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Preparation of Recombinant Bovine, Porcine, and Porcine W4R/R5K Leptins and Comparison of Their Activity and Immunoreactivity with Ovine, Chicken, and Human Leptins

Nina Raver,* Eugene E. Gussakovsky,†,‡ Duane H. Keisler,§ Radha Krishna,‖ Jehangir Mistry,‖ and Arieh Gertler*,[1]

**Institute of Biochemistry, Food Science and Nutrition, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University, Rehovot 76100, Israel; †Department of Life Sciences, Bar Ilan University, Ramat Gan 52900, Israel; ‡Institute of Horticulture, The Volcani Center, ARO, Bet Dagan 50250, Israel; §Animal Science Department, University of Missouri, Columbia Missouri 65211; and ‖Diagnostic System Labs, Webster, Texas 77598*

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Recombinant ovine Ala-leptin (GenBank Accession No. U84247, of ovine leptin), previously prepared in our laboratory in prokaryotic expression plasmid pMON3401, was mutated using a mutagenesis kit to prepare plasmids encoding for bovine (GenBank Accession No. U50365) and porcine (GenBank Accession No. U59894) leptins and for porcine leptin analogue W4R/R5K. *Escherichia coli* cells transformed with these plasmids overexpressed large amounts of these proteins upon induction with nalidixic acid. The expressed proteins, found in inclusion bodies, were refolded and purified to homogeneity using subsequently anion- and cation-exchange chromatography. All three purified proteins showed a single band of the expected molecular mass of 16 kDa in SDS–PAGE in the presence of reducing agent and were composed of 90–100% monomers. Proper refolding was evidenced by comparing their CD spectra to those of previously prepared chicken and ovine leptins and to commercially available human leptin. The amino acid content of the purified proteins closely resembled the predicted composition. The biological activity of bovine leptin, porcine leptin, and porcine leptin analogue W4R/R5K was evidenced by their ability to stimulate proliferation of leptin-sensitive BAF/3 cells transfected with a long form of human leptin receptor. All three proteins, as well as ovine and chicken leptins, but not human leptin, exhibited a very high degree of cross-immunoreactivity against antiserum raised against ovine leptin in rabbits. In contrast, none or very low cross-immunoreactivity was observed against antiserum raised against ovine leptin in goats.

Key Words: **recombinant; bovine; porcine; analogue; leptin; bioactivity; cross-immunoreactivity.**

Obese protein (OB), also known as leptin, is a 146-amino-acid-long hormone secreted by adipose tissue that serves as a protein signal, acting on the central nervous system to regulate food intake and the body's energy balance (1–4) through specific receptors. This protein, which belongs to the cytokine family (5,6), exhibits high homology in mammalian species. Although the sequence of various leptins from 10 mammalian species was recently compiled (7), only four recombinant leptins (mouse, human, ovine, and chicken) have been prepared to date. So far, studies of the effect of leptin have been mainly limited to rodents, and the effect of this protein on the metabolism and nutritional behavior of farm animals, which require large quantities of the recombinant leptin, has not yet been investigated. It was suggested that the profound effects of leptin on regulating body energy balance make it a prime candidate for drug therapies for humans and farm animals (8). To answer these challenges, a rather simple and inexpensive method that allows large-scale preparation of biologically active ovine (9) and chicken (10) leptins was developed in our laboratory. In order to allow further study of the effects of leptin in other species of farm animals and to create immunoassays aimed at detecting leptin in body fluids, we have used recombinant ovine Ala-leptin (9) (GenBank Accession No. U84247) as a template to prepare bovine (GenBank Accession No. U50365) and porcine (GenBank Accession No. U59894) leptins by site-directed mutagenesis. This approach was chosen due to

[1] To whom correspondence should be addressed. Fax: (972) 89-476-189. E-mail: gertler@agri.huji.ac.il.

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TABLE 1

Composition of Primers Used for Preparation of Bovine and Porcine Leptins

Primer[a]	Primer sequence	New restriction site (underlined)
bL-1	S (5′-CAACAGATCCTC**A**CCAGTCTGCCTTCT**T**AGAAATGTG**G**TCCAAATATC-3′)	*Xba*I
	A (5′-GATATTTGGACCACATTTCTAGAAGGCAGACTGGTGAGGATCTGTTG-3′)	
pL-1	S (5′-GCAGTGCCGAT**T**CG**AA**G**G**GTCCAGGATGAC-3′)	*Bsp*119I
	A (5′-GTCATCCTGGACCCTTCGAATCGGCACTGC-3′)	
pL-2	S (5′-GCAGTGCCGATC**T**G**GCGT**GT**A**CAGGATGAC-3′)	*Bsp*1407I
	A (5′-GTCATCCTGTACACGCCAGATCGGCACTGC-3′)	
pL-3	S (5′-CAGGATCA**G**TGA**T**ATCTCACACA**T**GCAGTCCGTC-3′)	*Eco*32I
	A (5′-GACGGACTGCATGTGTGAGATATCACTGATCCTG-3′)	
pL-4	S (5′-GGCTCCACCCT**G**TC**TTA**AGTTTGTCCAAGATGGACCAG-3′)	*Bsp*TI
	A (5′-CTGGTCCATCTTGGACAAACTTAAGACAGGGTGGAGCC-3′)	
pL-5	S (5′-CTACCAACAGATCCTC**A**C**T**AGTCTGCCTTCCA-3′)	*Bsu*I
	A (5′-TGGAAGGCAGACTAGTGAGGATCTGTTGGTAG-3′)	
pL-6	S (5′-CTGCT**A**GCC**T**CCTCCAAGAGCTGCCCCTTGCCGCAGG**C**CAGG-3′)	*Eco*130I
	A (5′-CCTGGCCTGCGGCAAGGGGCAGCTCTTGGAGGAGGCTAGCAG-3′)	
pL-7	S (5′-GGGCCCT**C**GAGA**C**CTTGGAGAGCCTGGGCG**G**CGTCCTGG-3′)	*Xho*I
	A (5′-CCAGGACGCCGCCCAGGCTCTCCAAGGTCTCGAGGGCCC-3′)	
pL-8	S (5′-GGCTACAGG**G**G**G**C**C**CTACAGGACATGT-3′)	*Bsp*120I
	A (5′-ACATGTCCTGTAGGGCCCCCTGTAGCC-3′)	

[a] S, sense primer; A, antisense primer; all mutations are in bold letters.

the fact that ovine and bovine leptins differ by two amino acids only and that the corresponding difference between ovine and porcine leptins is 11 amino acids (7). Furthermore, preparation of these proteins as well as porcine leptin mutant that differs by two amino acids from the nonmutated protein allowed us to compare the biological activity and cross-immunoreactivity of the five recombinant leptins prepared in our laboratory to a commercially available human leptin. To minimize the difference between the native and recombinant proteins, constructs encoding for Ala-leptins rather than for Met-leptins were preferred, as in our expression system the amino-terminal Met–Ala bond was efficiently cleaved in the bacteria, leading respectively to over 95 and 93% of alanyl ovine and chicken leptins (9,10). Since the N-terminus of leptins is relatively more variable than other portions, to test its importance, we have also prepared porcine leptin mutant with the N-terminal sequence corresponding to that of ovine and bovine leptins.

MATERIALS AND METHODS

Materials. Ovine (fraction SP) and chicken leptins were prepared in our laboratory as described previously (9,10) and recombinant human leptin (lot no. B24800) was purchased from Calbiochem–Novabiochem Corp. (San Diego, CA). Molecular weight markers for gel electrophoresis, RPMI-1640 medium, interleukin-3 (IL-3), polyethylene glycol (PEG), bovine immunoglobulin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Thiazol Blue, MTT), and nalidixic acid were obtained from Sigma Chemical Co. (St. Louis, MO). SDS–PAGE reagents were purchased from Bio-Rad Laboratories (Richmond, CA). Fetal calf serum (FCS) was purchased from Biolab Co. (Jerusalem, Israel), and the Superdex75 HR 10/30 column, Q-Sepharose, and SP-Sepharose (fast flow) were purchased from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Carrier-free $Na^{125}I$ was purchased from New England Nuclear Corp. (Boston, MA), and DNA primers were obtained from GIBCO BRL, NV Life Technologies S.A. (Ghent, Belgium). All other chemicals were of analytical grade.

Construction of bovine leptin expression plasmid. In order to mutate ovine leptin to bovine leptin, which differs from the former by two amino acids only (A66T and I74V), the insert encoding for ovine leptin (9) was subcloned into the pTRC expression vector. Then the latter plasmid was modified with the Stratagene Quickchange mutagenesis kit (La Jolla, CA) according to the manufacturer's instructions, using two complementary primers (Table 1, primer bL-1). Those primers were designed to contain a specific restriction site for *Xba*I (underlined), still conserving the same amino acid sequence, for colony screening and two base changes (marked in bold) for performing two-amino-acid mutation. The procedure included 16 PCR cycles and the use of *Pfu* polymerase enzyme for the reaction. The mutated construct was then digested with *Dpn*I restriction enzyme, which is specific to methylated and hemimethylated DNA (target sequence: 5′-G^{m6}ATC-3′), in

order to digest the template and to select for mutation-containing synthesized DNA. The plasmid was then transfected into XL1 competent cells. Ten colonies were then screened for mutation, using the specific restriction site designed, and revealed 90% efficiency. Two colonies were sequenced and confirmed to contain the mutation and no undesired misincorporation of nucleotides. The insert was then removed from the pTRC plasmid, ligated to pMON3401 vector between *Nco*I/*Hin*dIII restriction sites, and transfected into MON105 competent cells.

Construction of porcine leptin and porcine leptin mutant expression plasmid. The pTRC plasmid containing the ovine leptin-encoding sequence (see former paragraph) was modified with the Stratagene Quickchange mutagenesis kit according to the manufacturer's instructions. To achieve the 11 mutations required for conversion of ovine into porcine leptin, eight different couples of complementary primers were designed. The general strategy was to mutate one or two amino acids and in parallel to induce a new restriction site in order to identify whether the mutation(s) had occurred. Therefore each couple of primers was designed to contain a specific restriction site (underlined), still conserving the same amino acid sequence and changes aimed at performing the mutation (marked in bold). The mutations were performed in the following order (Table 1): mutation 1, S106T and V112G using primer pL-7; mutation 2, A92S and V101A using primer pL-6; mutation 3, N22S and T27M using primer pL-3; mutation 4, L48V using primer pL-4; mutation 5, A66T using primer pL-5; mutation 6, S132A using primer pL-8; mutation 7, R4W and K5R using primer pL-2 or mutation 7a, K5R using pL-1. As a result of those mutations, three final plasmids were created: porcine leptin plasmid, in which all 11 amino acids that differed from ovine leptin were mutated (mutations 1–7), porcine leptin mutant, in which only mutations 1–6 plus 7a were performed, and porcine leptin mutant, in which only mutations 1–6 were performed, namely, in which 9 amino acids were mutated and the amino acids existing in ovine leptin (R4 and K5) were not changed. This porcine mutant was respectively designed as porcine leptin W4R/R5K. After each mutation the respective plasmids were sequenced and confirmed to contain the mutation and no undesired misincorporation of nucleotides. The porcine leptin mutant in which mutations 1–6 plus 7a were performed, designed as porcine leptin mutant W4R, was also expressed but the protein was not further explored.

The procedure for preparation of the plasmids was identical to that described above for preparation of bovine leptin, except that the number of PCR cycles varied between 12 and 18.

Expression, refolding, and purification of bovine leptin, porcine leptin, and porcine leptin analogue W4R/R5K. A preliminary experiment was performed with four clones of each leptin in a 250-ml flask containing 30 ml of Terrific Broth (TB) medium (11). Four hours after nalidixic acid induction, *E. coli* MON105 cells transformed with the respective expression plasmids expressed leptin (~16 kDa) as a major cell protein. One of the colonies expressing each protein was selected for large-scale preparation in 5 × 500 ml TB medium, conducted in 2.5-liter flasks incubated at 200 rpm at 37°C. After an A_{600} of 1.0 was reached, 25 mg of freshly prepared nalidixic acid dissolved in 2.5 ml of 0.1 N NaOH was added to each flask. Bacteria were incubated for an additional 4 h, harvested by 10-min centrifugation at 10,000*g*, and frozen at −20°C. Over 95% of the leptin protein was found in the inclusion bodies (not shown). It was extracted according to the detailed procedure previously used for the preparation of bovine placental lactogen (12), except that two additional washings with 1% Triton X-100 followed by two washings with water were added. The inclusion-body pellet obtained from one fermentation was solubilized in 500 ml of 4.5 M urea buffered with 10 mM Tris base, cysteine (1 mM), pH 11.3. The clear solution was gently stirred at 4°C for 6 h, diluted with three volumes of 0.67 M L-arginine, and dialyzed for 48 h against 5 × 10 liters of 10 mM Tris–HCl, pH 8. Then the respective leptins were purified using anion- and cation-exchange chromatography according to the protocol described previously for ovine leptin fraction SP (9).

Determination of purity and monomer content. SDS–PAGE was carried out according to Laemmli (13) in a 15% polyacrylamide gel under reducing and nonreducing conditions. Gels were stained with Coomassie brilliant blue R. HPLC gel filtration chromatography was performed on a Superdex75 HR 10/30 column with 0.2-ml aliquots of Q-Sepharose- or SP-Sepharose-column-eluted fractions using 25 mM Tris–HCl buffer, pH 8, containing 150 mM NaCl. Freeze-dried samples were dissolved in H_2O.

Determination of amino acid composition. Lyophilized samples of ovine, chicken, human, bovine, and porcine leptins and porcine W4R/R5K leptin analogue were hydrolyzed in gas-phase 6 N HCl and 0.1% phenol under N_2 at 110°C for 24 h, dried, and analyzed using an amino acid analyzer (Biotronik, Model LS5000, Eppendorf Co., Frankfurt, Germany).

Determination of circular dichroism (CD) spectra. The CD spectra, in millidegrees, were measured with an Aviv Model 62A DS circular dichroism spectrometer (Aviv Associates, Lakewood, NJ) using a 0.020-cm rectangular QS Hellma cuvette. The spectrometer was calibrated by camphorsulfonic acid (14). Lyophilized protein samples were dissolved in 55–65 mM sodium

carbonate buffer, pH 7.5–7.8, at concentrations of 0.55–0.75 mg/ml and cleared by centrifugation at 11,000*g* for 10 min. The CD measurements were done at 25.0°C, controlled to an accuracy of 0.1°C by thermoelectric Peltier elements. The CD spectra were measured in five repetitions, resulting in an averaged spectrum for each protein. The standard deviation of the averaged CD signal at 222 nm was in the 0.5–2.0% range. For the secondary structure determination, the CD data were expressed in deg cm^2 $dmol^{-1}$ per mean residue, whose molecular weight was assumed to be 110 at the molecular weight of leptins of 16.0 kDa calculated from the 147 amino acids. The protein concentration of the cleared solutions was determined by the biuret method (15) in five repetitions at different dilutions for each protein, using bovine pancreatic ribonuclease A as a reference (1 mg/ml gives OD = 0.715 at 277 nm). The standard deviation of the concentration determination was in the 3.2–7.8% range. Estimation of the protein concentration using the expected extinction coefficient according to Pace *et al.* (16) gives a higher uncertainty. The secondary structure of leptins was calculated from the CD spectra by applying a procedure and the computer program CONTIN developed by Provencher and Glöckner (17). The program determined α-helix, β-strands, and β-turns as a percentage of amino acid residues involved in these ordered forms. The constraint that the sum of all elements of the secondary structure in a protein must equal unity resulted in a remainder, which may be interpreted as the content of random coil (18). In the present study, for calculations by the CONTIN program, a set of standard CD spectra of 17 proteins by Sreerama and Woody (19) was employed.

Mass spectrometry. The mass analysis was done using matrix-assisted laser-desorption time-of-flight (MALDI-TOF) mass spectrometry (2E, Micromass, UK). The proteins were deposited on a metal target as cocrystals with sinapic acid (Fluka) (20) and the mass spectrum was determined in the positive ion mode.

BAF/3 cell proliferation bioassay. The proliferation rate of leptin-sensitive BAF/3 1442-CI 4 cells transfected with the long form of human leptin receptor was used as an *in vitro* leptin bioassay. The original bioassay (21) was modified as described below. In stationary cultures, these cells were grown in RPMI-1640 medium supplemented with 5% fetal calf serum (Biolab, Jerusalem, Israel) and 100 ng of murine IL-3/ml at 37°C in 95%/5% air/CO_2. Prior to the experiment, the cells were collected by centrifugation, washed, and resuspended in the same medium without IL-3. The cells were then diluted to 500,000 cells/ml and added in triplicate, and the cells were grown for an additional 48 h. Then 20 μl of the MTT solution (0.625% in PBS) was added to each well and the plates were left at 37°C. Two hours later, 100 μl of the solubilization/stop solution (containing 20 g of SDS in 100 ml of 50% dimethylformamide and 2.5 ml of 80% acetic acid) was added and the plates were left overnight at 37°C. Cell proliferation was determined by monitoring the absorbance at 595 nm, using an ELISA plate reader (ELx800, Bio-Tek Instruments, Inc., Winooski, VT). 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. Ovine, human, and chicken leptins were used as positive controls.

Preparation of antiserum against ovine leptin. Recombinant ovine leptin antiserum was produced in rabbits as follows. One hundred micrograms of GST-ovine leptin (J. Simmons and D. H. Keisler, unpublished) was solubilized in 0.5 ml of saline and emulsified in 0.5 ml of Freund's complete adjuvant and injected. Three weeks later the rabbits were reimmunized with 100 μg of recombinant ovine leptin (9) solubilized in 0.5 ml of saline and emulsified in 0.5 ml of Freund's incomplete adjuvant. Beginning on the sixth week after initial immunization, antiserum was collected at weekly intervals and rabbits reimmunized every third week. Standard SDS–PAGE and Western blot techniques were used to document that the antiserum recognized the recombinant ovine leptin. An additional procedure for preparation of anti-ovine leptin antibodies in goats was also employed. Goats were immunized with ovine or chicken leptin antigens reconstituted with water and injected at 1, 0.5, 0.25, and 0.1 mg per goat at day 1, day 30, day 60, and day 90, respectively. The first injection was with Freund's complete adjuvant and subsequent injections were with Freund's incomplete adjuvant. First bleed was collected on day 100. Subsequent vaccinations were done every month at 0.1 mg per goat and bleeds were collected every 10th day after the injection. Serum was separated from bleeds and tested for antibody quality and characteristics by using RIA.

Cross-immunoreactivity. The cross-immunoreactivity of various recombinant leptins with ovine leptin was determined by radioimmunoassay using anti-ovine leptin serum raised in rabbits and goats. Radiolabeled ovine leptin served as a ligand and all other nonlabeled proteins as competitors. In experiments conducted with antiserum raised in rabbits, each tube contained 50 μl of 10 mM Na barbiturate, pH 8.8 buffer (reaction buffer) containing 0.1% bovine serum albumin, 50 μl of ^{125}I-ovine leptin (55,000 cpm), and 50 μl of different leptin solutions (providing 0–1000 ng/tube) in the reaction buffer. Addition of 50 μl of anti-ovine leptin antiserum to a final dilution of 1:1500 started the reaction. The tubes were incubated for 48 h at room temperature. Then the leptin–antibody complex was

precipitated by adding 50 μl of 1% (w/v) bovine immunoglobulin and 500 μl of 25% (w/v) polyethylene glycol. Tubes were thoroughly mixed, incubated for 20 min at 4°C, and centrifuged at 12,000*g* for 15 min at 4°C. Then supernatant was carefully aspirated and the precipitates were counted in a Kontron γ counter. A slightly different protocol was employed for testing the cross-immunoreactivity with the antiserum prepared in goats. One hundred microliters each of ovine leptin standards (prepared using PBS + 1% BSA + 0.09% sodium azide and containing 0–1000 ng of ovine leptin), 100 μl of 1:100 diluted antiserum, and 100 μl of ^{125}I-ovine leptin were incubated overnight at 4°C. Then 1 ml of donkey anti-goat antibody/PEG was added and the tubes were further incubated for 20 min and centrifuged at 4°C for 20 min. The supernatant was decanted and the tubes containing the pellet were counted in a γ counter for 1 min. Other leptins were prepared at 100 ng/ml concentrations and run as unknowns in the assay to see if any of the leptins cross-reacted (by competing with and displacing the original ^{125}I-labeled tracer). From the computer printout, the calculated concentration was divided by the actual (100 ng/ml) concentration and multiplied by 100 to obtain the % cross-reactivity. Iodination of ovine leptin was performed according to a protocol previously described for iodination of human growth hormone (22).

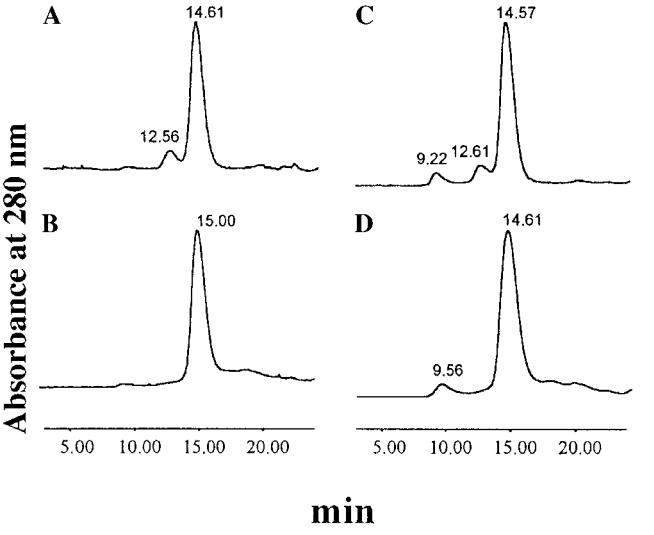

FIG. 1. Gel filtration of purified bovine leptin (A), porcine leptin (B), porcine leptin analogue W4R/R5K (C), and human leptin (D). Aliquots (200 ml) of lyophilized fractions (~1 mg/ml) were applied to a Superdex 75 HR 10/30 column and the eluate was monitored by absorbance at 280 nm. The column was developed with 25 mM Tris–HCl buffer, pH 8, containing 150 mM NaCl at 0.8 ml/min and calibrated with bovine serum albumin (66 kDa, RT = 11.13 min), egg albumin (45 kDa, RT = 12.58 min), extracellular domain of hGH receptor (28 kDa, RT = 13.52 min), and ovine placental lactogen (23 kDa, RT = 13.92 min).

RESULTS

Purification and Characterization of Bovine Leptin, Porcine Leptin, and Porcine Leptin Analogue W4R/R5K

The protein contained in the inclusion bodies prepared from 2.5 liters of induced cells was refolded in 4.5 M urea as described under Materials and Methods. The refolded and dialyzed protein (total volume 2000 ml) was applied on a Q-Sepharose column (2.6 × 6 cm), equilibrated with 10 mM Tris–HCl buffer, pH 8.0. In all three cases, over 80–90% of the monomeric leptin was not absorbed and was fully recovered in the breakthrough fraction (not shown). The column was subsequently washed with 0.05 and 0.5 M NaCl, and small amounts of leptins, as evidenced by SDS–PAGE and biological activity, were eluted. Gel filtration chromatography of these fractions on a Superdex75 column revealed, however, that the 0.05 M eluate contained a fraction composed of a mixture of monomeric (50–70%) and dimeric (30–50%) leptins. A small quantity of dimeric and oligomeric leptin was eluted with 0.5 M NaCl (not shown). Because of small amounts and lack of homogeneity, these fractions were not further explored. The breakthrough fraction of the three leptins obtained from the Q-Sepharose column contained over 90% monomeric leptin. It was adjusted to pH 5 with 10% HAc and applied to an SP-Sepharose column (1.2 × 8 cm) previously equilibrated with 25 mM NaAc buffer, pH 5. All protein was absorbed. Elution was carried out using a discontinuous NaCl gradient in the same buffer at a rate of 120 ml/h, and 5-ml fractions were collected (not shown). Fractions, containing over 90% pure monomeric leptin (as evidenced by gel filtration on a Superdex column), were eluted as a wide peak with 100 mM NaCl in the case of bovine leptin, with 200 mM NaCl in the case of porcine leptin analogue W4R/R5K, or with both 100 and 200 mM NaCl in the case of porcine leptin. They were pooled, dialyzed against 0.2% $NaHCO_3$, and lyophilized. The corresponding fractions eluted with 500 mM NaCl consisted of a mixture of oligomers, dimers, and monomers (not shown). Inclusion bodies prepared from a 2.5-liter fermentation culture yielded respectively 125, 90, and 195 mg of bovine, porcine, and porcine W4R/R5K leptins. Since almost all expressed protein was found in the inclusion bodies in an inactive form, a standard purification table cannot be presented. Therefore aliquots of inclusion body suspension were separated on SDS–PAGE gels. Semiquantitative estimation based on density scanning was conducted, leading to approximate values of ~500, 400, and 700 mg for bovine, porcine, and porcine W4R/R5K leptins, respectively, expressed in 2.5 liters of fermentation mixture. Calculation of the corresponding yields on monomeric leptins indicated, respectively, 25, 22, and 28% yield of the expressed

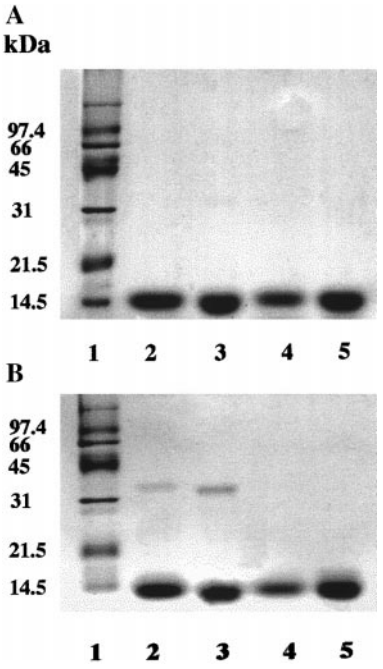

FIG. 2. SDS–PAGE analysis of recombinant leptins on 15% gel run after (A) or without (B) pretreatment with reducing agent. Lane 1, molecular mass markers in kilodaltons; lane 2, bovine leptin; lane 3, porcine leptin analogue W4R/R5K; lane 4, porcine leptin; lane 5, ovine leptin.

protein. As shown in Fig. 1B porcine leptin consisted of pure monomer, whereas in bovine leptin (Fig. 1A), a small amount (less than 5%) of dimers was also seen. A similar profile was also obtained for porcine leptin analogue W4R/R5K, but in this fraction, a small amount of oligomers was also observed (Fig. 1C). Commercial human leptin (Fig. 1D) was composed of monomer and a small amount of oligomers. Bovine leptin, porcine leptin, and porcine leptin analogue W4R/R5K as well as ovine leptin yielded only one band of ~15–16 kDa following 15% SDS–PAGE performed in the presence of reducing agent (Fig. 2A). In the absence of reducing agents, small amounts of dimers could be seen in bovine leptin and porcine leptin analogue W4R/R5K (Fig. 2B). Since the results from SDS–PAGE gave only approximate molecular mass, the purified protein was also analyzed by mass spectrometry (Table 2), yielding a main peak close to the predicted molecular mass. The small difference may be attributed to the fact that the purified protein was lyophilized in the presence of $NaHCO_3$ and thus some molecules could carry Na^+ ion. As shown, minor peaks with higher molecular masses (122–159 Da) and relative intensities of approximately 6–12% compared to the main peak were also observed. Those peaks most likely represent the noncleaved Met-Ala-leptin molecules. The predicted molecular masses for those molecules should be 129 Da higher than that of Ala-leptin, close to the observed values. The mass spectrometry analysis also revealed the existence of small amounts of dimers, in agreement with the gel filtration results.

Figure 3 shows the CD spectra of six leptins at neutral pH. The difference between the spectra of bovine, porcine, porcine analogue W4R/R5K, and chicken leptins was negligible. The spectrum for ovine leptin had a slightly higher absolute intensity in the 210- to 230-nm range, and the absolute value of the molar ellipticity of human leptin was lower at 190–210 nm. The spectra had a shape resembling the spectrum of an α-helix, which shows characteristic negative bands at 208 and 222 nm (18). The spectra measured in the present work had negative bands at about 209 and 220 nm. The deviation from the classic spectrum of an α-helix indicated the existence of other ordered forms contributed to the observed spectra. The secondary structures of leptins calculated from the CD spectra are shown in Table 3. High content of α-helix, no β-strands, and 12–20% content of β-turns are clearly characteristic for all the proteins. In fact, no difference in the secondary structure of bovine, porcine, porcine analog, and chicken leptins is the reason for the identity of the CD spectra. The difference in the spectra of

TABLE 2

Comparison of the Predicted and MALDI-TOF-MS-Determined Molecular Masses (in Daltons) of Purified Recombinant Ovine, Bovine, Chicken, Porcine, and Porcine W4R/R5K Leptins

Species	Predicted	Main peak	Minor peaks[a]	Dimers[a]	Trimers
Ovine	16,106	16,122	16,268	32,244	None
Bovine	16,122	16,134	16,231	32,253	48,468[b]
Chicken	15,966	16,147	16,247	32,276	48,165[b]
Porcine	16,127	16,180	16,338	32,429	None
Porcine W4R/R5K	16,071	16,167	16,388	32,234	None

[a] Though an accurate estimation of the relative amount of the minor peak is impossible, the approximate amounts calculated respectively for ovine, bovine, chicken, porcine, and porcine W4R/R5K were 7–9, 6–8, 7–9, 10–12, and 6–8%. The corresponding approximate values for the relative amounts of dimers were 1.6, 5.5, 4.4, 2.2, and 2.7%.

[b] Traces (less than 10% of the amount of dimers).

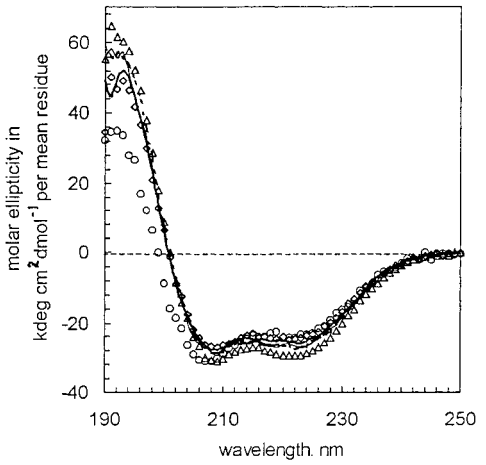

FIG. 3. Circular dichroism (CD) spectra of bovine (dotted line), porcine (regular line), porcine W4R/R5K analogue (bold line), human (circles), ovine (triangles), and chicken (rhombs) leptins at pH 7.5 in 65 mM Na-carbonate buffer.

human and ovine leptins led to a lower α-helix content in human leptin and a higher α-helix content in ovine leptin, but this difference was not crucially high. Table 4 compares the primary structures of the different recombinant leptins to that of human leptin, in which the secondary structure was determined on the basis of X-ray diffraction (PDB 1AX8). The difference concerned 18–26 residues. Each of the various residues has a frequency of appearance in the α-helix ranging from about 0.5 for very low frequency to 1.5 for very high frequency (23). When the frequencies of appearance were summed for human and bovine or porcine or other studied leptins, the sums differed by 1.0–1.8, which was close to the frequency for one residue. This means that the expected variation of the secondary structure was about $^1/_{147} = 0.7\%$, which is less than the error of the secondary structure determination. Hence one could not expect to measure a difference in the secondary structure of different leptins using the CD method.

The amino acid content of bovine, porcine, and porcine analogue W4R/R5K leptins was compared to ovine, chicken, and human leptins (Table 5). As can be seen, a high correlation between the experimental and the theoretical values was observed. The determined values for Thr, Ser, and Tyr were, in most cases, lower than expected as those amino acids undergo partial destruction during acid hydrolysis (24). In most cases, lower than expected values were also found for branched amino acids. Branched amino acids (Val, Ile, and Leu) account for 30–35% of total amino acids in leptins and there are 9–10 peptide bonds formed between them. Those peptide bonds are more stable to acidic hydrolysis and therefore up to 96 h of hydrolysis is required to achieve full recovery of free amino acids (24).

Biological Activity in Vitro

The biological activity of bovine, porcine, and porcine analogue W4R/R5K leptins was compared to that of ovine, chicken, and human leptins using a BAF/3 proliferation bioassay (Fig. 4). The results were analyzed by PRISM software (25), according to a nonlinear regression, using a sigmoidal dose–response curve. In all analyses, the degree of match for the nonlinear correlation was very high ($r^2 > 0.97$). All leptins exhibited the same maximal response (Fig. 4). Half of the maximal responses (EC_{50}) of bovine, porcine, and porcine analogue W4R/R5K leptins were respectively (mean $\pm$ SE, in pM) 51.8 $\pm$ 7.4, 64.1 $\pm$ 9.3, and 37.3 $\pm$ 4.1. These values are comparable to the corresponding EC_{50} values of 37.2 $\pm$ 2.8, 16.8 $\pm$ 1.6, and 113 $\pm$ 10.5 obtained respectively for ovine, human, and chicken leptins. Statistical comparison of these results revealed that human leptin was significantly ($P > 0.05$) more potent than other leptins. In contrast, chicken leptin was less potent, whereas the differences between the ovine, bovine, porcine, and porcine analogue W4R/R5K leptins were not significantly different.

TABLE 3

Secondary Structure of Recombinant Leptins at Neutral pH

Secondary structure (%)[a]	Bovine	Porcine	Porcine analogue W4R/R5K	Human[b]	Ovine	Chicken
α-Helix	78 $\pm$ 0.9	76 $\pm$ 0.5	76 $\pm$ 4.7	70 $\pm$ 1.1 (61)	87 $\pm$ 1.4	74 $\pm$ 1.2
β-Strands	0	0	0	0 (0)	0	0
β-Turns	12 $\pm$ 0.8	14 $\pm$ 0.5	20 $\pm$ 3.1	18 $\pm$ 0.9 (11)	7 $\pm$ 1.2	16 $\pm$ 1.1
Remainder	10 $\pm$ 0.8	10 $\pm$ 0.5	5 $\pm$ 3.1	12 $\pm$ 1.0 (28)	6 $\pm$ 1.0	10 $\pm$ 0.9

[a] The results are given as means $\pm$ SD. The errors arose only from an uncertainty of the fitting of the experimental CD spectrum by the set of standard protein CD spectra in the CONTIN program. The errors of both the CD measurement and the protein concentration determination were not included.

[b] The values in parentheses are taken from the Protein Data Bank (PDB code 1AX8), which were deduced from the X-ray data with 2.40-Å resolution. The value of % α-helix is a sum of 54% α-helix and 7% 3_{10}-helix.

TABLE 4

Comparison of the Primary and Secondary Structures of Ovine, Bovine, Chicken, Porcine, Porcine W4R/R5K, and Human Leptins[a]

```
Ovine          1  ...R..... .......... D......... ....R..... .........S
Bovine         1  ...R..... .......... .......... ....R..... .......L.S
Chicken        1  ..C.IF.... .......... ....... .. .A..R..... .........S
Porcine        1  ...WR..... .......... .S....M... ....R..... .......V.S
Porcine
W4R/R5K        1  ...R..... .......... ......M... ....R..... .......V.S
Human          1  VPIQKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHPILT
                     HHHHHHH HHHHHHHHHH HHH                   S   SS  SS


Ovine         51  .........I .....A.L.. .......... ........L. .A....P..Q
Bovine        51  .........I .......L.. ...V...... ........L. .A....P..Q
Chicken       51  .......... ...V...L.. Q..L..A... ........L. ......S..Q
Porcine       51  .........I .......L.. .......... ........L. .S....P..Q
Porcine
W4R/R5K       51  .........I .......L.. .......... ........L. .S....P..Q
Human         51  LSKMDQTLAV YQQILTSMPS RNVIQISNDL ENLRDLLHVL AFSKSCHLPE
                    HHHHHHHHHH HHHHHHTS   HHHHHHHHHH HHHHHHHHHH HHHTT


Ovine        101  VRA....E.. .V.....L.. .......... .......R.. ......
Bovine       101  VRA..S.E.. .V.....L.. .......... .......R.. ......
Chicken      101  T...QKPE.. D......L.. .......... .....I.Q.. .I..E.
Porcine      101  RRA....E.. .......L.. .......... .A.....R.. ......
Porcine
W4R/R5K      101  RRA....E.. .......L.. .......... .A.....R.. ......
Human        101  ASGLETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC
                        S SSTT HHHH  TTS  HHHHHHHHHH HHHHHHHHHG GG
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[a] The secondary structure of human leptin was obtained from the Protein Data Bank (PDB code 1AX8). H, α-helix; G, 3_{10}-helix (3-helix); T, hydrogen-bonded turn; S, bend. Residues not identical to human leptin are in bold letters and identical residues are marked by dots. Chicken leptin lacks residue 28.

Cross-immunoreactivity

Cross-immunoreactivity between the newly prepared bovine, porcine, and porcine analogue W4R/R5K leptins, formerly prepared ovine and chicken leptins, and commercially available human leptin was studied using two antisera raised against recombinant ovine leptin in rabbits or goats. As shown in Fig. 5, anti-ovine leptin antibodies raised in rabbits almost equally recognized the bovine, porcine, porcine analogue W4R/R5K, ovine, and chicken leptins, with respective IC_{50} values of 0.79, 2.08, 2.35, 1.90, and 1.23 ng/tube. Surprisingly, bovine leptin was even more potent than ovine leptin and chicken leptin, though the differences were not statistically significant. In contrast, the ability of human leptin to displace the ^{125}I-ovine leptin was two orders of magnitude less than other leptins, with a respective IC_{50} value of 98 ng/tube. This difference cannot be, however, attributed to the method of preparation, because identical results were obtained with recombinant human leptin prepared in our laboratory (N. Raver and A. Gertler, unpublished data). Similar very high cross-immuoreactivity between the five farm animals' leptins was also observed using different antiserum raised against ovine leptin in rabbits (D. Keisler, personal communication). In contrast, the cross-reactivity of different leptins using anti-ovine leptin serum raised in goats was very low. Whereas in a standard curve using ^{125}I-ovine leptin and unlabeled ovine leptin, the IC_{50} value for the latter was 6.5 ng/tube, the cross-reactivities for bovine, porcine, porcine analogue W4R/R5K, chicken, and human leptin were respectively 3.0, 1.8, 2.9, 9.0, and 0%.

DISCUSSION

Three electrophoretically highly pure recombinant bovine, porcine, and porcine W4R/K5R alanyl-leptins were prepared using prokaryotic expression plasmid. The yield was comparable to that of previously prepared ovine and chicken leptins (9,10). Whereas porcine leptin consisted of close to 100% monomer, small amounts (less than 5%) of dimers were found in bovine

TABLE 5

Comparison of the Determined and Predicted (in Parentheses) Amino Acid Compositions of Pure Recombinant Bovine, Chicken, Human, Ovine, Porcine, and Porcine W4R/R5K Leptins

	Amino acid (mol/mol of leptin)[a]					
Amino acid	Bovine	Chicken	Human	Ovine	Porcine	Porcine W4R/R5K
Asp + Asn	12.88 (13)	11.83 (14)	12.79 (14)	12.33 (13)	11.43 (12)	11.65 (12)
Thr	8.38 (9)	7.44 (10)	9.01 (11)	7.11 (8)	8.07 (9)	8.17 (9)
Ser	14.76 (18)	11.94 (17)	12.45 (17)	15.24 (18)	15.14 (18)	14.62 (18)
Glu + Gln	14.30 (16)	15.24 (19)	12.74 (15)	14.91 (16)	14.78 (16)	14.71 (16)
Pro	7.34 (7)	6.43 (7)	5.69 (6)	6.49 (7)	6.71 (7)	7.21 (7)
Gly	4.99 (5)	5.34 (5)	7.42 (8)	5.27 (5)	6.09 (6)	5.87 (6)
Ala	7.00 (7)	7.00 (7)	5.00 (5)	8.00 (8)	8.00 (8)	8.00 (8)
Val	7.24 (12)	7.22 (10)	7.90 (11)	7.82 (11)	7.53 (10)	8.00 (10)
Ile	8.87 (9)	8.14 (10)	7.69 (10)	8.60 (10)	8.66 (10)	8.91 (10)
Leu	25.43 (27)	19.21 (25)	18.20 (23)	23.08 (27)	22.83 (26)	22.58 (26)
Tyr	1.56 (2)	1.77 (2)	1.63 (2)	1.74 (2)	1.77 (2)	1.75 (2)
Phe	1.12 (1)	2.76 (3)	1.98 (2)	1.11 (1)	1.09 (1)	1.02 (1)
His	3.21 (3)	2.94 (3)	3.84 (4)	3.00 (3)	2.81 (3)	2.99 (3)
Lys	5.38 (6)	5.28 (6)	5.69 (7)	6.24 (6)	5.21 (5)	6.25 (6)
Arg	9.11 (8)	3.21 (4)	3.04 (4)	6.24 (8)	5.96 (8)	6.46 (8)

[a] Cys, Met, and Trp were not included in the table due to their partial or full destruction in acid hydrolysis. The predicted values of amino acid composition were obtained from the respective GenBank Accession Nos. U84273, AF012727, NM000230, U84247, and U59894 for bovine, chicken, human, ovine, and porcine leptins. All recombinant leptins prepared in our laboratory have an additional Ala residue.

leptin and porcine leptin analogue W4R/R5K (Figs. 1A and 1C). Those dimers likely result from improper formation of disulfide bonds, since they disappear in SDS–PAGE performed in the presence of reducing agent (Fig. 2). Small amounts of oligomers were found in porcine leptin analogue W4R/R5K (Fig. 1C). As they were not seen in SDS–PAGE, we conclude that they were formed by a noncovalent interaction. MALDI-TOF analysis confirmed, in general, the data obtained by other methods and indicated that 6–12% of the recombinant molecules represent Met-Ala-leptin. In order to validate proper refolding, the secondary structure of the three leptins was studied by CD spectra. According to Protein Data Bank data for human leptin (PDB code 1AX8), which were deduced from X-ray data with 2.40-Å resolution, this protein contains 61.0%

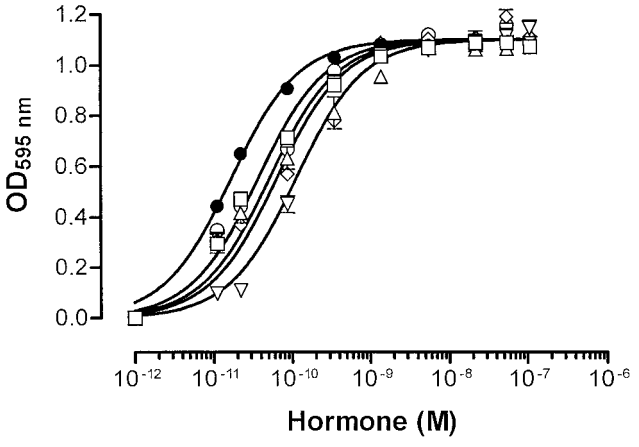

FIG. 4. Biological activity of bovine leptin (△), porcine leptin (◇), porcine leptin analogue W4R/R5K (○), ovine leptin (□), chicken leptin (▽), and human leptin (●) in BAF/3 cells transfected with the long form of human leptin receptor. Synchronized cells were grown for 48 h in the presence of various concentrations of the various leptins and the number of cells was determined subsequently by the MTT method (see text).

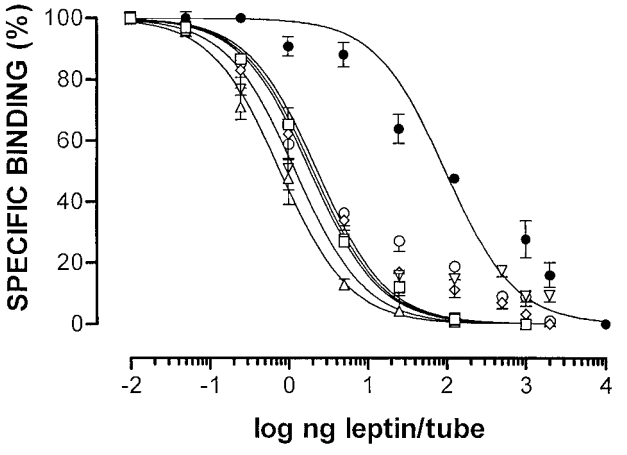

FIG. 5. Cross-immunoreactivity of bovine leptin (△), porcine leptin (◇), porcine leptin analogue W4R/R5K (○), ovine leptin (□), chicken leptin (▽), and human leptin (●).The cross-reactivity of various recombinant leptins with ovine leptin was determined by radioimmunoassay using anti-ovine leptin serum raised in rabbits. Radiolabeled ovine leptin served as a ligand and all other nonlabeled leptins as competitors.

α-helix (54% α-helix plus 7% 3_{10}-helix), 11.0% β-turns, and no β-strands. Our data on the secondary structure of leptins closely correlated with our previous unpublished data for ovine, chicken, and chicken C4S leptins (E. E. Gussakovsky, N. Raver, and A. Gertler, unpublished data) and the PDB data for human leptin. The overall helix content reported in the PDB data consists of 61% helix, which is the sum of α-helix and 3_{10}-helix. The CD method averages three or four turns per helical segment and hence cannot distinguish between the two types of helices (18). The difference in the CD-measured α-helix content could result from both low resolution of the X-ray data and errors in the CD spectra and protein concentration determination. In addition, the CD-based method of the secondary structure determination allows itself an error for any structure content of about $\pm$5% (18). So at present, there is no evidence that the secondary structures of leptins studied in the present work are significantly different. Even if there are such differences, their extent may not exceed about 10–15% of α-helix and about 9% of β-turns. Definitely, all leptins do not contain β-strands. These data, along with the study of biological activity (see below), prompt us to suggest that the bovine, porcine, and porcine analogue W4R/R5K leptins are properly folded.

All three purified fractions were capable of activating the growth of BAF/3 cells transfected with the long form of human leptin receptor. Their activity was comparable to that of ovine leptin but 2- to 4-fold lower than that of human leptin. It should be noted that in our former paper (9) we reported that the activity of human and ovine leptins is equal. The present work utilized the same cell line but used the MTT method instead of thymidine incorporation. It is therefore not clear whether the 2-fold difference can be attributed to this fact or more likely results from different batches of human leptin used in two experiments. In any case, the differences in the activity are relatively small, indicating that ruminant or porcine leptins bind and activate human leptin receptors with affinity equal to or slightly less than human leptin. In contrast, the activity of chicken leptin was considerably lower, confirming the former results (10). It should be also noted that the biological activity of chicken and ovine leptins was previously demonstrated in *in vivo* experiments in homologous species (10,26). Full mutational analysis of leptin has not yet been done, but the present results concerning the porcine W4R/R5K leptin analogue as well as the C4S analogue of chicken leptin (27) hint that the residues located at the N-terminus play little role in binding to the receptors. As the small differences in the activity could not be related to Met-Ala-leptin content, it seems that the additional amino acid at the N-terminus does not affect the biological activity, confirming this suggestion.

The cross-immunoreactivity of recombinant leptins prepared in this and former studies (9,10) was compared using antisera raised against recombinant ovine leptin in rabbits or goats. Whereas the antisera raised in goats exhibited rather high species specificity, the sera raised in rabbits recognized with almost equal potency the ovine, bovine, porcine, porcine W4R/R4K, and chicken leptins. The only exception was human leptin. As no epitope mapping of human or other leptins has been done to date, no valid explanation for this difference can be proposed. One major structural difference that characterizes human leptin is the WAS (amino acids 101–103) epitope (7), which is different in other leptins and lacks the Trp in position 101. Whether this difference may account for low cross-reactivity of human versus other leptins remains, however, to be clarified.

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