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Subcloning, Expression, Purification, and Characterization of Recombinant Human Leptin-binding Domain*

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A subdomain of the human leptin receptor encoding part of the extracellular domain (amino acids 428 to 635) was subcloned, expressed in a prokaryotic host, and purified to homogeneity, as evidenced by SDS-PAGE, with over 95% monomeric protein. The purified leptin-binding domain (LBD) exhibited the predicted β structure, was capable of binding human, ovine, and chicken leptins, and formed a stable 1:1 complex with all mammalian leptins. The binding kinetics, assayed by surface plasmon resonance methodology, showed respective k_{on} and k_{off} values (mean ± S.E.) of $1.20 \pm 0.23 \times 10^{-5}$ mol^{-1} s^{-1} and $1.85 \pm 0.30 \times 10^{-3}$ s^{-1} and a K_d value of 1.54×10^{-8} M. Similar results were achieved with conventional binding experiments. LBD blocked leptin-induced, but not interleukin-3-induced, proliferation of BAF/3 cells stably transfected with the long form of human leptin receptor. The modeled LBD structure and the known three-dimensional structure of human leptin were used to construct a model of 1:1 LBD·human leptin complex. Two main residues, Phe-500, located in loop L3, and Tyr-441, located in L1, are suggested to contribute to leptin binding.

Leptin is a hormone produced by fat cells. It acts in specific parts of the brain and is an important regulator of food intake. Its discovery in 1994 by Friedman and co-workers (1) in an obese mutant mouse line (*ob/ob*), in which the active form of leptin is not expressed, indicated its importance as a metabolic signal from body fat deposits for many physiological functions, *e.g.* reproduction. This role has been increasingly documented in rodents, as well as in humans (2, 3). The effects of leptin on these functions may be mediated centrally via changes in hypothalamic neuropeptide Y expression, which in turn regulates the secretion of gonadotropic hormones (4) and food intake (5). Metabolic changes induced by alterations in food intake affect various hormone systems indirectly. In addition to its systemic effects, direct peripheral leptin actions have been demonstrated in several target tissues. Thus, leptin has been shown to modulate insulin activity in hepatocytes *in vitro* (6). Leptin modulates ovarian steroidogenesis *in vitro* (7, 8) and affects angiogenesis, acting in some tissues as a positive angiogenic factor (9), whereas it is angiostatic in adipose tissues (10).

Our group recently prepared recombinant leptins from several farm animals, such as sheep (11), chicken (12), cow, and pig (13), and from humans (14). A variety of *in vivo* experiments performed with leptin-deficient *ob/ob* and normal mice (for review see Refs. 3, 5, and 15), as well as our experiments with chicken and sheep (16–18), indicate that administration of leptin by direct intraventricular, intramuscular, or intraperitoneal injections leads to a remarkable decrease in food intake and subsequent weight loss. The main target of leptin's action is located in the brain, and as leptin is produced in adipose tissue, it has to be transferred through the blood-brain barrier. This transfer is mediated mainly through the short form of the leptin receptor located in the choroid plexus (3, 5). In addition to central activity, leptin also affects several peripheral actions and is involved in reproduction (19). We have shown recently that in rat ovary, leptin attenuates apoptosis and thus enhances sexual maturation (20). We have also found that leptin regulates several functions in the pituitary cells (21). In the blood of humans and mice, leptin is found in both free and bound forms (22–25); the main binding protein is the extracellular domain (ECD)[1] of the leptin receptor (26).

Its seems logical that blocking leptin receptors that are responsible for its transfer through the blood-brain barrier or for its action in the hypothalamus would lead to increased food intake and antagonize other brain-mediated leptin actions (27). This could be achieved by neutralizing the peripheral leptin with soluble leptin receptors similar to many known interleukin-soluble receptors (28, 29). The leptin receptor belongs to the cytokine receptor superfamily (30). Its ECD consists of ~800 amino acids, making its preparation in large quantities problematic. However, it has been suggested that only the cytokine homology subdomain I (~200 amino acids) is responsible for binding (31). To verify this notion, the present paper describes subcloning of this subdomain, its expression in a prokaryotic host, and its subsequent purification and characterization.

EXPERIMENTAL PROCEDURES

Materials—Ovine leptin (fraction SP), chicken leptin, and human leptin (hLEP) were prepared in our laboratory as described previously (11, 12, 14); pET29a expression vector was purchased from Novogene

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[1] The abbreviations used are: ECD, extracellular domain; LEP, leptin; LBD, leptin-binding domain; GH, growth hormone; SPR, surface plasmon resonance; RT, retention time; h, human; IL, interleukin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; WT, wild-type.

Inc. (Madison, WI). Restriction enzymes used in the molecular biology experiments were from Fermentas (Vilnius, Lithuania) and New England Biolabs (Beverly, MA). DNA primers were ordered from Invitrogen. Lysozyme, urea, arginine, radioimmunoassay-grade bovine serum albumin, Triton X-100, RPMI 1640 medium, interleukin-3 (IL-3), isopropyl β-D-thiogalactopyranoside (IPTG), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue) were purchased from Sigma, fetal calf serum was from Biolab Co. (Jerusalem, Israel), and Superdex™75 HR 10/30 column, Q-Sepharose, and SP-Sepharose (fast flow) were from Amersham Biosciences. A research-grade CM5 sensor chip, *N*-hydroxysuccinimide, *N*-ethyl-*N*′ (3-dimethylaminopropyl)-carbodiimide hydrochloride, ethanolamine hydrochloride, and HBS-EP running buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, and 0.005% (v/v) surfactant P20, pH 7.4) were purchased from Biacore, AB (Uppsala, Sweden). All other chemicals were of analytical grade.

Preparation of LBD Expression Plasmid—A DNA insert encoding the LBD fragment, consisting of amino acids 428–635 of the leptin receptor, was prepared by PCR using the following primers: the 5′-sense primer, 5′-GGAATTCCATATGATTGATGTCAATATCAATATCTC-3′ containing an *Nde*I restriction site (underlined) and the antisense 3′-end primer, 5′-CATAGGAAGCTT**TCA**ATCCATGACAACTGTGTAGGCTGG-3′ containing a stop codon (bold letters) followed by a *Hind*III site (underlined). The resulted PCR product was cloned into the pGEM-T vector, sequenced to ensure lack of mutations, digested with *Nde*I/*Hind*III, and subcloned into the pET29a plasmid, predigested with the same restriction enzymes. The expression plasmid was then transformed into BL21 cells.

Expression, Refolding, and Purification of LBD—BL21 cells (500 ml) were grown in a 2.5-liter flask in Terrific Broth (TB) medium at 37 °C to an A_{600} of 0.9, and IPTG was then added to a final concentration of 1 mM. Cells were grown for an additional 4 h and then harvested by centrifugation at 16,000 × g for 10 min and frozen. The bacterial pellet from 3 liters of culture was thawed on ice and resuspended in lysis buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8) containing 0.5 mg lysozyme/ml. Inclusion bodies were then prepared as described previously and frozen (11). Subsequently, inclusion bodies obtained from 3 liters of bacterial culture were solubilized in 600 ml of 4.5 M urea, pH 11.5, in the presence of 10 mM cysteine. After 1 h of stirring at 4 °C, the solution was diluted with 2 vol of 0.75 M L-Arg to a final concentration of 0.5 M and stirred for an additional 10 min, and then the clear solution was dialyzed against 5 × 10 liters of 10 mM Tris-HCl, pH 9. The protein was then applied to a Q-Sepharose column (2.5 × 6 cm) pre-equilibrated with 10 mM Tris-HCl, pH 9. The breakthrough fraction (which contained no LBD) was discarded, the absorbed protein was eluted in a stepwise manner by increasing concentrations of NaCl in the same buffer, and 5-ml fractions were collected. Protein concentration was determined by absorbance at 280 nm.

Determination of the Amino-terminal Sequence—Automated Edman degradation technique was used to determine the amino-terminal protein sequence. Degradation was performed on an ABI Model 470A gas-phase sequencer (Foster City, CA) using the standard sequencing cycle. The respective phenylthiohydantoin derivatives were identified by reverse phase-high pressure liquid chromatography analysis, using an ABI Model 120A phenylthiohydantoin analyzer fitted with a Brownlee 2.1-mm inner diameter phenylthiohydantoin-C_{18} column.

Determination of Purity and Monomer Content—SDS-PAGE was carried out according to Laemmli (32) in a 15% polyacrylamide gel under reducing and non-reducing conditions. Gels were stained with Coomassie Brilliant Blue R. Gel filtration chromatography was performed on a Superdex™75 HR 10/30 column with 0.2-ml aliquots of the Q-Sepharose column-eluted fractions using 25 mM TN buffer (Tris-HCl buffer, pH 8, containing 150 mM NaCl). Freeze-dried samples were dissolved in H_2O.

Determination of CD Spectra and Extinction Coefficients—The CD spectra in millidegrees were measured with an AVIV model 62A DS circular dichroism spectrometer (Lakewood, NJ) using a 0.020-cm rectangular QS Hellma cuvette. The spectrometer was calibrated with camphorsulfonic acid. The absorption spectra were measured with an AVIV model 17DS UV-visible IR spectrophotometer using a 1.000-cm QS cuvette and correction for light scattering. Lyophilized protein was dissolved in water, dialyzed against 50 mM phosphate buffer, pH 7.5, for 20 h, and then centrifuged at 11,000 × *g* for 10 min. The CD measurements were performed at 25.0 °C as controlled by thermoelectric Peltier elements to an accuracy of 0.1 °C. The CD spectra were measured in five repetitions resulting in an average spectrum for each protein. Standard deviation of the average CD signal at 222 nm was in the 5% range. For the secondary structure determination, the CD data were expressed in degree cm^2/dmol per mean residue, based on a molecular mass of 24.6 kDa calculated for the protein from the 208 amino acids. The protein concentration was determined by the Biuret method (33) in five repetitions at different dilutions for each protein, using lysozyme as a reference (A_{280} = 0.388 at 1 mg/ml) (34). The obtained protein concentration values were applied for both extinction coefficient determination at 280 nm and for secondary structure determinations using CD spectra. The secondary structure of the protein was calculated by applying the procedure and computer program CONTIN developed by Provencher and Glöckner (35). The program determines α-helices, β-strands, and β-turns as percentage of amino acid residues involved in these ordered forms. Unordered conformation was determined as unity minus the sum of all elements of the secondary structure (36). In the present study, for calculations by the CONTIN program, a set of standard CD spectra of 17 proteins (37) was employed.

Determination of Complex Stoichiometry—Complexes between LBD and hLEP were prepared at various molar ratios in TN buffer. After a 20- to 30-min incubation at room temperature, 200-μl aliquots were applied to a Superdex™75 HR 10/30 column. To determine the molecular mass of the complex, the column was calibrated with several pure proteins.

Binding Assays—Radiolabeled human ^{125}I-leptin served as a ligand, and all other (human, ovine, and chicken) nonlabeled leptins served as competitors. The experiments were conducted using either recombinant LBD or homogenates of BAF/3 cells stably transfected with the long form of hLEP receptor. In the latter case, the cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum in the presence of IL-3 to minimize leptin-receptor down-regulation until a concentration of 10^6 cells/ml was reached. Then the cells were spun and stored at −70 °C. Prior to each experiment, the cells were thawed, suspended at 10^6 cells/150 μl of reaction buffer (12.5 mM sodium barbiturate, pH 8.6, buffer containing 0.1% (w/v) bovine serum albumin, 7.5 mM EDTA, 150 mM NaCl, and 0.1% (w/v) Triton X-100), and homogenized with a Polytron for 30 s at 10,000 rpm on ice. Each tube contained 150 or 200 μl of reaction buffer in the case of the assay with the cells or recombinant LBD, respectively, 100 μl of ^{125}I-hLEP (100,000 cpm for cells or 180,000 cpm for binding domain assays), and 100 μl of different leptin solutions (providing 0–5000 ng/tube) in the reaction buffer, and the reaction was started by addition of 150 μl of cell homogenate or 100 μl of LBD (20 ng). The tubes were incubated for 24 h at room temperature. Then the leptin·receptor complex was precipitated by adding 250 μl of 1% (w/v) bovine immunoglobulin and 500 μl of 20% (w/v) polyethylene glycol. After thorough mixing, the tubes were incubated for 20 min at 4 °C and centrifuged at 12,000 × g for 15 min at 4 °C. Then supernatant was carefully aspirated, and the precipitates were counted in a Kontron γ-counter. Human leptin was iodinated according to a protocol described previously for the iodination of human growth hormone (hGH) (38).

Kinetic Measurements of LBD-hLEP Interactions—All experiments were performed at 25 °C using surface plasmon resonance (SPR) methodology. The kinetics and equilibrium constants for the interaction between hLEP and LBD were determined using the Biacore 3000 system (Uppsala, Sweden). hLEP was immobilized in a flow cell of a research-grade CM5 sensor chip using amine-coupling chemistry (39). The immobilization steps were carried out at a flow rate of 10 μl/min in HBS-EP buffer. The surface was activated for 7 min with a mixture of *N*-hydroxysuccinimide (0.05 M) and *N*-ethyl-*N*′ (3-dimethylaminopropyl)-carbodiimide hydrochloride (0.2 M). hLEP was injected at a concentration of 50 μg/ml in 10 mM acetate, pH 3.5, until the desired level (1000 resonance units) was achieved. Ethanolamine (1 M, pH 8.5) was injected for 7 min to block the remaining activated groups. A control surface was prepared by activating the carboxyl groups and then blocking the activated groups by ethanolamine as described. For the binding studies, the LBD, resuspended in HBS-EP buffer, was passed at different concentrations (31.25, 62.5, 125, and 250 nM) through both flow cells at a rate of 30 μl/min. Regeneration of the surface after each interaction was performed by using a 10-μl pulse of 10 mM glycine buffer, pH 2. The experiment was done using the kinetics Wizard of the Biacore control software, which corrects automatically for refractive index changes and nonspecific binding by subtraction of the responses obtained for the control surface from the data obtained for the interaction with hLEP. The obtained binding curves were fitted to the association and dissociation phases at all leptin receptor concentrations simultaneously using evaluation software from Biacore. The best fit was obtained for a simple bimolecular interaction (Langmuir model).

BAF/3 Proliferation Assay—The proliferation rate of leptin-sensitive BAF/3 1442-CI4 cells stably transfected with the long form of human leptin receptor was used to estimate self- and antagonistic activity of

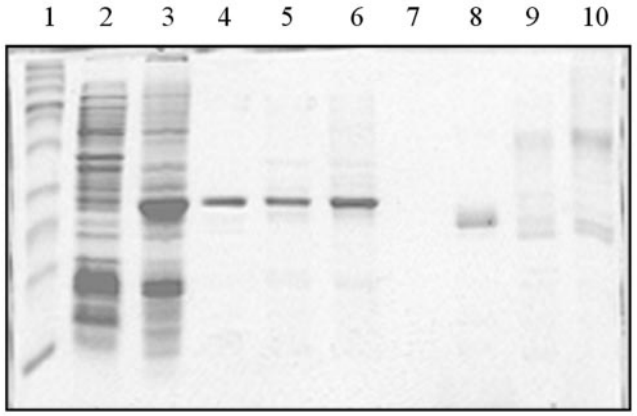

FIG. 1. **SDS-PAGE analysis of recombinant hLBD on a 15% gel.** *Lane 1*, molecular mass markers (172, 111, 79.6, 61.3 (the strongest band), 49, 36.4, 24.7, 19.2, 13.1, 9.3 kDa); *lane 2*, IPTG-induced bacteria; *lane 3*, inclusion bodies; *lanes 4–6*, pooled 100, 125, and 150 mM NaCl eluates (see legend to Fig. 2) following pretreatment with reducing agent; *lanes 8–10*, the same but without pretreatment with reducing agent; *lane 7*, empty.

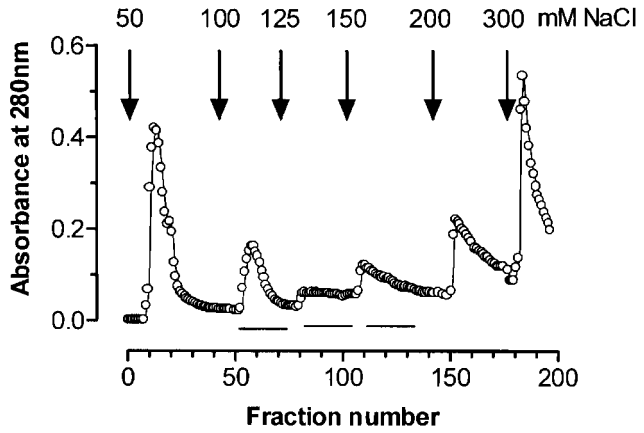

FIG. 2. **Purification of hLBD extracted and refolded from inclusion bodies on a Q-Sepharose column.** The column (2.5 × 7 cm) was equilibrated with 10 mM Tris-HCl, pH 9.0, at 4 °C. The dialyzed solution of refolded protein was applied to the column at a rate of 120 ml/h. Elution was carried out using a discontinuous NaCl gradient in the same buffer at 120 ml/h, and 5-ml fractions were collected. Protein concentration was determined by absorbance at 280 nm. Every fifth tube was assayed for hLBD content by gel filtration in a Superdex^{TM}75 HR column (see text). Tubes 51–75, 78–104, and 110–135 were pooled (pools 100, 125, and 150 mM, respectively).

recombinant LBD, using the thiazolyl blue method as described previously (13). To determine antagonistic activity of LBD, human, ovine, or chicken leptin were added to each well (to a final concentration of 0.57 nM) with various concentrations of recombinant LBD. The average absorbance in wells with wild-type leptins after subtraction of the negative control was used as a positive control to calculate percent inhibition caused by LBD.

RESULTS

Purification and Characterization of LBD—Induction of *Escherichia coli* cells by IPTG led to the appearance of a weak band corresponding to LBD, which appeared as a main band in the inclusion bodies (see Fig. 1, *lanes 2* and *3*). Inclusion bodies collected from IPTG-induced cells were solubilized and refolded as described under "Experimental Procedures." Subsequently, the LBD protein was purified by one-step ion-exchange chromatography on a Q-Sepharose column. Every fifth fraction was tested for LBD appearance by gel filtration on a Superdex^{TM}75 HR column. Three fractions containing LBD protein, eluted, respectively, with 100, 125, and 150 mM NaCl from the Q-Sepharose column, were collected and pooled (*underlined* in Fig. 2). Each of those pools was analyzed by gel filtration on a Superdex^{TM}75 HR column. Only the fraction eluted with 100 mM contained over 95% monomeric protein and 5% dimers, whereas fractions eluted with higher NaCl concentrations contained higher amounts of dimers and oligomers (not shown). These results were also verified by SDS-PAGE, showing that only the first fraction contained monomeric LBD under both reducing and non-reducing conditions (Fig. 1, *lanes 4* and *8*) with an approximate molecular mass of 25 kDa, close to the predicted value of 24,616 Da, calculated for Met-LBD. Pools eluted at 125 and 150 mM contained a mixture of monomers and dimers, the latter formed by S-S links (see Fig. 2, *lanes 5* and *6 versus lanes 9* and *10*). The yield of the monomeric fraction (100 mM NaCl eluate) was 4 mg from 3 liters of bacterial culture. The amino-terminal sequence of the purified LBD was Met-Ile-Asp-Val-Asn-Ile-Asn-Ile-Ser-Xaa-Glu, as predicted from the primary structure (40), with an additional Met residue. The unidentified amino acid at position 10 is most likely Cys, which could not be identified by the present method. The results of the CD analysis are presented in Fig. 3. The secondary structure calculations revealed the contents of α-helices, β-strands, β-turns, and unordered forms to be (mean ± S.D.) 6.6 ± 0.4, 37 ± 1.2, 25 ± 1.0, and 31 ± 1.6%, respectively, indicating strong similarity to the structure observed in the ECDs of hGH, human prolactin, and rat prolactin receptors

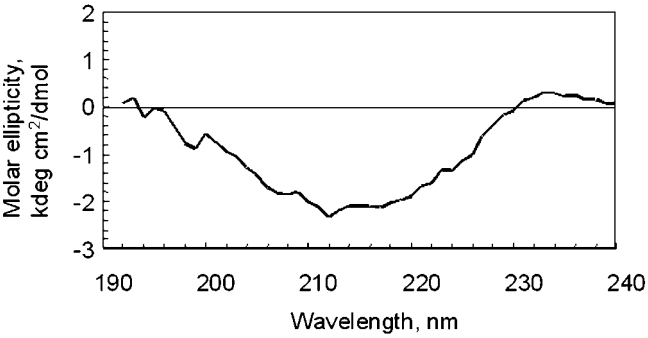

FIG. 3. **CD spectra of purified recombinant leptin-binding domain in 65 mM sodium carbonate buffer, pH 7.5.**

(41–43). The specific absorbance of the protein (1 mg/ml at A_{280}) was 1.95, calculated according to Perkins (44), and this value was used in the calculations in other experiments. LBD lyophilized in the presence of excess $NaHCO_3$ retained its monomeric form, and after solubilization (at 0.5 mg/ml), no dimerization or oligomerization was observed in a solution kept at 4 °C for several days.

Detection of LBD·hLEP Complex by Gel Filtration—The experiment was performed using either a constant concentration of hLEP and increasing concentrations of LBD or *vice versa*. As shown in Fig. 4, both components added alone were eluted from the column as monomers at the respective RTs of 15.45 and 13.93 min. Their molecular masses calculated from the standard curve were 15.3 and 24.8 kDa, respectively, close to the predicted theoretical values. Mixing the two components in a 1:1 molar ratio resulted in a new single peak with an RT corresponding to molecular mass of 39.9 kDa, indicating 1:1 complex formation. Changing the molar ratio by adding excess hLEP or LBD did not change the RT of this peak, further proving that under the present experimental conditions, formation of LBD·hLEP complexes at a 2:1 molar ratio cannot be detected.

Binding Experiments—To evaluate whether the binding properties of LBD are similar to those of the full-size membrane-embedded leptin receptor, we compared the binding of radio-iodinated hLEP to the purified LBD and to a homogenate

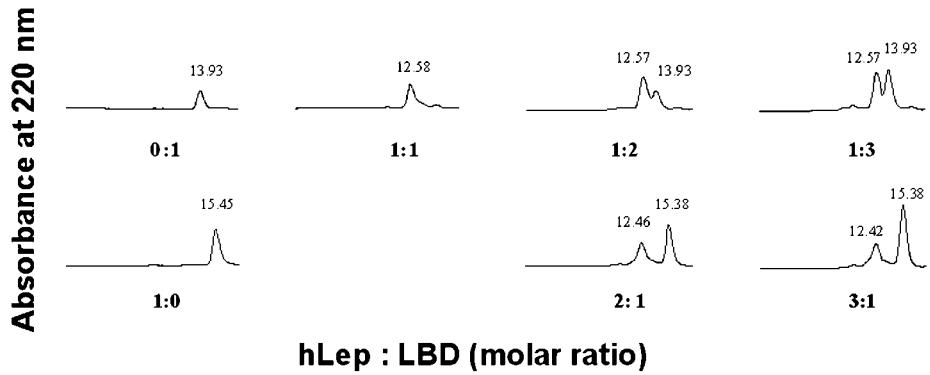

FIG. 4. **Gel filtration of complexes of hLEP and on a Superdex™75 HR 10/30 column.** Complex formation was carried out during a 20- to 30-min incubation at room temperature in TN buffer using various hLEP:LBD molar ratios and then aliquots (200 μl) of the incubation mixture were applied to the column, pre-equilibrated with the same buffer. The initial hormone concentration (2 μM) was constant in all cases in the *upper row*, whereas in the *lower row* the LBD concentration was held constant (4 μM). The column was developed at 0.8 ml/min and calibrated with bovine serum albumin (66 kDa, RT = 10.78 min), egg albumin (45 kDa, RT = 12.11 min), extracellular domain of hGH receptor (28 kDa, RT = 13.52 min), and ovine placental lactogen (23 kDa, RT = 14.12 min). Protein concentration in the eluate was monitored by absorbance at 220 nm. Each experiment was conducted at least three times.

of BAF/3 cells stably transfected with the long form of human leptin receptor. In addition to hLEP, ovine and chicken leptins were also employed to displace the radioactive ligand. Results shown in Fig. 5 highlight two differences: (i) the K_d for binding of hLEP to LBD was 7-fold higher than to the BAF/3 homogenate (5.91 ± 1.10 *versus* 0.83 ± 0.14 nM, mean ± S.E.), and (ii) chicken leptin could displace binding of hLEP to BAF/3 homogenate (though its capacity was ~ 20-fold lower than that of hLEP) but not to LBD. In contrast, the differences between human and ovine leptins were minimal.

SPR Determination of the Interaction between hLEP and LBD—The interactions of hLEP and LBD were analyzed by comparison with a theoretical model using Chi-square analysis. In all cases, the interactions proved to be best suited to the 1:1 model (not shown). Analysis of the data presented in Fig. 6 resulted in a k_{off} constant (mean ± S.E.) of $1.85 \pm 0.30 \times 10^{-3}$ s^{-1}, indicating a complex half-life of 6.24 min. The k_{on} calculated by averaging the results obtained at five concentrations of LBD was $1.2 \pm 0.30 \times 10^5$ mol^{-1} s^{-1} and the corresponding K_d value was calculated as 1.54×10^{-8} M.

Inhibition of Human, Ovine, and Chicken Leptin-induced Proliferation of BAF/3 Cells by LBD—BAF/3 cells stably transfected with the long form of human leptin receptor (45) were chosen to test this activity, because proliferation of those cells can be stimulated by both leptin from various sources (11–13) and by IL-3 (45). LBD inhibited the proliferation of BAF/3 cells stimulated, respectively, by human, ovine, and chicken leptins in a dose-dependent pattern, but the molar excess required to achieve 50% inhibition in cells stimulated by human, ovine, or chicken leptins was rather large, namely 200, 200, and 600 molar excess, respectively (Fig. 7). The inhibitory effect was, however, very specific, as no inhibition was observed in cells stimulated by IL-3 even at a 10^5 molar excess of LBD.

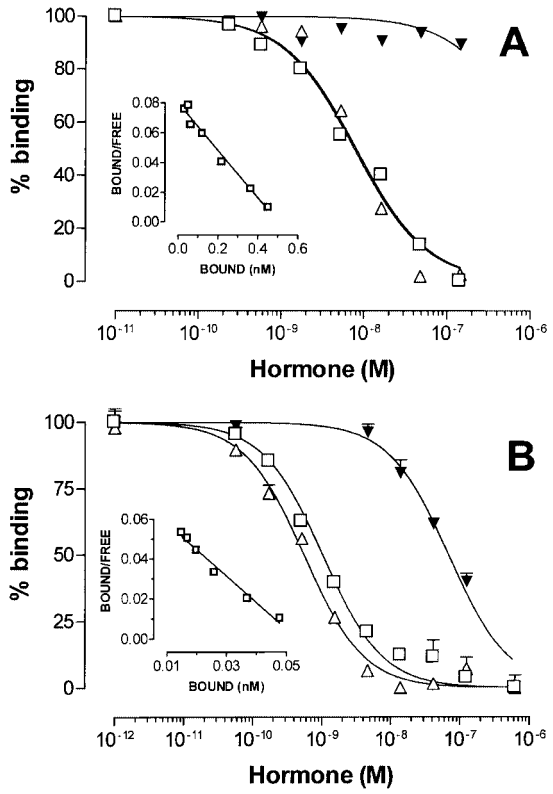

FIG. 5. **Competition of unlabeled human leptin (□), ovine leptin (△), and chicken leptin (▼) with ^{125}I-human leptin (80,000 cpm/tube) for binding to LBD (*A*) and to homogenate of BAF/3 cells (*B*).** The specific binding (%) in experiments performed with human, ovine, and chicken leptins and their mutants were, respectively, 7.3% in *A*, and 8.1% in *B*, and the nonspecific binding was respectively, 5.4 and 14%. All values for specific binding were normalized, and the *solid lines* and the IC_{50} values were calculated using the PRIZMA curve-fitting program (59).

DISCUSSION

The present work clearly indicates the feasibility of producing recombinant LBD, a 208-amino acid fragment of the ECD of human leptin receptor (corresponding to residues 428 to 635 of the full-size WT receptor), which has the ability to bind human and other leptins. Though the yield is rather low at present, further experiments aimed at scaling up its production will enable an increase in yield and the production of enough material for both structural and *in vivo* studies. The electrophoretically pure monomeric protein was capable of forming a stable 1:1 complex with hLEP. Preparation of LBD capable of binding leptin raises two questions. (i) Does it bind leptin at an affinity similar to that of the full-size leptin receptor ECD? (ii) Are the affinities of the soluble and membrane-embedded leptin receptor comparable? To answer those questions we performed several binding experiments using either classical methods or SPR with pure recombinant LBD and membrane-embedded leptin receptor in BAF/3 cells stably transfected with this protein. Our results are compiled in Table I and compared with results reported by other groups. To answer the first question, comparison of the binding of LBD to full-size leptin

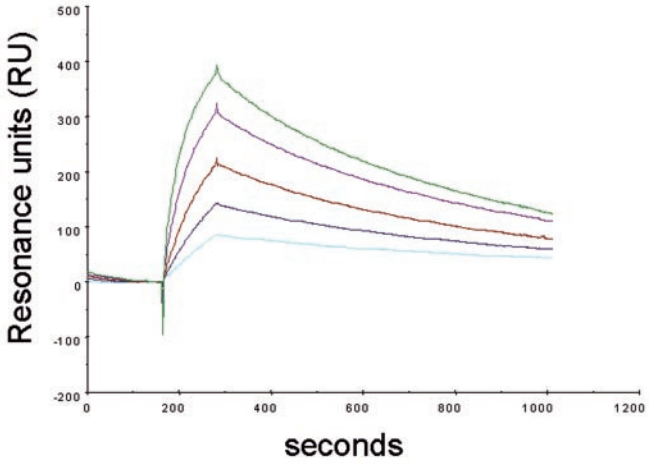

FIG. 6. **Association and dissociation kinetics between LBD and hLEP linked covalently to carboxy-methylated dextran through amino groups.** For other details see text.

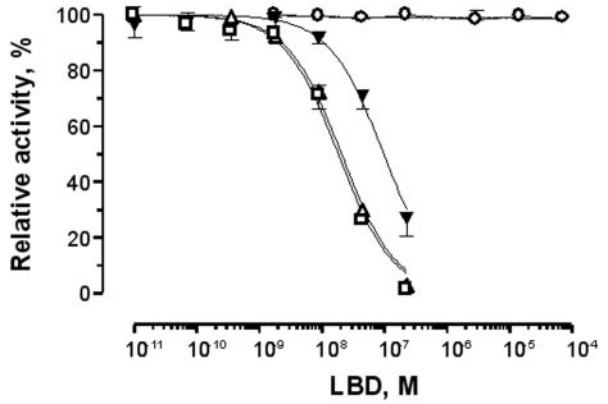

FIG. 7. **Inhibition of human (□)-, ovine (△)-, chicken (▼)-, and interleukin-3 (○)-stimulated proliferation of BAF/3 cells transfected with the long form of human leptin receptor.** Synchronized cells were grown for 48 h in the presence of human, ovine, or chicken leptin (0.57 nM) or interleukin-3 (6 nM) and various concentrations of LBD. The number of cells was determined subsequently by the thiazolyl blue method (see text). *Full lines* and IC_{50} values were calculated using the PRIZMA curve-fitting program (59).

TABLE I
Comparison of K_d values for interaction of human leptin with human leptin receptors

Leptin receptor	K_d or IC_{50}	Method	Reference
	nM		
WT in BAF/3 cell homogenate	0.83	Binding	Present work
WT in BAF/3 cell homogenate	1.03[a]	Binding	Present work
LBD	5.93	Binding	Present work
LBD	15.3	SPR	Present work
LBD	7.6[a]	Binding	Present work
WT in COS cells	0.6[a]	Binding	31
Minimal BD in COS cells[b]	1.3[a]	Binding	31
WT in COS7 cells	~0.2[a]	Binding	24
ECD secreted by COS7 cells[c]	~0.2[a]	Binding	24
WT COS7 cells	0.9	Binding	57
ECD secreted by Sf9 cells[d]	9.5	SPR	46
ECD in human serum	0.42	Binding	58

[a] IC_{50} values.
[b] The minimal binding domain (BD) consisting of leptin BD (LBD) with upstream immunoglobulin domain anchored in COS cells.
[c] Full-size extracellular domain (ECD) engineered to be secreted.
[d] Partially purified His_6 and FLAG-tagged ECD prepared in Sf9 cells using baculovirus expression system.

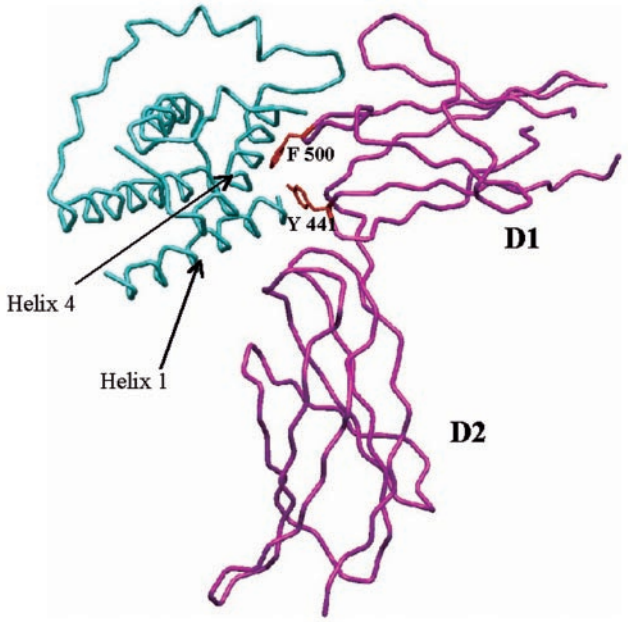

FIG. 8. **Schematic representation of the human leptin·LBD 1:1 complex.** The amino- and carboxyl-terminal domains of LBD are denoted as *D1* and *D2*, respectively. Tyr-441 and Phe-500, which may be crucial for leptin binding, are labeled and shown in *red*.

receptor ECD (46) is most relevant, because both experiments were conducted by a similar method, SPR. This comparison shows that the affinities are quite similar (15.3 *versus* 9.5 nM) and suggests that other parts of the ECD beyond the LBD region play only a minor, if any, role in binding of the hormone. This conclusion is also supported by others (31) who have shown a rather minor difference (0.6 *versus* 1.3 nM) in the affinity of the WT receptor as compared with the minimal binding domain that consists of the LBD region flanked by the upstream 100-amino acid long immunoglobulin domain. In contrast, other data (24) are not consistent with this conclusion, as the IC_{50} for LBD is 38-fold higher than that of the full-size ECD. However, this comparison should be made with caution, because the methodology applied during the precipitation step in the binding experiments, in particular in those studying the interaction of soluble proteins, may affect the experimental results. Most of the results also suggested that the affinity of the membrane-embedded receptors is higher than that of the soluble domain. This is similar to an analogous situation existing with several prolactin receptors (47–49), with the exception of rabbit prolactin receptor ECD (50). Again, this conclusion has to be approached with caution, because as already stated, the methodology applied during the precipitation step may affect the results. It has been also suggested that the *N*-glycosylated Asn-624 located near the WS*X*WS motif may affect the refolding of the receptor. Our present data using LBD produced in bacteria, and thus non-glycosylated, do not support this suggestion.

To better understand the LBD-hLEP interaction, a model of the 1:1 complex based on the known three-dimensional x-ray structures of the cytokine-binding region of gp-130 and the hGH receptor-ECD (PDB accession codes 1BQU and 1AXI, respectively) was built. Based on the sequence alignments of these proteins with that of LBD, amino acid mutations, insertions, and deletions were applied by using the graphic program O (51). The modeled LBD structure and the known three-dimensional structure of hLEP (PDB accession code 1AX8) (52) were used to construct the 1:1 LBD·hLEP complex. The 1:1 model was then minimized via CNS software (53). The resulting model was then utilized to assess plausible amino acid

residues that may either enhance or reduce binding to the leptin hormone, and the final model is presented in Fig. 8.

The ligand-binding determinants of cytokine receptor ECDs consist of six segments denoted L1–L6 (41, 54). These segments are positioned in three loop regions, L1–L3 situated in the amino-terminal domain, L4 in the interdomain linker, and L5 and L6 in two main loops, located in the carboxyl-terminal domain. Previous structural and mutational research with the hGH and hGH receptor ECD system has indicated that the binding epitope consists of many interacting residues, some of which are crucial for ligand binding (55). One of these residues is Phe-500, located in loop L3, where an aromatic residue is conserved throughout the sequences of the cytokine receptor superfamily. An additional residue that may have an impact on leptin binding is Tyr-441, located in L1 (Fig. 8). Preliminary results indeed indicate that mutation each of those amino acids to Ala leads to loss of ability to bind leptin.[2] The WS motif consisting of residues WSNWS (622–626) in the LBD, and regarded as a signature sequence of the cytokine receptor superfamily (56), is located toward the last strand (β-G) of the carboxyl-terminal domain (D2). An additional Trp (Trp-583) extends the WS motif into the LBD. Two arginine residues (Arg-612 and Arg-573) are sandwiched between each tryptophan pair to form an extended π-cation system.

Although the affinity of LBD toward hLEP is somewhat lower than that of the full-length, membrane-embedded receptor-soluble system could be useful as a model for mapping of the binding epitope of both receptor and hormone. A short fragment of the receptor with high affinity binding capabilities to the hormone provides a higher potential system for crystallization and subsequent structural studies. Furthermore, extensive mutagenesis and subsequent binding assays would identify the crucial amino acid residues in the binding sites and may provide a platform for the design of small molecules and/or peptidic high affinity binders of leptin receptor.

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