Mouse Leukemia Inhibitory Factor Suggest a Complex Binding Interaction*

(Received for publication, December 16, 1993, and in revised form, April 11, 1994)

Meredith J. Layton, Peter Lock, Donald Metcalf, and Nicos A. Nicola

From the Walter and Eliza Hall Institute for Medical Research and the Cooperative Research Centre for Cellular Growth Factors, P.O. Royal Melbourne Hospital, Parkville, Victoria, 3050, Australia

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine whose activities appear to be mediated through a single heterodimeric receptor complex. Human LIF (hLIF) can bind to and activate mouse LIF (mLIF) receptors but mLIF is unable to bind to hLIF receptors. Cross-species competition of mLIF and hLIF for binding to the mLIF receptor was found to be dependent on which ligand was used as the radioactive tracer (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), and this phenomenon was investigated in the present study. We found that hLIF bound to the low affinity mLIF receptor with a 100-500-fold higher primary affinity and lower kinetic dissociation rate than mLIF, but both ligands displayed a single rate of ligand dissociation. In contrast, the binding of hLIF to low and high affinity hLIF receptors revealed two classes of binding site. The observed tracer-dependent phenomena suggested that both mLIF and hLIF interfere with the binding of each other to the mLIF receptor. A model is presented in which hLIF binds to two sites on mLIF and hLIF receptors, one of which interferes with the common site for mLIF. This model may reconcile some of the observed complexities of LIF/LIF receptor interactions.

Leukemia inhibitory factor (LIF)* is a glycoprotein that was originally purified (Hilton et al., 1988a; Tomida et al., 1984) and cloned (Gearing et al., 1987) on the basis of its ability to induce terminal macrophage differentiation of the M1 myeloid leukaemic cell line. It has since been shown to have a variety of activities on a wide range of cell types including megakaryocytes, osteoblasts, hepatocytes, adipocytes, neurons, embryonal stem cells, and primordial germ cells (Metcalf, 1991).

Specific receptors for LIF have been found on a range of mouse cells known to respond to LIF (Hilton et al., 1988b, 1991) and on some human cell lines (Godard et al., 1992). Most mouse cells express only high affinity mouse LIF (mLIF) receptors ($K_D = 20–100$ pM) although certain activated macrophage populations express both high and low affinity ($K_D = 1–2$ nM) mLIF receptors (Hilton and Nicola, 1992). The majority of human cell lines examined also express only high affinity human LIF (hLIF) binding sites ($K_D = 30–100$ pM), although a low affinity ($K_D = 1–4$ nM) binding component was identified on some human cell lines using high concentrations of labeled hLIF at low specific radioactivity (Godard et al., 1992).

Recently, cDNAs encoding a membrane-bound hLIF receptor (hLIF-R) and a soluble mLIF receptor (mLIF-R) have been isolated (Gearing et al., 1991). The human and mouse LIF receptors are members of the cytokine receptor family, contain two hemopoietin domains (Bazan, 1990), and have weak sequence similarity to gp130, a component of the interleukin-6 receptor complex, and to the granulocyte-colony stimulating factor receptor. The soluble mLIF-R shares 70% amino acid sequence identity with the hLIF-R (Gearing et al., 1991). Both receptors bind their cognate ligands with low affinity ($1–2$ nM), and so have been designated the α-chains of the LIF receptor complex. A soluble mouse LIF-binding protein (mLBP) has been isolated from normal mouse serum and found to have an N-terminal amino acid sequence identical to that of the α-chain of the mLIF-R, although it appears to be truncated at both the N- and C-terminal ends. Mouse mLBP also binds mLIF with low affinity ($0.5–2$ nM) (Layton et al., 1992), which is similar to the affinity observed for mLIF binding to detergent-solubilized mLIF receptors or to the low affinity mLIF receptors on certain activated macrophage populations (Hilton and Nicola, 1992).

A cDNA that encodes the high affinity converting subunit of the hLIF-R, designated the β-subunit, has also been isolated and was found to be identical with gp130, the β-subunit of the IL-6 receptor complex. The gp130 protein alone did not bind hLIF, but was able to confer high affinity binding of hLIF when co-expressed with the hLIF-R α-chain in COS cells (Gearing et al., 1992).

Recently, we observed that purified mLBP exhibited unusual cross-species binding characteristics, where mLIF and hLIF showed a dramatic difference in their ability to compete for binding to mLBP depending on whether $^{125}$I-mLIF or $^{125}$I-hLIF was used as a radioactive tracer (Layton et al., 1992). In the present study, we have carried out detailed equilibrium binding and kinetic analyses of the interaction of mLIF and hLIF with mLBP and cellular mouse and human LIF receptors, and found that, although isologous competition curves were consistent with the apparent individual equilibrium binding constants, nonisologous competition curves were not. These data suggest that the presence of one ligand may interfere with the binding of the other ligand, leading to behavior that is not consistent with a model of simple competitive binding. A model is proposed that attempts to explain the mechanism behind this complex interaction of LIF with its receptor, and which may help elucidate the structure of the receptor binding sites.

*This work was supported by the National Health and Medical Research Council, Canberra, Australia, the Anti-Cancer Council of Victoria, AMRAD Corporation, Melbourne, the J. D. and L. Harris Trust Fund, the Philip Bushell Trust, National Institutes of Health Grant CA2256, and the Australian Government Cooperative Research Centre scheme. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed. Tel.: 61-3-345-2559; Fax: 61-3-343-2616.

The abbreviations used are: LIF, leukemia inhibitory factor; LIF-R, LIF receptor; mLBP, LIF-binding protein; m, mouse; h, human; FCS, fetal calf serum; RFH, RPMI 1640 medium supplemented with Hepes and fetal calf serum; PBS, phosphate-buffered saline.
MATERIALS AND METHODS

Radioiodination of LIF—1–2 μg of recombinant mouse LIF (mLIF) or human LIF (hLIF) produced in Escherichia coli was purified and iodinated, as previously described (Hilton and Nicola, 1992; Hilton et al., 1991). The binding (the percentage of radioactivity capable of binding specifically to its receptor) of each LIF was estimated by measuring the specific binding of a constant amount of radiolabeled LIF to increasing amounts of receptor as previously described (Nicola and Metcalfe, 1988). The binding was approximately 100% for both preparations of both 125I-mLIF and 125I-hLIF. The specific radioactivity was determined by a self-consistent binding analysis (Calvo et al., Nicola, 1992; Nicola et al., 1988). The specific radioactivities ranged between 2 and 5 × 10^6 cpm/pmol for 125I-mLIF and 4 and 15 × 10^6 cpm/pmol for 125I-hLIF.

Human LIF Receptor α-Chain cDNA—A 4.1-kilobase cDNA encoding the hLIF-R α-chain was isolated, subcloned into the mammalian expression vector pCDM8 (Seed, 1987), and transiently expressed in COS cells (Gluzman, 1983) as previously described (Owczarek et al., 1993).

A plasmid encoding a truncated soluble form of the hLIF-R that includes both hemopoietin domains and two of the three fibronectin repeat structures, was constructed from the plasmid encoding the full-length hLIF-R. The C terminus of this soluble hLIF-R includes 10 amino acid residues encoded by vector sequences (Gly-Arg-Gly-Arg-His-Ser-Arg-Gly-Ser-Leu).

Binding of mLIF and hLIF to LIF Receptors on the Cell Surface—A monocytic cell line, U937, was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). PC.13 (Bernini et al., 1993), was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). PC.13 cells were detached by agitation in ice-cold RPMI 1640 medium buffered at pH 7.4 with 20 mM HEPES and containing 10% FCS (RHF), harvested by centrifugation at 300 × g, and resuspended in RHF. Allen 1 cells, derived from a human Ewing's sarcoma (a gift from Dr. G. Kraemer, Royal Children's Hospital, Melbourne), were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FCS. Allen 1 cells were harvested by centrifugation at 300 × g and resuspended in RHF. COS cells were maintained in RPMI 1640 medium containing 10% FCS. COS cells were transfected with the hLIF-R a-chain cDNA by electroporation as previously described (Owczarek et al., 1993). Seventy-two hours post-transfection, cells were detached in the presence of RHF containing 0.02 mM EDTA and 0.1 mg/ml chondroitin sulfate, harvested by centrifugation and resuspended in RHF.

Saturation binding experiments and competitive binding experiments were carried out in Falcon 2054 tubes (Becton Dickinson Labware) to which were added 50–100 μl aliquots of cells, resuspended in RHF at 2 × 10^5 to 5 × 10^5 cells/ml and 10 μl of radiolabeled LIF. Non-specific binding was determined from incubations containing at least 5 ng/ml unlabeled LIF. For competitive binding experiments, a 50–100 μl aliquot of unlabeled LIF was added simultaneously to the incubation mixture containing the cells and the labeled LIF. After incubation at 4°C overnight, bound and free labeled LIF were separated by centrifugation at 15000 rpm of bovine serum at 10000 g for 1 min and counted in a γ-counter (Packard Instruments). Scatchard analyses of saturation binding isotherms were performed using the curve-fitting program LIGAND (McPherson, 1985; Munson and Rodbard, 1980).

For kinetic association experiments, 1 × 10^5 to 5 × 10^5 cells/ml were mixed on ice with radiolabeled LIF at a final concentration of approximately 10^7 cpm/100 μl in the presence or absence of at least 5 ng/ml unlabeled LIF. At various times after addition of labeled LIF, 100 μl aliquots of the cell suspension were removed, and bound and free labeled LIF were separated and counted as described above. The number of specific counts bound had reached a plateau, the incubation mixture was centrifuged at 10000 g, the supernatant was removed, and dissociation of the labeled LIF was initiated by resuspending the cell pellet in the same volume of RHF containing 50 μg/ml unlabeled LIF. At various times after resuspension, 100-μl aliquots of cells were removed, and bound and free labeled LIF were separated as described for Scatchard analyses. Estimates of the kinetic rate constant governing dissociation (k_d) and association (k_a) were made using the curve-fitting program KINETIC (McPherson, 1986).}

Purification of Soluble Receptors—The soluble form of the hLIF-R α-chain, mouse LIF binding protein (mLBP), was purified from mouse serum, either as previously described (Layton et al., 1992) or as described below, and had a final specific activity of greater than 0.1 μg/mLBP/mg of mLIF binding capacity. In some experiments, normal mouse serum was used as a source of mLBP.

To obtain soluble hLIF-R α-chain, COS cells were transfected with a plasmid encoding a truncated soluble form of the hLIF-R by electroporation as described above. Approximately 96 h post-transfection, the conditioned medium was harvested and concentrated 5–10-fold using a 10,000 molecular weight cutoff dialysis membrane.

The soluble hLIF-R α-chain was purified by affinity chromatography. A high affinity column was prepared by immobilizing Affi-Gel-10 (Bio-Rad) with 3.5 mg of hLIF and 5 mg of ovalbumin (used as a filler protein), in 15 ml of 0.1 M NaHCO_3, pH 8, at 4°C for 24 h, with constant mixing. A control column was also synthesized using 5 mg of ovalbumin alone. Derivatized sites were blocked using 5 ml of 1 M ethanolamine, pH 8; the gel was then washed alternately with phosphate-buffered saline (PBS; this and all subsequent buffers contained 0.02% (v/v) Tween 20 and 0.02% (w/v) sodium azide) and 6 mM guanidine HCl in PBS. The hLIF-Affi-Gel column was equilibrated in PBS, and either 20 ml of COS cell-conditioned medium containing the soluble hLIF-R α-chain or 20 ml of normal mouse serum containing mLBP were loaded at 0.5 ml/min. The column was washed with 40 ml of PBS at the same flow rate, then bound protein was eluted with a 30-mI gradient from 0 to 6 M guanidine HCl in PBS at 0.5 ml/min. Fractions of 2.5 ml were collected and exchanged into PBS using Sephadex G-25 Medium columns (Pharmacia Biotech Inc.).

Binding of mLIF and hLIF to Soluble Receptors—Saturation binding experiments for soluble receptors were performed as previously described, using concanavalin A-Sepharose beads to precipitate the soluble receptor complexes (Layton et al., 1992). Determinations of non-specific binding, separation of bound and free labeled LIF, and Scatchard analyses were performed as described for cells.

For competitive binding experiments, 20-μl aliquots of soluble receptor in PBS were added to 96-well filtration assay plates containing a 0.65-mM Durapore membrane (Millipore) with 10 μl of radiolabeled LIF, 50 μl of unlabeled LIF, and 25 μl of concanavalin A-Sepharose beads (Pharmacia; diluted 1 in 4 in 0.1 M sodium acetate, pH 6.0, containing 1 mM each MgCl_2, MnCl_2, and CaCl_2) and incubated at room temperature, overnight, with agitation. Bound and free radioactivity were separated by vacuum filtration of the supernatant, and the concanavalin A-Sepharose pellet was washed once with 200 μl of cold PBS. Assay plates containing the concanavalin A-Sepharose pellet were dried and exposed to a phosphor screen for 2–3 days, and the results were quantitated with a computer software package.

For kinetic association and dissociation binding experiments, aliquots of soluble receptor in PBS, preincubated with concanavalin A-Sepharose, were mixed with radiolabeled LIF at a final concentration of approximately 10^7 cpm/100 μl in the presence or absence of excess unlabeled LIF. Time courses of labeled LIF association and dissociation were followed as described for cells.

Bioassay of LIF on M1 Cells—Samples for bioassay were exchanged into normal saline containing 5% FCS using pre-packed Sephadex G-25 Medium columns and sterilized by filtration through a 0.45-μm filter. M1 differentiation assays were performed as described by Metcalfe et al. (1988).

Size Exclusion Chromatography—Aliquots of molecular weight standards or samples containing 125I-mLIF or 125I-hLIF that had been incubated with PBS or with samples of mLBP or soluble hLIF-R α-chain in PBS, overnight at room temperature, were applied to a Superose-12 column (Pharmacia), equilibrated in PBS. Samples were eluted with PBS at a flow rate of 0.5 ml/min, and 0.5-min fractions were collected and counted in a γ-counter.

RESULTS

Binding of mLIF and hLIF to mLBP—We reported recently that, while unlabeled mLIF and hLIF had a similar ability to compete with 125I-mLIF for binding to mLBP (a soluble form of the mLIF-R α-chain), unlabeled hLIF was consistently 1000–10000-fold more effective than unlabeled mLIF in competing with 125I-hLIF for binding to mLBP (Layton et al., 1992). At equilibrium, binding curves for simple competitive inhibition should not be tracer-dependent, so we investigated the basis of the phenomenon by obtaining equilibrium binding and kinetic analysis of mLIF and hLIF-R binding to mLBP.

Scatchard analyses of 125I-mLIF binding to mLBP in normal mouse serum or to purified mLBP showed that mLBP exhibited a single class of low affinity mLIF binding site (K_d = 1–4 nM) (Fig. 1A). This affinity was similar to that reported for 125I-mLIF binding to the low affinity mLIF-R prepared from detergent solubilized mouse liver membranes (Hilton and Nicola,
Receptor Binding of Human and Mouse LIF

**Fig. 1.** A, Scatchard plot of $^{125}$I-mLIF binding to 6 nm purified mLBP (○) and $^{125}$I-hLIF binding to mLBP in normal mouse serum diluted 1/100 (□), 1/300 (△), or 1/1000 (■). Normal mouse serum contains ~50–250 nm mLBP. B, natural log plots of time courses of association of 900 pm $^{125}$I-mLIF (○) and 450 pm $^{125}$I-hLIF (x) with 350 pm mLBP in normal mouse serum. C, natural log plots of time courses of dissociation of $^{125}$I-mLIF (○) and $^{125}$I-hLIF (x) from 350 pm mLBP in normal mouse serum. $S_B$, $S_{B1}$, and $S_{B2}$ are the amounts of radioactivity specifically bound to the receptor at time 0, at time $t$, and at equilibrium, respectively. D, Scatchard plots of $^{125}$I-mLIF (○) and $^{125}$I-hLIF (x) binding to PC.13 cells (15 × 10⁶ cells/ml for $^{125}$I-mLIF; 12 × 10⁶ cells/ml for $^{125}$I-hLIF). E, natural log plots of time courses of association of 2.2 nm $^{125}$I-mLIF (○) and 2 nm $^{125}$I-hLIF (x) with 9 × 10⁶ cells/ml PC.13 cells. F, natural log plots of time courses of dissociation $^{125}$I-mLIF (○) and $^{125}$I-hLIF (x) from 9 × 10⁶ cells/ml PC.13 cells. G, Scatchard plots of $^{125}$I-hLIF binding to 45 × 10⁶ cells/ml Allen1 cells (•) to 3 × 10⁶ cells/ml COS cells transfectected with a plasmid encoding the hLIF-R α-chain (○), or to conditioned medium collected 4 days after transfection of COS cells with a plasmid encoding a truncated soluble form of the hLIF-R α-chain (□). H, natural log plots of time courses of association of 1.25 nm $^{125}$I-hLIF with 7 × 10⁵ cells/ml Allen1 cells (•), 1.25 nm $^{125}$I-hLIF with 6 × 10⁶ cells/ml COS cells expressing an average of 20,000 hLIF receptor α-chains/cell (○), and 1.25 nm $^{125}$I-hLIF with 1 nm purified soluble hLIF-R α-chain (□). I, natural log plots of time courses of dissociation of $^{125}$I-hLIF from 7 × 10⁵ cells/ml Allen1 cells (•); 6 × 10⁶ cells/ml COS cells expressing an average of 20,000 hLIF receptor α-chains/cell (○) and 1 nm soluble hLIF-R α-chain (□).

In contrast, Scatchard analyses of $^{125}$I-hLIF binding to similar concentrations of mLBP revealed a convex curvilinear plot (Fig. 1A). Convex Scatchard plots can reflect a positively cooperative binding interaction (Boeynaems and Dumont, 1980; Cuatrecasas and Hollenberg, 1976), but we failed to find evidence of ligand or receptor isomerization or polymerization, or more than one ligand-binding site per receptor molecule. Size exclusion chromatography, under non-dissociating conditions, of a reaction mixture containing high concentrations of mLBP (90–100 kDa) and $^{125}$I-LIF (22 kDa) showed that the apparent molecular masses of both the $^{125}$I-mLIF and $^{125}$I-hLIF/mLBP complexes were approximately 110–130 kDa (Fig. 2), indicating that both mLIF and hLIF probably form a 1:1 complex with mLBP. Density ultracentrifugation also indicated that the size of the LIF/mLBP complex was the same for both mLIF and hLIF, and contained one mLBP molecule and one LIF molecule in each case. In addition, no difference was apparent in the total number of binding sites for $^{125}$I-mLIF and $^{125}$I-hLIF in an identical sample of mLBP, as estimated by Scatchard analysis.

A variety of artefacts can also result in convex Scatchard plots, including high receptor concentration, failure to reach equilibrium at the lower concentrations of ligand, heterogeneity of the labeled ligand, and inaccurate estimation of the true free ligand concentration or of nonspecific binding (Boeynaems and Dumont, 1980; Cuatrecasas and Hollenberg, 1976). The shape of the $^{125}$I-hLIF/mLBP Scatchard curve was dependent on the receptor concentration (Fig. 1A), suggesting that this curve shape may be an artefact of high receptor concentration (Boeynaems and Dumont, 1980; Cuatrecasas and Hollenberg, 1976). Binding of $^{125}$I-hLIF to mLBP at low receptor concentrations produced a linear Scatchard plot, enabling estimation of the affinity of hLIF for mLBP ($K_D = 10–20$ pm), which was approximately 50–400-fold higher than the affinity of $^{125}$I-mLIF for mLBP.

Association kinetics for these two molecules appeared essentially equivalent (Fig. 1B; Table I); however, there was an approximately 500-fold difference in kinetic dissociation rates.

---

2 G. Howlett and M. Layton, unpublished results.
Receptor Binding of Human and Mouse LIF

Table 1
Summary of equilibrium and kinetic data

<table>
<thead>
<tr>
<th>Factor</th>
<th>mLBP (mLIF-Ra)</th>
<th>PC.13 cells (mLIF-Ra β)</th>
<th>CO8 hLIF-R cells (hLIF-Ra)</th>
<th>Soluble hLIF-R α-chain (hLIF-Ra)</th>
<th>Allen1 cells (hLIF-Ra β)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mLIF K_d</td>
<td>1-4 nm</td>
<td>150-250 pm</td>
<td>0.2-1 nm</td>
<td>0.7-1 nm</td>
<td>ND</td>
</tr>
<tr>
<td>K_d (min^-1)</td>
<td>3 x 10^8</td>
<td>3 x 10^9</td>
<td>1.9 x 10^9</td>
<td>0.0001-0.004</td>
<td>0.0001-0.002</td>
</tr>
<tr>
<td>k_off (min^-1)</td>
<td>0.5</td>
<td>0.002</td>
<td>0.7-2 x 10^9</td>
<td>2-4 x 10^9</td>
<td>0.001-0.01</td>
</tr>
<tr>
<td>k_off/s</td>
<td>2 nm</td>
<td>9 pm</td>
<td>0.05-10 nm</td>
<td>10-20</td>
<td>10 PM</td>
</tr>
<tr>
<td>ID_50 (mLIF)</td>
<td>~20 pm</td>
<td>~0.1 nm</td>
<td>~2000 nm</td>
<td>~100 nm</td>
<td></td>
</tr>
<tr>
<td>ID_50 (hLIF)</td>
<td>~500 pm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hLIF K_d</td>
<td>10-30 pm</td>
<td>20-90 pm</td>
<td>0.2-1 nm</td>
<td>0.7-1 nm</td>
<td>~8 pm</td>
</tr>
<tr>
<td>K_d (min^-1)</td>
<td>4 x 10^8</td>
<td>8 x 10^9</td>
<td>1.9 x 10^9</td>
<td>0.0001-0.004</td>
<td>0.0001-0.002</td>
</tr>
<tr>
<td>k_off (min^-1)</td>
<td>0.0008</td>
<td>0.0004</td>
<td>0.7-2 x 10^9</td>
<td>2-4 x 10^9</td>
<td>0.001-0.01</td>
</tr>
<tr>
<td>k_off/s</td>
<td>2 pm</td>
<td>0.5 pm</td>
<td>0.05-10 nm</td>
<td>10-20</td>
<td>10 PM</td>
</tr>
<tr>
<td>ID_50 (mLIF)</td>
<td>~8 pm</td>
<td>~0.01 nm</td>
<td>~2000 nm</td>
<td>~100 nm</td>
<td></td>
</tr>
<tr>
<td>ID_50 (hLIF)</td>
<td>~1 nm</td>
<td>~0.01 nm</td>
<td>~2000 nm</td>
<td>~100 nm</td>
<td></td>
</tr>
</tbody>
</table>

a F, fast dissociating component.
b S, slow dissociating component.
c ND, not determined.

Dissociation of 125I-mLIF from mLBP was fast, with essentially all of the LIF dissociating from the receptor within 3 min, while dissociation of 125I-hLIF from mLBP was very slow, with less than 10% of the LIF dissociated after 2 h. Transformation of these data revealed that both 125I-mLIF and 125I-hLIF binding to mLBP is consistent with a simple bimolecular interaction between LIF and its low affinity receptor (Fig. 1C; Table 1). Estimation of the equilibrium dissociation constants (K_d) from the ratio of k_off/k_on gave a 500-1000-fold higher affinity of hLIF for mLBP compared to mLBP (Table 1).

The difference in the rates at which mLIF and hLIF dissociate from mLBP was also discernible in the elution profiles from the size-exclusion chromatography column (Fig. 2). The peak for the 125I-hLIF-mLBP complex was sharp, indicating a tightly bound complex, while the peak for 125I-mLIF-mLBP complex was diffuse, suggesting that ligand dissociation occurred during the time of elution of the complex from the column.

The above data demonstrated that hLIF has a 100-500-fold higher affinity for mLBP compared to mLIF, but failed to explain the differences in the 50% inhibitory dose (ID_50) values observed when 125I-hLIF rather than 125I-mLIF was used as the radioactive tracer. Several possible artefacts were considered that might have explained the tracer-dependent competition curves. 1) The binding behavior of mLIF or hLIF may be altered differentially by radiodination, leading to tracer-specific effects. This was considered unlikely because the bindable fractions of both 125I-mLIF and 125I-hLIF were approximately 100%, and the binding affinities of labeled and unlabeled LIF were indistinguishable by self-displacement analysis (Calvo et al., 1983) (results not shown). 2) The slower dissociation rate of hLIF compared to mLIF may give it a kinetic advantage in cross-competition experiments and give rise to tracer-specific effects if the system did not reach equilibrium. When 125I-hLIF was the labeled ligand, the competitor curves for mLIF and hLIF were closer together at shorter incubation times (Fig. 3B), reflecting the dominance of kinetic association rates. However, increasing the incubation time accentuated the tracer-dependent differences between the ID_50 values for mLIF and hLIF (Fig. 3B), suggesting that, even at equilibrium, the tracer-dependent difference would not disappear. 3) The higher affinity of hLIF for mLBP may lead to selective ligand depletion of hLIF at high receptor concentrations. The absolute ID_50 values were dependent on the total receptor concentration (Fig. 3, C and D) as expected (Chang et al., 1975), but the tracer-dependent differences in the ID_50 values for mLIF and hLIF were maintained.

The Cheng-Prusoff relationship for nonisologous competition at equilibrium (Cheng and Prusoff, 1973) was applied to this system to test how the observed competition curves differed from the predicted ID_50 values. This relationship is: ID_50 = K_d + (K_d/K_a) where ID_50 is the dose of unlabeled ligand required to inhibit 50% of labeled ligand binding, K_d is the free concentration of the radiolabeled or tracer ligand, and K_a and K_d are the equilibrium dissociation constants for the unlabeled and labeled ligands, respectively. Using experimentally derived values for these parameters (K_d = 2 nm for mLIF, K_d = 10 pm for hLIF, L = 1.9 nm for 125I-mLIF, L = 980 pm for 125I-hLIF), the theoretical competition curves for mLIF and hLIF were calculated and compared to the experimental curves (Fig. 3, E and F). The isologous competition curves agreed quite well with the predicted curves, but the nonisologous ligand was a weaker competitor than would be expected for a simple competitive inhibitor in both cases. This tracer-dependent phenomenon therefore implied that each of the ligands was interfering with the binding of the other, in a manner that was not consistent with simple competition for a common binding site.

Inhibition of LIF Biological Response by mLBP—The higher affinity and slower dissociation kinetics of hLIF binding to mLBP compared to mLIF had implications for LIF action in biological systems. As described previously, mLIF and hLIF have similar abilities to induce differentiation of M1 myeloid leukemic cells in culture (Fig. 4A), and mLBP was able to act as a competitive inhibitor of LIF action in this assay. A concentration of approximately 1 µg/ml mLBP was sufficient to block the induction of differentiation of M1 colonies by up to 90 units/ml of mLIF (Layton et al., 1992). In contrast, only 0.04 µg/el purified mLBP was required to inhibit completely the differentiation of M1 colonies induced by 300 units/ml of hLIF (Fig. 4B). Similarly, approximately 300-fold less mouse serum, a source of mLBP, was required to inhibit completely M1 cell differentiation induced by hLIF compared to that induced by mLIF (Fig. 4C).

The 100-500-fold difference in the concentration of mLBP required to inhibit differentiation of M1 cells induced by mLIF or hLIF is consistent with the higher primary affinity of mLBP
for hLIF compared to mLIF and results in mLBP being a much more effective competitive inhibitor of hLIF than mLIF in vitro.

**Binding of mLIF and hLIF to PC.13 Cells**—High affinity LIF receptors are reported to be comprised of a low affinity LIF-binding α-chain plus a β-subunit, gp130 (Gearing et al., 1992). PC.13 cells are derived from a mouse embryonal carcinoma and have high affinity receptors for mLIF (Hilton and Nicola, 1992).

Scatchard analyses of 125I-LIF binding to PC.13 cells demonstrated that these cells exhibited a single class of high affinity mLIF binding site ($K_D = 150-250$ pm) and a single class of hLIF binding site of approximately 10-fold higher affinity ($K_D = 20-30$ pm) (Fig. 1D), which was essentially the same as the affinity of hLIF for mLBP, the mLIF-R α-chain. The total number of mLIF and hLIF binding sites on the surface of PC.13 cells was also approximately the same (5000–8000 receptors/cell).

Similar association kinetics were observed for both 125I-mLIF and 125I-hLIF binding to PC.13 cells (Fig. 1E and Table 1). The relatively slow rate of dissociation of 125I-mLIF was consistent with the presence of a high affinity mLIF-R on PC.13 cells; however, dissociation of 125I-hLIF was 5–10-fold slower. Transformation of kinetic data gave rise to mon-exponential rate curves for both molecules (Fig. 1F and Table 1). The 5–10-fold difference in the dissociation rates of mLIF and hLIF from the high affinity mLIF-R was consistent with the Scatchard data, but contrasted with the 100–500-fold difference in the dissociation rate of mLIF and hLIF from the low affinity mLIF-R. The equilibrium dissociation constants ($K_D$) were estimated from the ratio of $h_D/h_D$ for mLIF and hLIF, and the difference was again approximately 10-fold (Table 1).

The cross-species competition curves observed for labeled and unlabeled mLIF and hLIF binding to the low affinity mLIF-R α-chain (mLBP) (Layton et al., 1992) were essentially reproduced on the high affinity mLIF-R displayed on PC.13 cells (Fig. 5, A and B). Again, the difference in $ID_{50}$ for mLIF and hLIF did not reflect the difference in their primary affinity for binding to the high affinity mLIF-R, as determined by Scatchard analyses. When 125I-mLIF was used as a tracer in competition experiments, hLIF was an approximately 10-fold better inhibitor than mLIF, but when 125I-hLIF was the tracer,
hLIF was 1000–10,000-fold more effective.

**Binding of hLIF to Human LIF Receptors**—The binding properties of hLIF to the high affinity hLIF-R on mammalian cells and to the low affinity hLIF-R α-chain expressed on COS cells were also investigated. As expected, unlabeled mLIF was unable to compete for 125I-hLIF binding to Allen1 cells, which are derived from a human Ewing’s sarcoma and are reported to display high affinity binding sites for hLIF. Similarly, no binding of mLIF was detected to the COS cells expressing the hLIF-R α-chain (Fig. 5C), consistent with the reported inability of mLIF to bind to the hLIF receptor (Gearing et al., 1991). In contrast, unlabeled hLIF competed with 125I-hLIF for binding to both low and high affinity hLIF receptors, with an ID50 of approximately 1 nM for the low affinity hLIF-R α-chain expressed on COS cells and 5 pm for the high affinity hLIF-R on Allen1 cells (Fig. 5C).

Scatchard analysis of 125I-hLIF binding to Allen1 cells revealed an apparent single class of high affinity hLIF binding site (Kd = 8 pm) with an average of 1000 receptors on the surface of each cell (Fig. 1G). Scatchard analysis of 125I-hLIF binding to the low affinity hLIF-R α-chain expressed on COS cells revealed an equilibrium dissociation constant of 0.3–1 nM and an average expression level of approximately 20,000 receptors/cell. A truncated, soluble form of the α-chain of the hLIF-R, expressed in COS cell-conditioned medium, exhibited an affinity for 125I-hLIF similar to the transmembrane form (Kd = 0.7–1 nM) (Fig. 1G and Table I).

The kinetics of hLIF binding to the hLIF-R were markedly different from those observed for mLIF binding to the mLIF-R. Association kinetics for 125I-hLIF binding to the high affinity hLIF-R on Allen1 cells, the low affinity hLIF-R on COS cells, and the truncated, soluble hLIF-R α-chain were monophasic and were similar for each receptor type (Fig. 1H and Table I). The dissociation kinetics for 125I-hLIF from the same receptors were, however, bimodal and biphasic (Fig. 1I). Transformation by the KINETIC program revealed two dissociation rate constants for each receptor (Table I), with only the proportion of fast versus slow dissociating LIF differing between receptor type. Allen1 cells had 20–40% of the fast dissociating component and 60–80% of the slowly dissociating component, while both COS cells expressing the hLIF-R α-chain and the soluble hLIF-R α-chain had 50–60% of the fast dissociating component and 40–50% of the slowly dissociating component.

The equilibrium dissociation constant (Kd) estimated from the ratio of k1/k2 was calculated for both the fast and slowly dissociating components. Allen1 cells had a low affinity, fast dissociating site (Kd = 1–2 nM) and high affinity, slowly dissociating site (Kd = 4–7 pm). The hLIF-R α-chain expressed on COS cells and the soluble hLIF-R α-chain also displayed two sites of Kd = 0.5–10 nM and Kd = 20–150 pm (Table I). This observation is apparently at odds with the single class of binding site determined from Scatchard analyses.

**DISCUSSION**

The tracer-dependent cross-species competition binding curves observed for mLIF and hLIF led us to investigate the binding characteristics of mouse and human LIF to low and high affinity mouse and human LIF receptors. The tracer-dependent behavior can best be summarized by stating that, in the presence of mLIF, hLIF is a less effective competitor than mLBP would be expected from its equilibrium dissociation constant, and in the presence of hLIF, mLIF is a less effective competitor than mLBP would be expected from its equilibrium dissociation constant (Fig. 3, E and F).

We first compared the primary binding characteristics of 125I-hLIF and 125I-mLIF to purified mLBP. While 125I-mLIF bound to mLBP with low affinity and rapid dissociation kinetics, 125I-hLIF bound with higher affinity due to a very slow rate of dissociation. The kinetic rate constants governing the rate of mLIF and hLIF association with mLBP were essentially the same. The 100–500-fold difference in primary binding affinity and kinetic dissociation rate explains why mLBP is a better biological inhibitor of hLIF than mLIF in vitro, but does not resolve the anomalous competition curves.

Despite the binding of hLIF to mLBP being characterized by convex Scatchard plots, there was no evidence of receptor or ligand polymerization. It also seemed difficult to account for the observed competition curves by known influences on binding phenomena, such as alteration of the behavior of the ligand as a consequence of radiiodination, failure to reach equilibrium, ligand depletion, or the Cheng-Prusoff relationship.

The anomalous behavior of the competition curves was reproduced on PC.13 cells, which display high affinity mLIF receptors, and so presumably express both the low affinity

---

3 G. Kannourakis, unpublished data.
mLIF-R α-chain and the β-chain, mouse gp130 (mgp130). The difference in both the primary affinity and kinetic dissociation rate of mLIF and hLIF for PC.13 cells was only 5–10-fold, due primarily to a higher affinity of mLIF, but not hLIF, for the α-β receptor complex compared to the isolated mLIF-R α-chain. Since the affinity of hLIF for the low-affinity mLIF-R α-chain or the high affinity mLIF-R on PC.13 cells was essentially the same, as were the anomalous competition curves, this implied a mechanism that is independent of equilibrium binding or kinetic characteristics of either mLIF or hLIF, and so must be mediated through the α-chain of the mLIF-R.

Binding of mLIF and hLIF to hLIF receptors had quite different characteristics than binding to the mLIF-R. The human sarcoma cell line, Allen1, was found to bind hLIF with a higher affinity than the hLIF-R α-chain alone, so these cells presumably express human gp130 (hgp130). Mouse LIF was completely unable to bind to either high or low affinity hLIF receptors.

In contrast to the monophasic association and dissociation kinetics of both mLIF and hLIF binding to both low and high affinity mLIF receptors, binding of mLIF to both high and low affinity hLIF receptors was characterized by biphasic dissociation kinetics. The high affinity hLIF-R on Allen1 cells had a larger proportion of the slower dissociating component compared to low affinity hLIF-R α-chain on COS cells. Receptor or ligand polymerization or the presence of more than one ligand-binding site per receptor molecule was unlikely to be responsible for these kinetics, as the molecular weight of the 125I-hLIF-soluble hLIF-R α-chain complex determined by size-exclusion chromatography suggested a 1:1 complex was formed. It is therefore possible that heterogeneity of binding sites in this system may be a result of receptor isomerization between high affinity and low affinity forms. If this was the case, there may be no simple relationship between kinetic association and dissociation rates and the apparent equilibrium dissociation constants. The receptor isomerization constant would be expected to also contribute to the binding equilibrium and may explain why the linear Scatchard plot apparently does not correlate with the biphasic kinetic data.

Biphasic dissociation of hLIF from cells expressing high affinity hLIF-R has been reported previously and has been attributed to a reversible interconversion step between an hLIF-hLIF-R α-chain complex and an hLIF-hLIF-R α-chain-hLIF-R β-chain complex on the cell surface (Godard et al., 1992). However, the biphasic dissociation of hLIF from the low affinity hLIF-R α-chain, expressed either on the surface of COS cells or as a truncated soluble form in COS cell conditioned medium, suggests that the hLIF-R α-chain, and not the β-chain (hgp130), mediates the observed kinetics.

Recently, we constructed a series of mouse/human LIF chimeric molecules to determine the contribution of each hLIF amino acid residue to both the unexpectedly high affinity binding to mLIF-R α-chain and to the species-specific binding of hLIF to the hLIF-R α-chain (Owczarek et al., 1993). The residues on the hLIF molecule that mediated the binding of hLIF to the hLIF-R α-chain also conferred the higher affinity binding of hLIF to the mLIF-R α-chain (Fig. 6, site b). However, mLIF and hLIF were able to cross-compete for binding to the soluble mLIF-R α-chain, indicating that they must also contain a common binding site for the mLIF-R α-chain (Fig. 6, site a). It is proposed that this model can be extended to explain the unusual cross-species binding phenomena described in this report.

In the proposed model (Fig. 6), mLIF binds to the mLIF-R α-chain with low affinity through site a. This complex can then associate with mgp130 to form the additional interaction sites that result in high affinity mLIF binding. Human LIF binds to the mLIF-R α-chain through site a, or through site b which is absent in mLIF, and this leads to rapid and complete receptor isomerization to result in further interactions of both site a and site b on hLIF with site A and site B on the mLIF-R α-chain to generate an unexpectedly high affinity of hLIF for the mLIF-R α-chain. The presence of mgp130 does not increase this affinity, suggesting that either the prior interaction of hLIF and the mLIF-R α-chain prevents the recognition of hLIF by mgp130, or that mgp130 replaces one of the hLIF contacts with the mLIF-R α-chain, A or B, with similar interaction energy. This behavior is consistent with the presence of two receptor binding sites on many cytokines that induce receptor oligomerization (Kastelein and Shanafelt, 1993).

This proposed model is not only consistent with the binding of m-hLIF chimeras, but also provides a rationale for the unusual tracer-specific cross-competition behavior of mLIF and hLIF with either the mLIF-R α-chain or high affinity mLIF receptors. The model proposes that receptor isomerization, which leads to high affinity hLIF binding, would be impeded if site A on the LIF-R α-chain was occupied by mLIF and that mLIF would be expelled from site A by the receptor isomerization induced by hLIF, resulting in apparent interference of the binding of one ligand by the other ligand. This situation might be considered a special, unimolecular case of "half-of-sites reactivity" (Boeynaems and Dumont, 1980). This model is not dependent on the identity of sites A and B, but since LIF receptors contain a duplicated hemopoietin domain, it is tempting to speculate that sites A and B could reside within different hemopoietin domains.

The proposed model can be further extended to explain the biphasic kinetic behavior of hLIF with the hLIF-R α-chain. The mechanism of hLIF binding to the hLIF-R α-chain would be similar to that of hLIF binding to the mLIF-R α-chain, except that the receptor isomerization is less rapid or complete. The model predicts that the fast and slow components observed in the hLIF dissociation kinetics result from dissociation of hLIF from receptors in different isomerization states. The hLIF-hLIF-R α-chain complex interacts with hgp130 to form a
complex that contains more hLIF bound with a higher affinuity and therefore a larger proportion of slow dissociating hLIF.

The soluble mLIF-R α-chain can function as a high affinity specific inhibitor of hLIF biological action in vitro, and has been observed in this report and by others (Yamaguchi et al., 1993) to be approximately 100-fold more active as an inhibitor of hLIF than of mLIF in in vitro bioassays. The soluble mLIF-R α-chain is also likely to be a better inhibitor of hLIF than the soluble hLIF-R α-chain, given the lower affinity of hLIF for the soluble hLIF-R α-chain compared to mLBP and the relatively fast rate of hLIF dissociation from the soluble hLIF-R α-chain. This unexpected observation suggests that mLBP or a modified form of this protein could have some potential therapeutic importance in the design of inhibitors of hLIF action. Humanization of the mLBP molecule with retention of its unusual kinetic characteristics for the binding of hLIF might allow the development of a highly specific and potent antagonist of unwanted activities of hLIF in vivo.

Acknowledgments—We thank S. Mifsud for skilled technical assistance and G. Kannourakis for the Allen 1 cell line.

REFERENCES
