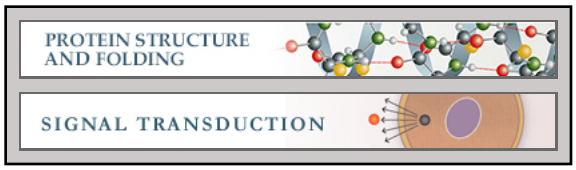
Protein Structure and Folding:

Development and Characterization of High Affinity Leptins and Leptin Antagonists



Michal Shpilman, Leonora Niv-Spector, Meirav Katz, Chen Varol, Gili Solomon, Michal Ayalon-Soffer, Eric Boder, Zamir Halpern, Eran Elinav and Arieh Gertler

J. Biol. Chem. 2011, 286:4429-4442.
doi: 10.1074/jbc.M110.196402 originally published online November 30, 2010

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 6, pp. 4429–4442, February 11, 2011

Development and Characterization of High Affinity Leptins and Leptin Antagonists*[S]

Received for publication, October 25, 2010, and in revised form, November 26, 2010 Published, JBC Papers in Press, November 30, 2010, DOI 10.1074/jbc.M110.196402

Michal Shpilman[‡1], Leonora Niv-Spector[‡1], Meirav Katz[§], Chen Varol[§], Gili Solomon[‡], Michal Ayalon-Soffer[¶], Eric Boder[‖], Zamir Halpern[§], Eran Elinav[§2], and Arieh Gertler[‡2,3]

From the [‡]Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University, Rehovot 76100, Israel, the [§]Research Center for Digestive Tract and Liver Diseases, Tel-Aviv Sourasky Medical Center and the Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv 64239, Israel, [¶]Bioline Innovations, Jerusalem 91450, Israel, and the [‖]Department of Chemical and Biomolecular Engineering, University of Tennessee, Knoxville, Tennessee 37996-2200

Leptin is a pleiotropic hormone acting both centrally and peripherally. It participates in a variety of biological processes, including energy metabolism, reproduction, and modulation of the immune response. So far, structural elements affecting leptin binding to its receptor remain unknown. We employed random mutagenesis of leptin, followed by selection of high affinity mutants by yeast surface display and discovered that replacing residue Asp-23 with a non-negatively charged amino acid leads to dramatically enhanced affinity of leptin for its soluble receptor. Rational mutagenesis of Asp-23 revealed the D23L substitution to be most effective. Coupling the Asp-23 mutation with alanine mutagenesis of three amino acids (L39A/D40A/F41A) previously reported to convert leptin into antagonist resulted in potent antagonistic activity. These novel superactive mouse and human leptin antagonists (D23L/L39A/D40A/F41A), termed SMLA and SHLA, respectively, exhibited over 60-fold increased binding to leptin receptor and 14-fold higher antagonistic activity *in vitro* relative to the L39A/D40A/F41A mutants. To prolong and enhance *in vivo* activity, SMLA and SHLA were monopegylated mainly at the N terminus. Administration of the pegylated SMLA to mice resulted in a remarkably rapid, significant, and reversible 27-fold more potent increase in body weight (as compared with pegylated mouse leptin antagonist), because of increased food consumption. Thus, recognition and mutagenesis of Asp-23 enabled construction of novel compounds that induce potent and reversible central and peripheral leptin deficiency. In addition to enhancing our understanding of leptin interactions with its receptor, these antagonists enable *in vivo* study of the role of leptin in metabolic and immune processes and hold potential for future therapeutic use in disease pathologies involving leptin.

Leptin, a 16-kDa protein, is a central regulator of body weight (1), as well as a pleiotropic hormone whose involvement in many physiological processes has been well established (2). The three-dimensional structure of leptin was elucidated shortly after its discovery (3), but so far no structural information on its complex with leptin receptor has been published. Although several theoretical models of such a complex have been proposed (4–6), lack of a valid crystallographic structure hampers reliable structural interpretation of any new leptin mutations. In the last decade, leptin has also been documented as a major regulator of the innate and adaptive immune response and a modulator of the onset and progression of autoimmunity in several animal models of disease (7), including rheumatoid arthritis, experimental autoimmune encephalomyelitis (4), and immune-mediated colitis (8). Work published from our and others' laboratories has also shown that leptin enhances thioacetamide-induced liver fibrosis (9) and liver inflammation in several mouse models (10). In addition, leptin acts as a mitogenic agent in many tissues and is suggested to promote cancer cell growth. In fact, leptin has been shown to act as a growth factor for prostate (11, 12), breast (13), and ovarian (14) cancer cells *in vitro*, to induce increased migration of prostate cancer cells and expression of vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF-β1), and basic fibroblast growth factor, and to enhance prostate cancer growth *in vivo* (11, 12).

Consequently, the ability to block the activity of leptin may hold potential for diverse therapies. We previously reported the development of leptin antagonists in four animal species (15, 16) by alanine mutagenesis of residues LDF (amino acids 39–41) or LDFI (amino acids 39–42). Using mouse leptin antagonist (MLA),[4] we demonstrated that inhibition of leptin signaling is beneficial in several mouse models of fibrosis and inflammation (9, 10). Pegylation of the antagonist resulted in further improvement of the *in vivo* antagonistic activity via inhibition of the transport of endogenous leptins through the brain-blood barrier (17).

The affinity of leptin antagonists toward the leptin receptor is equivalent to that of wild-type (WT) leptin. The thermodynamic consequence of the similar affinity is that occupation of 90% of the receptor necessitates a 10-fold molar excess of an-

* This work was supported by the Israel Science Foundation Grant 521/07 (to A. G. and E. E.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Table S1.

[1] Both authors contributed equally to this work.

[2] Both authors should be considered as senior authors.

[3] To whom correspondence should be addressed: Institute of Biochemistry, Food Science and Nutrition, Robert H. Smith Faculty of Agriculture, Food, and Environment, Hebrew University of Jerusalem, P. O. Box 12, Rehovot 76100, Israel. Tel.: 972-8-948-9006; Fax: 972-8-948-9006; E-mail: gertler@agri.huji.ac.il.

[4] The abbreviations used are: MLA, mouse leptin antagonist; hLBD, human leptin binding domain; PEG, pegylated; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; IB, inclusion body.

tagonist. Enhancing the affinity of the antagonist by 10-fold would lead to similar receptor binding at a 1:1 molar antagonist/agonist ratio. The feasibility of such an approach has been demonstrated with pegvisomant, a human growth hormone mutein carrying nine mutations as follows: one (G120R) converting the agonist into antagonist and eight aimed at increasing affinity toward its receptor. Pegylation of this mutein resulted in an effective drug for the treatment of acromegaly (18–20). Here, we report the discovery of Asp-23 as a residue in the leptin or leptin antagonist, whose mutation to non-negatively charged amino acids confers dramatically enhanced affinity to the receptor. Subjecting this mutein to further mutagenesis and pegylation resulted in the creation of a potent long acting leptin antagonist that is capable of inducing a dramatic weight increase in naive animals.

EXPERIMENTAL PROCEDURES

Materials—Recombinant soluble human leptin binding domain (hLBD) (21), human leptin triple antagonist, and mouse leptin were prepared in our laboratory as described previously (15, 16). Synthetic mouse leptin WT cDNA optimized for expression in *Escherichia coli* was synthesized by Entelechon Co. (Rensberg, Germany). Human leptin and mouse interleukin-3 were purchased from Protein Laboratories Rehovot (Rehovot, Israel). Restriction enzymes used in the molecular biology experiments were from Fermentas (Vilnius, Lithuania). Highly pure DNA primers were ordered from Syntezza (Jerusalem, Israel). Lysis buffer, nalidixic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue), puromycin, and kanamycin were purchased from Sigma. Superdex 75 HR 10/30 and 26/60 and Superdex 200 HR 10/30 columns and Q-Sepharose and SP-Sepharose were from GE Healthcare. Molecular markers for SDS-gel electrophoresis and Bradford protein assay were purchased from Bio-Rad. Bacto-tryptone was from Conda Laboratories (Madrid, Spain). Bacto-yeast extract and Bacto-casamino acids (−Trp and −Ura) were from Difco (BD Biosciences). Sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin) was purchased from Pierce. Plasmid pCT302 and strain EBY100 of the yeast *Saccharomyces cerevisiae* were provided by Dr. E. T. Boder. mAb 9e10 was purchased from Covance (Emeryville, CA); FITC-labeled $F(ab')_2$ goat anti-mouse IgG was from Chemicon (Temecula, CA), and streptavidin-phycoerythrin conjugate was from Pharmingen. p-STAT-3 (Tyr-705) and STAT-3 antibodies were from Cell Signaling (Danvers, MA) and methoxy PEG-propionylaldehyde-20 kDa was purchased from Jenkem Technology, Inc. (Allen, TX). Fetal bovine serum (FBS), penicillin/streptomycin (10,000 units/ml and 10,000 mg/ml), and enhanced chemiluminescence (ECL) reagent were from Biological Industries Ltd. (Beit Haemek, Israel). RPMI 1640 medium and DMEM were from Invitrogen; PelletPaint co-precipitant was from Novagen, EMD Biosciences. Luciferase assay reagent was from Promega (Madison, WI); peroxidase-conjugated streptavidin was from Jackson ImmunoResearch (West Grove, PA), and 3,3′,5,5′-tetramethylbenzidine was from Dako (DakoCytomation, Copenhagen, Denmark). Other reagents (Tris, cysteine, arginine, NaOH, HCl, boric acid, Tween 20, ultra pure urea, and skim milk) were all of analytical grade.

The following kits were purchased: GeneMorph® kit, QuikChange mutagenesis kit and XL-1 Blue cells (Stratagene, La Jolla, CA); Qiagen miniprep and QIAquick gel extraction kit (Qiagen, Valencia, CA), and Zymoprep II yeast plasmid miniprep kit (Zymo Research, Orange, CA). The following reagents were prepared in our laboratory: LB (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl, sterilized), TB (80 g/liter tryptone, 160 g/liter yeast extract, 33.3 g/liter glycerol, sterilized), YPD media (10 g/liter yeast extract, 20 g/liter peptone, 20 g/liter dextrose, sterilized), SD-CAA media (20 g/liter dextrose, 6.7 g/liter Difco yeast nitrogen base, 5 g/liter Bacto casamino acids, 5.4 g/liter Na_2HPO_4, and 8.56 g/liter NaH_2PO_4, sterilized), SG-CAA media (as for SD-CAA, but with 20 g/liter galactose instead of dextrose), FACS/PBS buffer (8 g/liter NaCl, 0.2 g/liter KCl, 1.44 g/liter Na_2HPO_4, 0.24 g/liter KH_2PO_4) adjusted to pH 7.4 and supplemented with 5% (w/v) bovine serum albumin (BSA) and 0.05% (w/v) azide, lysis buffer for luciferase activity (25 mM Tris phosphate, 2 mM DTT, 2 mM CDTA, 10% (w/v) glycerol, 1% w/v Triton X-100, pH 7.8), TN buffer for gel filtration experiments (25 mM Tris-HCl, pH 8 or 9, containing 300 mM NaCl).

Biotinylation of hLBD— hLBD (0.12 mg/ml) was dialyzed against PBS, pH 7.5, and incubated with a 10-fold molar excess of the biotinylation reagent sulfo-NHS-LC-biotin for 40 min at room temperature. Excess nonreacted biotin was removed by dialyzing against PBS buffer. Biotinylated leptin binding domain was equally capable of forming a 1:1 complex with leptin or leptin antagonists as nonbiotinylated leptin binding domain (not shown).

Yeast Surface Display of Mouse Leptin—Mouse leptin WT cDNA was modified by PCR to introduce NheI and BamHI restriction sites at the 5′ and 3′ ends, respectively, enabling subsequent subcloning into acceptor vector pCT302 linearized with NheI and BamHI. The primers used in the PCR were 5′-GTACGCAAGCTAGCGCTGTTCCGATCCA-GAAAGTTCAGG-3′ to the 5′ end and 5′-CGTAGGATC-CGCATTCCGGAGAAACGTCCAACTG-3′ to the 3′ end. The PCR product of mouse leptin was digested with NheI and BamHI, extracted, and ligated into linearized pCT302 expression vector. XL-1 Blue cells were transformed with the new plasmid and plated on LB agar plates containing 100 μg/ml ampicillin. Four *E. coli* colonies were isolated and confirmed to contain the mouse leptin cDNA by digestion with NheI and BamHI restriction enzymes. All of the colonies were positive, and one of them was sequenced. Mouse leptin was expressed as an Aga2p protein fusion in *S. cerevisiae* strain EBY100 by induction in medium containing galactose (22). Hemagglutinin (HA) epitope tag was expressed upstream of the 5′ end of the leptin-encoding DNA, and a c-Myc epitope tag was attached to the 3′ end of the Aga2p-leptin fusion construct; the schematic structure is presented in Fig. 1*A*. The c-Myc epitope tag can be detected by mouse mAb 9e10 and a goat anti-mouse antibody conjugated with FITC. Detection of the c-Myc epitope tag at the C terminus of the Aga2p-leptin fusion is indicative of display of the full-length leptin fusion on the yeast cell surface.

Yeast cells transformed with pCT302/mouse leptin WT were grown overnight at 30 °C with shaking in 3 ml of selective glucose medium SD-CAA. After ~18–20 h, Aga1p (a membrane yeast protein) + Aga2p-leptin expression was induced at 30 °C with shaking in 5 ml of selective galactose medium SG-CAA. Cultures were harvested after ~20–24 h (1–2 doublings) by centrifugation, washed with PBS containing 5% BSA and 0.05% azide (FACS buffer), and incubated for 60 min on ice with anti-c-Myc mAb 9e10 (1:100 dilution) and biotinylated hLBD (final concentration of ~50 nM), washed with PBS, and incubated for 30 min on ice with either FITC-labeled $F(ab')_2$ goat anti-mouse IgG (1:50), a streptavidin/phycoerythrin conjugate (1:50), or both. Labeled yeast cells were analyzed on a BD Biosciences FACSCalibur flow cytometer at the Flow Cytometry Center of the Weizmann Institute of Science.

Construction of the Mouse Leptin Library—The mouse leptin WT gene was subjected to random mutagenesis using the Stratagene GeneMorph kit to give a high mutagenesis rate. As described in the study by Raymond *et al.* (23), to obtain the best transformation efficiency, homologous recombination primers were designed so that the inserts would have an ~50-bp overlap at each end with the cut acceptor vector. The primer used to make inserts with 5′ homology to the cut vector was 5′-GTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGCTAGCGCTGTTCCGATCCAGAAAGTTC-3′, and the primer used to make inserts with 3′ homology to the cut vector was 5′-GATCTCGAGCTATTACAAGTCCTCTTCAGAAATAAGCTTTTGTTCGGATCCGCATTCCGGAGAAACGTCCAACTG-3′. We used 0.1 ng of target DNA in the first PCR using 40 cycles, and the PCR product was gel-purified and extracted using the QIAquick gel extraction kit. The resultant PCR product was further amplified with random mutagenesis kit using an aliquot of the first PCR and re-extracted. Then the mutagenized gene was amplified by doing a set of normal PCRs (100 reactions of 50 μl each) and re-extracted. The final PCR product was transformed into the yeast along with linearized pCT-mouse leptin. Homologous recombination *in vivo* in yeast between the 5′- and 3′-flanking 50 bp of the PCR product with the gapped plasmid resulted in a library of $\sim 5 \times 10^5$ mouse leptin variants. Mutagenic DNA insert (41 μg) and 8.2 μg of restriction enzyme-linearized pCT302 vector backbone were concentrated with Pellet Paint (Novagen) and transformed into EBY100-competent yeast cells (22) by five electroporations (24). A library of 5×10^5 yeast transformants was obtained, as estimated by plating aliquots of the library and colony counting. The ratio of total insert fragments to cut acceptor vector was maintained at 5:1 for all transformations. Sequencing of 10 clones from the unscreened library indicated mutation frequency (per gene) of 2.6 in the DNA level and 1.9 in amino acid level.

Preparation of Electrocompetent Yeast for Homologous Recombination—Yeast preparation closely followed the method described by Meilhoc *et al.* (24). First, 50 ml of YPD was inoculated with *S. cerevisiae* strain EBY100 (22) to an absorbance 600 nm (A_{600}) of 0.1 from an overnight culture of EBY100 in YPD. Next, the cells were grown with shaking at 30 °C to an absorbance of 1.3–1.5 (~6 h of growth). Cells were harvested by centrifugation and suspended in 50 ml of cold sterile water. The cells were washed with 25 ml of cold sterile water and suspended in 2 ml of 1 M ice-cold sterile sorbitol. Cells were harvested by centrifugation and suspended in 50 μl of 1 M sterile sorbitol. Electroporation of the suspended cells was then carried out using a gene pulser from Bio-Rad with a 0.2-cm cuvette (voltage 1.5 kV, capacitance 25 microfarads). After pulsing, the cell aliquots were immediately resuspended in 1 ml of cold 1 M sorbitol, and the entire transformation mix was transferred to 50 ml of SD-CAA selective medium containing kanamycin (25 μg/ml) and penicillin/streptomycin (1:100 dilution) for growth at 30 °C. A small aliquot of cells was removed and plated on SD-CAA plates to determine transformation efficiency.

Mouse Leptin Library Screening by Flow Cytometry—A 50-ml volume of pooled transformed cells in SD-CAA selective media was grown overnight at 30 °C with shaking, diluted to an A_{600} of 0.05, and grown overnight at 30 °C to A_{600} >1.0. A 5-ml volume of SG-CAA was then inoculated to A_{600} ~0.5 and grown overnight to an A_{600} of 3 or 4. Detailed protocols for screening yeast polypeptide libraries have been described (25). Briefly, 3×10^8 induced yeast cells were then labeled with biotinylated hLBD at a concentration of 50 nM for 1 h at 37 °C in FACS buffer. To detect expression of the C-terminal c-Myc epitope tag, mAb 9e10 (at 1:100 dilution) was added simultaneously to the incubation mix. Then yeast cells were washed with ice-cold FACS buffer and resuspended in FACS buffer containing excess cold nonbiotinylated hLBD at a concentration of 2000 nM for 2 h at 37 °C. The cells were washed and labeled for 1 h with secondary antibodies as follows: streptavidin conjugated with (*R*)-phycoerythrin (1:50) and a goat anti-mouse antibody conjugated with FITC (1:50). Cells were washed and screened by dual color flow cytometric sorting for yeast on a BD Biosciences FACSAria III cell sorter to isolate clones with improved binding to soluble hLBD, relative to WT leptin. Collected yeast cells were cultured and induced for expression. Three rounds of sorting by flow cytometry were carried out, with regrowth and reinduction of surface expression between each sorting round. A total of about 1×10^7 cells was examined during the first sorting round, and ~5% of the population was collected. After a second round of sorting, 1.6×10^5 cells were collected with 0.5–1% stringency, and after the third round of screening, 5000 cells with 0.1% stringency were collected. Each library was frozen and saved at −80 °C according to the protocol in Chao *et al.* (26).

DNA Isolation and Sequencing—After the three rounds of sorting, collected cells were plated on selective medium plates to isolate individual clones. DNA from 40 individual clones was extracted using a Zymoprep kit according to the manufacturer's protocol. The DNA was amplified by transforming into XL-1 Blue cells. Cells were plated on selective LB plates supplemented with 100 μg/ml ampicillin. Colonies from these plates were grown overnight at 37 °C in LB media plus 100 μg/ml ampicillin, and DNA was isolated using a Qiagen miniprep kit according to the manufacturer's instructions, and DNA was sequenced.

Determination of Affinity for Soluble hLBD in Yeast Clones Selected after the Third Round of Screening—Individual yeast cells cloned after the third screen, and WT mouse leptins were grown and induced. Cells (1×10^6) were labeled using biotinylated hLBD at different concentrations (1000, 333, 111, 37, 12, 4, and 1.37 nM) along with anti-c-Myc antibody and secondary fluorescent antibodies as already described. Fluorescence data of c-*myc*-positive yeast cells were obtained using a FACSCalibur flow cytometer. To calculate the dissociation constant (K_d), the mean fluorescence intensity, obtained with each of the biotinylated hLBD concentrations tested, was then plotted against the hLBD concentration using a nonlinear regression (curve fit) with hyperbola equation, analyzed by Prism software (Prisma, GraphPad Prism™ version 4.0, GraphPAD Software, San Diego).

Preparation of Bacterial Expression Plasmids Encoding Mouse Leptin Muteins—PCR was carried out to introduce NcoI and HindIII restriction sites at the 5′ and 3′ ends, respectively, enabling subsequent subcloning of the best mutant mouse leptin binder DNA into the pMON3401 vector linearized with NcoI and HindIII. The primers used were AAAAA-ACCATGGCTGTTCCGATCCAGAAAG for the 5′ end and AAAAAAAAGCTTTCAGCATTCCGGAGAAACGTCC for the 3′ end of the leptin mutant. The cDNA of the mouse leptin muteins in pCT302 was digested with NcoI and HindIII, 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 leptin cDNA by digestion with NcoI/HindIII restriction enzymes. All of the colonies were positive, and one of them was sequenced.

Insertion of the L39A/D40A/F41A Mutations into Mouse Leptin Muteins—The procedure used to insert mutations into leptins to create leptin antagonists is disclosed in International Application PCT/IL2005/001250, herein incorporated as a reference (see Ref. 27). To prepare the leptin mutants with antagonistic activity, the pMon3401 expression plasmids encoding six selected mouse leptin muteins were used as starting material. The leptin inserts were modified with the Stratagene QuikChange mutagenesis kit according to the manufacturer's instructions, using two complementary primers (supplemental Table 1). The primers were designed to contain base changes (marked in boldface) 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 DpnI 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 ml of 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.

Insertion of the Asp-23 Mutation into Plasmid Encoding Mouse and Human Leptin Antagonist—The D23A, D23G, D23L, D23R, D23K, D23F, and D23W mutants were prepared as described above, and the pMon3401 expression plasmids encoding the MLA were used as starting material. Preparation of the expression plasmid of the human D23L/L39A/D40L/F41A leptin mutant (termed super human leptin antagonist, SHLA) was performed similarly. The primers used were 5′-CAATTGTCACCAGGATTAATCTGATTTCACACACG-CAG-3′ (modified restriction site = VspI (+)) (sequence identification number 29) and 5′-CTGCGTGTGTGAAATC-AGATTAATCCTGGTGACAATTG-3′ (sequence identification number 30), as shown in supplemental Table 1.

Expression, Refolding, and Purification of Mouse Leptin Muteins—The recombinant mutated mouse leptins with an extra Met-Ala (methionine is cleaved by the bacteria) at the N terminus were expressed in 2.5 liters of 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 0.3 liter of bacterial culture were solubilized in 50 ml of 4.5 M urea and 40 mM Tris base containing 1 mM cysteine, adjusted to pH 11.3 with NaOH. After 2 h of stirring at 4 °C, 3 volumes of 0.67 M arginine 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 liters 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 100 mM, and the protein solution was then applied at maximal flow rate (400–500 ml/h) onto a Q-Sepharose column (5-ml bead volume), pre-equilibrated with 10 mM Tris-HCl, pH 9, containing 100 mM NaCl. The breakthrough fraction, which contained the respective leptin mutein, was collected and concentrated to 2–3 mg protein/ml. Then 18-ml portions were applied to a preparative Superdex 75 column (26/60 cm) pre-equilibrated with 25 mM Tris-HCl, pH 9, containing 300 mM NaCl (TN buffer). Fractions containing the monomeric protein as determined by gel filtration on analytical Superdex 75 HR 10/30 column (1/30 cm) were pooled, dialyzed against $NaHCO_3$ to ensure a 4:1 protein-to-salt ratio, and lyophilized.

Large Scale Purification of Mouse and Human Leptin D23L/L39A/D40A/F41A Antagonist—The procedure was essentially as described above with the following changes: IBs obtained from 3 liters of bacterial culture were solubilized in 400 ml of 4.5 M urea and 40 mM Tris base containing 1 mM cysteine, adjusted to pH 11.3 with NaOH. After 48 h of stirring at 4 °C, 3 volumes of 0.67 M arginine were added to a final concentration of 0.5 M. Then the solution was dialyzed against 10 liters of 10 mM Tris-HCl, pH 9, for 60 h, with five or six external solution exchanges. 150 mM NaCl was then added, and the protein was applied on a pre-equilibrated Q-Sepharose column (30-ml bead volume). The flow-through fraction was collected, and the monomeric SMLA was isolated using preparative gel filtration in TN buffer, pH 9, followed by dialysis, and lyophilization was conducted as described previously for

MLA (16). The protocol used for preparation of SHLA was identical to that published for human L39A/D40A/F41A (15).

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-250. 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, 300 mM NaCl, pH 8).

Pegylation of SMLA and SHLA—20 kDa of methoxy PEG-propionylaldehyde was used for pegylation under conditions that favor pegylation of the N-terminal amino group. SMLA or SHLA (150 mg) was dissolved in 111 ml of 0.1 M sodium acetate buffer, pH 5, and centrifuged at 35,000 × *g* for 10 min to remove the insoluble material. Then −1 M $NaBH_3CN$ (2.7 ml) was added, and the dissolved protein was conjugated with 1.5 g of methoxy PEG-propionylaldehyde-20 kDa dissolved in 15 ml of 1 mM HCl. After 20 h of stirring at 4 °C, 160 μl of acetic acid (17 M) was added. The solution was stirred for a few seconds, diluted with 1 liter of double distilled H_2O, and applied at a maximal flow rate (400–500 ml/h) onto an SP-Sepharose column (20-ml bead volume), pre-equilibrated with 10 mM sodium acetate, pH 4. The column was then washed with 400 ml of 10 mM sodium acetate, pH 4, and the pegylated protein was eluted in 10 mM sodium acetate, pH 5, containing 50 and 75 mM NaCl. Fractions containing the mono-pegylated protein as determined by gel filtration on an analytical Superdex 200 column (1/30 cm) were pooled, dialyzed against $NaHCO_3$ to ensure a 2:1 protein-to-salt ratio, and lyophilized. Protein concentrations were determined by absorbance at 280 nm using an extinction coefficient (for 0.1% solution of pegylated protein of 0.200 mg/ml). These values apply to the protein part of the pegylated product.

Binding Assay—In all competitive binding experiments, the biotinylated mouse leptin served as a ligand that could be competed off by the respective mouse or human leptins or leptin antagonists. The hLBD was used as the receptor source. Polystyrene 96-well microtiter plates were coated overnight at 4 °C with 100 μl of 40 pM hLBD in PBS, pH 7.4. Wells were then washed once with PBST (PBS containing 0.05% (w/v) Tween 20) and blocked with PBS containing 3% (w/v) skim milk for 2 h at room temperature. All further incubations were carried out at room temperature. Wells were washed again once with PBST and incubated with different concentrations of unlabeled leptins (50 μl/well) for 30 min, and then 50 μl of 62.5 pM biotinylated mouse leptin was added to each well for another 2 h. The wells were then washed three times with PBST and incubated with 1:30,000 streptavidin/HRP in PBS containing 1% Tween 20 for 1 h. Wells were washed three times with PBST, and the reaction was quantified at 450 nm by ELISA Micro-Plate Reader ELx808, BioTek Instruments Inc. (Winooski, VT) using 3,3′,5,5′-tetramethylbenzidine according to the manufacturer's instructions.

Kinetics Measurements of SMLA- and SHLA-hLBD Interactions—The kinetics and equilibrium constants for the interactions between hLBD and SMLA or MLA or SHLA were determined by surface plasmon resonance methodology using a Biacore 3000 instrument (Neuchatel, Switzerland) at 25 °C (31). SMLA, MLA, or SHLA were immobilized in a flow cell on a research grade CM5 sensor chip using amine-coupling chemistry. Immobilization steps and experimental procedure are described in Niv-Spector *et al.* (15). For the binding studies, hLBD was dissolved in HBS-EP buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, and 0.005% (v/v) surfactant P20 at pH 7.4) and passed at different concentrations (0, 0.4, 0.8, 1.9, 3.9, 7.8 and 15.6 nM) through flow cells carrying MLA or SMLA or SHLA at a rate of 30 μl/min. The surface was regenerated after each interaction with a 10-μl pulse of 4 M $MgCl_2$. The experiments were analyzed using the Kinetics Wizard (Biacore control software). The resultant binding curves were fitted to the association and dissociation phases at all hLBD concentrations simultaneously, using Biacore evaluation software.

BAF/3 Proliferation Assays—The proliferation rate of leptin-sensitive BAF/3 cells stably transfected with the long form of human leptin receptor was used to estimate both agonistic and antagonistic activity of leptins and leptin muteins as described previously (29). To determine antagonistic activity, 0.05 ng of 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 Prisma (4.0) nonlinear regression sigmoidal one-site competition program, and the IC_{50} values were calculated. Note that all mammalian leptins are capable of activating human leptin receptor to an almost identical degree (28, 29, 32).

Determination of Biological Activity by Activating Luciferase Reporter Gene—The H-49 cell line, received from Dr. M. Einat (ARO, Israel), consists of HEK-293 cells stably transfected with three constructs as follows: phOB-Rb (long form of human leptin receptor), pAH32 (luciferase reporter construct), and pgkPuro (expression vector containing the puromycin resistance gene) at a ratio of 4:4:1 as described previously (33). H-49 cells were briefly dissociated with trypsin and resuspended in DMEM supplemented with 10% (v/v) FBS, 50 μg/ml streptomycin, 50 units/ml penicillin, and 2 μg/ml puromycin. Resuspended cells were plated in 24-well tissue culture plates at 5×10^5 cells per well in a final volume of 500 μl. After 16 h, the medium in each well was replaced with 300 μl of DMEM. Mouse leptin mutants were added at different concentrations with a constant concentration of WT mouse leptin. The dilutions were made in DMEM supplemented with 0.5% BSA. Three replicates were used for each concentration, and a triplicate without leptin served as a negative control. After 18 h of incubation at 37 °C (CO_2/O_2, 5:95), the cells were harvested with 100 μl of lysis buffer and frozen at −80 °C. Each cell lysate (50 μl) was mixed with luciferase assay reagent (Promega), and luciferase activity was determined. The measured luminescence was normalized to the amount of protein in each well. Protein concentration was measured by Bradford assay according to the manufacturer's protocol. The

results were analyzed by Prism software, according to a non-linear regression one-site competition curve.

STAT-3 Phosphorylation Inhibition—CHO cells (0.5×10^5 cells per well) stably expressing the long form of mouse leptin receptor (ObRb) were seeded in 24-well plates, grown to 80% confluence, and then in serum-deprived medium for 16 h before stimulation with hormones. The cells were then incubated in serum-free medium in the presence or absence of various concentrations (0.2–12.8 μg/ml) of MLA or SMLA and one concentration of WT mouse leptin (0.1 μg/ml) for 20 min in 0.5 ml in 24-well plates. Following these treatments, cells were harvested in 75 μl of ice-cold lysis buffer. Lysates were clarified by centrifugation at 35,000 $\times$ *g* for 10 min, and supernatants were kept for Western blot analyses. Protein concentrations of supernatants were determined using the Bradford assay. Cell proteins (20 μg) were resolved in SDS-PAGE followed by Western blot using p-STAT-3 (Tyr-705) (catalog no. 9138) and STAT-3 (catalog no. 9132) antibodies. Western blot bands were revealed by ECL.

In Vivo Experiments—Male C57Bl mice were intraperitoneally administered with pegylated MLA (PEG-MLA) or superactive MLA (PEG-SMLA) at 6.25 mg/kg/day for a period of 20 days. During this period, food intake and weight gain were recorded daily and averaged for a period of 7 days. In the weaning part of the experiment, the treatment was ceased after 20 days, and reversibility of the leptin deficiency phenotype was recorded. The second experiment was carried out in a similar manner using four doses of either PEG-MLA or PEG-SMLA as follows: 20, 6.7, 2.2, and 0.72 mg/kg/day and lasted 17 days. In all experiments, animals were maintained under 12-h light/dark cycles, in accordance with regulations of the institutional animal and care authority of the Tel Aviv Sourasky Medical Center.

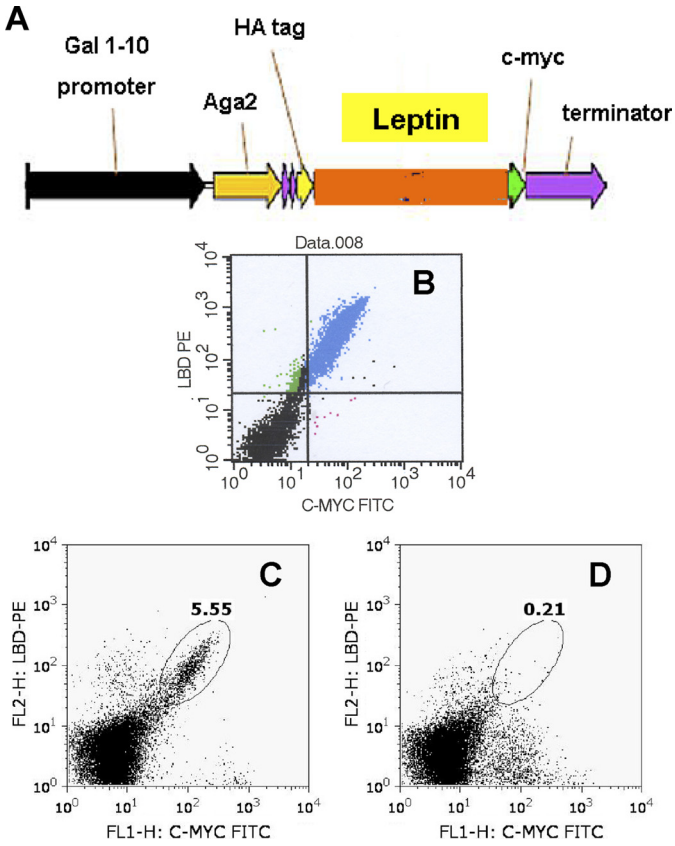


FIGURE 1. *A*, schematic structure of the leptin-expressing plasmid in yeast cells. For yeast surface display of mouse leptin, the c-Myc epitope tag was detected using mouse mAb 9e10 and goat anti-mouse antibody conjugated with FITC, and the expressed leptin was detected using biotinylated hLBD and streptavidin/phycoerythrin (*SA-PE*) conjugate. *B*, representative figure from one of the cell sorting experiments. *C*, library prior to competition with nonlabeled hLBD; *D*, library after competition. Yeast cells that were not competed off were collected for an additional cycle of growth and selection.

RESULTS

Nonbiased Screening for Leptin Mutants with Improved Binding to Soluble Human Leptin Receptor—To generate a leptin mutant yeast display library, mouse leptin was expressed on the surface of yeast cells as a fusion to the Aga2p agglutinin subunit and a c-Myc tag, on the surface of yeast (22), as shown in Fig. 1*A*. In-frame surface expression of the full-length Aga2p + leptin fusion protein was established by flow cytometric c-Myc labeling, whereas affinity to leptin receptor was confirmed by binding to a fluorescently labeled hLBD (Fig. 1*B*). As a negative control, yeast displaying an irrelevant EGF receptor did not show any signal (data not shown). For construction of the yeast-displayed leptin mutant library, leptin cDNA was randomly mutated (Stratagene GeneMorph random mutagenesis kit), followed by cloning into the pCT302 vector to form a leptin mutant-Aga2p agglutinin subunit-c-Myc fusion transcript. Electroporation into the yeast yielded a leptin mutant library with 5×10^5 clones. This library was screened by three rounds of flow cytometric sorting, with regrowth and reinduction of surface expression between each sorting, to isolate clones with improved binding to hLBD. In each round of selection, the yeast library was screened by dual fluorophore flow cytometry for clones that display both full-length leptin (as determined by expression of the c-Myc epitope tag) and binding to biotinylated hLBD. The screening approach used a kinetic binding screen, by labeling yeast to saturation with fluorescently labeled hLBD and then incubating in the presence of excess nonfluorescent hLBD competitor (Fig. 1, *C* and *D*). Cells that exhibited fluorescence after being competed off by nonbiotinylated hLBD were collected for regrowth (Fig. 1*D*). Forty mutants obtained in the third selection cycle (38 mutants with higher gating stringency (0.1%) and two with lower stringency (0.5%)) were sequenced resulting in the identification of 13 new and distinct sequences, with a total of 23 amino acid changes ranging from 1 to 6 amino acids (Table 1). The most frequent mutation that occurred in 18 out of 40 clones was exchange of Asp-23 to Gly, His, or Asn.

The approximate affinity of the surface-displayed mouse leptin mutants was determined *in situ* on the cell wall by titrating whole cells with varying concentrations of biotinylated hLBD. Equilibrium binding was measured by analyzing cell-bound hLBD and c-Myc-positive labeling in 13 distinct clones (identified in Table 1) by flow cytometry. Nonlinear regression (curve fit) of these data indicated up to 3-fold increased affinity in 7 out of 13 leptin mutants (H30, H15, H22, H12,

TABLE 1
Sequence changes in 40 clones selected after three screening cycles

Clone number[a]	Sequence change[b]
H1, H23	S25F/L49M
H2	A125T/S132Y
H3, H4, H5, H8, H9, H11, H13, H15, H21, H26, H31, H35, L2	D23G/L68M/S97F/S132Y
H6	D23G/V30D/Y119H
H7, H18, H27, H33	K11R/Q34R/T37A/F92C/S97Y/I136V
H12, H20, H24, H25, H28, H29, H32, H36, H37, H38, H39, H40	S97Y
H16	D23G/G112S
H17	S109F
H19	D23G/T37A/G44D
H22	T12I
H30	D23H
H34	No change
L1	D23N/Q34L/L114P

[a] H indicates higher stringency clones, and L indicates lower stringency clones. All clones were sequenced with c-Myc primer, so the end of C-terminal sequence (12 last amino acids in helix D of leptin) was not sequenced.
[b] The most frequent mutation change (Asp-23) that was found in 18 clones is underlined.

TABLE 2
Binding properties and biological activity in the seven mutants of mouse leptin and six mutants of mouse leptin antagonist prepared in *E. coli*

Mouse leptin			
Mutant	Binding assay[a]	BAF/3 bioassay[a]	Luciferase bioassay[a]
H30	35.0 ± 2.00 (2)	0.3 ± 0.06 (3)	0.6 (1)
H15	9.7 ± 1.01 (2)	0.5 ± 0.08 (3)	0.8 (1)
H22	11.7 ± 0.20 (2)	0.8 ± 0.21 (3)	0.7 (1)
H12	1.5 ± 0.00 (2)	0.9 ± 0.16 (3)	0.7 (1)
H16	20.3 ± 1.25 (2)	0.8 ± 0.04 (3)	0.9 (1)
H19	10.4 ± 2.90 (2)	0.9 ± 0.19 (3)	1.5 (1)
H7	0.83 ± 0.17 (2)	0.5 ± 0.12 (2)	0.3 (1)
Mouse leptin antagonists			
Mutant	Binding assay[a]	BAF/3 bioassay[a]	Luciferase bioassay[a]
H30 (antagonist)	34 ± 3.15 (8)	5 ± 0.34 (5)	5.7 ± 1.42 (4)
H15 (antagonist)	12.4 ± 0.60 (2)	4.8 ± 1.09 (3)	4.2 ± 0.69 (3)
H22 (antagonist)	11.2 ± 0.80 (2)	2.4 ± 0.51 (2)	3.3 ± 0.20 (3)
H12 (antagonist)	1.4 ± 0.48 (2)	0.8 ± 0.21 (3)	0.1 ± 0.04 (3)
H16 (antagonist)	11.0 ± 3.90 (2)	3.3 ± 0.21 (3)	3.3 ± 0.64 (3)
H19 (antagonist)	6.1 ± 1.05 (2)	3.5 ± 0.26 (2)	2.9 ± 1.35 (3)

[a] The numbers show the fold increase in affinity or bioactivity as compared with the WT mouse leptin or WT mouse leptin antagonist and are given as mean ± S.E. when the number of experiments were three or more and as S.D. when the number of experiments was two. The numbers of performed experiments are given in parentheses.

H16, H19, and H7) as shown in supplemental Fig. 1. H12 was included despite its marginal increase in the affinity.

Preparation and Characterization of Mutants of Mouse Leptin and Leptin Antagonists—Seven mouse leptin mutants that were selected by yeast surface display screening (see above and in Table 2) were expressed in *E. coli*, refolded, and purified as recombinant proteins by consecutive anion exchange and gel filtration chromatography as described under "Experimental Procedures." In the initial experiments, the H7 mutant did not show any increase in affinity, and the corresponding L39A/D40A/F41A mutant was therefore not prepared. To obtain six other respective MLAs, all six leptin mutants were also inserted with the L39A/D40A/F41A mutations and purified. All 13 proteins (seven leptins and six mutated MLAs) were tested for purity using SDS-PAGE and found to be over 99% pure with over 95% monomer content (data not shown). It should be noted that all the recombinant proteins prepared in *E. coli* are untagged proteins devoid of HA or c-Myc sequences.

The 13 recombinant muteins were subsequently tested for an eventual change in binding properties by binding to immobilized hLBD and for their agonistic or antagonistic activity using the BAF/3 cell-proliferation assay and activation of luciferase reporter gene in H-49 cells. As shown in Table 2, the most potent mutein was derived from clone H30 bearing the mutation D23H. However, elevated activity was also found in proteins derived from clones H15, H16, and H19, all having the Asp-23 mutated to glycine but also having additional mutations (Table 2). In contrast, proteins derived from clone H12 featured no increase in affinity or bioactivity. Only one protein derived from clone H22, lacking the Asp-23 mutation and having only one mutation (T12I), showed a moderate increase in affinity and bioactivity (the latter as antagonist only). To examine whether the T12I mutation may have an additive effect to that of Asp-23, we prepared three double mutants (T12I/D23H, T12I/D23R and T12I/D23L) of MLA, but neither their inhibitory activity in BAF/3 bioassay nor their binding affinity toward hLBD was superior to that of D23H or D23R or D23L MLA respectively (data not shown). Interestingly, although the changes in the binding properties of the agonists and antagonists were highly comparable, the biological activity as determined in BAF/3 bioassays was elevated only in the antagonists.

Assessment of the Role of Asp-23 by Rational Mutagenesis of MLA—To further assess the role of the D23H mutation in increasing the biological activity of MLA, Asp-23 was replaced by small (Gly and Ala), hydrophobic (Leu, Phe, and Trp), or positively charged (Lys and Arg) amino acids using rational mutagenesis. All seven mutants were purified as recombinant proteins by consecutive refolding, dialysis, and anion exchange and gel filtration chromatography. They were found pure by SDS-PAGE and contained over 98% monomers as evidenced by analytical gel filtration (Figs. 2 and 3). These mutants were tested for binding affinity toward hLBD and for their biological inhibitory potency in a BAF/3 proliferation assay, and D23L (Table 3) was found to exhibit the highest affinity. Therefore, we chose to use this mutant in all further experiments.

Large Scale Purification, Pegylation, and Characterization of Mouse and Human Leptin D23L/L39A/D40L/F41A Super Antagonists—Prolonging the refolding in 4.5 M urea from 2 to 48 h prior to dialysis (see under "Experimental Procedures") dramatically improved the yield of the monomeric fraction of the D23L/L39A/D40A/F41A (SMLA), and the final yield of the protein that was purified from the IBs corresponding to 3 liters of fermentation mixture varied between 300 and 400 mg. The protein, designated superactive MLA (SMLA), was over 99% pure according to SDS-PAGE and contained more than 95% monomers (see the respective D23L mutant in Figs. 2 and 3). Its binding affinity toward hLBD was increased by 64-fold, and its biological inhibitory activities *in vitro* in BAF/3 or H-49 cell bioassays were increased by 14.7- and 13.4-fold, respectively (see Table 3 and Fig. 4, *A* and *E*). Similar results were obtained for SHLA (Fig. 4, *C*, *D*, *G*, and *H*), and as shown, the biological activities of SHLA and SMLA in

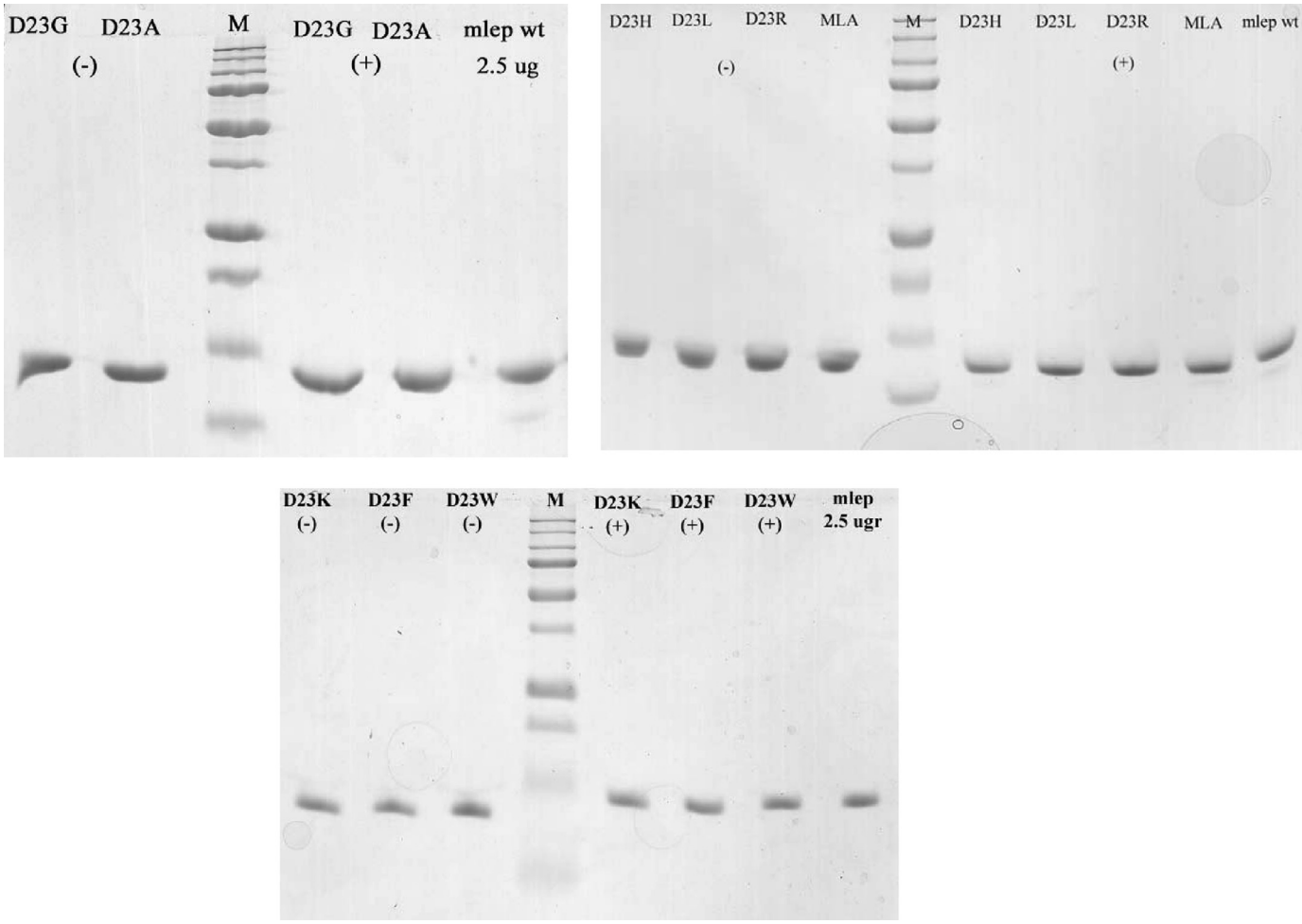


FIGURE 2. **SDS-PAGE of the eight variants of MLA mutated at Asp-23 and run on a 15% gel in the presence (+) or absence (−) of β-mercaptoethanol.** WT mouse leptin (*mlep*) was run as a control. The sizes of the molecular mass markers (*M*) (from *top* to *bottom* in kDa) are as follows: 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10.

H-49 cell bioassays (Fig. 4*C*) and in binding assays (Fig. 4*G*) were identical. To better characterize the nature of the increased affinity, SMLA and MLA interactions with hLBD were also compared by surface plasmon resonance methodology. The respective kinetic constant k_{on} for MLA and SMLA was 1.32×10^5 and 2.69×10^5 M/s, whereas the respective k_{off} constant was 1.19×10^{-3} and 2.01×10^{-4} s^{-1}, and the calculated thermodynamic association constant K_a was 1.11×10^8 and 1.34×10^9 M, respectively. This result clearly indicated that the increased affinity of SMLA originates mainly from a longer occupation of the receptor. Similar results ($k_{on} = 1.68 \times 10^5$ M/s, $k_{off} = 2.55 \times 10^{-4}$ s^{-1}, and $K_a = 6.59 \times 10^8$ M) were also obtained for SHLA.

The biological activities of MLA and SMLA were also tested in a semi-quantitative STAT3 phosphorylation bioassay. A representative experiment (one out of three) shows at least 5-fold higher activity of SMLA (Fig. 5). Pegylation of this protein carried out by the method described resulted in a yield, varying between 55 and 75 mg of pegylated protein from 150 mg of nonpegylated SMLA. The final preparation of PEG-SMLA, shown in Fig. 6 (a representative experiment out of 15 performed), was pure by SDS-PAGE criteria and contained ~9% double-pegylated SMLA, 90% mono-pegylated SMLA, and less than 1% nonpegylated SMLA. N-terminal amino acid analysis indicated that the N terminus was blocked suggesting the pegylation occurred mainly at the N terminus as suggested by the pegylation protocol of the manufacturer. The nature of the double-pegylated SMLA was not tested, but it likely exists as an N-terminal pegylated species with a single or a few partially pegylated lysines in 1 out of 10 molecules. As shown by a representative experiment (Fig. 4, *A, B, E,* and *F*), the apparent affinity for hLBD and the biological activity in H-49 cells of PEG-SMLA were reduced by 9.1- and 6.1-fold, respectively, compared with the nonpegylated SMLA. These changes were comparable with the pegylation effect on MLA shown in Fig. 4, *B* and *F* (7- and 6.3-fold reduction, respectively), and in our recent publication (17). The effects of pegylation on the biological activity of SHLA (Fig. 4*D*) and its apparent affinity toward leptin receptor were also similar (Fig. 4*H*).

Mouse and Human Leptin Antagonists (D23L/L39A/D40A/F41A) Induce a Dramatic Weight Increase—To assess the *in vivo* activity of SMLA, 6.25 mg/kg PEG-MLA or PEG-SMLA were intraperitoneally administered daily to 7-week-old male C57BL mice. As depicted in Fig. 7 and as published previously (17), administration of PEG-MLA induced a statistically sig-

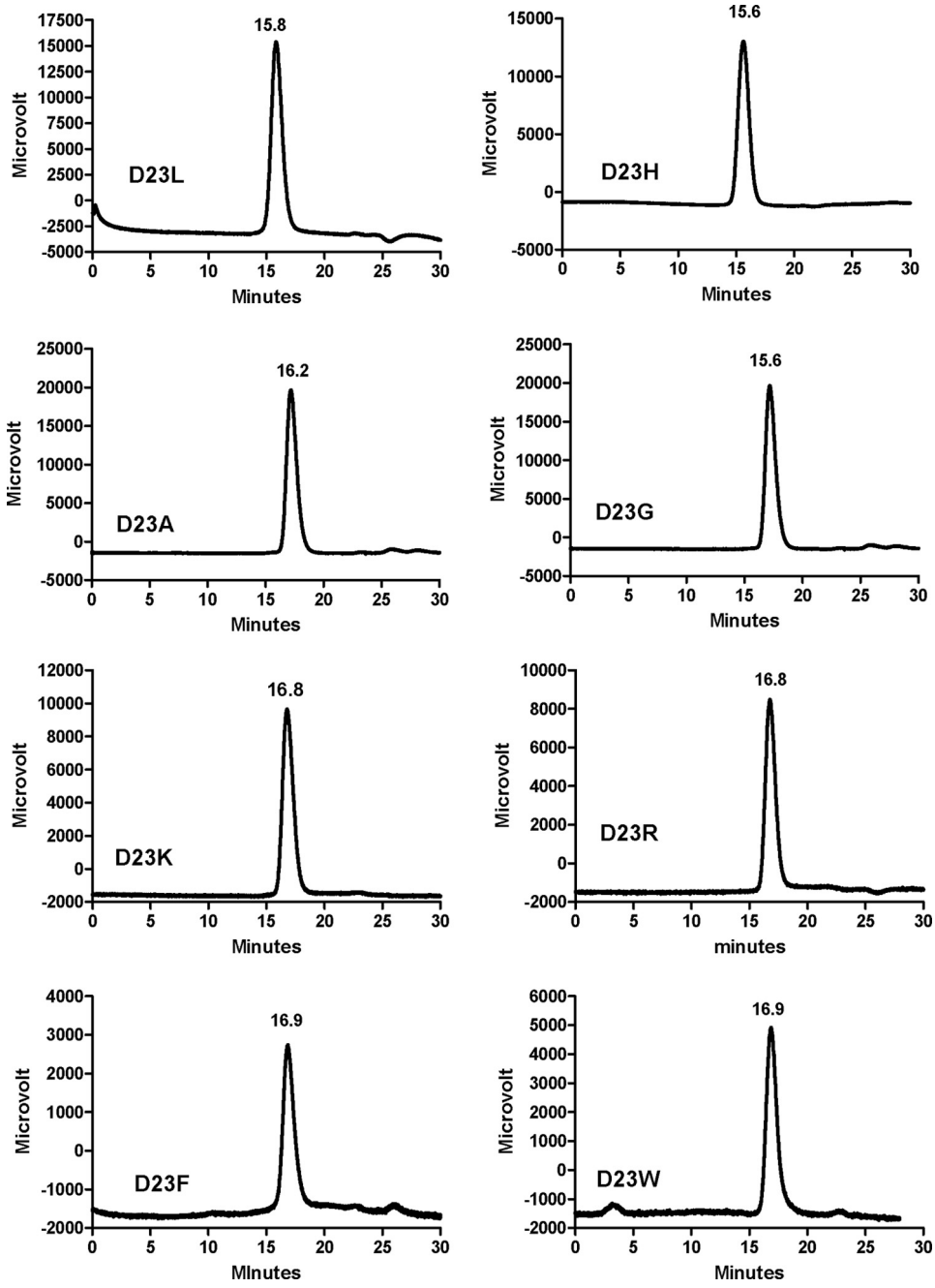


FIGURE 3. **Gel filtration analysis of eight variants of MLA mutated at Asp-23 developed on a Superdex 75 column at 0.8 ml/min in TN buffer, pH 8.**

TABLE 3
Activity summary of mouse leptin antagonists D23 mutants

Mutant	Binding assay[a]	BAF/3 bioassay[a]	Luciferase bioassay[a]
D23H	34 ± 3.15 (8)	5 ± 0.34 (5)	NT
D23G	17.5 ± 4.50 (2)	2.5 ± 0.05 (2)	NT
D23A	21 ± 1.00 (2)	3.3 ± 0.75 (2)	NT
D23L	64 ± 5.82 (6)	14.7 ± 0.43 (4)	13.4 ± 1.50 (7)
D23K	19 ± 9.00 (2)	3.7 ± 0.35 (2)	NT
D23R	51 ± 3.95 (6)	8.6 ± 1.15 (2)	NT
D23F	28 ± 3.56 (4)	3.7 ± 0.25 (2)	NT
D23W	36 ± 6.02 (4)	4.2 ± 1.7 (2)	NT

[a] The numbers show the fold increase in affinity or bioactivity as compared with the WT mouse leptin or WT mouse leptin antagonist and are given as mean ± S.E. when the number of experiments were three or more and as S.D. when the number of experiments was two. The numbers of performed experiments are given in parentheses. NT indicates not tested.

nificant weight gain by day 8, reaching a level of 121 ± 1.4% (mean ± S.E.) and stabilizing after 8 days of treatment. In comparison, PEG-SMLA induced a weight gain that peaked at 143.4 ± 3.15% on day 17, and stabilized thereafter. The differences in weight gain between PEG-MLA and PEG-SMLA were statistically significant from day 6. Weight gain was mediated by differences in food consumption, which was significantly higher in PEG-SMLA-treated mice (4.02 ± 0.17 g/day per mouse) than in both PEG-MLA (3.17 ± 0.56 g/day per mouse) and control mice (2.54 ± 0.14 g/day per mouse, $p <$ 0.01). Throughout the experiment, mice looked healthy and active with no obvious signs of stress. On day 20, three mice from each group were sacrificed, whereas the remaining mice

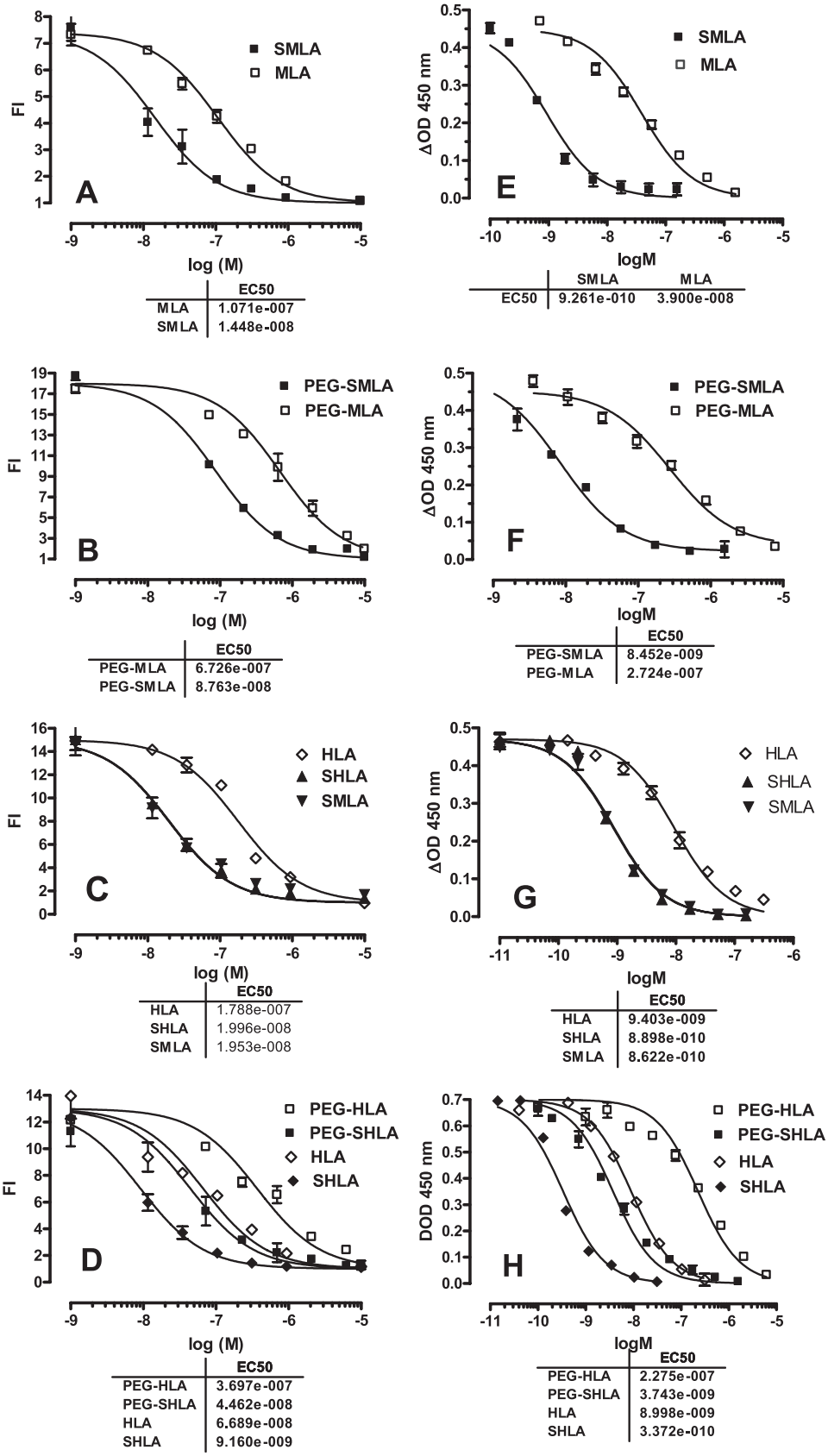


FIGURE 4. **Comparison of biological activity in H-49 cells (*A–D*) and binding properties (*E–H*) of MLA and SMLA (*A, C, E,* and *G*), of PEG-MLA and PEG-SMLA (*B* and *F*), and of HLA, SHLA (*C, D, G,* and *H*), and PEG-SHLA (*D* and *H*).**

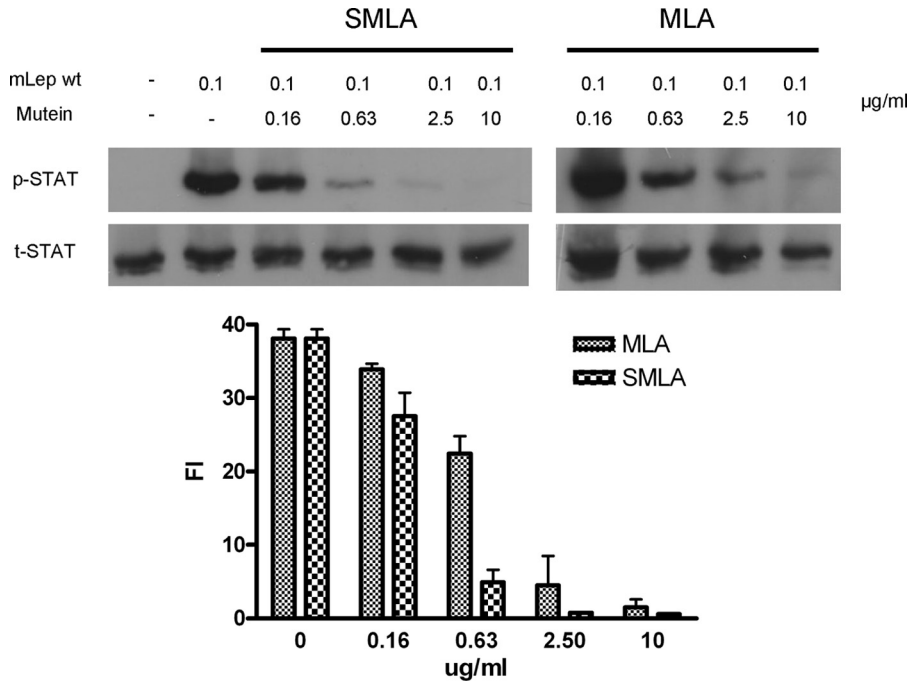


FIGURE 5. **Comparative inhibition of STAT3 phosphorylation by SMLA and MLA in CHO cells stably transfected with the long form of mouse leptin (*mLep*) receptor.**

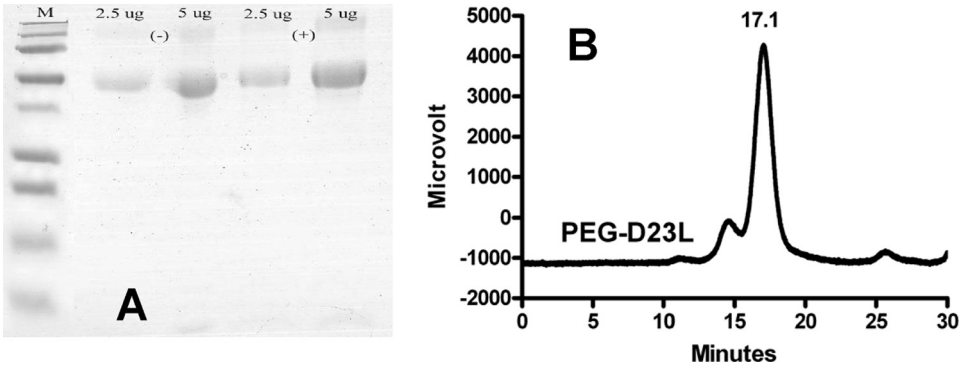


FIGURE 6. *A*, SDS-PAGE of PEG-SMLA run on a 12% gel in the presence (+) or absence (−) of β-mercaptoethanol. The molecular mass markers (*M*) (from *top* to *bottom* in kDa) are as follows: 150, 100, 75, 37, 25, 20, 15, and 10. *B*, gel filtration analysis of PEG-SMLA on a Superdex 200 column at 0.7 ml/min in TN buffer, pH 8.

in each group were taken off the treatments and observed for resolution of the weight gain. During the following 17 days (Fig. 7), mouse weights gradually decreased. A representative figure featuring mice treated with vehicle, PEG-MLA, or PEG-SMLA is depicted in Fig. 7 (*lower panel*).

To assess the dose-related differences between PEG-MLA and PEG-SMLA, several daily doses of both proteins (20, 6.7, 2.2, and 0.72 mg/kg) were directly compared (Fig. 8). At all four concentrations, the weight gain induced by PEG-SMLA was significantly higher than the respective effect of PEG-MLA. A near-identical weight gain (45 ± 3.8%) was obtained by PEG-SMLA at 20 and 6.7 mg/kg. On day 15, the lowest dose of PEG-SMLA (0.72 mg/kg) induced weight gain comparable with that from 20 mg/kg PEG-MLA, indicating that PEG-SMLA efficacy is up to 27-fold greater than that of PEG-MLA. There was a strong correlation between weight gain and food intake. The average food intake (g/day) for the mice injected with PEG-SMLA (20, 6.7, 2.2, and 0.72 mg/kg) was, respectively, 4.82 ± 0.29, 4.54 ± 0.26, 4.01 ± 0.23, and 3.34 ± 0.16 (mean ± S.E.), and the corresponding values for mice injected with PEG-MLA were 3.58 ± 0.24, 3.36 ± 0.18, 3.12 ± 0.15, and 3.05 ± 0.13 compared with 2.87 ± 0.13 of the control. In contrast, there was almost no difference in water intake, confirming the specific effects on appetite- rather than thirst-regulatory pathways for the leptin antagonist. Finally, we assessed the *in vivo* effect of PEG-SHLA to that of the mouse counterpart (PEG-SMLA). In both mouse and human superactive leptin antagonists, daily injections of 6.25 mg/kg/day resulted in a comparably significant ($p < 0.01$) increase in food intake (40–41%) and weight gain (30–32%) in 16 days (data not shown).

DISCUSSION

We report the discovery of the Asp-23 residue as a site that modulates leptin binding to its receptor, and we show that mutagenesis of this residue is associated with a dramatic in-

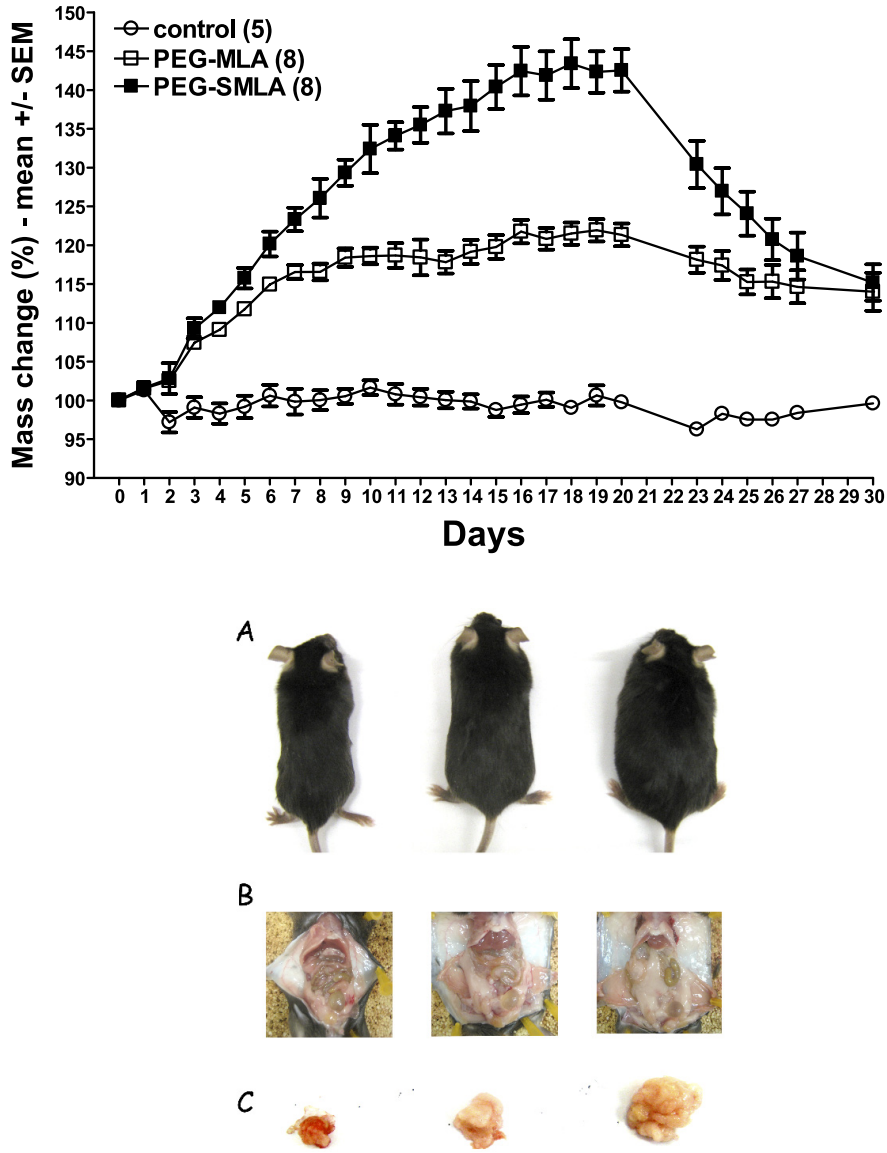


FIGURE 7. *Top panel,* comparison of the effect of PEG-MLA and PEG-SMLA on weight gain in male mice. Both materials were injected daily at 6.25 mg/kg. After 20 days, the injections were ceased, and mouse weight was checked for another 10 days ($n = 8$ and control $n = 5$). *Lower panel,* representative male mice treated with vehicle (*left*), PEG-MLA (*center*), or PEG-SMLA (*right*). Depicted are the general habitus of mice (*A*) and appearance of the intact (*B*) and dissected (*C*) abdominal fat.

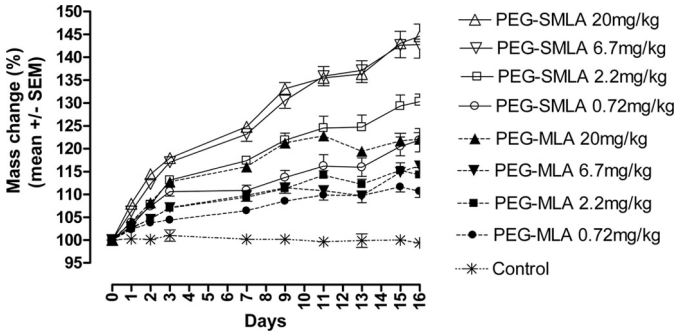


FIGURE 8. **Comparison of the effect of PEG-MLA and PEG-SMLA on weight gain in male mice.** Each material was injected daily at 20, 6.7, 2.2, and 0.72 mg/kg, $n = 8$.

crease in leptin binding affinity. Although a three-dimensional leptin structure was reported 13 years ago (3), no crystallized complex between leptin and leptin receptor has yet been elucidated. Lack of such a structure hampers valid structural interpretation of the presently reported D23L or other Asp-23 mutations. Our suggested interpretation is therefore based on theoretical complex models reported in the last few years (4–6), suggesting that leptin has three binding sites that interact with leptin receptor. Site I is poorly described, and its importance is connected to formation of the putative hexameric complex composed of two molecules of leptin reacting with four leptin receptors. Binding site II, which is a major binding site, is found on the surface of helix A and C and binds to the CHR2 (cytokine homology region II) subdomain of the leptin receptor. This subdomain of human leptin receptor was subcloned in our laboratory and expressed as a soluble recombinant protein termed leptin binding domain, capable of forming high affinity 1:1 complex with human or other mammalian leptins (21). This protein, which is also termed CHR2, was used in this study as a hook to fish out the high

affinity leptin mutants expressed on the surface of yeast cells. Site III presumably binds to the Ig-like domain of the leptin receptor (4) and is necessary for its activation. Using a molecular modeling and mutagenesis approach (4), it was shown that Asp-9, Thr-12, Lys-15, Thr-16, and Arg-20 located on helix A and Gln-75, Asn-82, Asp-85, and Leu-86 located on helix C (see Fig. 6*B* in Ref. 5) are structurally similar and face the same orientation, and they are most likely involved in interacting with CHR2. Mutations of those residues, such as D9S, T12Q, K15S, T16N, R20N, Q75S, N82S, D85S and L86A, L86S, L86N, and L86Q, significantly lowered the affinity of leptin for CHR2 and affected both binding and leptin receptor signaling (4, 5). To date, no report regarding the putative role of Asp-23 has been published; nevertheless, our data (see Table 3) strongly indicate that replacement of Asp-23 by any non-negatively charged amino acid is sufficient to increase affinity for hLBD (CHR2) and subsequent biological activity. The strongest effect was observed with the D23L mutant in binding and cell assays and was confirmed *in vivo* in weight gain experiments in mice. Although the increase in affinity determined by binding assays was up to 60-fold, the increases in the *in vitro* bioassays were only ~13–14-fold, likely because the cells used in those assays have an excess of spare receptors. The increased binding and resultant elevated antagonistic activity are more difficult to calculate *in vivo,* but as shown above, they are in the range of 9–27-fold. Interestingly, we performed an identical D23L mutation in human leptin triple antagonist, and we obtained *in vitro* and *in vivo* binding results identical to those obtained with SMLA.

Asp-23 is located at the C-terminal end of helix A and is oriented in the same direction as Arg-20, Thr-16, and Thr-12 (see Fig. 6*B* in Ref. 5). Its replacement by non-negatively charged amino acids probably abolishes some as yet unidentified repulsive effect and therefore increases the interaction with the leptin binding domain. Notably, the amino acid sequence of helix A in all known mammalian leptins is almost identical, and Asp-23 is preserved in all of them, allowing us to speculate that similar mutations on other mammalian leptins will lead to a similar increase in affinity. The increase in affinity occurred in both the antagonist and agonist mutants, but in contrast to antagonists, the biological activity of agonists in the *in vitro* cell-based assay was not increased. This is similar to the case of human growth hormone, whose mutant, selected by phage display, also exhibited increased affinity for human growth hormone receptor but was not more active in a cell bioassay (34, 35). The reason for this discrepancy is likely related to the fact that the increase in affinity of SMLA and SHLA originates mainly from a decrease in k_{off}, rather than an increase in k_{on}, leading to prolonged receptor occupancy. Such prolonged occupancy makes the antagonist more effective but likely does not increase the activity of the agonist.

When tested *in vivo*, the resultant superactive antagonist featured dramatic leptin-antagonistic biological activity, which was manifested as significant weight gain driven by an increase in appetite and food consumption. Although pegylation decreased the biological activity *in vitro*, the *in vivo* activity was elevated due to prolonged persistence in circulation, consistent with the previously published results (17). The potency and reversibility of the superactive antagonist make it a prime tool for the study of the peripheral and central activities of leptin in adult animals in the steady state as well as in disease conditions. This tool is far superior to the current main research tool, the leptin-deficient mouse model, in which leptin deficiency in the embryonic and neonatal stages is associated with developmental aberrations that carry limited relevance to WT physiology (36, 37). In addition, the superactive antagonists may be studied as potential future therapeutic agents in a myriad of disease conditions in which leptin signaling has been implicated, including solid tumors and auto-inflammatory disorders (38–40). Under these conditions, the use of the superactive leptin antagonist may enable effective and reversible long acting leptin inhibition with low doses and tolerable frequencies of injection.

Acknowledgment—We thank Dr. M. Einat (Agricultural Research Organization, Israel) for HEK-293 cells stably transfected with the three constructs.

REFERENCES

1. Friedman, J. M., and Halaas, J. L. (1998) *Nature* **395,** 763–770
2. Fantuzzi, G., and Faggioni, R. (2000) *J. Leukocyte Biol.* **68,** 437–446
3. Zhang, F., Basinski, M. B., Beals, J. M., Briggs, S. L., Churgay, L. M., Clawson, D. K., DiMarchi, R. D., Furman, T. C., Hale, J. E., Hsiung, H. M., Schoner, B. E., Smith, D. P., Zhang, X. Y., Wery, J. P., and Schevitz, R. W. (1997) *Nature* **387,** 206–209
4. Peelman, F., Van Beneden, K., Zabeau, L., Iserentant, H., Ulrichts, P., Defeau, D., Verhee, A., Catteeuw, D., Elewaut, D., and Tavernier, J. (2004) *J. Biol. Chem.* **279,** 41038–41046
5. Iserentant, H., Peelman, F., Defeau, D., Vandekerckhove, J., Zabeau, L., and Tavernier, J. (2005) *J. Cell Sci.* **118,** 2519–2527
6. Peelman, F., Iserentant, H., De Smet, A. S., Vandekerckhove, J., Zabeau, L., and Tavernier, J. (2006) *J. Biol. Chem.* **281,** 15496–15504
7. La Cava, A., and Matarese, G. (2004) *Nat. Rev. Immunol.* **4,** 371–379
8. Karmiris, K., Koutroubakis, I. E., and Kouroumalis, E. A. (2008) *Mol. Nutr. Food Res.* **52,** 855–866
9. Elinav, E., Ali, M., Bruck, R., Brazowski, E., Phillips, A., Shapira, Y., Katz, M., Solomon, G., Halpern, Z., and Gertler, A. (2009) *Hepatology* **49,** 278–286
10. Elinav, E., and Gertler, A. (2009) in *Leptin and Leptin Antagonists* (Gertler, A., ed) pp. 133–140, Landes Bioscience, Austin, TX
11. Frankenberry, K. A., Skinner, H., Somasundar, P., McFadden, D. W., and Vona-Davis, L. C. (2006) *Int. J. Oncol.* **28,** 985–993
12. Somasundar, P., Frankenberry, K. A., Skinner, H., Vedula, G., McFadden, D. W., Riggs, D., Jackson, B., Vangilder, R., Hileman, S. M., and Vona-Davis, L. C. (2004) *J. Surg. Res.* **118,** 71–82
13. Ray, A., and Cleary, M. P. (2010) *Expert Opin. Ther. Targets* **14,** 443–451
14. Choi, J. H., Choi, K. C., Auersperg, N., and Leung, P. C. (2004) *J. Clin. Endocrinol. Metab.* **89,** 5508–5516
15. Niv-Spector, L., Gonen-Berger, D., Gourdou, I., Biener, E., Gussakovsky, E. E., Benomar, Y., Ramanujan, K. V., Taouis, M., Herman, B., Callebaut, I., Djiane, J., and Gertler, A. (2005) *Biochem. J.* **391,** 221–230
16. Salomon, G., Niv-Spector, L., Gussakovsky, E. E., and Gertler, A. (2006) *Protein Expr. Purif.* **47,** 128–136
17. Elinav, E., Niv-Spector, L., Katz, M., Price, T. O., Ali, M., Yacobovitz, M., Solomon, G., Reicher, S., Lynch, J. L., Halpern, Z., Banks, W. A., and Gertler, A. (2009) *Endocrinology* **150,** 3083–3091
18. Muller, A. F., Kopchick, J. J., Flyvbjerg, A., and van der Lely, A. J. (2004) *J. Clin. Endocrinol. Metab.* **89,** 1503–1511
19. van der Lely, A. J., and Kopchick, J. J. (2006) *Neuroendocrinology* **83,**

264–268
20. Kopchick, J. J., Parkinson, C., Stevens, E. C., and Trainer, P. J. (2002) *Endocr. Rev.* **23,** 623–646
21. Sandowski, Y., Raver, N., Gussakovsky, E. E., Shochat, S., Dym, O., Livnah, O., Rubinstein, M., Krishna, R., and Gertler, A. (2002) *J. Biol. Chem.* **277,** 46304–46309
22. Boder, E. T., and Wittrup, K. D. (1997) *Nat. Biotechnol.* **15,** 553–557
23. Raymond, C. K., Pownder, T. A., and Sexson, S. L. (1999) *BioTechniques* **26,** 134–138
24. Meilhoc, E., Masson, J. M., and Teissié, J. (1990) *Bio/Technology* **8,** 223–227
25. Boder, E. T., and Wittrup, K. D. (2000) *Methods Enzymol.* **328,** 430–444
26. Chao, G., Lau, W. L., Hackel, B. J., Sazinsky, S. L., Lippow, S. M., and Wittrup, K. D. (2006) *Nat. Protoc.* **1,** 755–768
27. Gertler, A. (2006) *Trends Endocrinol. Metab.* **17,** 372–378
28. Gertler, A., Simmons, J., and Keisler, D. H. (1998) *FEBS Lett.* **422,** 137–140
29. Raver, N., Vardy, E., Livnah, O., Devos, R., and Gertler, A. (2002) *Gen. Comp. Endocrinol.* **126,** 52–58
30. Laemmli, U. K. (1970) *Nature* **227,** 680–685
31. Gertler, A., Grosclaude, J., Strasburger, C. J., Nir, S., and Djiane, J. (1996) *J. Biol. Chem.* **271,** 24482–24491
32. Raver, N., Gussakovsky, E. E., Keisler, D. H., Krishna, R., Mistry, J., and Gertler, A. (2000) *Protein Expr. Purif.* **19,** 30–40
33. Gertler, A., Niv-Spector, L., and Reicher, S. (2007) *Nat. Med.* **13,** 18–21
34. Lowman, H. B., and Wells, J. A. (1993) *J. Mol. Biol.* **234,** 564–578
35. Pearce, K. H., Jr., Cunningham, B. C., Fuh, G., Teeri, T., and Wells, J. A. (1999) *Biochemistry* **38,** 81–89
36. Udagawa, J., Hashimoto, R., Suzuki, H., Hatta, T., Sotomaru, Y., Hioki, K., Kagohashi, Y., Nomura, T., Minami, Y., and Otani, H. (2006) *Endocrinology* **147,** 647–658
37. Holness, M. J., Munns, M. J., and Sugden, M. C. (1999) *Mol. Cell. Endocrinol.* **157,** 11–20
38. Ramani, K., Yang, H., Xia, M., Ara, A. I., Mato, J. M., and Lu, S. C. (2008) *Hepatology* **47,** 521–531
39. Gonzalez-Perez, R. R., Xu, Y., Guo, S., Watters, A., Zhou, W., and Leibovich, S. J. (2010) *Cell. Signal.* **22,** 1350–1362
40. Siegmund, B., Lehr, H. A., and Fantuzzi, G. (2002) *Gastroenterology* **122,** 2011–2025