Polymorphism in the Ovine Leptin Gene (*oLEP*) Affects Leptin-Binding Affinity and Activity

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Introduction

The leptin gene contains three exons (Zhang *et al.* 1997). The mature hormone, which contains 146 aa residues, is translated mainly from the third exon. Leptin is synthesized and secreted mainly by white adipocytes and regulates body-weight homeostasis and energy balance. In farm animals, leptin has been found to be involved in regulating various economically important productive and reproductive traits (van der Lende *et al.* 2005).

A study of bovine leptin gene polymorphism revealed four non-synonymous mutations in the coding region of the gene: R4C, A59V, Q62R and N78S (van der Lende *et al.* 2005; Orru 2006). The R4C mutation was associated with increased milk yield and milk somatic cell count, increased carcass fat content, fat deposition rate and body fat reserves, as well as with higher leptin mRNA expression level and lower serum leptin during late pregnancy. The mutation A59V was found to be associated with elevated serum leptin levels during late pregnancy and increased average daily gain (van der Lende *et al.* 2005). It was also found to be associated with higher calving interval and number of days open, as well as with lower number of inseminations per conception (Komisarek and Antkowiak 2007). An association between polymorphisms in the leptin gene and manifested production traits has also been found in swine, as reviewed by van der Lende *et al*. (2005).

While polymorphism in the leptin gene has been thoroughly investigated in bovine and swine, limited information is available on polymorphism in the ovine leptin gene (*oLEP*) (Zhou *et al.* 2008). In the present communication, we describe the genetic variation in *oLEP*, and by generating recombinant muteins, show the biochemical and biological consequences of oLEP protein diversity.

Materials and methods

Animals. Polymorphism in the *oLEP* gene was investigated mainly in the Assaf breed - a synthetic dairy sheep breed developed from a cross between the Middle Eastern Improved Awassi (IA) fat tail dairy breed and the European East Frisian dairy breed. In addition, genotyping for the *oLEP* gene was conducted for several sheep belonging to the Awassi, the IA and the Dorper breeds.

Genomic DNA and RNA extraction. Genomic DNA and total RNA from abdominal fat tissue were extracted, and were used as a template for PCR. Fragments covering the exon 3 region of *oLEP* were amplified using specific primers. PCR products were then size-separated by electrophoresis on 1.2% agarose gels and excised. Subsequently, the amplicons were sequenced and mutations were detected by comparing the sequencing results to the *oLEP* sequence available in Genbank (accession number U84247).

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Mutein purity and monomer content. Muteins carrying mutations found in the present study (ΔQ28, N78S, V123L and R138Q), and recombinant oLEP muteins carrying the substitutions R84Q and P99Q (Zhou *et al.* 2008) were purified. SDS-PAGE was performed (Laemmli 1970) in a 15% (w/v) polyacrylamide gel under reducing conditions. The gel was stained with Coomassie Brilliant Blue R. Gel-filtration chromatography was performed on a Superdex™ 75 HR 10/30 column with 0.2-ml aliquots of the Q-Sepharose-column-eluted fraction using Tris-HCL, NaCl buffer (TN).

Preparation of oLEP muteins. To prepare leptin mutants, the pMon3401 expression plasmid encoding wild-type (WT) oLEP (Gertler *et al.* 1998) was used as the starting material. The *LEP* inserts were modified with the QuickChange mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Plasmids were transfected into expression plasmid Mon-105-competent cells, which expressed the muteins in inclusion bodies. Monomers of each mutein were purified by gel-filtration chromatography on a Superdex 75 HR 10/30 column and kept lyophilized.

oLEP complexes with chicken leptin-binding domain (chLBD): kinetics and equilibrium constants. oLEP-chLBD complexes were detected by gel filtration, as described previously (Salomon *et al.* 2006). The kinetics and equilibrium constants for the interactions between chLBD and WT oLEP or its muteins were determined by surface plasmon resonance (SPR) methodology, using a Biacore 3000 instrument (Neuchatel, Switzerland) (Gertler *et al.* 1996).

***In-vitro* biological activity in Baf-3 bioassay.** The proliferation rate of leptin-sensitive Baf-3 cells transfected with the long form of human leptin receptor was used to estimate the activities of WT oLEP and its muteins (Raver *et al.* 2000).

Results

Leptin gene polymorphism. Three synonymous and three non-synonymous mutations were found in exon 3 of *oLEP*. Sites of polymorphism and an alternative splicing event (deletion of Q28 in the abdominal fat tissue) are presented in Table 1.

Table 1: Polymorphisms and alternative splicing detected in *oLEP* exon 3

	Alternative splicing	Single-nucleotide polymorphism (SNP)					
Nucleotide no.*	81-83	225	228	232-3	312	367	413
Transition	CAG	A/G	A/C	AA/TC	G/A	G/T	G/A
Amino acid change	deletion	-	-	N78S	-	V123L	R138Q

* Nucleotide number refers to the sequence available in Genbank, accession no. U84247.

Preparation of oLEP muteins. The yields of all muteins, namely: ΔQ28, N78S, R84Q, P99Q, V123L and R138Q, expressed in an *Escherichia coli* system varied from 70 to 130 mg from 0.5 liter of bacterial culture.

Mutein purity and monomer content. All muteins were pure monomers as documented by SDS-PAGE under both reducing and non-reducing conditions: only one band of ~16 kDa was obtained for all muteins. Gel filtration under native conditions yielded a main monomeric peak consisting of at least 95% monomer and corresponding to a molecular mass of ~16 kDa.

oLEP-chLBD complexes. As expected, WT oLEP and all of its muteins formed 1:1 (molar ratio) complexes with chLBD (Mistrik *et al.* 2004).

Kinetics of oLEP-chLBD interactions. SPR experiments revealed reduced affinity of all oLEP muteins examined to chLBD relative to the WT oLEP (Table 2).

Table 2: Calculation of kinetics and thermodynamic constants for the interaction of immobilized oLEPs with soluble chLBD measured by SPR methodology

Analog	Ka (mol/s) × 10^6	Kd (s^{-1} × 10^{-3})	KD (M × 10^{-9})	X^2	Relative affinity
WT oLEP	3.62 ± 0.135	9.14 ± 0.29	2.53	0.7	1.00
ΔQ28	0.65 ± 0.005	3.05 ± 0.02	4.72	0.6	0.54
N78S	1.05 ± 0.013	7.08 ± 0.07	6.73	0.6	0.38
R84Q	0.54 ± 0.004	3.77 ± 0.02	6.93	0.9	0.36
P99Q	0.56 ± 0.007	2.56 ± 0.03	4.57	1.7	0.55
V123L	1.00 ± 0.012	3.66 ± 0.03	3.65	1.0	0.69
R138Q	1.14 ± 0.010	4.83 ± 0.03	4.23	0.7	0.60

***In-vitro* biological activity in Baf-3 bioassay.** Significant differences in proliferative activity were found only for N78S (1.8-fold higher) and R138Q (4.2-fold lower) relative to WT oLEP.

Discussion

To verify the biochemical significance of polymorphisms in *oLEP*, we generated recombinant muteins carrying novel mutations, namely: ΔQ28, N78S, V123L and R138Q, as well as muteins representing the R84Q (designated 105 $^{Arg/Gln}$) and the P99Q (designated 120 $^{Pro/Gln}$) mutations described by Zhou *et al.* (2008). Mutein yields ranged from 70 to 130 mg from 0.5 liter of bacterial culture, similar to the yields obtained in our lab for human, mouse, rat, bovine and porcine leptins (Solomon *et al.* 2006). All muteins were folded properly and were able to form a 1:1 complex with chLBD.

SPR analysis, using chLBD as the binding protein, indicated that all muteins manifest reduced affinity to the chLBD, which varied from 0.7 to 0.3 of the affinity of the WT oLEP. As an increase in food intake and decrease in energy utilization are major aims in intensive animal husbandry, it can be speculated that reduced leptin bioactivity manifested by a decrease in its affinity to its receptor would confer a selective advantage for carrying the mutations in environments where enhanced growth, enhanced breeding activity and enhanced milk production are selective advantages.

The muteins varied in their proliferative activity, as measured by Baf-3 cell assay. Muteins ΔQ28, P99Q and V123L were similar to the WT oLEP in their ability to induce proliferation. On the other hand, N78S had relatively lower somatogenic activity while muteins R84Q and R138Q manifested enhanced ability to induce cell proliferation. It is not yet clear why the muteins, which manifested reduced binding affinity, varied in their proliferative ability.

Different rankings of leptin muteins according to their affinity to their receptor and their biological activity have also been reported for ovine, human, rat and mouse leptin muteins (Solomon *et al.* 2006). In that study, while the leptin muteins' affinity to the leptin receptor was similar to that of the WT oLEP, they lacked biological activity, being practically leptin antagonists.

In conclusion, we identified novel non-synonymous mutations in the Assaf and IA sheep breeds. Following the production of oLEP muteins representing the products of all known *oLEP*-mutated genes, we showed that all of these muteins have reduced affinity to leptin receptor and that some have higher biological activity as compared to the WT oLEP. It is hypothesized that under artificial selection for enhanced productivity, these characteristics may provide a selective advantage for carrying these mutations.

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