protein purification and verification of protein structure

Each hybrid protein was expressed in *Escherichia coli* as a glutathione S-transferase fusion protein, purified by affinity chromatography on a glutathione Sepharose matrix, and cleaved from glutathione S-transferase by thrombin. Chimeras were further purified by cation-exchange chromatography, which takes advantage of the high isoelectric point of the unglycosylated LIF protein. The purity of each hybrid was then assessed by both reversed-phase HPLC (data not shown) and SDS–PAGE (Figure 3).

The protein concentration of each purified sample was determined by amino acid analysis, and the amount of biologically active protein in each sample was estimated by its ability to induce differentiation in mouse M1

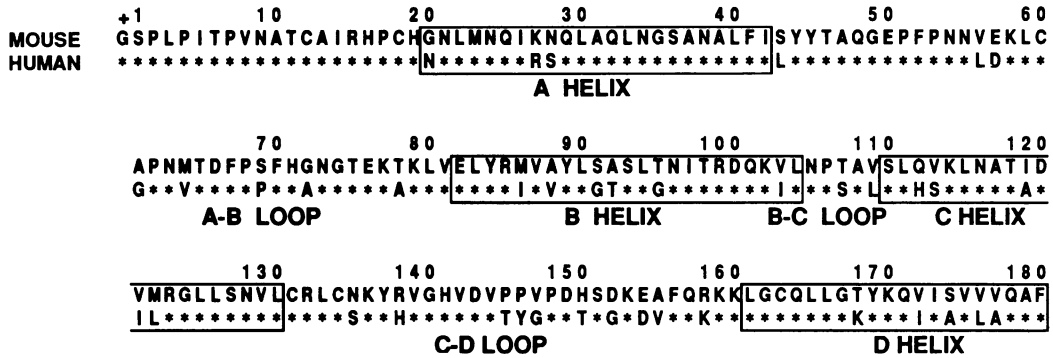


Fig. 2. Comparison of mouse and human LIF amino acid sequences. Amino acid residues that are identical between mLIF and hLIF are indicated by asterisks and predicted α-helices A,B, C and D are surrounded by boxes. Numbering of the amino acid residues of each protein is indicated as starting at +1 (serine) of the mature, native proteins; however, the thrombin cleavage results in an additional glycine residue at position −1 in recombinant mLIF, hLIF and all chimeras. The single-letter amino acid code is used throughout.

myeloid leukaemic colonies. An internal standard of mLIF (10^4 U/ml) was used to normalize all M1 cell bioassays (50 U/ml of LIF is defined as the concentration of LIF required for half maximal stimulation). The specific biological activity of each hybrid on mouse cells was defined by the number of units of LIF activity per milligram of protein and was found to be within the normal range for both mLIF and hLIF ($1-3 \times 10^8$ U/mg) (Figure 1B) indicating that joining structural elements of these homologous proteins did not significantly disrupt their tertiary structure, nor affect their activity on mouse cells.

The structural integrity of all m-hLIF chimeras was also verified by evaluating their ability to bind correctly to the mLIF-R α-chain. Mouse LIF, hLIF and all hybrids had a similar ability to compete for [^{125}I]mLIF binding to mLBP (Figure 4A).

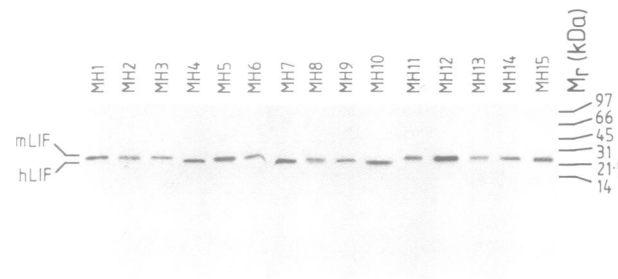


Fig. 3. SDS−PAGE analysis of purified hybrid LIF proteins (MH1−MH15). Approximately 50 ng of each protein was analysed on a 15% polyacrylamide gel and silver-stained. The relative positions of the mLIF and hLIF protein bands are shown at the left of the gel. Low molecular weight (M_r) standards (Bio-Rad) are indicated in kDa.

Competitive binding assays

The first feature of hLIF that distinguishes it from mLIF, i.e. its ability to bind to the hLIF-R α-chain, was evaluated by the ability of each hybrid to compete with [^{125}I]hLIF for binding to COS cells expressing the hLIF-R α-chain (COS hLIF-R cells). The second feature of hLIF that distinguishes it from mLIF, i.e. its ability to bind to the mLIF-R with a higher affinity than mLIF, was evaluated by the ability of each hybrid to compete with [^{125}I]hLIF for binding to mLBP and by its sensitivity to biological inhibition by mLBP.

The 1000- to 5000-fold difference in the ability of mLIF and hLIF to compete with [^{125}I]hLIF for binding to mLBP provided a large window in which to measure the degree of hLIF-like specific binding of each chimeric protein. In each assay, the doses of hLIF, mLIF and m-hLIF hybrid required to inhibit 50% of [^{125}I]hLIF binding to mLBP (ID_{50}) were measured, and hLIF and mLIF were defined as having 100% and 0% hLIF-like binding activity respectively. Assays could then be normalized for inter-assay variations by using a logarithmic scale to convert the ID_{50} for hLIF and mLIF to a score of 100% and 0% respectively, then converting the ID_{50} for each hybrid to a percentage score between these two extremes.

Initial experiments found that a hybrid LIF molecule (MH2) in which the amino-terminal half contained mLIF amino acid residues and the carboxy-terminal half consisted of hLIF residues, had 87 ± 4% (mean ± SEM) hLIF-like activity in terms of its ability to compete with [^{125}I]hLIF for binding to mLBP, and had gained the capacity to bind to the hLIF-R α-chain. In contrast, the reverse hybrid (MH1) had only ~23 ± 3% hLIF-like binding to mLBP (Figure 4B) and could not bind to COS hLIF-R cells (Figure 5A). These results defined the carboxy-terminal half of hLIF as containing a major receptor binding determinant.

Hybrids MH3, MH4, MH5 and MH6 were constructed to resolve which structural modules were involved in the receptor binding site. When the predicted D-helix was composed of hLIF residues (MH3), the hybrid had no hLIF-like binding to mLBP. When the predicted C-helix was composed of hLIF residues (MH4), the hybrid displayed 26 ± 1% hLIF-like activity in its ability to compete for [^{125}I]hLIF binding to mLBP (Figure 4C). Hybrid MH5, which contained the predicted C−D loop region substituted for the equivalent hLIF residues, displayed 52 ± 1% hLIF-like activity in the same assay. The combination of hLIF residues in both the predicted C-helix and the C−D loop (MH6) resulted in a hybrid with 78 ± 2% hLIF-like activity as assessed by its ability to compete for [^{125}I]hLIF binding to mLBP (Figure 4C). Hybrids MH7, MH8 and MH9 were constructed in order to test whether the D-helix co-operated with either the C-helix or the C−D loop to enhance hLIF-like binding specificity. Hybrid MH7, which contained hLIF sequences in the C−D loop and in the D-helix, behaved like hybrid MH5, which contained hLIF residues in the C−D loop only. Hybrid MH9, which contained hLIF residues in the C-helix and the D-helix, behaved like hybrid MH4, in which only the C-helix comprised hLIF sequence. Hybrid MH8, which contained hLIF residues in the C-helix, C−D loop and D-helix,

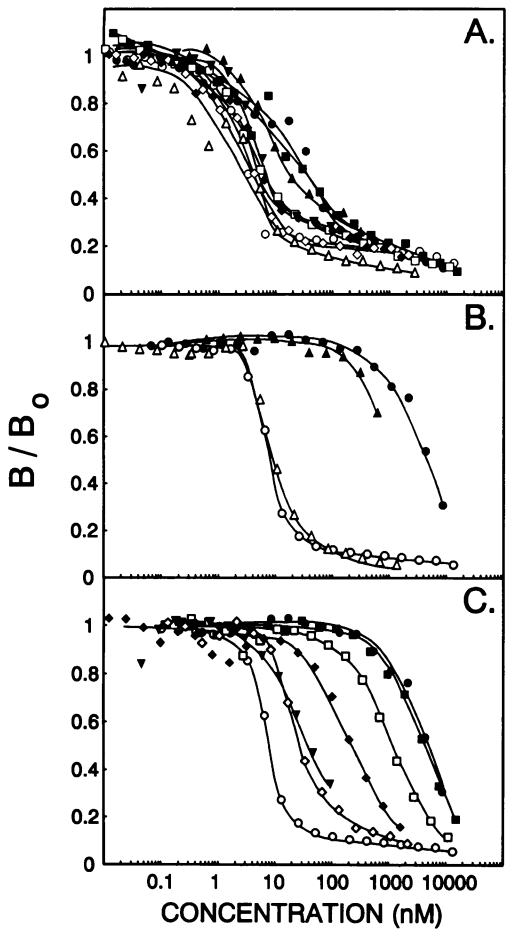


Fig. 4. (**A**) Competitive inhibition of [^{125}I]mLIF binding to mLBP (soluble mLIF-R α-chain) by mLIF, hLIF and a selection of m-hLIF hybrids. (●) mLIF; (○) hLIF; (▲) MH1; (△) MH2; (■) MH3; (□) MH4; (◆) MH5; (◇) MH6; (▼) MH14. Results for all competition assays were expressed as the number of counts bound to the receptor at a particular concentration of unlabelled competitor (B) divided by the number of counts bound to the receptor when no unlabelled competitor was present (B_0). (**B**) Competitive inhibition of [^{125}I]hLIF binding to mLBP by mLIF, hLIF and a selection of m-hLIF hybrids. (●) mLIF; (○) hLIF; (▲) MH1; (△) MH2. (**C**) Competitive inhibition of [^{125}I]hLIF binding to mLBP by mLIF, hLIF and a selection of m-hLIF hybrids. (●) mLIF; (○) hLIF; (■) MH3; (□) MH4; (◆) MH5; (◇) MH6; (▼) MH14.

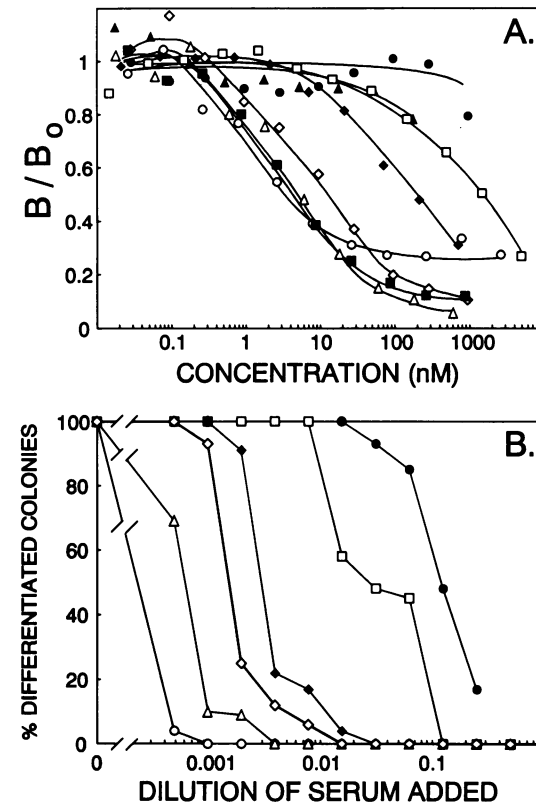


Fig. 5. (**A**) Competitive inhibition of [^{125}I]hLIF binding to COS cells transfected with the hLIF-R α-chain by mLIF, hLIF and a selection of m-hLIF hybrids. (●) mLIF; (○) hLIF; (▲) MH1; (△) MH2; (□) MH4; (◆) MH5; (◇) MH6; (■) MH14. (**B**) Competitive inhibition by normal mouse serum of M1 colony differentiation induced by 200 U/ml mLIF, hLIF or m-hLIF hybrids. (●) mLIF; (○) hLIF; (△) MH2; (□) MH4; (◆) MH5; (◇) MH6.

behaved like hybrid MH6, in which only the C-helix and C−D loop comprised hLIF sequence (Figure 1B).

All chimeras were also tested for their ability to compete with [^{125}I]hLIF for binding to COS hLIF-R cells. A typical example of a competitive binding assay between the chimeras and [^{125}I]hLIF on COS hLIF-R cells is shown in Figure 5A. When the hybrids were ranked according to the dose required to produce 50% inhibition (ID_{50}) of [^{125}I]hLIF binding to both mLBP and COS hLIF-R cells, the hierarchy was the same in each assay (Figure 1B), indicating that the two features of hLIF that distinguish it from mLIF map to the same regions on the hLIF molecule.

Single amino acid substitutions were then used to identify the individual amino acids within the C-helix that were critical for hLIF-like binding to both mLBP and COS hLIF-R cells. Hybrid MH8, which had 77 ± 3% hLIF-like binding to mLBP, was used as a framework for these constructs. The residues most likely to contribute to hLIF-specific binding in the C-helix are those that are different in mLIF and hLIF, and are predicted on the basis of a helical wheel projection to be on the external (hydrophilic) surface of the molecule (Figure 6). There were therefore three amino acids in the C-helix of hLIF (H112, S113 and I121) that were candidates for investigation by site-directed mutagenesis, although residue I121 represented a conservative substitution between mLIF (A) and hLIF (I) and so was discounted. Residues H112 and S113 were mutated individually to their equivalent mLIF residue in hybrids MH11 and MH12 respectively. If either residue was important for hLIF-like binding, its substitution to the equivalent mLIF residue should reduce the hLIF-like binding of the hybrid, perhaps to a level similar to that of hybrid MH5, in which only the C−D loop contains hLIF residues. The hLIF-like binding of hybrids MH11 and MH12 to mLBP was scored as 67 ± 1% and 73 ± 3% respectively, indicating that the substitution H112Q was the more significant of these mutations, but alone was insufficient to abrogate completely the contribution of the C-helix to hLIF-like binding to mLBP. Hybrid MH10, which comprised an MH8 framework with a residue in the D-helix, K168, swapped to its mLIF equivalent (T168), showed that changing the least conserved residue on the external face of the D-helix did not affect hLIF-like binding.

In order to test whether hLIF-like binding activity of hybrid MH5, in which the C−D loop contains hLIF sequence, could be restored to the level of hybrid MH6, in which both the C−D loop and the C-helix consist of hLIF

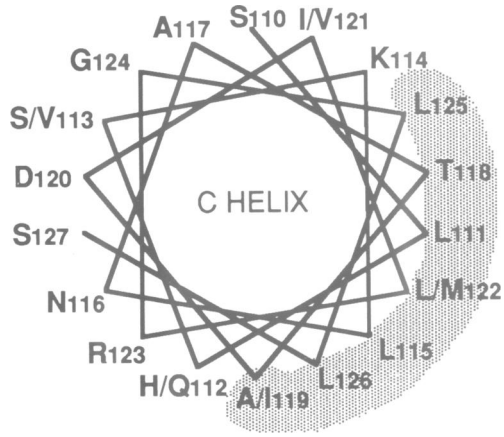


Fig. 6. Helical wheel projection of the C-helix, showing the predicted hydrophobic (shaded grey) and hydrophilic (unshaded) faces. The residues shown are numbered according to the LIF amino acid sequence (see Figure 2). Where there are differences between the mLIF and hLIF sequences, the hLIF residue is first, followed by the mLIF residue in the equivalent position.

residues, mLIF amino acids Q112, V113 in the C-helix and T168 in the D-helix were substituted with their equivalent hLIF residues. Hybrid MH14, which was based on hybrid MH5 but with additional substitutions Q112H and V113S, showed 71 ± 2% hLIF-like binding to mLBP, indicating that these two residues define the contribution of the C-helix to hLIF-specific binding (Figure 4C). Hybrid MH13, which was identical to hybrid MH14 except for an additional T168K mutation, also had 73 ± 3% hLIF-specific binding to mLBP, confirming that the mutated residues in the C-helix were sufficient to restore MH6-like mLBP binding. These chimeras were also tested for their ability to compete with [^{125}I]hLIF for binding to COS hLIF-R cells. Again, ranking this set of hybrids in order of their ID_{50} values for inhibition of [^{125}I]hLIF binding to both mLBP and COS hLIF-R cells gave qualitatively the same results in both assays (Figure 1B). This strategy has therefore identified two residues within the C-helix, H112, and S113, as critical for hLIF-like binding to both mLBP and the hLIF receptor.

Hybrids MH6 and MH8 showed hLIF-like binding to mLBP of ~78% whereas hybrids MH2 and MH15 demonstrated hLIF-like binding to mLBP of 87 ± 4% and 92% respectively. These differences in hLIF-like binding must be due to substitutions within the region between I103 and L109, which is predicted to form the B–C loop as well as the last two residues of the B-helix. The three amino acids in this region that are different between hLIF and mLIF are either conservative hydrophobic (I103V and L109V) or conservative hydrophilic (S107T) substitutions, suggesting that the contribution of this region to hLIF-specific binding is subtle.

Biological inhibition assays

A 1:4 to 1:8 dilution of normal mouse serum (equivalent to ~0.5–1 μg/ml mLBP) is required to block 50% of the M1 differentiation-inducing activity of up to 200 U/ml mLIF, whereas, due to the higher affinity of hLIF for mLBP, a 1:8192 dilution of serum (~0.6 ng/ml mLBP) is sufficient to block 50% of the M1 activity of up to 200 U/ml hLIF. This ability of mLBP to inhibit the differentiation-inducing activity of LIF in a species-specific manner was utilized as a second assay that distinguished hLIF from mLIF. Hybrids were also assessed for hLIF-like activity in this assay according to the dose of serum required to inhibit 50% of their M1 cell differentiation-inducing activity when they were present in the culture dish at a just maximal stimulatory concentration (~200 U/ml). An example of a typical serum dilution M1 assay is shown in Figure 5B. All data from this assay were consistent with the data obtained from the competitive binding assays with mLBP and [^{125}I]hLIF (Figure 1B), thus eliminating the possibility that the higher affinity of hLIF for mLBP was an artefact of the binding assays themselves or of the use of iodinated hLIF.

Discussion

We have been able to exploit the inability of mLIF to bind to the hLIF-R α-chain and the ability of hLIF to bind to the soluble mLIF-R α-chain (mLBP) with higher affinity than mLIF to map LIF receptor α-chain-binding determinants on the hLIF molecule. The human and mouse LIF molecules were divided into structural domains based on the predictions of Bazan (1991), and a series of mouse–human LIF chimeras was constructed according to this model.

The advantage of using the mLIF-R as well as the hLIF-R to map a binding site on hLIF was that it enabled each hybrid protein to be tested for correct folding (inhibition of [^{125}I]mLIF binding to mLBP) and allowed quantification of the degree of conversion to hLIF-specific activity (inhibition of [^{125}I]hLIF binding to mLBP) within the same assay. If gain of hLIF-like binding to the hLIF-R α-chain had been the only criterion used to map the site conferring hLIF-like binding specificity, it would have been difficult to distinguish whether a chimeric protein was unable to bind to the hLIF-R α-chain because it was incorrectly folded and therefore biologically inactive, or because it was behaving like mLIF.

The site that confers the unusual high-affinity binding of hLIF to mLBP was localized by assaying each hybrid for its ability to compete with [^{125}I]hLIF for binding to mLBP, and by determining the dose of mLBP (at a concentration of 1–5 μg/ml in normal mouse serum) required to inhibit the differentiation-inducing activity of each hybrid in a culture of M1 cells. Both assays measure essentially the same phenomenon, but the latter has the advantage that it is not necessary to use a particular radiolabelled ligand in order to observe a difference between mLIF and hLIF. The ability of each hybrid to compete with [^{125}I]hLIF for binding to COS cells expressing a transfected hLIF-R α-chain was used to localize the site that conferred normal hLIF binding to the hLIF-R α-chain. These two sites, which were mapped in this study, corresponded exactly, thus indicating that the same site on the hLIF molecule mediates both binding to the hLIF-R α-chain and the unusual high affinity of hLIF for the mLIF-R α-chain. The strength of this approach is demonstrated by the consistency, both qualitative and quantitative, of the behaviour of all the m-hLIF chimeras in each different assay.

We have shown that the carboxy-terminal half (I103–F180) of the hLIF molecule contains the amino acid residues that constitute the hLIF receptor α-chain-binding determinant. Substitution of this region of hLIF into the homologous region of mLIF resulted in a chimeric protein (MH2) that displayed 87 ± 4% hLIF-like ability to compete for [^{125}I]hLIF binding to mLBP. Hybrid MH5, which contained residues C131–K160 (the C–D loop) of hLIF

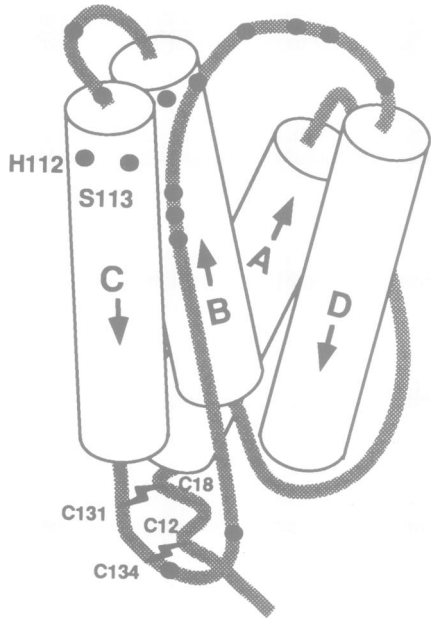


Fig. 7. Predicted three-dimensional structure of the hLIF protein, modelled as an anti-parallel four α-helical bundle. The α-helices (A, B, C and D) are depicted as cylinders and the connecting loops are shaded in grey. Amino acid residues that are different between mLIF and hLIF and are in the region which has been defined to confer hLIF-like binding activity are indicated as black dots, including the histidine at position 112 and the serine at position 113 in the C-helix. Disulphide bonds between residues C131 and C18, and C134 and C12, are indicated. The disulphide bond between residues C60 and C163 is obscured by helices A and D.

substituted into the mLIF sequence, showed a gain of hLIF-like activity of 52 ± 1%, indicating that this region contains the major receptor-binding determinant. Further mutational analysis defined two amino acid residues in the C-helix (H112 and S113) and the short loop between helices B and C as being of importance (Figure 7). Hybrid MH1, in which the amino-terminal half of the molecule comprises hLIF sequence, demonstrated a small amount of hLIF-like binding to mLBP, although it was unable to interact with the hLIF-R α-chain, so it is unclear whether any amino acids in the amino-terminal half of the hLIF molecule have some involvement in conferring hLIF-R binding specificity.

A possible interpretation of these results is shown in a model of mLIF and hLIF binding to receptors from both species (Figure 8). Since mLIF, hLIF and all m-hLIF chimeras had an approximately equal ability to compete with [^{125}I]mLIF binding to the soluble mLIF-R α-chain, and had nearly equal biological activities in a mouse cell bioassay, they must contain a common binding site for the α-chain of the mLIF-R. This binding site on the ligand (site a, oval in Figure 8) presumably comprises conserved amino acid residues in the mLIF and hLIF proteins and so is invisible in our assay system. This site, however, is not sufficient to mediate binding to the hLIF-R α-chain, or to generate enhanced binding to the mLIF-R α-chain, which are exclusive properties of hLIF. A second site on the hLIF molecule, which we have mapped in these studies (site b, triangle in Figure 8), is comprised of a small number of residues in the predicted C−D loop, C-helix and B−C loop of hLIF and is proposed to mediate the exclusive properties of hLIF. These residues form a cluster on one face of the predicted three-dimensional structure of hLIF (Figure 7).

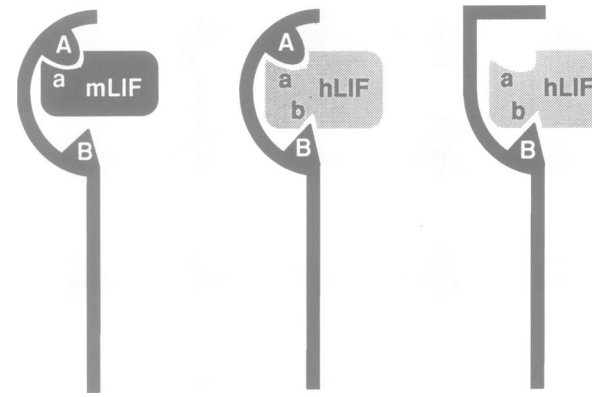


Fig. 8. Proposed model of mLIF and hLIF binding to mouse and human LIF-R α-chains. A binding site is present on both the mLIF and hLIF ligands (a), for which there is a complementary site (A) on the mLIF receptor α-chain only. A second site (b), which is present on the hLIF ligand only, and which is proposed to mediate both binding to the hLIF receptor α-chain and the unusual high-affinity binding to the mLIF receptor α-chain, interacts with a site (B) on both the mLIF receptor α-chain and the hLIF receptor α-chain. The site mapped in this study is the site on the hLIF ligand indicated by 'b'.

The primary interaction site on the LIF ligand for its isologous receptor α-chain is not the same in the human and mouse systems. It is proposed that hLIF but not mLIF is able to recognize a site which is conserved in both the hLIF and mLIF receptor α-chains (site B, Figure 8), while the primary binding site for mLIF on the mLIF-R α-chain (site A, Figure 8) is not present on the hLIF-R α-chain. The proposed model potentially explains the low-affinity binding of mLIF and hLIF to their isologous receptor α-chains, the inability of mLIF to bind to the hLIF-R α-chain, the extra interactions which must be involved in the unusual high-affinity binding of hLIF to the mLIF-R α-chain, and the apparent identity of the sites on the hLIF molecule that mediate both the high-affinity binding to the mLIF-R α-chain and binding to the hLIF-R α-chain. This appears to be the simplest model consistent with the data. Since both human and mouse LIF-R α-chains contain duplicated haemopoietin domains, more complex models of LIF ligand−receptor interactions are possible. These could include a situation where sites A and B are located in different haemopoietin domains on the receptor, or models involving ligand and/or receptor dimerization. Recent findings have suggested that homodimerization of gp130 occurs as a consequence of IL-6 binding to the IL-6-R α-chain (Davis and Yancopoulos, 1993) and that heterodimerization of the LIF-R and gp130 occurs upon ligand binding in the LIF and CNTF receptors (Ip *et al.*, 1992), implying that receptor dimerization is a common mechanism of activation for this family of receptors.

The ligands for the receptors of the haemopoietin or cytokine family display little homology in their primary amino acid sequences; however, they have been proposed to exhibit a common tertiary fold (Bazan, 1991; Parry *et al.*, 1991). Growth hormone (GH) (Abdel-Meguid *et al.*, 1987; de Vos *et al.*, 1992), IL-2 (Brandhuber *et al.*, 1987; Bazan, 1992), IL-4 (Powers *et al.*, 1992) and GM-CSF (Diederichs *et al.*, 1991) are known to form anti-parallel four α-helical bundle structures, a common motif for helical proteins, and other members of this family are modelled as having similar overall topologies (Bazan, 1990a).

The crystal structure of the GH/soluble GH receptor (sol

Table I. Construction of mouse–human chimeric LIF proteins

Hybrid	Amino acid sequence specifications
H	H1–180
M	M1–180
MH1	H1–102:M103–180
MH2	M1–102:H103–180
MH3	M1–160:H161–180
MH4	M1–109:H110–130:M131–180
MH5	M1–130:H131–160:M161–180
MH6	M1–109:H110–160:M161–180
MH7	M1–130:H131–180
MH8	M1–109:H110–180
MH9	M1–109:H110–130:M131–160:H161–180
MH10	M1–109:H110–167:M168:H169–180
MH11	M1–109:H110–111:M112:H113–180
MH12	M1–109:H110–112:M113:H114–180
MH13	M1–111:H112–113:M114–130:H131–160:M161–167:H168:M169–180
MH14	M1–111:H112–113:M114–130:H131–160:M161–180
MH15	M1–102:H103–160:M161–180

Amino acid sequences are designated according to the following example. M1–102:H103–180 (hybrid MH1) denotes that amino acid residues 1–102 (see Figure 2) are derived from mLIF sequence and amino acid residues 103–180 are derived from hLIF sequence.

GH-R) complex is the paradigm for cytokines and receptors of this family, and defines two receptor binding sites on the GH ligand. The first comprises the exposed face of the D-helix, the loop between helices A and B and some residues in the A-helix, while the second site comprises the exposed faces of the A-helix and the C-helix (de Vos *et al.*, 1992). Studies using mutagenesis, deletion analysis and neutralizing monoclonal antibodies for IL-3 (Lokker *et al.*, 1991a), IL-4 (Kruse *et al.*, 1992; Morrison and Leder, 1992), IL-5 (McKenzie *et al.*, 1991), IL-6 (Fiorillo *et al.*, 1992; Leebeek and Fowlkes, 1992; Leebeek *et al.*, 1992; Yasueda *et al.*, 1992), OSM (Kallestad *et al.*, 1991) and G-CSF (Kuga *et al.*, 1989) have defined the D-helix as being important for the interaction of each cytokine with either its low-affinity receptor (α-chain) or its receptor complex. GM-CSF, IL-3 and IL-5 are proposed to interact with a common receptor β-subunit through acidic residues in the A-helix, while GM-CSF is thought to bind to its unique receptor α-chain through the D-helix (reviewed in Kastelein and Shanafelt, 1993). The first or A-helix in these structures has also been implicated in the binding of prolactin (Luck *et al.*, 1989), IL-3 (Lokker *et al.*, 1991b), IL-4 (Morrison and Leder, 1992) and IL-6 (Fiorillo *et al.*, 1992) to their receptors. Interleukin-2 binds (i) to a non-haemopoietin receptor α-subunit through the B-helix and a helical region in the A–B loop; (ii) to a β-subunit, which is a member of the haemopoietin receptor family, through the A-helix; and (iii) to the recently described γ-subunit through the D-helix (reviewed in Bazan, 1992). The interferons and their receptors are distantly related to the haemopoietin family of cytokines and receptors, but are also thought to have similar secondary and tertiary structures (Bazan, 1990b). Interestingly, mouse interferon-β is proposed to bind to its receptor through the A–B loop and the C–D loop, which contains some helical structure (Senda *et al.*, 1992).

It has been proposed that most of the protein fold of a helical cytokine is a structural scaffold for presenting a common recognition site that varies between cytokines only in the shapes and sizes of the presented amino acid side chains. A unifying model of receptor–cytokine interaction has been suggested in which the generic structure of the cytokine or haemopoietin receptors (Bazan, 1990a) interacts with a structurally conserved recognition helix. The D-helix has been implicated as fulfilling this role in a range of cytokines (Bazan, 1990a) including IL-6 and OSM. The D-helix has no apparent role in the interaction described in this study (site b in Figure 8) although the close functional relationship between LIF, IL-6 and OSM suggests that it may be involved in the primary binding of mLIF to its own receptor (site a in Figure 8). The present study has not attempted to define the site on the LIF molecule that is involved in generating high-affinity binding by interacting with the gp130 molecule. Analogy with GM-CSF, IL-3 and IL-5 suggests that the A-helix may be involved in this interaction.

The LIF receptor α-chain-binding determinant on the hLIF ligand comprises several residues in the predicted C–D loop, up to three residues in the B–C loop and two residues in the C-helix. The predominant contribution to this interaction does not seem to be from a helical structure, suggesting that the receptor-binding sites on haemopoietin ligands are not entirely conserved. The proposed change in the LIF-R α-chain-binding site between mLIF and hLIF (Figure 8) suggests that receptor-binding sites may not even be conserved through the evolution of a particular cytokine. The definition of such binding sites in molecular detail should prove useful in refining our ideas of the structural paradigms for cytokine–receptor interactions and help in the design of specific agonists and antagonists of cytokine action.

Materials and methods

Construction of hybrid LIF proteins

Hybrid cDNAs, in which regions of mLIF sequence were replaced with homologous regions of hLIF sequence, were synthesized either from discrete restriction fragments of the two cDNAs (resulting in hybrids MH1 and MH2) or were generated by a PCR-based technique, splicing by overlap extension (Ho *et al.*, 1989), using *Pfu* polymerase (Stratagene) (hybrids MH3 to MH15). In order to facilitate cloning of the PCR-generated fragments into the bacterial expression vector pGEX-2TH, a *Bam*HI restriction site was introduced at the predicted translational start site and an *Eco*RI restriction site was inserted immediately after the stop codon of each cDNA. All constructs were sequenced in both directions using T7 DNA polymerase (Pharmacia) and a Promega dideoxy sequencing kit. The hybrid proteins

were constructed so that the amino terminus (+1, Figure 2) of each mature protein was at the serine at position 23 relative to the initiating methionine. An additional amino acid (glycine) is present at the amino terminus of each hybrid as the result of thrombin cleavage from the GST fusion protein. The exact specification of mLIF and hLIF sequences present in each hybrid is listed in Table I.

Protein expression and purification

All cDNAs were expressed as fusion proteins with glutathione S-transferase (GST) in *E.coli* NM522 except for MH11 (*E.coli* strain TOPP4, Stratagene). Growth and induction of transformants, lysis of cells, adsorption of fusion proteins to glutathione–Sepharose 4B (Pharmacia, Uppsala) and thrombin (Sigma) cleavage was carried out essentially as described (Smith and Johnson, 1988; Gearing *et al.*, 1989) except that cultures were induced by 50 μM IPTG in exponentially growing bacteria at 30°C rather than 37°C.

All hybrids were further purified on a 10 × 2 cm S-Sepharose Fast Flow column (Pharmacia, Uppsala) equilibrated in 50 mM sodium phosphate, pH 7.0. Elution was carried out with a linear gradient over 45 min of 0.2–0.45 M NaCl in the same buffer at a flow rate of 2.5 ml/min. The purity of each hybrid was assessed by its elution as a single peak from a 100 × 4.6 mm C8 reversed-phase HPLC column (Brownlee) using a linear gradient over 60 min of 0–100% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min and by electrophoresis on 15% SDS–polyacrylamide gels (Laemmli, 1970) in a Mini-Protean II system (Bio-Rad) followed by silver staining (Butcher and Tomkins, 1985). The concentration of protein in each preparation of purified hybrid was quantified by amino acid analysis on a Beckman amino acid analyser (model 6300) (Simpson *et al.*, 1986) with norleucine added as an internal standard in all samples.

Bioassay of mouse–human hybrid LIF proteins

The functional activity of the m-hLIF hybrids was assayed by their ability to induce differentiation in murine M1 leukaemic colonies. M1 differentiation assays were performed as described (Metcalf *et al.*, 1988). The specific activity of each hybrid was expressed as the units of M1 differentiation-inducing activity per milligram of protein.

For serum dose inhibition assays, the specific activity of each LIF preparation was determined by titration in cultures of M1 cells. Aliquots of normal mouse serum (containing ~5 μg/ml mLBP) were then added in serial 2-fold dilutions to cultures of M1 cells that also contained a just maximal concentration (200 U) of mLIF, hLIF or m-hLIF hybrid. Assays including mLIF and hLIF gave identical results when either crude mouse serum or a highly purified preparation of mLBP was used (Layton *et al.*, in preparation). Hybrids were assessed for mLIF or hLIF character from the dilution of serum required to block 50% of their M1 cell differentiation-inducing activity.

Radioiodination of ligands

Recombinant mLIF or hLIF (1–2 μg) produced in *E.coli* was purified and iodinated as previously described (Hilton *et al.*, 1988). The iodinated materials retained biological activity and had specific activities of 3.5–4.5 × 10^5 c.p.m./pmol for [^{125}I]mLIF and 4–8 × 10^5 c.p.m./pmol for [^{125}I]hLIF.

Binding of mouse–human hybrid LIF proteins to mLIF-binding protein

Normal mouse serum was used as a source of mLBP because the results of competitive binding experiments including unlabelled mLIF and hLIF were identical regardless of whether crude mouse serum or a highly purified preparation of mLBP was used (Layton *et al.*, in preparation). For competitive binding experiments, 50 μl aliquots of unlabelled LIF or an m-hLIF hybrid were added to 96-well filtration assay plates containing a 0.65 μm Durapore membrane (Millipore, MA) with 10 μl radiolabelled ligand, 20 μl aliquots of 1/10 or 1/20 dilution of normal mouse serum and 25 μl Concanavalin A–Sepharose 4B (ConA–Sepharose, Pharmacia, Uppsala) diluted 1:4 in 100 mM sodium acetate, pH 6.0, containing 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The assays were agitated overnight at room temperature. Bound and free radioactivity were separated by vacuum filtration of the supernatant and the ConA–Sepharose pellet containing the bound fraction was washed once with 200 μl cold phosphate-buffered saline (PBS). Assay plates containing the Sepharose pellets were then exposed to a phosphor screen (Molecular Dynamics, CA) for 16–24 h and the results quantified using Imagequant version 3.0 software (Molecular Dynamics).

The plots generated were used to calculate the concentrations of hLIF, mLIF or m-hLIF hybrid required to inhibit 50% of [^{125}I]hLIF binding to mLBP (ID_{50}). Human LIF and mLIF were defined as having 100% and 0% hLIF-like binding activity respectively. In order to assign a convenient score for hLIF-specific activity of each hybrid, a logarithmic scale was used to convert the ID_{50} of a given hybrid to a percentage hLIF binding activity according the following formula:

$$\% \text{ human} = \frac{\log ID_{50}(\text{mLIF}) - \log ID_{50}(\text{hybrid})}{\log ID_{50}(\text{mLIF}) - \log ID_{50}(\text{hLIF})} \times 100\%$$

where the ID_{50} values for inhibition of [^{125}I]hLIF binding to mLBP by mLIF, hLIF and a given hybrid are expressed in units of μg/ml. The values obtained were derived from at least two independent assays, except for hybrids MH9 and MH15.

Binding of the mouse–human LIF hybrids to COS cells transfected with a hLIF-R cDNA clone

A 4.1 kb cDNA encoding the hLIF-R α-chain was isolated from a fetal liver cDNA library (Clonetech) by plaque hybridization with a ^{32}P-radio-labelled DNA fragment corresponding to amino acid residues 13–177. The nucleotide sequence corresponding to the coding region of this cDNA was determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using synthetic oligonucleotide primers. With the exception of a silent T to C nucleotide substitution at the third position of codon 555, the sequence of this cDNA was identical to that previously reported (Gearing *et al.*, 1991). The hLIF-R cDNA was subcloned into the mammalian expression vector pCDM8 (Seed, 1987) and designated pCDM8/16C.

Transient expression of the hLIF-R α-chain in COS cells was achieved by electroporation with plasmid DNA. Briefly, 2 × 10^7 cells were harvested in 0.8 ml of PBS, mixed with 20 μg of pCDM8/16C DNA at 4°C and subjected to electroporation at 300 V and 500 μF. Viable cells were harvested by centrifugation through a cushion of fetal calf serum (FCS) and incubated in 50 ml HEPES-buffered RPMI medium containing 10% FCS (HRF) at 37°C in an atmosphere of 10% carbon dioxide. Seventy-two hours post-transfection, cells were detached in HRF containing 0.2 M EDTA and 0.1 mg/ml chondroitin sulphate, and harvested by centrifugation.

For competitive binding experiments, 20 μl aliquots of cells, resuspended in HRF at 5 × 10^5–8 × 10^5 cells/ml, were added to 96-well filtration assay plates containing a 0.65 μm Durapore membrane (Millipore, MA) with 50 μl unlabelled LIF or m-hLIF hybrid and 25 000 c.p.m. [^{125}I]hLIF in 10 μl and incubated overnight at 4°C. Bound and free radioactivity were separated by vacuum filtration of the supernatant and the cell pellet containing the bound fraction was washed once with 200 μl cold PBS. Assay plates containing the cell pellet were then exposed to a phosphor screen and the results quantified as previously described.

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