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

Received for publication, July 26, 2002

Yael Sadowski‡†§, Nina Raver‡§, Eugene E. Gussakovsكي, Suzan Shochat‡**, Orly Dym**, Oded Livnah**, Menachem Rubinstein‡‡, Radha Krishna‡, and Arieh Gertler§§

From the ‡Institute of Biochemistry, Food Science and Nutrition, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University, Rehovot 76100, Israel, §Department of Life Sciences, Bar Ilan University, Ramat Gan 52900, Israel and Institute of Horticulture, The Volcani Center, ARO, Bet Dagan 56285, Israel, **The Institute of Life Sciences, Faculty of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel, ‡‡Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel and §§Diagnostic System Laboratories, Webster, Texas 77598

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_{eq} and k_{on} values (mean ± S.E.) of 1.20 ± 0.23 × 10^{-5} M^{-1} s^{-1} and 1.85 ± 0.30 × 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 Thr-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 gonadotrophic 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 to mice reduces body fat 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 leptin receptor located in the choroid plexus (3, 5).

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.

*This work was supported by Israeli Science Foundation Research Grant 594/02 (to A. G.). 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.

§ Contributed equally to this work.

† To whom correspondence should be addressed. Tel.: 972-89-489-006; Fax: 972-89-476-189; E-mail: gertler@agri.huji.ac.il.

‡ Contributed equally to this work.

§§ To whom correspondence should be addressed. Tel.: 972-89-489-561; Fax: 972-89-476-189; E-mail: gertler@agri.huji.ac.il.

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

46304 This paper is available on line at http://www.jbc.org
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’-O-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue) were purchased from Sigma, fetal calf serum was from BioIdeas Co. (Jerusalem, Israel), and SuperdexTM75 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 NaH2PO4, 200 mM ethanolamine hydrochloride, and 0.05% NaN3) were obtained 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’-GGAAATCTGATGTTGACATATGTTGATGTCAATATCAATATCTC-3’ containing an NdeI restriction site (underlined) and the antisense 3’-end primer, 5’-CATAGAACCTTCACATCTGACAATCTGTTGAGCTGG-3’ containing a stop codon (bold letters) followed by a HindIII site (underlined). The resulting PCR product was cloned into the pGEM-T vector, sequenced to ensure lack of mutations, digested with NdeI and HindIII, and subcloned into the pET28a 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 A562 of 0.9, and IPTG was then added to a final concentration of 0.4 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 batch cultures were solubilized in 600 ml of 6.5 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 i-Arg to a final concentration of 0.5 M urea 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 of the protein was used to determine the amino-terminal protein sequence. Degradation was performed on an ABI Model 470A degradate 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-C8 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 SuperdexTM75 HR 10/30 column with 0.2-ml aliquots of the Q-Sepharose fractions using as the elution buffer 100 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 125I-hLEP (100,000 cpm for cells or 180,000 cpm for binding domain assays), and 100 μl of different leptin solutions (providing 0, 1, 10, 100, and 1,000 μl of 125I-hLEP). 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 (tGH) (38).

Kinetic Measurements of BD-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 mM) and N-ethyl-N‘-(3-dimethylaminopropyl)-carbodiimide hydrochloride (0.2 mM). 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 mM, pH 8.5) was injected for 7 min to block the remaining activated groups. A control flow cell of the bare gold chip was injected with acidified ethanolamine (0.01 M) and blocking the activated groups by ethanolamine as described. For the binding studies, the LBD, reconstituted 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 1 M glycine buffer, pH 2. The BIA evaluation software was used for data evaluation. The best fit was obtained for a simple bimolecular interaction (Langmuir model).

BAG3 Proliferation Assay—The proliferation rate of leptin-sensitive BAG3 1442-C14 cells stably transected with the long form of human leptin receptor was used to estimate self- and antagonistic activity of
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 SuperdexTM75 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 SuperdexTM75 HR column. The column (2.5 cm × 70 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 SuperdexTM75 HR column (see text). Tubes 51–75, 78–104, and 110–135 were pooled (pools 100, 125, and 150 mM, respectively).

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 SuperdexTM75 HR column (see text). Tubes 51–75, 78–104, and 110–135 were pooled (pools 100, 125, and 150 mM, respectively).

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 A280) 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 NaHCO3 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 values of 15.2 and 24.6 kDa, respectively.

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.
resulted in a 1:1 model (not shown). Analysis of the data presented in Fig. 6
sis. In all cases, the interactions proved to be best suited to the
comparison with a theoretical model using Chi-square analy-
LBD—human and ovine leptins were minimal. hLEP) but not to LBD. In contrast, the differences between
chicken leptin could displace binding of hLEP to BAF/3 homo-
genate (though its capacity was
cell stably transfected with the long form of human
leptin receptor (45) were
fected with the long form of human leptin receptor (45) were
stimulated by IL-3 even at a 105 molar excess of LBD.
LBD inhibited the proliferation of BAF/3 cells
stimulated, respectively, by human, ovine, and chicken leptins
and by IL-3 (45). LBD inhibited the proliferation of BAF/3 cells
stimulated by both leptin from various sources (11–13)
and membrane-embedded leptin receptor in BAF/3 cells stably transfected with
The specific binding (%) in experiments performed with
human, ovine, and chicken leptins and their mutants were, respec-
tively, 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 normal-
ized, and the solid lines and the IC50 values were calculated using the
PRIZMA curve-fitting program (59).

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 106 molar excess of LBD.

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 homoge-
nate (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 homoge-
genate (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 analy-
sis. 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 $k_{on}$ constant (mean ± S.E.) of 1.85 ± 0.30 × 103 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 $2.2 ± 0.30 × 10^5$ mol–1 s–1 and the corresponding $K_d$
value was calculated as $1.54 × 10^{-8}$ M.

The present work clearly indicates the feasibility of produc-
ing 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 ma-
terial 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

FIG. 4. Gel filtration of complexes of hLEP and on a Superdex75 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 gH 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.

FIG. 5. Competition of unlabeled human leptin (□), ovine lep-
tin (○), and chicken leptin (•) with125I-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, respec-
tively, 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 normal-
ized, and the solid lines and the IC50 values were calculated using the
PRIZMA curve-fitting program (59).

DISCUSSION

affinity similar to that of the full-size leptin receptor ECD? (ii)
Are the affinities of the soluble and membrane-embedded lep-
tin receptor comparable? To answer those questions we per-
formed several binding experiments using either classical
methods or SPR with pure recombinant LBD and membrane-
embodied 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
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 IC50 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 may affect the 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 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 WSXWS 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

---

**TABLE I**

Comparison of Kd values for interaction of human leptin with human leptin receptors

| Leptin receptor                  | Kd or IC50 | Method | Reference |
|---------------------------------|-----------|--------|-----------|
| WT in BAF/3 cell homogenate     | 0.83 nM   | Binding| Present work |
| WT in BAF/3 cell homogenate     | 1.03 nM   | Binding| Present work |
| LBD                             | 5.93 nM   | Binding| Present work |
| LBD                             | 15.3 nM   | SPR    | Present work |
| LBD                             | 7.6 nM    | Binding| Present work |
| WT in COS cells                 | 0.6 nM    | Binding| 31         |
| Minimal BD in COS cells         | 1.3 nM    | Binding| 31         |
| WT in COS7 cells                | ~0.2 nM   | Binding| 24         |
| ECD secreted by COS7 cells      | ~0.2 nM   | Binding| 24         |
| WT COS7 cells                   | 0.9 nM    | Binding| 57         |
| ECD secreted by SF9 cells       | 9.5 nM    | SPR    | 48         |
| ECD in human serum              | 0.42 nM   | Binding| 58         |

---

**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 IC50 values were calculated using the PRIZMA curve-fitting program (59).

**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.
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 work 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 affects the binding affinity of LBD toward hLEP in the binding assays and bioassays.

Although the affinity of LBD toward hLEP is somewhat lower than that of the full-length, membrane-embedded receptor-soluble system could be used 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.

Acknowledgment—We thank Nava Chapnik-Cohen for technical help in the binding assays and bioassays.

REFERENCES

1. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994) Nature 372, 425–432
2. Campfield, L. A. (2000) Front. Horm. Res. 28, 120–220
3. Friedman, J. M. (2000) Science 287, 448–451
4. Cohen, B., Novick, D., and Rubinstei, N. (1996) Science 274, 1185–1188
5. Spicer, L. J., and Francisco, C. C. (1998) Gen. Comp. Endocrinol. 115, 1–11
6. Karsan, H., Zner, M. R., and Strasburger, C. J. (1995) J. Biol. Chem. 270, 3183–3186
7. Siess, H., Pitot, B., Chambard, J. C., and Strasburger, C. J. (2002) J. Biol. Chem. 277, 7659–7700
8. Gertler, A., Simons, J., and Keisler, D. H. (1998) FEBS Lett. 442, 137–140
9. Raver, N., Zoon, M., Drider, D., Zerouati, P., Simon, J., Robinson, B., Dijane, J., and Gertler, A. (2000) Protein Expression Purif. 19, 30–40
10. Raver, N., Vardy, E., Linnah, O., Devos, R., and Gertler, A. (2002) Gen. Comp. Endocrinol. 126, 52–58
11. Campfield, L. A., Smith, F. J., and Burn, P. (1996) Horm. Metab. Res. 28, 619–622
12. Dridi, S., Raver, N., Gussakovski, E. E., Dervosti, M., Picard, M., Gertler, A., and Zoon, M. (2002) Am. J. Physiol. Endocrinol. Metab. 279, E116–E123
Subcloning, Expression, Purification, and Characterization of Recombinant Human Leptin-binding Domain
Yael Sandowski, Nina Raver, Eugene E. Gussakovsky, Suzan Shochat, Orly Dym, Oded Livnah, Menachem Rubinstein, Radha Krishna and Arieh Gertler

J. Biol. Chem. 2002, 277:46304-46309.
doi: 10.1074/jbc.M207556200 originally published online September 10, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207556200

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

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

This article cites 57 references, 16 of which can be accessed free at http://www.jbc.org/content/277/48/46304.full.html#ref-list-1