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Biochemical and *in vitro* biological significance of natural sequence variation in the ovine *leptin* gene ☆

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ABSTRACT

The hormone leptin is involved in diverse biological processes, including regulation of food intake, body-weight homeostasis and energy balance. Sequence variation in the bovine *leptin* gene has been found to be associated with variations in carcass fat content and average daily gain, as well as in milk yield, milk somatic cell count and several traits governing reproduction. We sequenced genomic DNA and cDNA samples of individuals from three divergent sheep breeds and revealed synonymous as well as novel non-synonymous allelic variation at the third exon of the ovine *leptin* gene (*oLEP*) as compared to the sequence published at Accession No. U84247 (reference sequence). In addition, two alternatively spliced *oLEP* transcripts were found in the abdominal fat tissue. The biochemical and the *in vitro* biological significance of the sequence variation in the *oLEP* was examined by generating recombinant oLEP-protein variants namely: p.Q28del, p.N78S, p.R84Q, p.P99Q, p.V123L and p.R138Q, carrying the corresponding sequence variation. Surface plasmon resonance experiments revealed, in most cases, reduced affinity of the oLEP protein variants examined, to human leptin-binding domain (hLBD), relative to the reference variant, being 0.75, 0.60, 0.60, 0.89, 0.92 and 1.03, respectively. In competitive binding assays between biotinylated oLEP and the recombinant leptin protein variants, p.N78S and p.R84Q variants exhibited the lowest affinity to hLBD (0.18 and 0.41, respectively) as compared to the reference hormone. We then tested the protein variants' ability to induce proliferation in Baf-3 cells stably expressing the long form of the human leptin receptor: significant differences in proliferative activity were only found for p.N78S (1.8-fold higher) and p.R138Q (4.2-fold lower) relative to the reference oLEP variant.

1. Introduction

Leptin, a 16-kDa cytokine-like hormone that is synthesized and secreted mainly by white adipocytes, was originally identified as a factor regulating body-weight homeostasis and energy balance [36]. It later became evident that leptin is also secreted by other tissues [16], and plays an important role in various biological processes, such as lipolysis, angiogenesis, hematopoiesis, reproductive activity, fetal growth, hypothalamic–pituitary–adrenal axis function and immune system function [1,14]. In farm animals, leptin has been found to be involved in regulating various economically important productive and reproductive traits [9,31].

The *leptin* gene contains three exons and is conserved across species [7,35]. The mature protein contains 146 aa residues which are translated mainly from the third exon. In humans and mice, alternative splicing leads to the formation of a leptin variant that is shorter by one glutamine residue in comparison to the common hormone variant [12,36].

Sequence variation in the *leptin* gene may alter the expression of traits regulated by the hormone. Indeed, association studies in humans show that carrying sequence alternations in different regions of the gene is associated with various illnesses, mainly excess weight and obesity [31]. Site-directed mutagenesis experiments using recombinant protein variants suggested that impaired receptor-binding activity and altered signal transduction might be the biochemical consequences underlying the deleterious phenotypes [24,32].

Studies on human *leptin* have explored mainly the association between sequence variation in the gene and the expression of metabolic illnesses. Studies on wild and domestic animals have revealed an association between allelic variation at the *leptin* gene and traits of adaptive importance. For example, the association between *leptin* sequence variation and adaptation to extreme environmental cold stress was investigated in pikas (*Ochotona curzoniae*) [34]. Studying bovine *leptin* gene sequence variation

☆ Sequence data from this article regarding ovine *leptin* have been deposited in the DDBJ/EMBL GenBank Data Library under GenBank Accession Nos. FR688115, FR688116, FR688117 and FR688118.

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revealed four non-synonymous sequence variants within its coding region: p.R4C, p.A59V, p.Q62R and p.N78S [20,31]. Two of these sequence alternations were associated with variation in carcass fat content, fat deposition rate, body fat reserves and lean meat yield, as well as with several traits governing reproduction [13]. Association between sequence variation in the *leptin* gene and manifestation of production traits was also found in swine [31,33].

While sequence variation at the *leptin* gene has been thoroughly investigated in bovine, limited information is available on variation in ovine *leptin* (*oLEP*) [3,37]. The aims of this study were to further explore genetic variation at *oLEP* and by generating recombinant protein variants, to investigate the biochemical and the *in vitro* biological consequences of oLEP protein diversity.

2. Materials and methods

2.1. Materials

Plasmids of oLEP carrying the reference *oLEP* (sequence Accession No. U84247), human leptin-binding domain (hLBD) and chicken leptin-binding domain (chLBD) were obtained from Protein Laboratories Rehovot Ltd. (Rehovot, Israel). Baf-3 cells stably expressing the long form of hLBD were obtained from Dr. R. Devos (Roche, Gent, Belgium). Restriction enzymes used in the molecular biology experiments were from Fermentas (Vilnius, Lithuania). High-purity DNA primers were ordered from Syntezza Bioscience Ltd. (Jerusalem, Israel). RPMI-1640 medium and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue, MTT) were purchased from Sigma (St. Louis, MO, USA); Superdex™ 75 HR 10/30 column and Q-Sepharose were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Antibiotic–antimycotic solution (5×104 U/mL penicillin, 50 mg/mL streptomycin, 0.125 mg/mL fungisone), NaCl, Tris-base and fetal calf serum (FCS) were purchased from Bio-Lab Ltd. (Jerusalem, Israel). Bacto-tryptone, Bacto-yeast extract, glycerol, EDTA, HCl, Triton X-100 and urea were from ENCO Diagnostics Ltd. (Petah-Tikva, Israel). Molecular markers for SDS–PAGE were purchased from Bio-Rad (Hercules, CA). A research-grade CM5 sensor chip, NHS (*N*-hydroxysuccinimide), EDC [*N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride], ethanolamine/HCl and HBS-EP running buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA and 0.005% v/v surfactant P20 at pH 7.4) were purchased from Biacore AB (Uppsala, Sweden). All other materials were of analytical grade.

2.2. Animals

Experimental protocols were approved by the Volcani Center's Animal Care Committee. Sequence variation in the *oLEP* gene was investigated in Assaf sheep – a synthetic dairy breed developed from a cross between the Middle Eastern Improved Awassi (IA) fat-tail dairy breed and the European East Friesian dairy breed. In addition, genotyping for the *oLEP* gene was conducted for several sheep belonging to the IA dairy breed and the Dorper mutton breed. The Dorper, known for its hardiness and favorable carcass composition, is a synthetic breed developed in South Africa from a cross between the Dorset Horn and the Persian breeds.

2.3. Genomic DNA and RNA extraction

Genomic DNA was extracted from blood samples using DNeasy Blood & Tissue Kit (Qiagen, USA) according to the manufacturer's instructions. For RNA extraction, samples from abdominal fat tissue were collected immediately after slaughter and kept in liquid nitrogen. Later, the samples were kept at −80 °C until RNA extraction. Total RNA was extracted from those samples utilizing TRI-reagent (Sigma–Aldrich, Israel) according to the manufacturer's instructions. For cDNA preparation, total RNA (1 μg) was reverse-transcribed in a final volume of 20 μL containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM deoxy-NTP (dNTP), 0.5 μg oligo-dT/hexamer primers (Promega, Israel), and 1 U avian myeloblastosis virus reverse transcriptase (Promega, Israel). The reverse-transcription reaction temperatures were 42 °C for 1 h and 95 °C for 10 min.

2.4. Screening for sequence variation at the oLEP gene

To search for alternations within the region that codes for the mature leptin protein, fragments covering the exon 3 region of the *oLEP* gene were amplified from genomic DNA or from cDNA samples obtained from different individuals. The amplicons were then sequenced and sequence variations were detected following comparison of the sequencing results to the *oLEP* reference sequence available at GenBank (Accession No. U84247).

PCR was carried out in a Thermal Cycler 2720 (Applied Biosystems, Foster City, CA, USA) in a final volume of 40 μL containing genomic DNA or cDNA template (0.5–1.0 μg/reaction), 8 pmol of each primer, 2 U of Taq DNA polymerase (Fermentas, Burlington, Canada), 1× Taq Buffer with $(NH4)_2SO_4$ (Fermentas), final concentration of 0.2 mM of each dNTP, 2 mM $MgCl_2$ and 200 ng BSA. PCR conditions were: initial denaturation step at 94 °C for 3 min, 35 cycles of denaturation at 92 °C for 30 s, annealing at 64.5 °C for 30 s and extension at 72 °C for 60 s, and a final extension step at 72 °C for 10 min.

Amplification of *oLEP* exon 3 from genomic DNA or cDNA yielded fragments of 461 or 441 bp, respectively. Primers were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), based on the *oLEP* sequence available at GenBank (Accession No. U84247). Primers for genomic DNA amplification were: sense 5′-CTTGATGTCCCCTTCCTCCT-3′ and antisense 5′-CAGCCAGAAGCTCAGGTTTC-3′. Primers for cDNA amplification were: sense 5′-CCATGGCAGTGCCCATCCGCAAG-3′ and antisense 5′-AAGCTTCAGCACCCGGGACTGAGGTC-3′.

Following amplification, PCR products were size-separated by electrophoresis on 1.2% agarose gels containing 200 ng/mL ethidium bromide, using 1× TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM Na_2EDTA). These separated PCR products were then extracted from the gel using QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and were sent for direct sequencing.

2.5. DNA sequencing and sequence analysis

PCR products were sequenced using the ABI BigDye Terminator Sequencing Kit (Applied Biosystems) in an ABI 3730 at the Center for Genomic Technologies facility, the Hebrew University of Jerusalem, Israel. Sequence alignments, translations and comparisons were carried out using bioinformatics software (Gap.4) [30].

2.6. Preparation of oLEP protein variants

To prepare leptin protein variants, the pMon3401 expression plasmid encoding the reference oLEP [5,24] was used as the starting material. The *leptin* DNA inserts were modified with the QuickChange mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions, using two complementary primers (Table 1). The primers were designed to contain base changes yielding the respective sequence variants and to modify specific restriction sites for colony screening. The procedure included 18 PCR cycles with *Pfu* polymerase. To digest the template and select for sequence variants containing synthesized DNA, the alternated construct was then digested with *Dpn*I restriction enzyme. The

Table 1
Primers used for preparation of ovine leptin protein variants.

Protein variant	Sense and antisense primer sequence[a]	Modified restriction site[b]
p.Q28del	S 5′-GACATCTCACACACG**—**TCCGT**GAGCTC**CAAACAGAGGGTC-3′ A 5′-GACCCTCTGTTTGGAGCTCACGGACGTGTGTGAGATGTC-3′	*Sac*I
p.N78S	S 5′-AATGTGATCC**AGATCT**CT**TC**TGACCTGGAGAACCTCCGGG-3′ A 5′-CCCGGAGGTTCTCCAGGTCAGAAGAGATCTGGATCACATT-3′	*Bgl*II
p.R84Q	S 5′-CTAATGAC**CTCGAG**AACCTCC**A**GGACCTTCTCCAC-3′ A 5′-GTGGAGAAGGTCCTGGAGGTTCTCGAGGTCATTAG-3′	*Xho*I
p.P99Q	S 5′-GCCTCCAAGAGCTGCCCC**CTGCAG**CAGGTCAGGGCC-3′ A 5′-GGCCCTGACCTGCTGCAGGGGGCAGCTCTTGGAGGC-3′	*Pst*I
p.V123L	S 5′-GAGAGCTTGGAGAGC**TTAGGCCTG**GTCCTGGAAGCCTCCCTC-3′ A 5′-GAGGGAGGCTTCCAGGACCAGGCCTAAGCTCTCCAAGCTCTC-3′	*Stu*I
p.R138Q	S 5′-CCGAGGTGGTGGCCCTGAGC**CAGCTG**CAGGGGTCTCTACAG-3′ A 5′-CTGTAGAGACCCCTGCAGCTGGCTCAGGGCCACCACCTCGG-3′	*Pvu*II

[a] S, sense primer; A, antisense primer; All sequence varients in comparison to the reference sequence (GenBank Accession No. U84247) are in bold letters.
[b] Successful mutagenesis was monitored by appearance of the respective restriction site (underlined).

plasmids were transfected into Mon-105 expression-competent cells. Competent cells were grown in 5–10 mL Luria–Bertani broth (LB) medium and the plasmids were isolated. Six colonies of each sequence variant were screened for the desired sequence alternation, using the specifically designed restriction site (Table 1). Two colonies of each variant were sequenced and confirmed to contain the desired sequence alternation with no change in other nucleotides.

2.7. Expression, refolding and purification of oLEP protein variants

Preliminary experiments were performed for all seven clones (reference, p.Q28del, p.N78S, p.R84Q, p.P99Q, p.V123L and p.R138Q) in a 250-mL flask containing 50 mL Terrific broth (TB) medium (1.2% w/v Bacto-tryptone, 2.4% w/v Bacto-yeast extract, 0.4% v/v glycerol in ddH_2O) and salts (0.023% w/v KH_2PO_4, 0.125% w/v K_2HPO_4) cultured at 37 °C. After the bacterial culture reached an A_{600} of 0.9–1.1, nalidixic acid was added to a final concentration of 0.1 mM. The cells were harvested 3 to 4 h later and assayed for oLEP expression by SDS–PAGE. One of the colonies expressing the protein corresponding to each of the sequence variant was selected for large-scale preparation in 2 × 500 mL TB medium in 2.5-L flasks incubated at 200 rpm, 37 °C, for 4.5 h post-nalidixic acid addition. The bacterial culture was harvested by 5-min centrifugation at 10,000*g*, and frozen at −20 °C. The pellet was then suspended in 4 °C ddH_2O and sonicated on ice for 4 min, using the 50%-cycle program (Sonicator model: W-375; Heat systems – Ultrasonics, Inc.). The suspension was then sonicated on ice for 4 min using the 50%-cycle program and the inclusion bodies were precipitated by centrifugation at 12,000*g* for 15 min (4 °C). The pellet was then suspended in cold (4 °C) ultra-pure endotoxin-free water, sonicated and centrifuged as already described. This procedure was repeated twice. The pellet was then suspended in 1% (w/v) Triton X-100, sonicated and centrifuged. The last five or six washes were carried out with cold (4 °C) ultra-pure water; sonication and centrifugation were applied as described.

Half of the bacterial pellet obtained from 1-L fermentation was solubilized in 45 mL of 4.5 M urea and 40 mM Tris base containing 1 mM cysteine. The pH of the solution was adjusted to 11.3 with NaOH. After 2 h of stirring at 4 °C, three volumes of cold 0.67 M arginine were added to a final concentration of 0.5 M and stirred for an additional 2 h. Then, the solution was dialyzed against 2 L of 10 mM Tris–HCl, pH 10, for 60 h, with five or six external solution exchanges. The resultant solution was then loaded on a 20-mL Q-Sepharose fast-flow anion-exchange resin pre-equilibrated with 10 mM Tris–HCl, pH 10, at 4 °C on a 2.6-cm diameter column. Elution was carried out using increasing NaCl concentrations in the same buffer (pH 10), and 15-mL fractions were collected. Protein concentration was determined by absorbance at 280 nm and monomer content by gel-filtration chromatography on a Superdex 75 HR 10/30 column. Fractions containing the monomeric protein were pooled, dialyzed at 4 °C against $NaHCO_3$ (pH 10) in a 4:1 (w/w) protein-to-salt ratio and lyophilized.

2.8. Determination of purity and monomer content

SDS–PAGE was performed in a 15% (w/v) polyacrylamide gel (5.7 mL ddH_2O, 12.5 mL 30% acrylamide mix, 6.3 mL 1.5 M Tris pH 8.8, 0.25 mL 10% SDS, 0.25 mL 10% ammonium persulfate and 0.01 mL TEMED) under reducing conditions. Running buffer (TGS) contained 25 mM Tris (pH 8.3), 192 mM glycine and 0.1% (w/v) SDS. The gel was stained with Coomassie Brilliant Blue R. Gel-filtration chromatography was performed on a Superdex™ 75 HR 10/30 column with 0.2 mL aliquots of the Q-Sepharose-column-eluted fraction using TN buffer (25 mM Tris–HCl and 150 mM NaCl, pH 10). Reverse-phase chromatography was carried out on a Symmetry 300 C4 4.6/250 column connected to an HPLC using the following gradient: 5–30% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetate (TFA) (5 min), 30–60% acetonitrile with 0.1% TFA (30 min), and re-equilibration with 5% acetonitrile and 0.1% TFA (5 min).

2.9. Detection of oLEP complexes with chLBD by gel filtration

To characterize the binding stoichiometries between oLEP protein variants and chLBD, the respective ligands and chLBD were mixed in different molar ratios, incubated for 20 min at room temperature (25 °C) and then separated by gel filtration using an analytical Superdex 75 column, as described previously [26].

2.10. Kinetics measurements of oLEP-hLBD and oLEP-chLBD interactions

The kinetics and equilibrium constants for the interactions between hLBD or chLBD and the oLEP protein variants were determined by surface plasmon resonance (SPR) methodology using a Biacore 3000 instrument (Neuchatel, Switzerland) at 25 °C. Protein variants were immobilized in a flow cell on a research-grade CM5 sensor chip using amine-coupling chemistry [4]. Immobilization steps and experimental procedure are described elsewhere [25]. For the binding studies, the hLBD or chLBD was dissolved in HBS-EP buffer and passed at different concentrations (0, 0.4, 0.8, 1.9, 3.9, 7.8 and 15.6 nM) through flow cells carrying the protein

variants 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, obtained by the supplier). The resultant binding curves were fitted to the association and dissociation phases at all hLBD or chLBD concentrations simultaneously, using Biacore evaluation software.

2.11. Competition assays

Affinities of oLEP protein variants to hLBD or chLBD relative to the affinity of the reference oLEP were determined by competition assays, where biotinylated reference oLEP and the other protein variants compete for binding to hLBD or chLBD. Polystyrene 96-well microtiter plates were coated overnight at 4 °C with 100 μL of 40 pM hLBD or chLBD 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 (25 °C). Wells were washed once again with PBST and incubated with different concentrations of unlabeled ovine leptins (50 μL/well) for 30 min, and then 50 μL of 62.5 pM biotinylated ovine leptin were added to each well for another 2 h. Then the wells were 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 – Bio-Tek Instrument Inc. (Winooski, VT) using TMB according to the manufacturer's instructions.

2.12. In vitro biological activity in Baf-3 bioassay

The proliferation rate of leptin-sensitive Baf-3 cells stably expressing the long form of human leptin receptor was used to estimate oLEP protein variants activities. Cell maintenance and experimental protocol were as described previously [24]. Experiments were performed in 96-well plates in which 1.5×10^5 cells/well were seeded. Increasing concentrations of reference oLEP or oLEP protein variants (0–5 ng/10 μL) were added to each well containing 100 μL culture medium. Cell proliferation was determined by MTT assay method as described previously [8], 48 h after hormone addition. Each concentration for each protein variant was tested in triplicate. The growth curves for each experiment were drawn using the Prism (4.0) nonlinear regression sigmoidal dose–response curve [23] and the EC_{50} values were calculated. Five cell-proliferation experiments were conducted.

2.13. Statistical analysis

The relative affinity of the different protein variants to hLBD and chLBD, as compared to the reference oLEP's affinity, was analyzed using the General Linear Models procedure of the Jump In® computer package [27]. The model included the effects of experimental system (SPR, competition assay), receptor type (hLBD, chLBD) and protein variant type ($n = 6$). The relative biological activity of the different protein variants as compared to the biological activity of the reference oLEP was analyzed in a similar way. The model included the effects of experiment No. and protein variant type. All data are expressed as least-square means ± SEM. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Sequence variation at oLEP

3.1.1. Gene sequence variation

Sequencing the third exon of the *oLEP* gene from genomic DNA and cDNA of 79 Assaf sheep (Table 2) revealed several sites with allelic variation. The most frequent mutation (as compared to GenBank Accession No. U84247) was c.367G > T (GenBank Accession No. FR688115), which was found at a frequency of 0.24. This non-synonymous sequence alternation leads to substitution of valine (V) with leucine (L) at position 123 of the mature protein. Distribution of the genotype at position 367 was 47, 27 and 5 individuals, belonging to the GG, GT and TT genotypes, respectively. The c.367G > T variation was also detected in IA sheep at an estimated frequency of 0.21, where out of 12 animals genotyped, 3 were heterozygous GT, and 1 was homozygous TT.

Two Assaf sheep that did not carry the c.367G > T sequence variation, were homozygous for a rare haplotype that differs from the GenBank U84247 sequence by five substitutions: c.225A > G, c.228A > C, c.232A > T, c.233A > C and c.312G > A (GenBank Accession No. FR688116). Carrying together the c.232A > T and c.233A > C sequence alternations leads to an asparagine (N) to serine (S) substitution (p.N78S) in the oLEP molecule.

Screening IA sheep revealed, aside from the c.367G > T sequence alternation, an additional non-synonymous sequence variant c.413G > A – leading to an arginine (R) to glutamine (Q) substitution (p.R138Q) in the oLEP molecule (GenBank Accession No. FR688118). No sequence alterations at the *oLEP* gene were found in Dorper sheep. The positions of all known ovine sequence variations on the globular structure of the leptin protein are presented in Fig. 1.

3.1.2. Alternative splicing

An alternatively spliced *oLEP* variant (GenBank Accession No. FR688117) r.[=,81_83del] carrying a deletion of three nucleotides (CAG) leading to a p.Q28del in the mature protein was observed in all Assaf, IA and Dorper cDNA sequencing results (Fig. 2).

3.2. Expression, purification and characterization of oLEP protein variants

Recombinant proteins carrying the reference sequence, the sequence variants found in the present study (p.Q28del, p.N78S,

Table 2
Distribution of ovine *leptin* sequence variation in the third exon in Assaf, Improved Awassi and Dorper breeds.

Nucleotide substitution[a]				c. 225A > G	c.228A > C	c.232A > T	c.233A > C	c.312G > A	c.367G > T		c.413G > A	
Expected aa change						p.N78S			p.V123L		p.R138Q	
Breed	Sequenced material	Genotyped (n)	Carrying reference sequence (n)	Genotype and number of individuals carrying mutated allele(s) at specific sites								
				GG	CC	TT	CC	AA	GT	TT	GA	AA
Assaf	DNA	51	28						19	4		
Assaf	cDNA	28	19	2					8	1		
Improved Awassi	cDNA	12	6						3	1	1	1
Dorper	cDNA	7	7									

[a] According to GenBank Accession No. U84247.

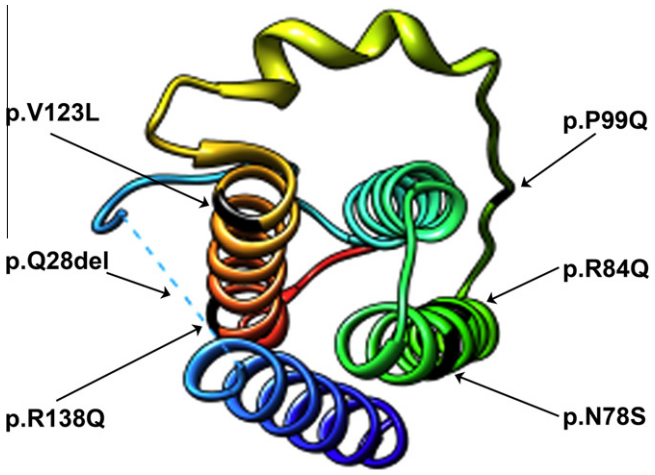

Fig. 1. All known ovine leptin protein variants are shown on the mature protein's structure (PDB ID: 1AX8).

p.V123L and p.R138Q), and the sequence variants p.R84Q and p.P99Q [37] were purified by anion-exchange chromatography. Fractions containing monomers that eluted in 50 mM NaCl were pooled, dialyzed in the presence of $NaHCO_3$ to ensure a 4:1 protein-to-salt ratio and lyophilized. The yields varied from 70 to 130 mg from 0.5 L of bacterial culture. The purity and homogeneity of all proteins were documented by SDS–PAGE. Only one band of ~16 kDa was obtained for all oLEPs under both reducing and non-reducing conditions (not shown).

As expected, in the absence of reducing agent, the protein exists in its globular state (50–60% α-helix), which allows it slightly higher mobility as compared to denaturated state. Gel filtration at pH 8 under native conditions yielded a main monomeric peak consisting of at least 95% monomer and corresponding to a molecular mass of ~16 kDa (Fig. 3, second column from the left). Reverse-phase chromatography also yielded a single peak (not shown).

3.3. Detection of oLEP complexes with chLBD by gel filtration

All recombinant oLEP proteins that were produced in this study formed 1:1 M ratio complexes with chLBD (Fig. 3, middle column), as expected [17]. This stoichiometry was evidenced by the appearance of a single main peak for the complex with shorter retention time (12.79–12.86 min), as compared to the higher retention times of chLBD (14.15–14.21 min) and the leptins (15.38–15.59 min). The highest main peak appeared when the components were mixed at a 1:1 M ratio, whereas an additional peak appeared when there was excess leptin or chLBD (Fig. 3, second and first columns from the right, respectively).

3.4. Kinetics measurements of oLEP-hLBD and oLEP-chLBD interactions

To characterize the binding capacities of the reference oLEP and the other protein variants to hLBD and chLBD, we used the SPR technique as well as competition assays of the ligands with biotinylated oLEP. Comparison of the variants' binding affinities gave similar results in the two experimental systems ($P = 0.53$), namely, all oLEP protein variants manifesting similar or reduced binding affinity to hLBD and chLBD, relative to the reference oLEP. Here, the results of the SPR and the competition assays are presented only for hLBD, since those obtained using chLBD as a receptor source were similar.

The thermodynamic kinetics association constant (k_{on}), the kinetics dissociation constant (k_{off}) and the dissociation constant (KD) values for all oLEP protein variants, as obtained in the SPR system following comparison to the 1:1 theoretical binding model [17], are presented in Table 3. The IC_{50} values obtained in the competition experiments are presented in Table 4.

Significant correlation ($P < 0.05$) was found between the rank of the different protein variants in their binding capacities to hLBD, in the SPR and the competition assays. Taking together, the protein variants p.Q28del, p.P99Q, p.V123 and p.R138Q had higher relative affinities, while the p.N78S and the p.R84Q variants had significantly lower ($P < 0.05$) relative affinity as compared to the affinity of the reference oLEP.

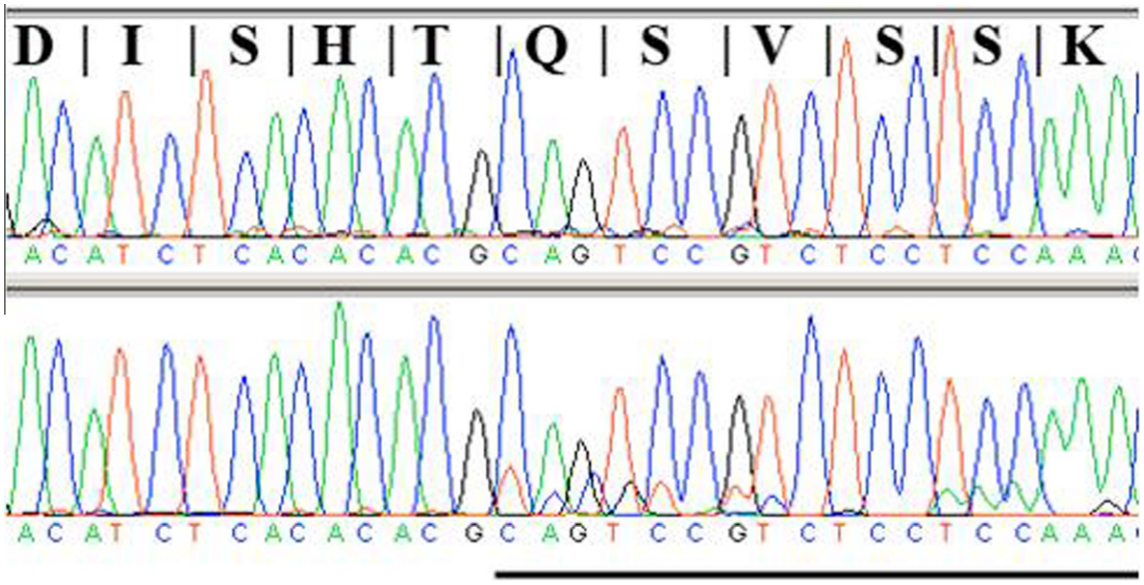

Fig. 2. Top trace: Profile of genomic DNA showing the consensus sequence of the ovine *leptin* gene (*oLEP*); Bottom trace: Profile of cDNA obtained from abdominal fat tissue, showing the consensus and an additional alternative spliced variant (underlined), that carry a deletion of amino acid Q at position 28.

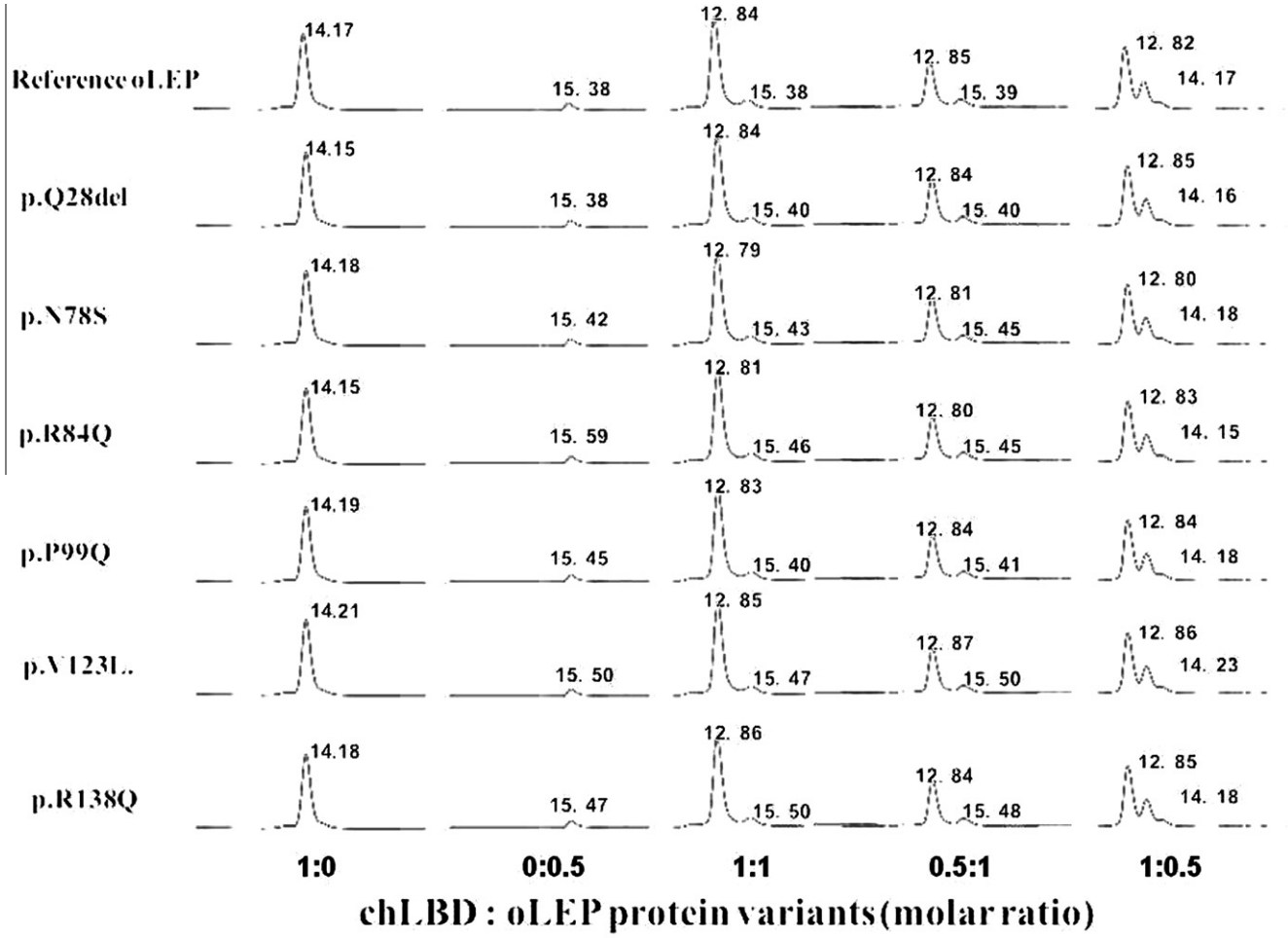

Fig. 3. Gel-filtration analysis of complexes between chicken leptin-binding domain (chLBD) and reference ovine leptin (oLEP) and the protein variants p.Q28del, p.N78S, p.R84Q, p.P99Q, p.V123L and p.R138Q on a Superdex 75 HR 10/30 column. Complex formation was achieved by 20-min incubation at room temperature in TN buffer using various molar ratios; 200-μL aliquots of the mixture were applied to the column, pre-equilibrated with the same buffer. The numbers above each peak indicate the retention time (RT) in minutes. The final concentrations (5 or 10 μM) of the respective hormones and 5 or 10 μM chLBD in a complex were used. When the separate proteins were applied, the final concentrations of the chLBD and oLEPs were, respectively, 10 and 5 μM. The column was developed at 0.8 mL/min. The ordinate axis reflects the concentration and the abscissa, the time course. The chLBD used for the complexes was over 95% monomeric (see left column).

3.5. Baf-3 bioassay

A bioassay measuring proliferative activity induced by the oLEP variants of a cell line stably expressing the long form of human leptin receptor (Baf-3) was employed. EC_{50} values (Fig. 4) obtained for the variants p.Q28del, p.P99Q and p.V123L were similar to those obtained for the reference oLEP. EC_{50} values for the p.N78S variant were significantly ($P < 0.05$) lower than those of the reference protein, indicating relatively high proliferative activity. The p.R84Q and the p.R138Q variants exhibited lower proliferative activity than the reference oLEP, but the difference was significant ($P < 0.05$) only for the p.R138Q variant. Table 5 summarizes the relative binding affinity (IC_{50}) and relative biological activity (EC_{50}) values of all leptin variants compared to the reference oLEP.

4. Discussion

4.1. Sequence variation at the oLEP gene

Leptin is secreted mainly by adipocytes and regulates appetite, energy partitioning, body composition and immune system function [1]. Interest in leptin in domestic animals emerged when it was found that allelic variation at the *leptin* gene is associated with variations in traits of economic importance, such as growth rate, milk yield and carcass content [3,29,31]. However, it remains unclear how sequence variation at the *leptin* gene affect hormone bioactivity, which in turn leads to the phenotypic variation. We addressed this question by studying the biochemical and the *in vitro* biological properties of oLEP recombinant proteins that carry natural occurring *oLEP* sequence variant and *oLEP* splice variant. Our results showed that relative to the reference hormone, recombinant oLEP protein variants exhibit decreased affinity toward the leptin receptor, as measured in heterologous systems.

By sequencing PCR products amplified from the third exon region of the *oLEP* gene, we found that the c.367G > T sequence variant segregates in the Assaf and IA fat-tail dairy breeds with estimated frequencies of 0.21 and 0.24, respectively (Table 2). The same sequence variant (designated $387^{G/T}$) has been identified in wool, meat and dual-purpose breeds in New Zealand [37]. The observation that the same mutation segregates in genetically remote sheep breeds suggests that it arose early in sheep domestication. The c.367G > T sequence alteration leads to a V-to-L substitution at position 123 of the mature protein, located within its binding region [6]. Although V and L are similar in their biochemical

Table 3
Calculation of kinetics and thermodynamic constants (mean ± SE) for the interaction of immobilized reference ovine leptin (oLEP) and the other oLEP protein variants with soluble human leptin-binding domain measured by surface plasmon resonance methodology (one or two experiments per protein variant were conducted).

Analogue	K*on* (mol/s) × 10^6	K*off* (s^{-1}) × 10^{-3}	KD (M × 10^{-9})	X^2	Relative affinity
oLEP reference	0.24 ± 0.0008	0.91 ± 0.010	3.79	0.7	1.00
oLEP p.Q28del	0.22 ± 0.0005	1.10 ± 0.007	5.04	0.3	0.75
oLEP p.N78S	0.30 ± 0.0041	1.91 ± 0.029	6.31	0.1	0.60
oLEP p.R84Q	0.18 ± 0.0008	1.15 ± 0.007	6.29	0.4	0.60
oLEP p.P99Q	0.22 ± 0.0014	0.94 ± 0.020	4.25	0.4	0.89
oLEP p.V123L	0.23 ± 0.0029	0.93 ± 0.022	4.10	0.1	0.92
oLEP p.R138Q	0.30 ± 0.0045	1.12 ± 0.038	3.68	0.2	1.03

properties, as both have non-polar, aliphatic R groups, carrying an a.a. substitution in the binding region may affect affinity of the hormone toward its receptor.

The two other non-synonymous sequence variants detected in the IA and Assaf sheep (Table 2), namely c.232A > T + c.233A > C and c.413G > A, are novel rare sequence variants that lead to p.N78S and p.R138Q a.a. substitutions, respectively. The p.N78S substitution located within the evolutionarily conserved C-helix region of the leptin protein involves the substitution of asparagine with serine, both of which have a polar uncharged R group. The p.N78S substitution has also been identified in bovine leptin [20]. Detailed analysis showed that the p.N78S substitution is analogous (similar but with different evolutionary origin) in bovine and

Table 5
Relative binding affinity (IC_{50}) and relative biological activity (EC_{50}) values of the ovine leptin (oLEP) protein variants relative to the reference oLEP (values given as percentage).

Analogue	Binding (affinity)		Biological activity
	SPR (Biacore)	Binding assay	Baf-3
oLEP reference	1.00	1.00	1.00
oLEP p.Q28del	0.75	0.67	0.98
oLEP p.N78S	0.60	0.18	1.83
oLEP p.R84Q	0.60	0.41	0.52
oLEP p.P99Q	0.89	0.93	0.88
oLEP p.V123L	0.92	0.73	1.03
oLEP p.R138Q	1.03	0.75	0.24

Table 4
The IC_{50} values (nM)[a] of all ovine leptin protein variants calculated from competitive-binding assays based on competition between biotinylated ovine leptin and the leptin protein variants on binding to human leptin-binding domain.

Analogue	Human Leptin-Binding Domain (hLBD)		
	IC_{50} (mean ± SE) (M × 10^{-9})	95% confidence intervals (M × 10^{-9})[b]	Relative to the reference oLEP
oLEP reference	4.16	2.83–5.99	1.0
oLEP p.Q28del	6.24	4.65–8.13	0.67
oLEP p.N78S	23.38	17.51–31.21	0.18
oLEP p.R84Q	10.0	6.77–14.94	0.41
oLEP p.P99Q	4.45	3.40–5.81	0.93
oLEP p.V123L	5.71	4.31–7.55	0.73
oLEP p.R138Q	5.56	3.96–7.80	0.75

[a] Each value is the mean of two experiments, each with three replicates ($n = 6$).
[b] Ninety-five percentage confidence intervals of the IC_{50} values were provided by Prism, 2003. GraphPad Prism™ version 4.0, GraphPad Software Inc., San Diego, CA.Prism (2003) software.

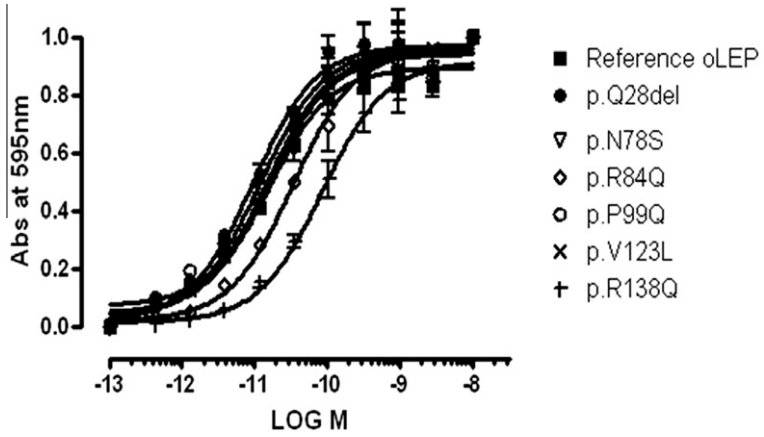

Fig. 4. Effect of reference ovine leptin (oLEP) and the oLEP protein variants p.Q28del, p.N78S, p.R84Q, p.P99Q, p.V123L and p.R138Q on proliferation of Baf-3 cells stably expressing the long form of human leptin receptor. Results of each experiment were normalized to the maximal response, and absorbance in wells not treated with oLEPs was taken as zero. Representative experiment (out of five performed for each analogue) is shown. EC_{50} values for the reference oLEP and for the protein variants: p.Q28del, p.N78S, p.R84Q, p.P99Q, p.V123L and p.R138Q were 18.14, 18.51, 9.91, 35.03, 20.67, 17.62 and 74.98 pM, respectively. In the figure, the results are presented as mean ± SEM, but the SEM values were in most cases too small to be visible on the graph. For more details, see text.

ovine: whereas in ovine, the substitution results from the cumulative effect of two mutations, c.232A > T + c.233A > C, in bovine, the p.N78S substitution results from the c.3157A > G substitution (GenBank Accession No. U50365). The analogous origin of the p.N78S mutation in the two species suggests its selective adaptive importance despite the biochemical similarity of N to S.

The p.R138Q substitution is located in the C-terminal region of the mature protein, within helix D. Though both amino acids are large and polar, arginine is positively charged while glutamine is uncharged. Interestingly, horse, mouse and rat leptins also have Q at position 138 (GenBank Accession Nos. NP_001157452, NP_032519 and NP_037208, respectively).

4.2. Post-translational modification and epigenetic gene regulation in oLEP

By sequencing cDNA-derived PCR products we observed that *oLEP* is subjected to alternative splicing, resulting in two leptin variants that differ in the presence or absence of a Q residue at position 28 of the mature hormone. Similar alternative splicing in leptin has been reported in humans and mice [12,36]. The absence of Q28 suggests only a small impact on the secondary structure of the protein and thus, whether the reference hormone and the spliced hormone differ in their function is not yet clear [19]. In addition, as the relative abundance of the alternative transcripts has not been determined in the present study, the physiological significance of the presence of two LEP variants in abdominal fat tissues has to be further investigated. However, it has been suggested that differential alternative splicing may affect organism phenotype under environmental constraints [15].

Imprinting, an epigenetic phenomenon in which maternal or paternal alleles are differentially expressed, can affect the manifestation of production traits in farm animals [2,28]. Thus, it was of interest to determine whether *oLEP* expression is imprinted. Sequencing the cDNA-originated amplicon revealed six heterozygous individuals (Table 2), suggesting that *oLEP* expression is not imprinted in the adipocytes of mature sheep. Whether the expression *oLEP* is imprinted in other tissues or at different developmental stages, however, warrants further investigation.

4.3. Biochemical and in vitro biological aspects of oLEP sequence variation

To verify the biochemical significance of sequence variation at the *oLEP* gene, we generated recombinant protein variants that carry the novel sequence variants, namely: p.Q28del, p.N78S, p.V123L and p.R138Q, as well as protein variants representing the p.R84Q (designated $105^{Arg/Gln}$) and the p.P99Q (designated $120^{Pro/Gln}$) mutations [37]. Following purification (Fig. 3), the specimens contained at least 95% monomeric protein, as evidenced by gel-filtration analyses (Fig. 3, middle column) and formed, as expected [17], 1:1 M ratio complexes with chLBD.

Affinity of the different protein variants relative to that of the reference hormone was tested by both SPR analysis and competition assay using hLBD and chLBD as binding proteins (results are presented only for hLBD, Tables 3 and 4). Interestingly, all of the protein variants, except for p.R138Q, manifested reduced affinity to hLBD as well as to chLBD, which varied from 0.6 to 0.9, compared to the affinity of the reference hormone.

Whereas all of the oLEP protein variants investigated here, except for p.R138Q, manifested reduced affinity to the receptor, they varied in their biological effect as measured by Baf-3 cell-proliferation assay. p.Q28del, p.P99Q and the p.V123L variants were similar to the reference oLEP in their proliferative activity in Baf-3 cells. On the other hand, p.N78S had relatively higher proliferative activity while the p.R84Q and the p.R138Q variants manifested reduced ability to induce cell proliferation (Fig. 4).

The p.N78S substitution, located on the surface of helix C, was the only alteration found to enhance the biological activity of the leptin, despite exhibiting lower affinity to both LBDs. Interestingly, these results are in agreement with those obtained for a similar protein variants of mouse leptin [22]. In that case, the mouse p.N78S variant was found to exhibit 4.4-fold lower affinity as detected by competitive binding assay to cytokine receptor homology domain 2 (CRH2) and yet, its biological activity was increased almost threefold compared to the reference leptin, as shown by rPAP1-luciferase reporter assay. CRH2 is the domain in leptin receptor responsible for binding leptin through the latter's site II. It consists of 210 amino acids and was subcloned, expressed and purified as a monomeric protein in our lab [18]. The mechanism enabling this phenomenon of higher biological activity along with lower binding ability, demonstrated in two independent experimental systems, is not yet clear. However it can be speculated that while affinity reflects the binding of leptin sites I and II [22], biological activity is dependent on the hormone's ability to form the hexameric complex, which involves interaction of leptin site III with the immunoglobulin-like domain (IGD) of the neighboring receptor [21]. Therefore, despite its lower affinity, the elevated biological activity of the p.N78S protein variant may originates from its increased affinity for hexamer formation.

Our findings of allelic sequence variation in the *oLEP* add to a growing list of sequence variants that have been identified for many candidate genes which control reproductive and productive traits, as well as health status in farm animals [10,11]. Regarding wild animals, adaptive evolution has been shown to have occurred in pika leptin in response to extreme cold environmental stress [34]. In farm animals, high production or reproduction ability, associated with increased food intake ability and efficient energy utilization are the selective forces. Investigation of the association between *oLEP* sequence variation in sheep and manifestation of production and reproduction traits is required to unveil the physiological relevance of oLEP sequence variation in farm animals.

In conclusion, our results extend our knowledge on natural sequence variation in *oLEP* with the identification of novel non-synonymous variants in the Assaf and IA breeds. Following the production of oLEP protein variants that represent products of all known *oLEP* gene sequence variations, we showed that most of these postulated products have reduced affinity toward the leptin receptor, and some have higher biological activity as compared to the reference hormone. It is hypothesized that under artificial selection for enhanced productivity, maintaining *oLEP* sequence variation may provide a selective advantage.

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