Human leukemia inhibitory factor (hLIF) binds to both human and mouse LIF receptors (LIFRs), while mouse LIF (mLIF) binds only to mouse LIFRs. Furthermore, hLIF binds with much higher affinity to the mouse LIFR (mLIFR) α-chain than does mLIF itself. To define the structural elements of the mLIFR α-chain conferring high affinity binding of hLIF and the species-specific interaction with mLIF, we first constructed C-terminally truncated extracellular domains of both the mLIFR and the human LIFR (hLIFR) α-chains, which contained only the two hemopoietin domains separated by an immunoglobulin-like domain. These recombinant truncated LIFR α-chains had identical binding and biological characteristics to either their naturally occurring or transfected counterparts. On the basis of this, we have generated eight interspecies receptor chimeras by combining different regions of the mouse and human LIFR sequence. Surprisingly, the immunoglobulin-like domain of the mLIFR α-chain played the predominant role in receptor-ligand interactions. Moreover, both high affinity binding for hLIF and the species-specific binding for mLIF mapped to the same domain of mLIFR molecule. These findings should enable the development of a “humanized” mouse LIFR that could act as a potent antagonist of hLIF biological activities in vivo.
Surprisingly, hLIF bound to the mLIF receptor (mLIFR) α-chain with a much higher affinity (Kₐ ~ 10–20 pM) than it did to the isologous human LIF receptor (hLIFR) α-chain or than mLIF binding to the mLIFR α-chain. Cross-competition studies using the mLIFR α-chain revealed that the competition curves were dependent on which LIF was used as the radioactive tracer, and this behavior was interpreted as an interference by each type of LIF in the binding of the other.

A model was proposed to rationalize these complex interactions. In this model, hLIF is bound to both mouse and human LIF receptors through two different sites, a primary binding site and a secondary site induced by ligand-dependent receptor isomerization. The overall binding affinity and the presence or absence of two kinetic sites were determined by the isomerization equilibrium, with rapid isomerization favoring high affinity and single-site kinetics, while slow isomerization favored low affinity and two-site kinetics. Ligand interference in this model could be explained by the ability of mLIF or hLIF to favor different isomerization states of the mLIFR α-chain (Fig. 1).

Recently, we have exploited this unusual cross-species reactivity to map the binding epitope on hLIF that is responsible, on the one hand, for binding to the hLIFR α-chain and, on the other, for binding with high affinity to the mLIFR α-chain (22, 23). These studies identified an identical set of six amino acids in hLIF, suggesting that the site B shown in Fig. 1 for interaction with the mLIFR α-chain is the same site as hLIF used as the primary binding site for the hLIFR α-chain.

A soluble form of the mLIFR α-chain occurs at high levels (2 μg/ml) in normal mouse serum and is dramatically elevated in pregnancy (24, 25). The very high binding affinity of this receptor for hLIF makes it a potent biological inhibitor of hLIF (21) and suggests that it could be useful in clinical situations like inflammatory disease where LIF levels are expected to be elevated.

The present studies were undertaken to identify the region of the mLIFR α-chain responsible for high affinity interactions with hLIF. Again, we have taken advantage of the structural homology of mouse and human LIF receptors and their different binding characteristics for mouse and human LIF to generate interspecies receptor chimeras. These studies should help to define the structural elements involved in LIF binding and in developing a “humanized” mouse LIFR α-chain that retains its high affinity binding to hLIF as a possible therapeutic agent.

**EXPERIMENTAL PROCEDURES**

**Construction of Soluble LIFRs and Hybrid mhLIFR cDNAs**—A cDNA encoding a soluble mouse LIFR α-chain was a gift from Dr. D. J. Hilton of the Walter and Eliza Hall Institute of Medical Research. The 5'-end was modified to encode an XhoI site and an in frame 12CA5 epitope. The 3'-end of the mLIFR cDNA was modified to encode an XbaI site, and a stop codon was introduced after amino acid residue 531 in the amino acid sequence described in Ref. 13. A cDNA encoding the hLIFR α-chain (22) was also altered at its 5'-end to encode an XhoI site and an in frame 12CA5 epitope (YPYDVPDYA) (26). The sequence at the N terminus of the recombinant mLIFR was GVQYPYDVPDYA, and the sequence at the N terminus of the recombinant hLIFR was GAPYPYDVPDYA. The 3'-end was also modified to encode an XbaI site, and a stop codon was introduced after position 536 in the amino acid sequence described by Gearing et al. (13). The recombinant LIFRs therefore lacked the cytoplasmic domain, transmembrane domain, and all three FNIII-like domains. The resulting cDNAs were subsequently ligated into the Pichia pastoris expression vector pPIC9, which carried a XhoI and AvrII site, as XhoI-XbaI fragments. Mutagenesis of the LIFR cDNAs and construction of hybrid mouse-human LIFRs were carried out using a polymerase chain reaction-based technique, splicing by overlap extension (27), and Pfu polymerase (Stratagene).

The nucleotide sequences of the resulting constructs were confirmed by dye-deoxy sequencing (28) using either a PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit on an Applied Biosystems 373a DNA sequencer or a T7-based Pharmacia Dideoxy sequencing kit.

**Expression of Soluble LIFRs in P. pastoris**—All cDNAs were expressed as soluble secreted proteins in the methylotrophic yeast *P. pastoris*. This expression system uses the promoter from the methanol-induced alcohol oxidase gene, *AOX1*. Stably expressing clones are selected using the HIs4 gene as a selectable marker. The recombinant plasmids were digested with either *Bgl*II or *Sal*I and integrated into host cells by transforming *his4* (GS115) *P. pastoris* spheroplasts as described (29). Digestion of a plasmid with *Bgl*II disrupts the *AOX1* gene and results in a strain that is phenotypically His⁺ Mut⁻ (methanol utilization sensitive). Because plasmids MH111LIFR, MH31LIFR, MH55LIFR, and MH77LIFR contained *Bgl*II sites, they were digested with *Sal*I prior to transformation into *P. pastoris* spheroplasts. The resulting strains were His⁻ Mut⁺. His⁺ transformants were patched first onto a nitrocellulose filter overlaid onto an agar plate (MM) containing 0.5% methanol, 1.34% yeast nitrogen base, and 4 x 10⁻⁵ M biotin and then onto another agar plate (MD) containing 1% dextrose instead of methanol as the carbon source. The plates were incubated at 30 °C. After 48 h, the clones on the MD agar plate were placed at 4 °C. The nitrocellulose filters containing the His⁺ transformants were then lifted off the MM plates and incubated in 10% (w/v) skim milk powder.
in PBS. Colonies that expressed recombinant LIF receptors were then detected using a 12CA5 antibody. Clones identified in this way were grown in a shaking incubator at 30 °C with an A600 of 2–6 in 10 ml of medium containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6), 1.34% yeast nitrogen base, 4 × 10–4% C10, and 1% glycerol for 2–5 days. After incubation, 100 μl of culture medium was centrifuged, the supernatants were removed, and bound and free 125I-hLIF were separated as described previously (21). Scatchard analyses of saturation binding isotherms were performed using the curve-fitting program LIGAND (31, 32).

Experiments to determine the kinetic dissociation rate (koff) of the recombinant receptors were performed in duplicate using 125I-hLIF at a final concentration of 106 cpm/60 μl in the presence and absence of 8 μg/ml unlabeled hLIF. When the specific interaction had reached an equilibrium, the precipitated receptor complexes were collected by centrifugation (3 × 30 min) and dissolved in 40 μl of SDS sample buffer. The cross-linked proteins were analyzed by 10% PAGE and then transferred to a prewetted polyvinylidene difluoride (PVDF-Plus, Micron Separations Inc.) membrane using a transfer apparatus (33). Proteins separated by SDS-PAGE were electroblotted onto nitrocellulose membranes (Bio-Rad). Blots were blocked in 1% bovine serum albumin (w/v) in PBS containing no sodium azide, washed with PBS, and incubated with mouse 12CA5 antibody and then horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako, Denmark). The receptor proteins were visualized using ECL substrate kit (Amersham Corp.) followed by autoradiography.

We have previously reported that mLIF binds to the mLIFR α-chain with low affinity (Kd of 1–4 nM) but does not detectably interact with the hLIFR α-chain (22, 23). Human LIF binds to the hLIFR α-chain with low affinity (Kd of 0.3–1 nM), but it is also able to bind to the mLIFR α-chain and does so with a much higher affinity (Kd of 10–20 pM) than mLIF. The higher affinity binding of hLIF to the mLIFR α-chain was found to be due almost exclusively to a slower kinetic dissociation rate compared with mLIF. There were thus several different characteristics that a LIF receptor that was composed of a combination of mouse and human sequence might acquire. Any combination of mouse and human LIFR sequence should, however, result in a hybrid molecule that is at least able to bind 125I-hLIF with low affinity. To assess the relative contributions of the different mouse or human LIFR receptor domains to these different features, we constructed a series of hybrid mouse-human LIF receptors in which the two hemopoietin domains and the Ig-like domain were present in various combinations of mouse or human LIFR sequence and expressed them as soluble recombinant proteins (Fig. 2). The boundaries between the different domains were delineated as described by Gearing et al. (13).

### RESULTS

We have previously reported that mLIF binds to the mLIFR α-chain with low affinity (Kd of 1–4 nM) but does not detectably interact with the hLIFR α-chain (22, 23). Human LIF binds to the hLIFR α-chain with low affinity (Kd of 0.3–1 nM), but it is also able to bind to the mLIFR α-chain and does so with a much higher affinity (Kd of 10–20 pM) than mLIF. The higher affinity binding of hLIF to the mLIFR α-chain was found to be due almost exclusively to a slower kinetic dissociation rate compared with mLIF. There were thus several different characteristics that a LIF receptor that was composed of a combination of mouse and human sequence might acquire. Any combination of mouse and human LIFR sequence should, however, result in a hybrid molecule that is at least able to bind 125I-hLIF with low affinity. To assess the relative contributions of the different mouse or human LIFR receptor domains to these different features, we constructed a series of hybrid mouse-human LIF receptors in which the two hemopoietin domains and the Ig-like domain were present in various combinations of mouse or human LIFR sequence and expressed them as soluble recombinant proteins (Fig. 2). The boundaries between the different domains were delineated as described by Gearing et al. (13).
Construction and Expression of Recombinant mLIFR, hLIFR, and Hybrid mLIFR—We initially expressed mouse LIFR and human LIFR as soluble proteins that were truncated 13 amino acid residues after the predicted membrane proximal hemopoietin domain. These receptors therefore did not contain the cytoplasmic domain, the transmembrane domain, and all three fibronectin type III repeats that are present in native cellular LIF receptors. The recombinant proteins were modified at their N termini to encode a 12CA5 epitope tag to monitor their expression and retained the yeast α-factor signal peptide to enable the proteins to be secreted into the culture medium after transformation into yeast. The molecular weight of these recombinant proteins is predicted to be approximately 65 kDa. Scatchard analysis of $^{125}$I-mLIF to the recombinant mLIFR α-chain showed a single class of mLIF binding site ($K_d$ of 6–7 nM), which is essentially the same as that for mLBP ($K_d$ of 1–4 nM) and the low affinity mLIFR formed by detergent solubilization of mLIFRs present on liver membranes or activated macrophages (34). The binding of $^{125}$I-hLIF to the recombinant soluble mLIFR α-chain displayed a $K_d$ value of 10–46 pM. Both of these results are essentially identical to the values obtained previously (21). The binding of $^{125}$I-hLIF to the recombinant soluble hLIFR α-chain showed a $K_d$ value of 0.3–0.93 nM, which again was in the normal range for $^{125}$I-hLIF binding (Table I). These results indicated that the minimum ligand-binding domain of both the human and mouse LIFR α-chains is composed of the membrane-distal and membrane-proximal hemopoietin domains plus the intervening Ig-like domain.

As shown in Fig. 3A, several bands could be detected in the expression medium of the majority of the transformant clones. There was no reaction with Fichia supernatants from transformants expressing unrelated proteins (data not shown), so all bands represent receptor fragments. In addition to differentially glycosylated forms of receptor, the extra bands with molecular masses below 60 kDa were present early in the time course of expression and also in the cell lysate (data not shown), suggesting that they may represent prematurely terminated translation products or are more likely to be proteolytically degraded products. Hybrid MH5LIFR is not shown because the expression levels were too low to be detected.

Chemical cross-linking (Fig. 3B) of the soluble receptor variants with $^{125}$I-hLIF demonstrated that only the species with molecular masses higher than 70 kDa could specifically interact with $^{125}$I-hLIF. The receptor-ligand complexes detected by chemical cross-linking as shown in Fig. 3B have a molecular mass between 100 and 140 kDa. Considering that the apparent molecular mass of hLIF on SDS-PAGE is 20–30 kDa, the apparent molecular mass of the active receptor molecules ranges from 70 to 110 kDa. Furthermore, the position of the $^{125}$I-hLIF binding peak at 70–100 kDa (Fig. 3C) by size exclusion chromatography of soluble receptor samples indicated that the hybrid LIFRs have an apparent molecular mass of 70–100 kDa and exist as monomers. Products with a molecular mass smaller than 70 kDa (Fig. 3A) did not specifically bind hLIF and hence are inactive. The binding kinetics therefore only refer to the full-length receptor mutants and are not affected by potential binding to proteolytic fragments.

The expression levels of the different receptors were variable, ranging from 10 μg to 1 mg of receptor protein/liter of expression medium as determined by Scatchard analysis. Hybrid LIFRs MH4 and MH5 were found to be difficult to detect by Western blot analysis, which may be due to either extremely low expression levels or cleavage of the 12CA5 epitope tag during protein production. However, the behavior of these two hybrid receptors was similar to that of the other recombinant receptors with respect to both chemical cross-linking with $^{125}$I-hLIF and size exclusion chromatography.

The Mouse LIFR Immunoglobulin-like Domain Determines High Affinity hLIF Binding to Hybrid LIF Receptors—The mouse LIFR α-chain binds hLIF with high affinity, whereas the human LIFR α-chain binds hLIF with low affinity (21). The hybrid LIFR receptors were characterized by performing binding assays and subsequent Scatchard analyses to determine their affinities of interaction with $^{125}$I-hLIF. As shown in Fig. 4 and Table I, the recombinant mouse and human LIFRs had $K_d$ values of 10–46 pM and 0.3–0.9 nM, respectively, which were similar to those observed for both the naturally occurring soluble mouse LIF receptor and a soluble form of receptor α-chain expressed in COS cell-conditioned medium (21).

Hybrids MH3LIFR, MH4LIFR, and MH5LIFR all contain an intact Ig-like domain from mouse LIF receptor but have either one hemopoietin domain (MH3LIFR and MH4LIFR) or two hemopoietin domains (MH5LIFR) from the human LIF receptor. Surprisingly, all of these three hybrids acquired high affinity $^{125}$I-hLIF binding ($K_d$ of 11–60 pM) similar to that seen for hLIF binding to the mLIFR (Fig. 4, Table I). This strongly suggested that the immunoglobulin-like domain from the mouse LIF receptor has the most important influence in conferring the high affinity binding of hLIF.

### Table I

| Receptor  | $^{125}$I-hLIF | $^{125}$I-mLIF | $K_d$ | $k_{off}$ | 1/$K_d$/Fast | 1/$K_d$/Slow | ID$_{50}$/hLIF | ID$_{50}$/mLIF |
|-----------|----------------|----------------|-------|-----------|-------------|-------------|-------------|-------------|
| mLIFR     | 10–46          | 6 nM           |       |           | 0.001       | 0.01        | 0.016       | 32          |
| MH1LIFR   | 190–400        | ND             |       |           | 0.03        | 0.001       | 0.20        | 40          |
| MH2LIFR   | 150–440        | ND             |       |           | 0.04        | 0.001       | 0.20        | 59          |
| MH3LIFR   | 11–60          | ND             |       |           | 0.02        | 0.1         | 0.018       | 54          |
| MH4LIFR   | 11–58          | ND             |       |           | 0.02        | 0.1         | 0.014       | 36          |
| MH5LIFR   | 13–61          | ND             |       |           | 0.02        | 0.1         | 0.018       | 28          |
| MH6LIFR   | 158–380        | ND             |       |           | 0.05        | 0.001       | 0.28        | 0.048       | ND          |
| MH7LIFR   | 230–455        | ND             |       |           | 0.07        | 0.001       | 0.70        | 0.034       | ND          |
| MH8LIFR   | 700–2600       | ND             |       |           | 0.2         | 0.002       | 0.80        | 0.089       | ND          |
| hLIFR     | 300–900        | ND             |       |           | 0.16        | 0.002       | 0.52        | 0.250       | ND          |

* These estimates of $K_d$ values are from 2–6 experiments.
* The $k_{off}$ values are the average of at least two experiments.
* Fast dissociation rate (±50%).
* Slow dissociation rate (±50%).
* $1/K_d$, isomerization constant, is the ratio of the fast dissociating form (F) over the slow dissociating form (S) at equilibrium.
* ID$_{50}$ values (±20%) are the average of at least two experiments.
* ND, not detectable.
In hybrid MH1LIFR, the N-terminal region, to approximately halfway down the Ig-like domain, was composed of hLIFR residues, and the C-terminal half was composed of mLIFR residues, while hybrid MH2LIFR was the converse. When these recombinant hybrid LIF receptors were tested for binding of $^{125}$I-hLIF by Scatchard analysis, both had intermediate affinities ($K_d$ of $190-400$ pM and $150-440$ pM, respectively) (Fig. 4, Table I).

The relative contributions of the membrane-distal and membrane-proximal domains from the mLIFR to $^{125}$I-hLIF binding were investigated next. Hybrid MH6LIFR was composed almost entirely of mLIFR residues, except that the Ig-like domain was derived from the hLIFR and it bound $^{125}$I-hLIF with intermediate affinity ($K_d$ of $260$ pM). MH7LIFR, in which only the membrane-proximal hemopoietin domain was composed of mLIFR residues, also bound $^{125}$I-hLIF with intermediate affinity ($K_d$ of $300$ pM) (Fig. 4, Table I). This result indicated that of the two mLIFR hemopoietin domains the major contribution...
Ligand Binding Determinants on the LIF Receptor

Fig. 4. Scatchard analyses of $^{125}$I-hLIF binding to chimeric LIF receptor variants. Saturation binding was performed by incubating aliquots of P. pastoris culture supernatants containing recombinant LIF receptors with increasing concentrations of $^{125}$I-hLIF. Specific binding assays and Scatchard transformations were performed as described under "Experimental Procedures." These Scatchard binding data are representative for several independently performed experiments, and the resulting $K_d$ values are shown in Table I. The receptor variants are shown in each panel.

Fig. 5. Kinetic dissociation of $^{125}$I-hLIF from chimeric LIFRs. Each chimeric LIFR (0.01–0.02 nM) was incubated at room temperature for 3–4 h with $^{125}$I-hLIF, and kinetic dissociation assays were performed as described under "Experimental Procedures." The plot of the natural log of the ratio of the amount of $^{125}$I-hLIF remaining bound after a given time ($SB_t$) to the amount bound initially ($SB_0$) versus time is shown. Estimates of the kinetic rate constant governing dissociation ($k_{off}$) of ligand and receptor were made using the curve-fitting program KINETIC and shown in Table I. The receptor variants are shown in each panel.

to high affinity $^{125}$I-hLIF binding was from the membrane-proximal hemopoietin domain. MH8LIFR had an almost identical affinity for $^{125}$I-hLIF to the hLIFR ($K_d$ of ~$2$ nM), indicating that the mouse LIFR membrane-distal hemopoietin domain is not involved in high affinity $^{125}$I-hLIF binding (Fig. 4, Table I).

Interestingly, when either mLIFR hemopoietin domain was present in conjunction with the mouse LIFR Ig-like domain, as in hybrids MH3LIFR and MH4LIFR, there was no increase in affinity for $^{125}$I-hLIF when compared with hybrid MH5LIFR, which had only the mouse LIFR Ig-like domain. A comparison of hybrid MH5LIFR and the hLIFR shows that simply swapping the hLIFR Ig-like domain results in binding of hLIF that is similar to the mLIFR. The reverse hybrid, MH6LIFR, shows binding close to that of hLIF to the hLIFR, further suggesting that the Ig-like domain from the mouse LIFR plays the dominant role in determining the high affinity binding for hLIF (Fig. 4, Table I). The presence of the membrane-distal hemopoietin domain from mouse LIFR had no effect in increasing the affinity of $^{125}$I-hLIF binding, as indicated by the similar $K_d$ values of hybrid MH6LIFR and MH7LIFR on the one hand and hybrid MH8LIFR and the hLIFR on the other (Fig. 4, Table I).

The difference in hLIF binding affinities of chimeric LIFRs was further explored by performing kinetic dissociation experiments (Fig. 5). The LIF receptor variants, which had high affinity binding for hLIF based on Scatchard analysis, including mLIFR, MH3LIFR, MH4LIFR, and MH5LIFR hybrid receptors, showed single slow dissociation rates ($k_{off}$ of ~$0.001–0.002$ min$^{-1}$) (Fig. 5, Table I). The receptors with low affinity hLIF binding, such as MH8LIFR and hLIFR, clearly exhibited biphasic dissociation kinetics, with one phase being rapid ($k_{off}$ of ~$0.16–0.2$ min$^{-1}$) and the other slow ($k_{off}$ of ~$0.002$ min$^{-1}$). In the receptor variants (MH1LIFR, MH2LIFR, MH6LIFR, and MH7LIFR) that had intermediate hLIF binding affinity, curvilinear kinetic dissociation curves were observed to a lesser extent, which comprised a slow dissociation rate ($k_{off}$ of ~$0.001$ min$^{-1}$) and a fast dissociation rate ($k_{off}$ of ~$0.03–0.07$ min$^{-1}$) (Fig. 5, Table I).

Binding of mLIF to Mouse-Human Hybrid LIF Receptors—The binding of mLIF and hLIF to each of the hybrid receptors was also evaluated by performing competitive inhibition assays. When $^{125}$I-hLIF was used as a tracer, mLIF was able to compete with $^{125}$I-hLIF for binding only on hybrid receptors that contained either an intact mLIFR Ig-like domain (hybrids MH3LIFR, MH4LIFR, and MH5LIFR) or part of an mLIFR Ig-like domain (hybrids MH1LIFR and MH2LIFR) (Fig. 6). The ID$_{50}$ values for either hLIF or mLIF competing with $^{125}$I-hLIF binding to these hybrid receptors were essentially the same. However, the effective concentration of mLIF required to displace $^{125}$I-hLIF bound to these receptors was 2000–3000-fold higher than that of hLIF (Fig. 6, Table I). $^{125}$I-mLIF was able to detectably bind to MH3LIFR, MH4LIFR, and MH5LIFR but only at 10–50-fold higher receptor concentrations compared with those used for $^{125}$I-hLIF binding (data not shown).

In those receptors that did not contain the mLIFR Ig-like domain, including hLIFR and hybrid receptors MH6LIFR, MH7LIFR, and MH8LIFR, mLIF was unable to compete with $^{125}$I-hLIF even at high ligand concentrations (100 μg/ml). The ID$_{50}$ values for hLIF competing with $^{125}$I-hLIF bound to these receptors were 2–10-fold higher compared with that obtained with the mLIFR. This is essentially consistent with the $K_d$
values obtained from the Scatchard analyses (Table I). These data indicate that the mouse LIFR Ig-like domain was primarily responsible for the species-specific interaction of mLIF with the mLIFR.

Blocking of M1 Differentiation-inducing Activity of hLIF by Recombinant LIF Receptors—The naturally occurring mLBP is able to block the M1 cell differentiation-inducing activity of LIF by competing for free LIF with the cellular receptor (24). To investigate the potential of the chimeric LIFRs to function as antagonists of LIF biological action, M1 agar assays, in the presence of either hLIF itself, hLIF plus each chimeric LIFR, or each chimeric LIFR alone were performed. The results from these assays demonstrated that recombinant mLIFR was indistinguishable from mLBP in blocking the ability of hLIF to induce M1 differentiation with an ID50 value of 1.2–3 ng/ml (Fig. 7, Table II). Hybrid MH6 and MH7 LIFRs also inhibited hLIF-induced M1 differentiation but at a concentration 50–100-fold higher than that seen for mLIFR. Surprisingly, hybrids MH3LIFR, MH4LIFR, and MH5LIFR, which displayed high affinity binding for hLIF, exhibited very weak or no inhibition on hLIF-induced M1 cell differentiation (Fig. 7, Table II).

Blocking of hLIF-induced STAT3 Phosphorylation—Because the inhibitory activities of the chimeric receptors in the 7-day M1 differentiation assays did not correlate with their hLIF binding affinities, we reasoned that receptor degradation in the bioassay may be occurring. Consequently, a short term assay was employed that involved stimulation of STAT3 tyrosine phosphorylation by hLIF in M1 cells. STAT3 activation is a critical step in gp130-mediated terminal differentiation of M1 cells (35), and as shown in Fig. 8, tyrosine phosphorylation of STAT3 was dramatically increased by hLIF stimulation of M1 cells within 5 min. This STAT3 phosphorylation was almost completely blocked by preincubation of hLIF with recombinant mouse LIFR and hybrid MH3LIFR (Fig. 8, Table II). In the same experiment, hybrids MH4LIFR, MH5LIFR, and MH6LIFR also showed a moderately inhibitory effect (65%) on hLIF-induced STAT3 phosphorylation, although it was not as significant as that seen for mLIFR and MH3LIFR. The same applied to hybrids MH1LIFR, MH2LIFR, and MH7LIFR but to a lesser extent. Little or no inhibition of hLIF-stimulated STAT3 phosphorylation was observed for both MH8LIFR and hLIFR, which could be correlated with their low binding affinity for hLIF. STAT3 phosphorylation in M1 cells was not affected by the addition of chimeric LIFRs alone (Fig. 8, Table II).

DISCUSSION

The mouse and human LIFRs share 76% amino acid identity (13), while the ligands are 78% identical (36). Despite this high
FIG. 7. Blocking of induction of differentiation in hLIF-stimulated colonies of M1 leukemic cells by chimeric LIF receptor variants. Either P. pastoris culture supernatants (mLIFR and MH6LIFR) or concentrated fraction pools from Superoxide-12 columns (other receptor variants) were desalted and sterilized as described under “Experimental Procedures.” The concentration of each receptor preparation was determined by Scatchard analysis. Serial 2-fold dilutions of each preparation were added to cultures of M1 cells that had a maximal induction of differentiation by hLIF (78 units/ml). Normal mouse serum (containing approximately 2 μg/ml of mLBP) was used as a positive control. The receptor variants are as follows: mLIFR (●), MH1LIFR (■); MH2LIFR (▲); MH3LIFR (○); MH4LIFR (□); MH5LIFR (●); MH6LIFR (▲); MH7LIFR (●); MH8LIFR (black ellipse); hLIFR (black rectangle) and normal mouse serum (○).

The binding affinity for hLIF was most strongly dependent on the presence of an intact mLIFR Ig-like domain irrespective of the species origin of the two hemopoietin domains. However, in the context of a hLIFR Ig-like domain, the species origin of the membrane-proximal hemopoietin domain was more important than the distal hemopoietin domain in determining hLIF binding affinity.

2) In all cases where high affinity binding of hLIF was observed, the dissociation kinetics were predominantly a single class with a slow dissociation rate (off rate). In cases where intermediate or low affinity binding of hLIF was observed, the dissociation kinetics were biphasic with a variable ratio of fast off and slow off components, depending on the affinity. Kinetic association rates (on rates) were similar for all receptor chimeras (data not shown).

3) The ability of mLIF to compete for hLIF binding (albeit weakly) was also strongly correlated with the presence of mLIFR Ig-like domain sequences in the receptor chimera. However, in this case, even half of a mLIFR Ig-like domain (either N- or C-terminal) was sufficient to allow for competition, and there was no apparent contribution from the membrane-proximal hemopoietin domain.

These data can best be rationalized by a model of the LIF receptor in which there are two potential ligand contact sites on the receptor but for both contacts to be made a ligand-dependent receptor isomerization step has to occur (Fig. 1). For hLIF binding to the hLIFR, isomerization is inefficient, resulting in two kinetically distinguishable bound states of the receptor (one- or two-contact), but for hLIF binding to the mLIFR, isomerization to the two contact state is nearly complete, giving rise to high affinity and a single kinetic dissociation rate. Analysis of this model for the binding of LIF to its receptor at equilibrium gives the following equation in the Scatchard transformation (40),

\[ B = (K_i + K_iK_h)R_T - B \]  

where \( B \) is the specifically bound LIF concentration, \( F \) is the free LIF concentration, \( R_T \) is the total concentration of LIF receptors, \( K_i \) is the equilibrium affinity constant for the first contact site of LIF with its receptor, and \( K_h \) is the equilibrium isomerization constant for receptor isomerization to form the second contact with LIF.

The form of this equation shows that, regardless of the value of \( K_h \), Scatchard plots of LIF equilibrium binding data will all be apparent one-site linear curves (see Fig. 4). However, the slopes of such curves will not be true affinity constants but the combined constants \( K_i + K_h \).

If this model is correct, the kinetic data suggest that \( K_i \) is not drastically different for any of the chimeras, since \( k_{on} \) and \( k_{off} \) (the fast off rate) are nearly the same and that the primary effect of domain exchange is to alter the receptor isomerization equilibrium via \( K_h \). The predominant effect of the intervening Ig-like domain can then be seen as regulating receptor isomerization, with the mLIFR Ig-like domain promoting receptor isomerization and the hLIFR Ig domain retarding it. Since the Ig-like domain bridges the two hemopoietin domains, it may serve as a flexible hinge that allows first the proximal hemopoietin domain to form site 1 interactions with LIF and then the distal hemopoietin domain to form site 2 interactions. Since hLIFR has a value of \( K_i + K_hK_h \) of \(-1.7\ nM^{-1} \) (1/0.6) and a ratio of fast over slow dissociating sites of \(-0.5 (K_i - 2) \) for hLIF binding, the value for \( K_h \) should be about 0.6 \( nM^{-1} \) (1/1.7 nM). If this is held constant, then the calculated \( K \) values for mLIFR are about 100, and for intermediate affinity receptors the \( K_i \) values are about 7. This agrees approximately with the rate of slow to fast off rates determined experimentally (Fig. 4, Table I).

In view of the demonstration that the ligand binding site in the related growth hormone receptor is exclusively in the hemopoietin domain (41), the most unexpected observation in this study was the predominant involvement of the Ig-like domain (between the two hemopoietin domains of the mLIFR) in determining ligand binding specificity and conferring high affinity hLIF binding. However, Ig-like domains are commonly involved in ligand recognition (42) and have been shown to be directly involved in ligand binding by tyrosine kinase receptors, e.g. neurotrophin, platelet-derived growth factor, and macrophage colony-stimulating growth factor (43–45). Similarly for...
TABLE II

| Receptor          | $K_d$ | Concentration$^a$ | Blocking of M1 cell differentiation$^b$ | ID$_{50}$ | Blocking of STAT3 phosphorylation$^d$ |
|-------------------|-------|-------------------|----------------------------------------|----------|---------------------------------------|
|                   | pm    | ng/ml             | %                                      | ng/ml    | %                                     |
| mLIFR             | 10–46 | 150               | 100                                    | 1.2–3.0  | 96                                    |
| MH1LIFR           | 190–400 | 140              | 20                                     | ND$^c$   | 47                                    |
| MH2LIFR           | 150–440 | 250              | 45                                     | ND       | 39                                    |
| MH3LIFR           | 11–60  | 90                | 10                                     | ND       | 95                                    |
| MH4LIFR           | 11–58  | 90                | 10                                     | ND       | 12                                    |
| MH5LIFR           | 13–61  | 60                | 0                                      | ND       | 62                                    |
| MH6LIFR           | 158–380 | 875              | 100                                    | 110      | 63                                    |
| MH7LIFR           | 230–545 | 150              | 65                                     | 100      | 52                                    |
| MH8LIFR           | 700–2800 | 400             | 0                                      | ND       | 12                                    |
| hLIFR             | 300–900 | 150              | 5                                      | ND       | 19                                    |
| Mouse serum       | 10–20  | 2000              | 100                                    | <2.4     | NT$^f$                                |

$^a$ The concentration of each receptor sample was determined by Scatchard analysis.

$^b$ Maximum percentage inhibition of hLIF-induced M1 cell differentiation at the receptor concentration listed.

$^c$ ID$_{50}$ values were obtained from Fig. 7.

$^d$ Percentage inhibition of hLIF-induced STAT3 phosphorylation was quantitated by densitometric analysis of the band intensities from Fig. 8.

$^e$ ND, not detectable.

$^f$ NT, not tested.

FIG. 8. Effect of chimeric LIFRs on hLIF-induced STAT3 tyrosine phosphorylation. M1 cells were incubated at 37 °C for 5 min in the presence of 1 ng of hLIF, 1 ng of hLIF together with 11 ng of chimeric LIFR, or 11 ng of chimeric LIFR alone and analyzed by immunoprecipitation and Western blotting as described under “Experimental Procedures”.

other receptors more closely related to the LIFR, it has been shown that the Ig-like domain of the G-CSF receptor makes significant contributions to high affinity binding of G-CSF (19, 46) and that the Ig-like N-terminal domain of the interleukin-6 receptor determines the species specificity of IL-6 binding (47). Consequently, we cannot rule out the possibility that the contribution of the Ig-like domain to the high affinity binding of hLIF may also involve direct contact of hLIF.

The naturally occurring soluble mLBP in mouse serum is a highly specific inhibitor of hLIF biological action in vitro, based on its ability to block M1 cell differentiation induced by hLIF. The recombinant mLIFR α-chain, which contained only two hemopoietin domains and the intervening Ig-like domain, was as effective as mLBP, which presumably also contains two fibronectin type III repeats (4), in blocking hLIF-induced M1 cell differentiation. This suggested that the two hemopoietin domains together with the Ig-like domain from the mLIFR α-chain are necessary and sufficient for high affinity hLIF interactions and biological activity. Surprisingly, hybrid receptors MH3LIFR, MH4LIFR, and MH5LIFR, which all retain high affinity binding for hLIF, showed little inhibition on hLIF-induced M1 cell differentiation. The inability to block hLIF biological action by MH3LIFR, MH4LIFR, and MH5LIFR variants in the M1 differentiation assay may be due either to their inherent instability or their proteolysis during 1 week in cell culture. If this is true, inhibition of short-term hLIF action on M1 cells by the high affinity chimeric LIFRs should be observed. STAT3 activation is the initial step in a cascade of events leading to terminal differentiation and growth arrest of M1 cells induced by IL-6 or IL-6 (35). Activation of STAT3 involves phosphorylation of specific tyrosines in response to LIF and other IL-6-related cytokines, including ciliary neurotrophic factor and oncostatin M, whose receptor complexes contain gp130. The observation of complete blockage by mLIFR and hybrid MH3LIFR, and a significant inhibition by hybrids MH4LIFR and MH5LIFR, of hLIF-induced STAT3 tyrosine phosphorylation in M1 cells, indicates that the high affinity hLIF-binding receptors can block hLIF action. Less inhibition of STAT3 phosphorylation by MH4LIFR and MH5LIFR variants compared with mLIFR and MH3LIFR suggest that the interactions of hLIF with mLIFR or MH3LIFR are different from those with MH4LIFR or MH5LIFR. Alternatively, other receptor components such as gp130 may be involved in the interaction.

This study of structure-function mapping of the LIFR α-chain has demonstrated that the Ig-like domain from the mouse LIFR plays a dominant role in determining both high affinity binding for hLIF and species-specific binding for mLIF. The binding properties of the chimeric LIFRs correlated only partly with the biological activities in both the M1 cell differentiation assay and the STAT3 phosphorylation assay. Hybrid MH5LIFR, which maintained most of the residues from the hLIFR α-chain (both hemopoietin domains) and only about one-fifth of the residues from the hLIFR (Ig-like domain), still retained high affinity binding for hLIF in vitro, suggesting that it is a potentially humanized mLIFR that could act as a specific and potent antagonist of hLIF. The improvement of the expression level and stability of this chimeric receptor, which is necessary for its application to the further study of its biological function and use in clinical trials, is currently under investigation.

Acknowledgments—We thank Dale Cary, S. Mifsud, and L. diRago for skilled technical assistance.

REFERENCES

1. Metcalf, D. (1991) Int. J. Cell Cloning 9, 95–108
2. Hilton, D. J. (1992) Trends Biochem. Sci. 17, 72–76
3. Stewart, C. L., Kaspar, P., Brunet, L. J., Bhat, H., Gadi, I., Kontgen, F., and Abdennadjar, S. J. (1992) Nature 356, 76–79
4. Geurin, M. R., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, C. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B., Ziegler, S. F., and Cosman, D. (1992) Science 255, 1434–1437
5. Geurin, M. R., and Bruce, A. G. (1992) New Biol. 4, 61–65
6. Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasokawa, K., Kishimoto, T., Anderson, D. J., Stahl, N., and Yanceopoulos, G. D. (1992) Cell 69, 1121–1132
7. Pennica, D., Shaw, K. J., Swanson, T. A., Moore, M. W., Shelton, D. L., Zonation, K. A., Rosenthal, A., Taga, T., Paoni, N. F., and Wood, W. I. (1995) J. Biol. Chem. 270, 19315–19222
8. Pennica, D., King, K. L., Shaw, K. J., Lu, E., Rullamas, J., Lush, S. M., Darbonne, W. C., Knutson, D. S., Yen, R., Chien, K. R., Baker, J. B., and Wood, W. I. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1142–1146
Ligand Binding Determinants on the LIF Receptor

9. Hibi, M., Murakami, M., Saite, M., Hirano, T., Taga, T., and Kishimoto, T. (1990) Cell 63, 1149–1157
10. Fourcin, M., Chevalier, S., Lebrun, J. J., Kelly, P., Poupard, A., Wijdenes, J., and Gascan, H. (1994) Eur. J. Immunol. 24, 277–280
11. Hilton, D. J., Hilton, A. A., Renshaw, B. R., Hunt, J. S., Liggitt, D., Koblar, S., Harrison-Smith, M., Gough, N. M., Begley, C. G., Metcalf, D., Nicola, N. A., and Willson, T. A. (1994) EMBO J. 13, 4765–4776
12. Ware, C. B., Horowitz, M. C., Renshaw, B. R., Hunt, J. S., Liggitt, D., Kohlar, S., Gliniak, B. C., McKenna, H. J., Papayannopoulou, T., Thoma, B., Cheng, L., Donovan, P. J., Peschon, J. J., Bartletti, P. F., Willis, C. R., Wright, B. D., Carpenter, M. K., Davison, B. L., and Gearing, D. P. (1995) Development 121, 1283–1299
13. Gearing, D. P., Thut, C. J., VandeBos, T., Gimpel, S. D., Delaney, P. B., King, J., Price, V., Cosman, D., and Beckmann, M. P. (1991) EMBO J. 10, 2839–2848
14. Tomida, M., Yamaguchi, Y. Y., and Hozumi, M. (1994) J. Biochem. 115, 557–562
15. Bazan, J. F. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6934–6938
16. Cosman, D. (1993) Cytokine 5, 95–106
17. Fukunaga, R., Ishizaka-Ikeda, E., Seto, Y., and Nagata, S. (1990) Cell 61, 341–350
18. Fukunaga, R., Ishizaka-Ikeda, E., and Nagata, S. (1990) J. Biol. Chem. 265, 14088–14095
19. Fukunaga, R., Ishizaka-Ikeda, E., and Nagata, S. (1991) EMBO J. 10, 2855–2860
20. Horsten, U., Schmitz-Van de Leur, H., Mullberg, J., Heinrich, P. C., and Rose-John, S. (1995) FEBS Lett. 360, 43–46
21. Layton, M. J., Lock, P., Metcalf, D., and Nicola, N. A. (1994) J. Biol. Chem. 269, 17048–17055
22. Ouwens, C. M., Layton, M. J., Metcalf, D., Lock, P., Willson, T. A., Gough, N. M., and Nicola, N. A. (1993) EMBO J. 12, 3487–3495
23. Layton, M. J., Ouwens, C. M., Metcalf, D., Clark, R. L., Smith, D. K., Treutlein, H. R., and Nicola, N. A. (1994) J. Biol. Chem. 269, 29891–29896
24. Layton, M. J., Cross, B. A., Metcalf, D., Ward, L. D., Simpson, R. J., and Nicola, N. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8616–8620
25. Tomida, M., Yamamoto-Yamaguchi, Y., and Hozumi, M. (1995) FEBS Lett. 357, 341–350
26. Wilson, I. A., Niman, H. L., Houghten, R. A., Cherenson, A. R., Connolly, M. L., and Lerner, R. A. (1984) Cell 37, 767–778
27. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
28. Sanger, F. A., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
29. Gregg, J. M., Barringer, K. J., Hesseler, A. Y., and Madden, K. R. (1985) Mol. Cell. Biol. 5, 3376–3385
30. Hilton, D. J., Nicola, N. A., and Metcalf, D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5971–5975
31. Munson, P. J., and Rodbard, D. (1980) Anal. Biochem. 107, 220–239
32. McPherson, G. A. (1985) KINETIC, EBDA, LIGAND, LOWRY: A Collection of Radioligand Binding Analysis Programs, Biosoft, Cambridge, UK
33. Metcalf, D., Hilton, D. J., and Nicola, N. A. (1988) Leukemia 2, 216–221
34. Hilton, D. J., and Nicola, N. A. (1992) J. Biol. Chem. 267, 10238–10247
35. Minami, M., Inoue, M., Wei, S., Takada, K., Matsumoto, M., Kishimoto, T., and Akira, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3963–3966
36. Gough, N. M., Gearing, D. P., King, J. A., Willson, T. A., Hilton, D. J., Nicola, N. A., and Metcalf, D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2623–2627
37. Vigon, I., Morron, J.-P., Cocault, L., Mitjavila, M.-T., Tambourin, P., Gisselbrecht, S., and Souyri, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5640–5644
38. Itoh, N., Yonehara, S., Schreurs, J., Gorman, D. M., Maruyama, K., Ishii, A., Yahara, I., Ari, K., and Miyajima, A. (1990) Science 247, 324–327
39. Gorman, D. M., Itoh, N., Kitamura, T., Schreurs, J., Yonehara, S., Yahara, I., Ari, K., and Miyajima, A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5459–5463
40. Boynaems, J. M., and Dumont, J. E. (1986) Outline of Receptor Theory, Elsevier/North Holland Biomedical Press, Amsterdam
41. de Vos, A. M., Ultsch, M., and Kassiaiakoff, A. A. (1992) Science 256, 305–312
42. Bork, P., Holm, L., and Sander, C. (1994) J. Mol. Biol. 242, 309–320
43. Urfer, R., Tessier-Peck, P., O’Connell, L., Shelton, D. L., Purday, L. F., and Presta, L. G. (1995) EMBO J. 14, 2795–805
44. Heidaran, M. A., Pierce, J. H., Jensen, R. A., Matsui, T., and Aaronson, S. A. (1990) J. Biol. Chem. 265, 18741–18744
45. Wang, Z. E., Myles, G. M., Brandt, C. S., Lioubin, M. N., and Rohrschneider, L. (1993) Mol. Cell. Biol. 13, 5348–5359
46. Hiraoka, O., Anaguchi, H., Asakura, A., and Ota, Y. (1995) J. Biol. Chem. 270, 25928–25934
47. Cornelis, S., Plaetinck, G., Devos, R., Van der Heyden, J., Tavernier, J., Sanderson, C. J., Guisez, Y., and Fiers, W. (1995) EMBO J. 14, 3395–3402
The Unusual Species Cross-reactivity of the Leukemia Inhibitory Factor Receptor α-Chain Is Determined Primarily by the Immunoglobulin-like Domain
Catherine M. Owczarek, Yu Zhang, Meredith J. Layton, Donald Metcalf, Bronwyn Roberts and Nicos A. Nicola

J. Biol. Chem. 1997, 272:23976-23985.
doi: 10.1074/jbc.272.38.23976

Access the most updated version of this article at http://www.jbc.org/content/272/38/23976

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 45 references, 22 of which can be accessed free at http://www.jbc.org/content/272/38/23976.full.html#ref-list-1