A major binding protein for leukemia inhibitory factor in normal mouse serum: Identification as a soluble form of the cellular receptor

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ABSTRACT A protein that specifically binds leukemia inhibitory factor (LIF) has been isolated from normal mouse serum by using four successive fractionation steps: chromatography on a LIF affinity matrix, anion-exchange chromatography, size-exclusion chromatography, and preparative native gel electrophoresis. The purified LIF-binding protein (LBP) is a glycoprotein with an apparent molecular mass of 90 kDa that specifically binds ¹²⁵I-labeled murine LIF with an affinity comparable to that of the low-affinity cellular LIF receptor ($K_d = 600$ pM). N-terminal sequencing has identified this protein as a soluble truncated form of the α chain of the cellular LIF receptor. LBP is present in normal mouse serum at high levels (1 μ g/ml) and these levels are elevated in pregnant mice and reduced in neonatal mice. Since normal serum concentrations of LBP can block the biological actions of LIF in culture, LBP may serve as an inhibitor of the systemic effects of locally produced LIF.

Leukemia inhibitory factor (LIF) is a polyfunctional glycoprotein with actions on a broad range of tissue and cell types, including induction of differentiation in myeloid leukemic cell lines, suppression of differentiation in normal embryonic stem cells, stimulation of proliferation of osteoblasts and DA-1 hemopoietic cells, and potentiation of the proliferative action of interleukin (IL) 3 on megakaryocyte precursors. Functionally, it is able to switch autonomic nerve signaling from adrenergic to cholinergic mode, stimulate calcium release from bones, stimulate the production of acute-phase proteins by hepatocytes, and induce loss of fat deposits by inhibiting lipoprotein lipase-mediated lipid transport into adipocytes (1).

Cellular receptors for LIF exist in both high- and low-affinity forms depending on the cell type examined (2). A cDNA clone for the cellular receptor for LIF has been isolated by expression screening of a human (h) placental cDNA library (3) and recognized as a member of the hemopoietin family of receptors (4, 5). The cloned LIF receptor contains two hemopoietin domains but only binds LIF with low affinity. Recently, it has been suggested that although the signal-transducing subunit of the IL-6 receptor (gp130) itself does not bind LIF, it can nevertheless transform the low-affinity LIF receptor into a high-affinity form (6).

A murine (m) LIF receptor cDNA cloned from a liver library encoded a C-terminally truncated form of the receptor that might represent a soluble receptor, as it lacked a transmembrane region. Soluble receptors have been described for other members of the hemopoietic receptor family including the IL-4 (7), IL-5 (8), IL-6 (9), IL-7 (10), granulocyte colony-

stimulating factor (11), and granulocyte/macrophage colony-stimulating factor (12) receptors, but their role *in vivo* is unclear.

This study reports the purification of a LIF-binding protein (LBP) from normal mouse serum and its identification as a soluble form of the mLIF receptor. Its presence at high levels in normal serum, its modulation during pregnancy, and its inhibitory activity *in vitro* suggest that it may have the ability to block unwanted systemic actions of locally produced LIF.

MATERIALS AND METHODS

Collection of Mouse Sera. Blood from C57BL/6J, C3H/HeJ, CBA f/Ca H, DBA/2J, and BALB/C/An Bradley mice was collected by exsanguination; serum was obtained by centrifugation at $3000 \times g$ for 10 min and stored at -20°C for up to 6 months.

Affinity Chromatography. LIF coupled to Sepharose 4B through tyrosine residues was prepared by the method of Cuatrecasas and Anfinsen (13) using 8 mg of *Escherichia coli*-derived mLIF (2) and chicken ovalbumin (Sigma) as a nonspecific filler protein in 10 ml of 0.2 M sodium tetraborate (pH 9.2) for 16 hr at 4°C . The mLIF-conjugated *p*-aminobenzamidoethyl-Sepharose 4B (LIF-pABAE-Sepharose 4B) was washed extensively with 20 mM sodium phosphate (pH 7.4) containing 150 mM NaCl (PBS) and 6 M guanidine hydrochloride in PBS before use.

Mouse serum (50 ml) or pooled fractions containing LBP activity in PBS were applied to a 13×0.7 -cm column of LIF-pABAE-Sepharose 4B equilibrated with PBS (this and all succeeding buffers contained 0.02% Tween 20 and 0.02% sodium azide) and material was eluted with 20 ml of equilibration buffer followed by a 15-ml linear gradient to 6 M guanidine hydrochloride in the same buffer. Fractions (2.5 ml) were collected at a flow rate of 0.5 ml/min and exchanged where necessary into equilibration buffer using a prepacked Sephadex G-25 column (PD10; Pharmacia).

Anion-Exchange Chromatography. Active fractions from affinity chromatography were pooled, diluted 1:20 with 20 mM Tris-HCl (pH 7.0), and then applied to a Mono Q HR 5/5 (Pharmacia) column equilibrated in the same buffer. Elution was carried out using 25 ml of equilibration buffer, followed by a 30-ml linear gradient to 1 M NaCl in equilibration buffer. The 0.5-ml fractions were collected at a flow rate of 0.5 ml/min.

Size-Exclusion Chromatography. Active fractions from anion-exchange chromatography were pooled and concentrated to 100 μ l using a Centricon-10 microconcentrator (Amicon).

Abbreviations: h, human; LIF, leukemia inhibitory factor; LBP, LIF-binding protein; m, murine; pABAE, *p*-aminobenzamidoethyl; IL, interleukin; ¹²⁵I-, ¹²⁵I-labeled.

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The sample was injected onto a Superose-12 10/30 (Pharmacia) column equilibrated in PBS, and elution was carried out isocratically using the same buffer. The 0.2-ml fractions were collected at a flow rate of 0.2 ml/min.

Preparative Native PAGE. Active fractions from size-exclusion chromatography were electrophoresed on a 20-ml 7.5% gel using standard Laemmli (14) buffers, but excluding the NaDodSO₄, in a model 491 prep cell (Bio-Rad) according to the manufacturer's instructions. Fractions (2.5 ml) were collected at a flow rate of 1 ml/min.

Analytical NaDodSO₄/PAGE. NaDodSO₄/PAGE was performed by the method of Laemmli (14) using 12.5% polyacrylamide gels in a Mini-Protein II system (Bio-Rad), and gels were silver-stained (15).

Iodination of LIF. Recombinant mLIF or hLIF (1–2 µg) produced in *E. coli* was iodinated and purified as described (2). The iodinated materials retained biological activity and had specific activities of 4–5 × 10⁵ cpm/pmol for ¹²⁵I-labeled mLIF (¹²⁵I-mLIF) and 2 × 10⁶ cpm/pmol for ¹²⁵I-hLIF.

Binding Assays. Binding assays for LBP were carried out essentially as described by Nicola and Cary (16) for soluble cytokine receptors. Aliquots of fractions were added to Eppendorf tubes containing 10 µl of ¹²⁵I-LIF (5–10 × 10⁴ cpm) and 50–100 µl of concanavalin A-Sepharose (Pharmacia) [diluted 1:4 in 100 mM sodium acetate (pH 6.0) containing 1 mM MgCl₂, 1 mM MnCl₂, and 1 mM CaCl₂] and incubated at room temperature for 16 hr with agitation. Bound and free labels were separated as described and radioactivity was measured in a γ counter. Variations of this assay were also used for competitive binding and Scatchard analyses of LBP and mouse liver membranes [prepared essentially as described (17) and solubilized in 1% Triton X-100].

For Scatchard analyses of 3T3-L1 cells, cells [maintained as described (18) and harvested using Hepes-buffered RPMI medium with 10% (vol/vol) fetal calf serum (HRF) containing 40 mM EDTA and chondroitin sulfate at 200 µg/ml] were resuspended in 60 µl of HRF in Falcon 2054 tubes (Becton Dickinson) with 10 µl of ¹²⁵I-mLIF and then incubated at 4°C for 3 hr.

Scatchard analysis of saturation binding isotherms was performed using the curve-fitting program LIGAND (19), after correction for the bindable fraction of radioligand as described (2). Scatchard analysis allows the derivation of the affinity of a labeled ligand for its binding site and the concentration of these binding sites, thereby permitting the quantitation of LBP in samples of known volume, assuming one mLIF binding site per LBP molecule and a molecular mass of 90 kDa for LBP.

Bioassay. Samples for bioassay were exchanged into normal saline using prepacked Sephadex G-25 M (PD10 columns; Pharmacia), concentrated using Centricon-10 microconcentrators (Amicon), and sterilized by passage through a 0.45-µm (pore size) filter (Millipore). M1 differentiation assays were performed as described (20).

Protein Assay. Protein was estimated by the method of Bradford using a Coomassie blue-based reagent (Pierce) according to the manufacturer's instructions.

Amino Acid Sequence Analysis. N-terminal amino acid sequence analysis was carried out by Edman degradation of proteins blotted onto a poly(vinylidene difluoride) membrane as described (21).

RESULTS

Identification of LIF-Binding Activity in Mouse Serum. LBP was detected in normal mouse serum by its ability to inhibit the binding of ¹²⁵I-mLIF to a neutralizing monoclonal antibody in a competition radioimmunoassay or to its cellular receptor on 3T3-L1 cells in a competition radioreceptor assay (data not shown). It was detected by either of these assays in

Table 1. Levels of LBP in serum

Serum type	Strain	Sex	Age, weeks	Dilution giving 50% inhibition	LBP, µg/ml
Mouse					
Adult	Mixed	Mixed	NA	1:4–1:8	1
	C57	M	6	1:4	1
	C57	F	6	1:4	1
	CBA	F	12	1:4	1
Neonatal	CBA	F	1	1:2	0.5
	CBA	M	1	1:1	0.25
	CBA	M	2	1:4	1
Pregnant	CBA*	F	12	1:128	32
Human	NA	Mixed	NA	ND	0
Rat	NA	Mixed	NA	1:4	1

Levels of LBP detected in serum are expressed as the dilution of serum required to inhibit 50% of specific binding of ¹²⁵I-mLIF to a neutralizing monoclonal antibody in a competition RIA. The number of ¹²⁵I-mLIF binding sites in normal mouse serum was determined by Scatchard analysis to be 1 µg/ml and was used as a standard to convert 50% inhibition in RIA to an LBP concentration. NA, not applicable; ND, not detected.

*Pregnant 14 days.

normal mouse serum at a 1:4 to 1:8 dilution, in the serum of pregnant mice but at a 20- to 30-fold greater dilution, and in neonatal mice at a 2- to 4-fold lower dilution (Table 1).

Direct binding of ¹²⁵I-mLIF to LBP was detected in two ways. By size-exclusion chromatography, ¹²⁵I-mLIF exhibits a molecular mass of ≈20 kDa. When ¹²⁵I-mLIF was chromatographed in the presence of normal mouse serum, under nondissociating conditions, a labeled complex was detected with an apparent molecular mass of ≈110 kDa (Fig. 1), signifying that the LIF-binding protein in serum had a molecular mass of 90 kDa. Concanavalin A-Sepharose was demonstrated to have the ability to precipitate the ¹²⁵I-mLIF-LBP complex from serum, implying that LBP was a glycoprotein, since the recombinant LIF labeled for these studies was produced in *E. coli* and so contained no carbohydrate

FIG. 1. Size-exclusion profile of ¹²⁵I-mLIF in the presence and absence of normal mouse serum. The Ultrogel AcA 44 (20 × 1 cm) column was equilibrated and run in PBS at 0.2 ml/min, 1-min fractions were collected, and radioactivity was measured in a γ counter. (A) ¹²⁵I-mLIF (100,000 cpm) in 100 µl of PBS. (B) ¹²⁵I-mLIF (100,000 cpm) preincubated with 100 µl of normal mouse serum for 16 hr at room temperature.

Table 2. Representative purification of LBP from normal mouse serum

Fractionation step	Volume, ml	Total LBP, μg	Total protein, μg	Yield, %	Purification, fold
Normal serum	85	102	2.24×10^6	—	—
LIF Sepharose 1	78	41	1.54×10^4	39.8	58
LIF Sepharose 2	38	18	2.73×10^3	19.4	147
Anion exchange	8.9	18	2.45×10^3	19.9	158
Size exclusion	3.2	6.0	573	7.8	232
Native gel	0.8	1.4	11.6	1.4	2620

Total LBP was calculated from the concentration of ^{125}I -mLIF binding sites derived from Scatchard analysis, assuming one binding site per LBP molecule and a molecular mass of 90 kDa for LBP.

groups. This property was used to separate bound from free ^{125}I -LIF as described for solubilized receptor assays (16).

Isolation of LBP. The purification of mLBP from normal mouse serum was achieved by sequential fractionation using affinity chromatography on an immobilized LIF column, anion-exchange chromatography, size-exclusion chromatography, preparative native PAGE, and by monitoring LBP by direct ^{125}I -mLIF binding assays.

LBP was found to be a very unstable protein that was completely inactivated by acidic conditions and in the presence of many of the chemicals usually used in protein purification procedures, such as acetonitrile, methanol, NaDodSO₄, and ammonium sulfate. This proved the major difficulty in the isolation of mLBP, as it precluded the use of many high-resolution purification techniques, and may be the basis for the relatively low yields obtained during fractionation (Table 2).

Chemical modification of lysine residues in the LIF molecule has been shown to destroy its biological activity (N.A.N. and D. Hilton, unpublished data), whereas iodination of tyrosine residues is not detrimental to LIF activity (2). A method of linking protein to Sepharose beads through

FIG. 2. Representative sequential fractionations of normal mouse serum (50 ml) used in the purification of LBP. Absorbance (dotted lines) and specific ^{125}I -mLIF binding (solid lines) of fractions in a concanavalin A-Sepharose binding assay are shown for affinity chromatography on LIF-pABAE-Sepharose 4B (A), anion-exchange chromatography on a Mono-Q HR 5/5 column (B), and size-exclusion chromatography on a Superose-12 10/30 column (C).

FIG. 3. (A) Preparative native PAGE of LBP on a 7.5% gel. Specific ^{125}I -mLIF binding of eluted fractions in a concanavalin A-Sepharose binding assay is shown. (B) NaDodSO₄/PAGE of 20 μl of pooled fractions containing LBP activity from various stages of purification. Lanes: S, low molecular mass standards (Pharmacia); a, normal mouse serum (1:50 dilution); b and c, first LIF-pABAE-Sepharose 4B column; d, second LIF-pABAE-Sepharose 4B column; e, Mono Q HR 5/5 column; f, Superose-12 10/30 column; g, preparative native PAGE gel (arrow shows band of interest). Molecular masses in kDa are shown.

tyrosine residues was, therefore, chosen to ensure that the LIF molecule would be active when attached to an affinity column matrix. Affinity chromatography (Fig. 2A) on an mLIF-pABAE-Sepharose 4B affinity matrix removed a large proportion of the contaminating serum proteins, although the guanidine used as an eluent had some denaturing effects on LBP activity, resulting in low recoveries. A second round of affinity chromatography, however, resulted in a significant additional purification. In contrast, LBP did not bind to a control ovalbumin-pABAE-Sepharose 4B column. Anion-exchange (Fig. 2B) and size-exclusion (Fig. 2C) chromatography achieved a further 1.6-fold purification.

Analytical NaDodSO₄/PAGE of fractions from the size-exclusion column revealed two bands that were barely separated from each other on a 12.5% polyacrylamide gel but were well separated from other contaminating bands. Both had an apparent molecular mass of ≈ 90 kDa and their distributions in size-exclusion column fractions, as determined by silver staining of analytical NaDodSO₄/PAGE gels, exactly matched that of LBP activity by direct ^{125}I -mLIF binding assay.

The two peak fractions were electrophoresed on a 7% NaDodSO₄/PAGE gel and blotted onto a poly(vinylidene difluoride) membrane, and the two bands of interest were subjected to N-terminal sequencing. The major band had a slightly lower molecular mass and yielded amino acid sequence at the 5-pmol level (23 amino acid residues), and the higher molecular mass band yielded sequence at the 3-pmol level (8 amino acid residues). Both revealed an N-terminal sequence identical to a sequence within the recently cloned soluble mLIF receptor, beginning at residue 48 of the published sequence (Gly-Val-Gln-Asp-Leu-Lys-Cys-Thr-Thr-Asn-Asn-Met-Arg-Val-Trp-Asp-Cys-Thr-Trp-Pro-Ala-Pro-

FIG. 4. Scatchard analysis of $^{125}\text{I-mLIF}$ binding to LBP, solubilized murine liver membranes, and 3T3-L1 cells. (A) Saturation isotherm for $^{125}\text{I-mLIF}$ binding to mouse serum (5 μl ; total reaction mixture, 210 μl) showing total cpm bound ($\blacktriangle$), nonspecific cpm bound ($\bullet$), and specific cpm bound ($\circ$). (B) Scatchard transformation of $^{125}\text{I-mLIF}$ binding to LBP in mouse serum (5 μl ; total reaction mixture, 210 μl) ($\circ$) and purified LBP (1 μl ; total reaction mixture, 90 μl) ($\bullet$). (C) Scatchard transformation of $^{125}\text{I-mLIF}$ binding to solubilized murine liver membranes (25 μl ; total reaction mixture, 175 μl) ($\circ$) and 3T3-L1 cells (6×10^5 cells; total reaction mixture, 80 μl) ($\bullet$).

Leu . . .) (3). The two bands of mLBP were not seen in every preparation and were presumed to be glycosylation variants, although the C terminus of mLBP is yet to be identified and differences at this end of the molecule could account for the slight size heterogeneity.

Preparative gel electrophoresis using native conditions (Fig. 3A) allowed high-resolution separation of LBP from contaminating proteins with only two major bands of 90 and 67 kDa revealed by analytical NaDodSO₄/PAGE after pooling active fractions from this step (Fig. 3B). The band at 67 kDa could be albumin contamination, although we cannot exclude the possibility that a protein other than the band at 90 kDa might have LIF-binding activity. Fractions pooled from this step were used in assays to determine the binding characteristics of LBP.

Binding Properties of LBP. The specific binding of $^{125}\text{I-mLIF}$ to purified LBP is shown in Fig. 4A. Scatchard analysis of the saturation binding isotherm of $^{125}\text{I-mLIF}$ to normal mouse serum or purified LBP showed that LBP contained a single class of mLIF binding site (K_d , 250 pM–1 nM) and indicated that normal mouse serum contains LBP at $\approx 1 \mu\text{g/ml}$ (Fig. 4B). The affinity of LBP for mLIF was comparable to that of the low-affinity LIF receptor solubilized by detergent from mouse liver membranes (K_d , 680 pM) and was significantly lower than that of the high-affinity cellular receptor on 3T3-L1 cells (K_d , 57 pM) (Fig. 4C).

FIG. 5. Displacement curves for unlabeled mLIF ($\circ$) and hLIF ($\bullet$) competing for the binding with $^{125}\text{I-mLIF}$ (A) and $^{125}\text{I-hLIF}$ (B) to 1.8 ng of purified LBP (total volume, 100 μl).

Although hLIF was able to bind to mLBP, its binding characteristics were rather different. Unlabeled mLIF and hLIF showed a similar ability to compete for $^{125}\text{I-mLIF}$ binding to mLBP (Fig. 5A), but mLIF was consistently 10,000-fold less effective than hLIF in competing for $^{125}\text{I-hLIF}$ binding to mLBP (Fig. 5B).

Blocking Activity of LBP. When affinity-purified LBP was combined with LIF in cultures of murine M1 myeloid leukemic cells, it blocked the ability of LIF to induce differentiation of M1 colonies in a dose-dependent manner with, for example, 50% inhibition of the activity of LIF at 90 units/ml (10^8 units/ml = 1 mg/ml) by LBP at 62–125 ng/ml (Fig. 6A). When a lower dose of mLIF (22 units/ml) was used, LBP did not alter the response, but intermediate doses of mLIF (stimulating 50% differentiation of M1 colonies) revealed inhibition by high doses of LBP, with possible augmentation at low doses of LBP (2–4 ng/ml; Fig. 6A). The presence of LBP at 125 ng/ml in the culture was able to displace the biological response curve 2-fold toward higher mLIF doses (Fig. 6B).

DISCUSSION

It is difficult to conceive of any situation that would require a coordinated response in all the known target tissues of LIF (1), so LIF actions are probably designed to be restricted by colocalization of LIF-producing cells and LIF-responsive cells, with tight regulation of LIF production. However, such an arrangement is likely to result in some release of LIF into the blood. The present observations have documented that normal mouse serum contains sufficiently high levels of LBP (1 $\mu\text{g/ml}$) to inhibit the biological activity of concentrations of LIF of up to 1 ng/ml, and LBP could, therefore, serve to prevent any systemic effects of locally produced LIF.

The levels of serum LBP themselves appear to be subject to regulation since they are low in neonates and highly elevated during pregnancy. These data are consistent with an inhibitory role for LBP since it is known that LIF is actively produced by the endometrial glands prior to blastocyst implantation (22) and it may be desirable to prevent the dissemination of LIF to other tissues. Conversely, low LBP levels may be required during the early postnatal period to permit systemic actions of LIF, for example, on neuron and muscle cell growth. At present, LBP has been detected at high levels in the serum of all strains of mice tested and in rat serum but not in human serum.

FIG. 6. Blocking of induction of differentiation in mLIF-stimulated colonies of M1 leukemic cells by affinity-purified LBP. (A) Titration of LBP in the presence of mLIF at 90 units/ml (○), 45 units/ml (●), and 22 units/ml (▲). (B) Titration of mLIF with LBP at 125 ng/ml (○), 32 ng/ml (●), and 4 ng/ml (▲).

The purification, binding properties, and N-terminal sequencing of LBP strongly suggest that it is a truncated form of the cellular LIF receptor. The existence of a soluble form of the mLIF receptor is not unanticipated since cDNAs cloned from a mouse liver library encoded a truncated, apparently soluble, form of the LIF receptor (3). The predicted leader sequence for this protein is unusually long, so that the mature protein is hypothesized to begin at residue 45 of the published sequence (3). LBP begins at residue 48 and the preceding amino acid residue is arginine, so it is uncertain whether this alternate starting point is a consequence of the leader sequence or additional proteolytic modification. The relationship of the published soluble LIF receptor sequence, which has a molecular mass of 130 kDa when expressed in COS-7 cells (3), to that of LBP (90 kDa) is not clear and requires further sequence analysis of LBP, especially at the C-terminal end.

The binding affinity of LBP for LIF is essentially identical to that of the cell-associated low-affinity LIF receptor (α chain) (3) and that of the LIF receptor solubilized from liver cells by detergents. This evidence, and the apparent molecular mass of the LIF-LBP complexes in serum (determined by size-exclusion chromatography), suggests that only the α chain and not the proposed β chain of the LIF receptor (gp130) (6) (which confers high-affinity binding) is involved in the LIF-binding activity detected in serum.

Like cellular mLIF receptors, mLBP was able to bind both mLIF and hLIF (conversely, human receptors do not recognize mLIF). However, this cross-species competition showed some unusual characteristics. When ^{125}I -mLIF was used as a tracer, unlabeled hLIF and mLIF showed nearly equivalent capacity for binding to mLBP but when ^{125}I -hLIF was used as a tracer, unlabeled mLIF was nearly 10,000 times

less effective than hLIF. There is no obvious explanation for this phenomenon and it may be that LIF binding to LBP is not a simple equilibrium at a single site or that the dissociation kinetics for mLIF and hLIF are markedly different. Further study will be required to resolve this apparent paradox.

The inhibitory activity of LBP on LIF action appears to be mediated by competitive binding of free LIF to LBP rather than to cellular receptors. Although cellular receptors for LIF are generally of higher affinity than LBP they are always low in numbers (a few hundred to a thousand per cell) so that the high levels of LBP in serum ($\approx 10^{10}$ molecules per μl) more than compensate for the 20 times lower affinity of LBP compared to high-affinity cellular receptors. However, the ability of LBP to interact with cellular gp130 molecules in either a stimulatory or inhibitory manner (9) remains to be investigated.

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