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Large-scale preparation of biologically active mouse and rat leptins and their L39A/D40A/F41A muteins which act as potent antagonists

Gili Salomon [a,b], Leonora Niv-Spector [a], Eugene E. Gussakovsky [c], Arieh Gertler [a,*]

[a] *Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University, Rehovot 76100, Israel*
[b] *Protein Laboratories Rehovot (PLR) Ltd., Rehovot 76349, Israel*
[c] *Department of Botany, University of Manitoba, Winnipeg, MB, Canada R3T 2N2*

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Abstract

Expression plasmids encoding mouse and rat leptins and their L39A/D40A/F41A muteins were prepared. The proteins were expressed in *Escherichia coli*, refolded and purified to homogeneity, yielding electrophoretically pure, over 98% monomeric protein. Circular dichroism (CD) analysis revealed that the mutations hardly affect the leptins' secondary structure, and they were similar to previously reported CD spectra for human leptin. Both mouse and rat leptins were biologically active in promoting proliferation in BAF/3 cells stably transfected with the long form of human leptin receptor. The mutations did not change the binding properties to BAF/3 cells as compared, respectively, to non-mutated mouse, rat or human leptins, or their ability to form 1:1 complexes with the leptin-binding domain of chicken leptin receptor. In contrast, their biological activity, tested in a BAF/3 proliferation assay, was abolished and both became potent antagonists. As the LDF (amino acids 39–41) sequence is preserved in all known leptins, the present results substantiate the hypothesis that this sequence plays a pivotal role in leptins' site III and that interaction of leptin with its receptors resembles the corresponding interactions of interleukin-6 and granulocyte colony-stimulating factor their receptors.

Keywords: Mouse leptin; Rat leptin; Leptin antagonists; Site-directed mutagenesis; Leptin site III

Since its discovery [1], leptin has been shown to play a key role in regulation of numerous body functions by serving as an afferent signal to the brain in a negative-feedback loop that regulates body weight [2–4]. A solid body of evidence has shown that genetic defects leading to either leptin deficiency (ob/ob mice) or mutation of leptin receptors (db/db mice, fa/fa rats) results in severe metabolic syndromes including hyperphagia, obesity, and diabetes [5–7]. Leptin, given either centrally or peripherally, causes a dramatic improvement of these symptoms in ob/ob mice via decreasing food intake and increasing energy expenditure [1,6,8] whereas animals with mutation of leptin receptors resist leptin treatments [8]. Leptin resistance is frequently seen among obese animals and humans without mutation in its receptors, where leptin concentrations are abnormally high [8–10]. It has been well documented that plasma leptin concentrations are positively correlated with adipose tissue mass and decrease with weight loss [9,10]. In addition to the availability of leptin and its functional receptors, the downstream signaling pathway of leptin is important and their dysfunctions have been shown to contribute to obesity and leptin resistance [11–13].

The extracellular domain (ECD) of human leptin receptor (LEPR)[1] consists of 841 amino acids divided into several subdomains: (i) signal peptide, amino acids 1–21; (ii) N-terminal cytokine receptor homology domain 1 (CRH1),

* Corresponding author. Fax: +972 8948 9006.
E-mail address: gertler@agri.huji.ac.il (A. Gertler).

[1] *Abbreviations used:* CD, circular dichroism; LEP, leptin; LEPR, leptin receptor; LBD, leptin-binding domain; ch, chicken.

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amino acids 22–328; (iii) immunoglobulin-like domain (IGD), amino acids 329–427; (iv) CRH2, amino acids 428–637, and (v) two consecutive F3 domains, amino acids 636–841 [14]. CRH2 has been identified as the major leptin-binding domain (LBD) [15]. Human and chicken (ch) LBDs have been subcloned and expressed as recombinant proteins which show a 1:1 molar interaction with leptin [16,17]. Several models for the interaction between leptin and LEPR have been suggested [18–20]. However, more recently, Tavernier and his group documented that leptin binding to its receptor resembles the interaction between interleukin 6 (IL6) and its receptor [21,22], and suggested the existence of a novel, previously unidentified leptin site III composed of Ser 120 and Thr 121, responsible for the formation of active 2:2 or 2:4 leptin/LEPR complex. Conversion of these amino acids to Ala resulted in the creation of a leptin antagonist [23]. To determine whether, in addition to the N-terminal part of helix D, other parts of the leptin molecule also contribute to leptin site III, we carefully analyzed the known structures of IL6-receptor complexes (viral (v) IL6/gp130 [24] and IL6/IL6Rα/gp130 complex [25]) in which site III was first identified. Using the sensitive bidimensional hydrophobic cluster analysis (HCA) [26], we identified an LDFI (Leu-Asp-Phe-Ile) sequence (amino acids 39–42), located in the loop connecting helices A and B, as a major sequence contributing to leptin site III. Mutations of some or all of those amino acids to Ala in human and ovine leptins did not change their binding properties, but abolished their biological activity and converted these muteins into antagonists [27]. As the LDF (amino acids 39–41) sequence is preserved in all known leptins and the respective L39A/D40A/F41A and L39A/D40A/F41A/I42A muteins exhibit similar properties [27], we decided to test the role of this sequence in other species by preparing the respective mouse and rat leptin mutants. Thus, we first subcloned and prepared mouse and rat leptins and then mutated them, resulting in a large-scale preparation of potent antagonists.

Materials and methods

Materials

Recombinant human chLBD [17], as well as human leptin, was prepared in our laboratory as described previously [28]. Restriction enzymes used in the molecular biology experiments were from Fermentas (Vilnius, Lithuania) and New England Biolabs (Beverly, MA). Highly pure DNA primers were ordered from Sigma Chemical (Rehovot, Israel). RPMI-1640 medium, interleukin-3 (IL3), nalidixic acid, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue, MTT) were purchased from Sigma (St. Louis, MO), fetal calf serum (FCS) was from Biolab (Jerusalem, Israel); a Superdex 75 HR 10/30 column and Q-Sepharose were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Molecular markers for SDS-gel electrophoresis were purchased from Bio-Rad (Hercules, CA, USA).

Preparation of expression plasmids encoding mouse and rat leptins

Synthetic cDNA encoding the sequences of mouse (GenBank Accession Number AAA64213) and rat (GenBank Accession Number NM_013076) leptins was ordered from Entelechon GmbH (Regensburg, Germany). The cDNA was modified to ensure better codon usage and expression in *Escherichia coli* (Table 1). The cDNA in pTOPO was digested with *Nco*I and *Hin*dIII, extracted, and ligated into linearized pMon3401 expression vector. *E. coli* MON105 competent cells were transformed with the new expression plasmid and plated on LB-agar plates containing 75 μg/ml spectinomycin for plasmid selection. Four *E. coli* colonies were isolated and confirmed to contain the mouse or rat leptin cDNA by digestion with *Nco*I/*Hin*dIII restriction enzymes. All of the colonies were positive and one of them was sequenced.

Table 1
Synthetic DNAs optimized for expression in *E. coli*, used for the preparation of mouse and rat leptins

cDNA encoding mouse leptin[a]

```
  1  CCATGGCTGTTCCGATCCAGAAAGTTCAGGATGACACCAAAACCCTGATCAAAACCATCG
 61  TTACCCGTATCAACGACATCTCTCATACCCAGTCTGTTTCTGCTAAGCAGCGTGTTACCG
121  GTCTGGACTTCATCCCGGGCCTGCATCCGATCCTGTCTCTGTCTAAAATGGACCAGACCC
181  TGGCTGTTTATCAGCAGGTTCTGACCTCTCTGCCGTCTCAGAACGTTCTGCAGATCGCTA
241  ACGACCTGGAAAACCTGCGTGACCTGCTGCATCTGCTGGCTTTCTCTAAATCTTGCTCTC
301  TGCCGCAGACCTCTGGCCTGCAGAAACCGGAATCTCTGGACGGCGTTCTGGAGGCTTCTC
361  TGTATTCTACCGAAGTTGTTGCTCTGTCTCGTCTGCAGGGCTCTCTGCAGGACATCCTGC
421  AGCAGTTGGACGTTTCTCCGGAATGCTGATGAAAGCTTGGATCC
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cDNA encoding rat leptin[a]

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  1  CCATGGCTGTTCCGATCCATAAAGTTCAGGATGACACCAAAACCCTGATCAAAACCATCG
 61  TTACCCGTATCAACGACATCTCTCATACCCAGTCTGTTTCTGCTCGTCAGCGTGTTACCG
121  GTCTGGACTTCATCCCGGGCCTGCATCCGATCCTGTCTCTGTCTAAAATGGACCAGACCC
181  TGGCTGTTTATCAGCAGATCCTGACCTCTCTGCCGTCTCAGAACGTTCTGCAGATCGCTC
241  ATGACCTGGAAAACCTGCGTGACCTGCTGCATCTGCTGGCTTTCTCTAAATCTTGCTCTC
301  TGCCGCAGACCCGTGGCCTGCAGAAACCGGAATCTCTGGACGGCGTTCTGGAGGCTTCTC
361  TGTATTCTACCGAAGTTGTTGCTCTGTCTCGTCTGCAGGGCTCTCTGCAGGACATCCTGC
421  AGCAGTTGGACCTGTCTCCGGAATGCTGATGAAAGCTTGGATCC
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[a] Initiation and stop codons are in bold letters and the six codons that are different in mouse and rat leptin are underlined.

Preparation of mouse and rat leptin muteins L39A/D40A/F41A

To prepare the leptin mutants, the pMon3401 expression plasmids encoding the wild-type (WT) rat or mouse leptin were used as starting material. The leptin inserts were modified with the Stratagene QuickChange mutagenesis kit (La Jolla, CA) according to manufacturer's instructions, using two complementary primers (Table 2), The primers were designed to contain base changes (marked in bold), to obtain the respective mutations but still conserve the appropriate amino-acid sequence, and to modify a specific restriction site (underlined) for colony screening. The procedure included 18 PCR cycles using *Pfu* polymerase. The mutated construct was then digested with *Dpn*I restriction enzyme, which is specific to methylated and hemi-methylated DNA (target sequence: 5′-G^{m6}ATC-3′), to digest the template and select for mutations containing synthesized DNA. XL-1 competent cells were transformed with the mutated plasmids and grown in 5–10 ml LB medium and the plasmids were isolated. Five colonies of each mutant were screened for mutation, using the specific designed restriction site, and revealed at least 80% efficiency. Two colonies of each mutant were sequenced and confirmed to contain the mutation but no unwanted misincorporation of nucleotides. Mon105 competent cells were then transformed with the plasmids and used for expression.

Expression, refolding, and purification of mouse and rat leptins and their muteins

The recombinant WT or mutated mouse and rat leptins with an extra Met-Ala (methionine is cleaved by the bacteria) at the N-terminus were expressed in a 2.5-L culture, upon induction with nalidixic acid, and grown for an additional 4 h. Inclusion bodies (IBs) were then prepared as described previously [28,29] and frozen. Subsequently, IBs obtained from 5 L of bacterial culture were solubilized in 200 ml (rat and mouse leptins) or 300 ml (rat and mouse leptin muteins) of 4.5 M urea, 40 mM Tris base containing 10 mM cysteine. The pH of the solution was adjusted to 11.3 with NaOH. After 2 h of stirring at 4 °C, three volumes of 0.67 M Arg were added to a final concentration of 0.5 M and stirred for an additional 1.5 h. Then, the solution was dialyzed against 10 L of 10 mM Tris–HCl, pH 9, for 60 h, with five or six external solution exchanges. NaCl was added to a final concentration of 150 mM and the protein solution was then applied at maximal flow rate (400–500 ml/h) onto a Q-Sepharose column (30-ml bead volume), pre-equilibrated with the 10 mM Tris–HCl, pH 9, containing 150 mM NaCl. The breakthrough fraction, which contained the respective leptin or leptin mutein, was collected and concentrated between 3 and 4 mg protein/ml. Then, 12-ml portions were applied to a preparative Superdex 75 column (2.6 × 60 cm) pre-equlibrated with 10 mM Tris–HCl, pH 9, containing 150 mM NaCl. Fractions containing the monomeric protein were pooled, dialyzed against $NaHCO_3$ to ensure a 4:1 protein-to-salt ratio and lyophilized.

Determination of purity and monomer content

SDS–PAGE was carried out according to Laemmli [30] in a 15% polyacrylamide gel under reducing and nonreducing conditions. The gel was 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 fraction using TN buffer (25 mM Tris–HCl, 150 mM NaCl, pH 8).

Determination of CD spectra and secondary structure

The CD spectra in the wavelength range of 200–240 nm were measured with an Jasco J-810 Spectropolarimeter (Tokyo, Japan) using a 0.020-cm rectangular QS Hellma cuvette with a spectral resolution of 1 nm and signal-to-noise ratio of about 1% at 210–220 nm. Solutions were prepared dissolving the lyophilized samples in 50 mM phosphate buffer, pH 7.6, followed by centrifugation. Protein concentration of 40–80 μM was determined spectrophotometrically at 280 nm using light scattering correction and extinction coefficient (one mg/ml) of 0.20. Secondary structure of proteins was determined applying procedure and computer program CONTIN developed by Provencher and Glöckner [31] to calculate α-helices, β-sheets, and β-turns as a percentage of amino acids involved in these ordered forms. The constraint that the sum of all elements of the secondary structure in a protein must equal unity has resulted in a remainder, which may be interpreted as the content of random coil [32]. In the present study, for calculations by the CONTIN program, a set of standard CD spectra of 21 proteins was employed [33].

Various binding assays

Determination of leptin:chLBD complex stoichiometry by gel-filtration chromatography and binding assays using radioactive leptin were carried out according to procedures described in our recent manuscript [27].

Table 2
Primers used for the preparation of rat and mouse leptin mutants

Primer	Primer sequence[a]	Modified restriction site[b]
L39A/D40A/F41A-5	S 5′-CAGCGTGTTACCGG**CGC**GG**CTGC**CATCCCGGGCCTGC-3′	*Bsh*TI(−)
L39A/D40A/F41A-3	A 5′-GCAGGCCCGGGATGGCAGCCGCGCCGGTAACACGCTG-3′	*Bsh*TI(−)

[a] S, sense primer; A, antisense primer; all mutations are in bold letters.
[b] Successful mutations were monitored by disappearance (−) of the respective restriction site (underlined).

BAF/3 proliferation assays

The proliferation rate of leptin-sensitive BAF/3 cells stably transfected with the long form of hLEPR was used to estimate both agonistic and antagonistic activity of leptins and leptin muteins as described previously [28]. To determine antagonistic activity, 6.25×10^{-11} M WT homologous leptin was added to each well, which also contained different concentrations of muteins. The average absorbance in wells without leptin (negative control) was used as a blank value and subtracted from other absorbance values to yield the corrected absorbance values. The average absorbance in wells with WT leptin after subtracting the negative control was used as a positive control to calculate percent inhibition. The inhibition curves were drawn using the Prizma (4.0) nonlinear regression sigmoidal dose–response or one-site inhibition program [34] and the IC_{50} values were calculated. It should be pointed out that all mammalian leptins are capable of activating human leptin receptor to almost identical degree [28,29,35].

Results

Purification and physico-chemical characterization of leptins and leptin muteins

Mouse and rat leptins and their L39A/D40A/F41A muteins were purified by consecutive anion-exchange and gel-filtration chromatography (see Materials and methods). The gel-filtration profiles of the four purified proteins show that the Q-Sepharose eluate is composed of a mixture of oligomers, dimers, and monomers in various ratios. In the case of the rat leptin mutein, and in particular in rat leptin, the relative amounts of oligomers and dimers were higher than those in mouse (Fig. 1). The fractions containing pure monomer were pooled, dialyzed against $NaHCO_3$, pH 8, at a 4:1 (w/w) protein/salt ratio and lyophilized. The yields of mouse and rat leptins and their muteins varied from 400 to 700 mg from 5 L of bacterial culture.

The purity and homogeneity of the purified mutants were documented by three independent methods. SDS–PAGE under reducing conditions yielded only one band of ~16 kDa, under both reducing and nonreducing conditions (Fig. 2), and reverse-phase chromatography also yielded a single peak (not shown). As expected, in the absence of reducing agent, the mobility of all four proteins was slightly higher, indicating a globular structure. However, all four bands appeared slightly below the 15-kDa protein marker despite the predicted 16-kDa molecular mass. Since bands of the same size were also observed in human and ovine leptins (not shown), this discrepancy was likely related to the slower

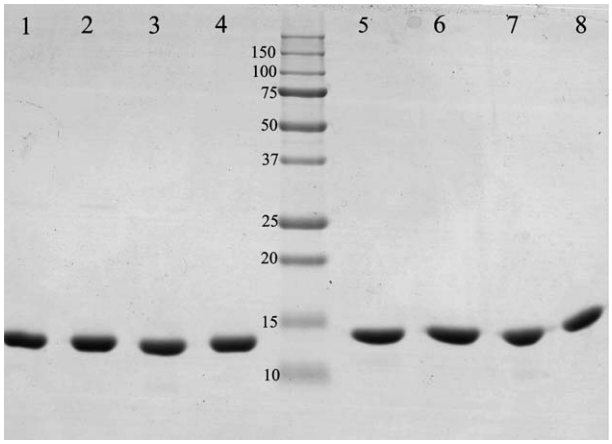


Fig. 2. SDS–PAGE (15%) of mouse and rat leptins and their L39A/D40A/F41A muteins. Lanes 1 and 5, mouse leptin; lanes 2 and 6, mouse leptin mutein; lanes 3 and 7, rat leptin; lanes 4 and 8, rat leptin mutein. Middle lane, molecular-mass markers (in kDa). Lanes 1–4 are without and lanes 5–8 with β-mercaptoethanol. Aliquots of 5 μg of each protein were applied per lane.

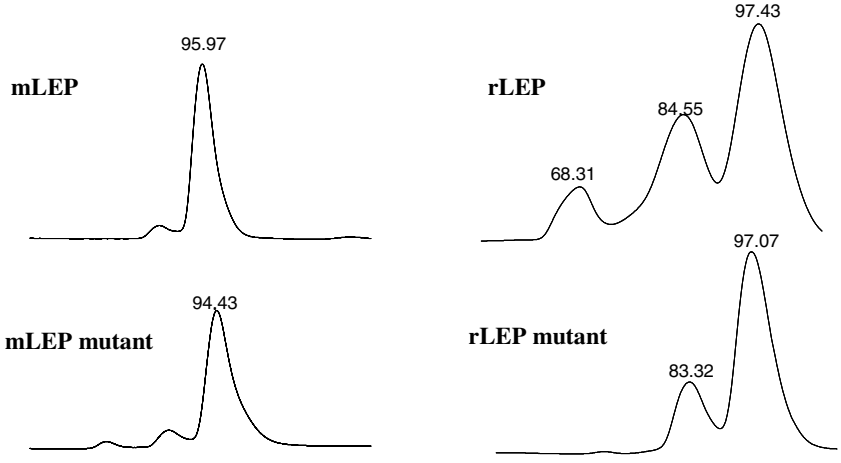


Fig. 1. Gel filtration of mouse (m) and rat (r) leptins (LEP) and their L39A/D40A/F41A muteins on a preparative Superdex 75 column connected to HPLC. The column (2.6 × 60 cm) was equilibrated with 10 mM Tris–HCl, pH 8.0, containing 150 mM NaCl at 4 °C. The breakthrough fractions obtained from anion-exchange chromatography Q-Sepharose column (see text) were concentrated to 3–4 mg/ml and 12-ml aliquots were applied to the column at a rate of 120 ml/h. Elution was carried out using the same buffer at 120 ml/h, and 2-ml fractions were collected. Protein concentration was determined by absorbance at 280 nm. The ordinate axis reflects the concentration and the abscissa axis the time course. Fractions with peak retention times of 94.43–97.43 min contained monomeric proteins and were pooled.

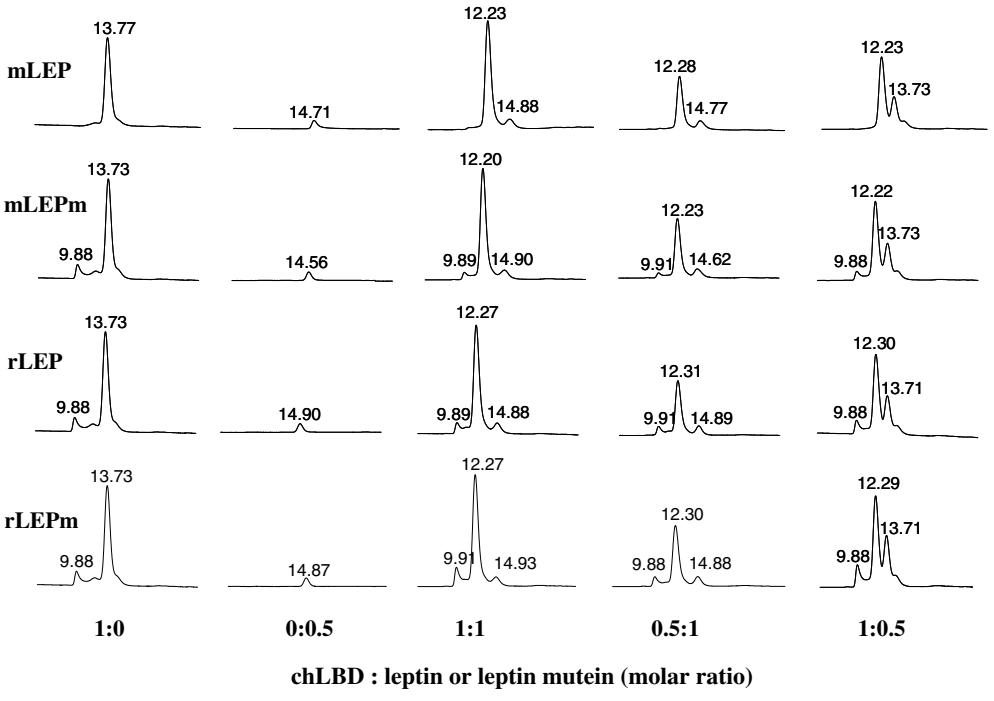


Fig. 3. Gel-filtration analysis of complexes between chLBD and mouse or rat leptin and their muteins on a Superdex 75 HR 10/30 column. Complex formation was achieved by 20-min incubation at room temperature in TN buffer using various molar ratios; 200-μl aliquots of the mixture were applied to the column, pre-equilibrated with the same buffer. The final concentrations (10 μM) of mouse and rat leptins (mLEP and rLEP) and leptin muteins (mLEPm and rLEPm) and of chLBD alone or in complex were marked as 1 in all cases and the final 5 μM concentration as 0.5. The column was developed at 0.8 ml/min and calibrated with bovine serum albumin (66 kDa, RT = 11.47 min), ovine placental lactogen (23 kDa, RT = 14.2 min) and lysozyme (14 kDa, RT = 21.45 min). The ordinate axis reflects the concentration and the abscissa axis the time course. The chLBD used for the complexes with mLEP was over 98% monomeric whereas that used for other experiments underwent partial (<10%) dimerization and oligomerization (see peak with retention time of 9.88 min).

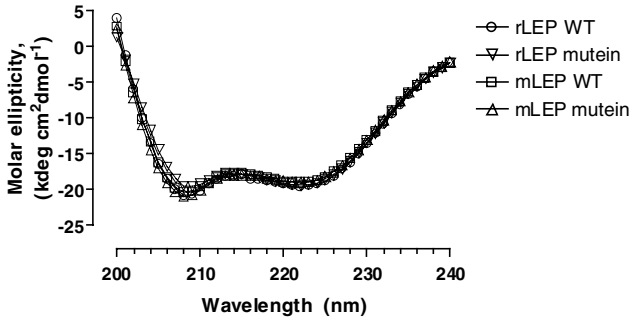


Fig. 4. CD spectra of purified recombinant mouse and rat leptins and their L39A/D40A/F41A muteins. Leptins and leptin muteins were dissolved in 50 mM KH_2PO_4 buffer, pH 7.6. For other details see text.

mobility of the marker. Gel filtration at pH 8 under native conditions yielded a single monomeric peak consisting of over 95% monomer, corresponding to a molecular mass of ~15–16 kDa (Fig. 3, second column from the left). The secondary structures of mouse and rat leptins and their muteins calculated from the CD spectra (see Fig. 4) and compared to human leptin are shown in Table 3. A high content of α-helix (54–56%), 0–1% β-sheets, and 14–18% β-turns were clearly characteristic of all proteins, similar to human leptin, indicating proper refolding. Specific extinction coefficients at 280 nm for a 0.1% solution, assuming an extra Ala at the N-terminus, were calculated according to Pace et al. [36], yielding the following values: 0.201, 0.200, 0.203, and 0.201, respectively, for mouse and rat leptins and their respective muteins. The stability of both mouse and rat leptins and their muteins in solution was tested at 4 and 37 °C. All four proteins could be stored at both temperatures as sterile 0.2 mM solutions for at least 30 days at pH 6 or 8 without undergoing any changes in their monomeric content and retaining their activity in the BAF/3 bioassay.

Table 3
Secondary structure of recombinant mouse (m) and rat (r) leptins (LEP) and their L39A/D40A/F41A muteins at pH 7.6

Secondary structure (%)[a]	mLEP WT	rLEP WT	mLEP mutein	rLEP mutein	hLEP WT[b]
α-Helix	55 ± 0.3	56 ± 0.3	54 ± 0.4	54 ± 0.4	63 ± 0.6
β-Strands	0 ± 0.0	1 ± 0.8	1 ± 0.8	2 ± 1.0	8 ± 1.1
β-Turns	15 ± 0.4	15 ± 0.5	14 ± 0.5	18 ± 0.7	17 ± 0.6
Remainder	30 ± 1.0	27 ± 0.9	31 ± 1.0	26 ± 1.3	12 ± 1.5

[a] Results are given as means ± SD. Errors only arose from uncertainty in the fitting of the experimental CD spectrum with the set of standard protein CD spectra in the CONTIN program. Errors in both the CD measurements and the protein concentration determination were not included.
[b] From [13].

Detection of chLBD-leptin or chLBD-leptin mutein complexes by gel filtration

To characterize the binding stoichiometry between mouse or rat leptins or their muteins and chLBD, the respective ligands and chLBD were mixed in different molar ratios and separated by gel filtration using an analytical Superdex 75 column to determine the molecular mass of the binding complex under nondenaturing conditions. The experiments were performed using a constant 10 or 5 μM of the respective ligand or chLBD. All four proteins formed a 1:1 molar ratio (Fig. 3, middle column) as shown before for other leptins [3,4,13–15]. This stoichiometry was evidenced by the appearance of a single main peak for the complex with shorter retention time (12.20–12.27 min), as compared to the higher retention times of chLBD (13.73–13.77 min) or the leptins (14.56–14.90 min). This main peak appeared when the components were mixed at a 1:1 molar ratio, whereas an additional peak appeared when there was an excess of leptin or chLBD. The complex's calculated molecular mass, based on the peak's retention time, was ~41 kDa in all cases, close to the predicted value of 40.5 kDa.

Binding experiments

Iodinated human leptin served as the ligand in all competitive experiments and the respective WT mouse and rat leptins and their muteins as competitors. Freshly prepared homogenate of BAF/3 cells stably transfected with the long form of human LEPR was used as the receptor source. Homogenate from 1.6×10^6 cells per tube gave 6–7% specific binding. The inhibition curves (average of two experiments) are presented in Fig. 5 and the respective IC_{50} values, calculated by Prism 4.0 program [34] (nonlinear regression, best fit for one-site competition), for human leptin (positive control), and for mouse and rat leptins and their muteins were, respectively, 1.431, 1.925, 2.259, 2.218, and 1.833 nM. Although the IC_{50} value of human leptin was slightly lower, the differences among the IC_{50} values were not statistically significant and all were within the 95% confidence limit, indicating that the mutation did not change the leptins' binding properties.

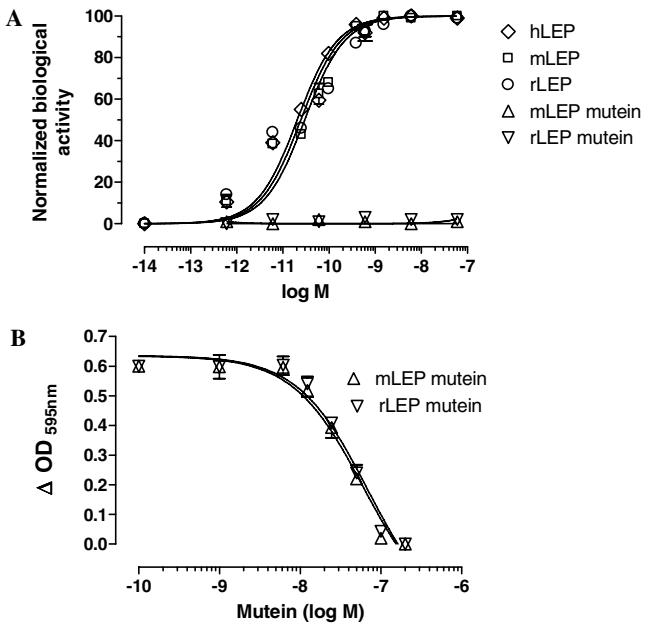
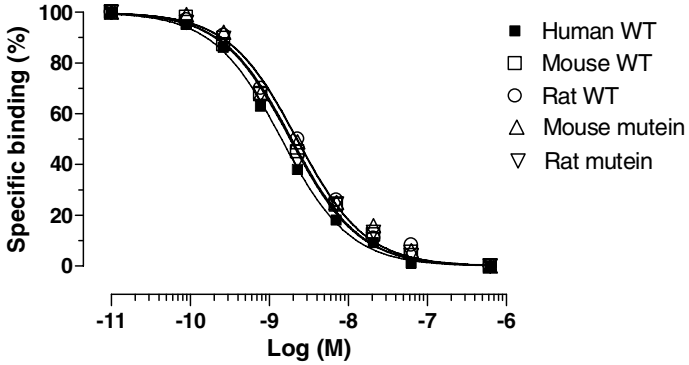


Fig. 6. Effect of mouse (m) and rat (r) leptins (LEP) and their L39A/D40A/F41A muteins on proliferation of BAF/3 cells stably transfected with the long form of human LEPR. Results of several experiments were pooled by normalizing the maximal response to 100 and the absorbance in wells not treated with leptin to zero (A). BAF/3 cells were stimulated with 62.5 pM of human leptin (hLEP) and various amounts of muteins. A representative experiment (out of five performed) is shown (B). In both figures, the results are presented as means ± SD, but the SD values were in most cases too small to be visible on the graph. For other details, see text.



	Human WT	Mouse WT	Rat WT	Mouse mutein	Rat mutein
EC50	1.431e-009	1.925e-009	2.259e-009	2.218e-009	1.833e-009

	Human WT	Mouse WT	Rat WT	Mouse mutein	Rat mutein
EC50	1.310e-009 to 1.564e-009	1.670e-009 to 2.220e-009	1.946e-009 to 2.623e-009	1.794e-009 to 2.742e-009	1.542e-009 to 2.179e-009

Fig. 5. Radio-receptor assay using homogenate of BAF/3 cells stably transfected with the long form of hLEPR. ^{125}I-labeled human leptin (8×10^5 cpm/tube) was used as a ligand and human, mouse, and rat leptins, and mouse and rat leptin muteins L39A/D40A/F41A as competitors. The results are averages of two experiments. The specific binding in two experiments was, respectively, 7.5 and 8.2×10^4 cpm, and was normalized to 100. The nonspecific binding was, respectively, 3.6 and 4.0×10^4 cpm. For other details, see text.

Biological activity in vitro in the BAF/3 bioassay

Human, mouse, and rat leptin exhibited almost identical activity in the BAF/3 bioassay with their respective EC_{50} values of 20.5, 29.7, and 24.5 pM, comparable to human leptin in previously published papers [17,27,28], whereas mouse or rat leptin muteins were devoid of any agonistic activity (Fig. 6A). The differences in the EC_{50} values were not significant as the corresponding 95% confidence intervals (in pM) were 13.3–31.6, 19.1–46.2, and 12.1–49.5. To determine antagonistic activity, BAF/3 cells were stimulated with 62.5 pM of human leptin in the presence (8–250 nM) or absence of the respective muteins. Both mouse and rat leptin muteins exhibited antagonistic activity and no differences between them were observed (Fig. 6B). To verify the specificity of inhibition, the proliferation of BAF/3 cells was stimulated by 55 pM IL3 in the presence or absence of mouse and rat leptin muteins. No inhibition observed, even at a 1.25 μM concentration for both muteins (not shown).

Discussion

Our recent work demonstrated the antagonistic effect of human and ovine leptins mutated to Ala in the LD, LDF or LDFI amino acids located in the loop connecting the A and B helices in positions 39–42 of leptin [27]. That work documented that the binding of the respective muteins is hardly affected, whereas their biological activity, as determined by several bioassays, is completely abolished. As the LDF (or LDFI) sequence is preserved in all known species except pig (LDFS), the present work was devoted to preparing similar triple muteins of mouse and rat leptins. In the first stage of this work, we prepared the WT leptins and to facilitate their high expression, the original DNA sequence was modified to ensure optimal codon usage of *E. coli*. Once the WT proteins were properly refolded and purified into biologically active proteins (using human leptin as a positive control), the DNA insert was mutated to encode the L39A/D40A/F41A variant. Due to the similarity between mouse and rat WT leptin and their respective muteins, we employed the same refolding and purification protocol. As in preliminary experiments, we found that unlike in other mammalian leptins [28,29,35], direct application of dialyzed refolded solution onto a Q-Sepharose column results in low yield, so we modified the protocol by applying the refolded solution in the presence of 150 mM NaCl. Non-protein contaminants were retained by the column while the WT leptins or their muteins were not absorbed. This non-absorbed solution containing oligomeric, dimeric, and monomeric leptins or leptin muteins was concentrated and the three species were resolved by preparative gel-filtration chromatography, yielding up to 40 mg of monomeric protein per run, each run lasting ~2 h. The overall yield was very high (up to 700 mg of pure monomer per 5 L of fermentation medium) and the purity of the isolated monomers was verified by gel filtration, reverse-phase chromatography, and SDS–PAGE under reducing and nonreducing conditions. The higher mobility observed under the nonreducing conditions was characteristic of globular proteins. Proper refolding was further evidenced by CD spectra yielding a characteristically high percentage of α-helices. Furthermore, the presently described procedure yielded protein with a higher 280:260 nm ratio, 1.8–1.9, as compared to the values of 1.5 or 1.6 observed with leptins purified by ion-exchange chromatography alone.

The binding properties of both mouse and rat WT leptins and their muteins to membrane-embedded, long-form hLEPR in BAF/3 cell homogenate were comparable to that of human leptin, indicating a lack of species specificity and showing that the L39A/D40A/F41A mutation of mouse or rat leptin does not affect its binding properties, similar to the respective ovine or human muteins [27]. In contrast, the biological activity of the muteins was completely abolished, although large excesses of the antagonists were required. However, we have shown that the IC_{50} concentration may be dependent on bioassay type: in a luciferase bioassay, the same level of inhibition can be achieved with a 9- to 13-fold antagonist excess [27]. The BAF/3 assay is a very sensitive bioassay, probably because occupation of only a small fraction of the receptors is sufficient to induce a maximal effect in terms of cell proliferation. Thus, it appears that the antagonistic effect is inversely related to the number of spare receptors. The in vivo biological activity of our recombinant rat leptin was also recently documented [37].

The present data, as well as previous reports from us [27] and others [23], confirm the hypothesis that like in IL6 and granulocyte colony-stimulating factor (G-CSF), leptin's site III plays a pivotal role in LEPR dimerization or tetramerization, and in its subsequent activation. This happens due to formation of an active multimeric complex through its interaction with the IGD of LEPR. The presently studied mutation of site III seems to be relevant to leptins from the four species examined to date (human, ovine, mouse, and rat), and in view of the high identity in this sequence, to all other known leptins as well. As shown before (see Fig. 2 in [27]) amino acids 39–41 that form leptin's site III are located in the A–B loop. The accurate position of those amino acids could not be located in the missing leptin chain within the A–B loop as no residues visible in the electron-density map of this fragment were published [38]. They are however located very close to Ser-120 and Thr-121, which are in the N-terminal part of helix D and were identified as contributors to site III [23]. Mutation of these amino acids to Ala converted leptin into leptin antagonist. Thus, it seems that leptin's site III consist of rather large hydrophobic fragment that interacts with the receptor immunoglobulin domain (IGD) in crystal structures of IL6/gp130 complexes [24,25]. Mutation of some (and not necessary all) amino acids of leptin's site III is sufficient to prevent its interaction with IGD and as the

binding site I and II are not affected the corresponding muteins became antagonists.

The muteins reported at the present and former [27] work act as potent antagonists, and are at present the only reagents capable of blocking endogenous leptin in intact animals. Preliminary results have shown their ability to enhance food intake and to block STAT3 phosphorylation in vivo (unpublished results). To facilitate their in vivo use, preparation of long-term-acting variants of leptin antagonists is now in progress.

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