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Leptin and leptin receptor genes in Atlantic salmon: Cloning, phylogeny, tissue distribution and expression correlated to long-term feeding status

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ABSTRACT

The present study reports the complete coding sequences for two paralogues for leptin (sLepA1 and sLepA2) and leptin receptor (sLepR) in Atlantic salmon. The deduced 171-amino acid (aa) sequence of sLepA1 and 175 aa sequence for sLepA2 shows 71.6% identity to each other and clusters phylogenetically with teleost Lep type A, with 22.4% and 24.1% identity to human Lep. Both sLep proteins are predicted to consist of four helixes showing strong conservation of tertiary structure with other vertebrates. The highest mRNA levels for sLepA1 in fed fish (satiation ration = 100%) were observed in the brain, white muscle, liver, and ovaries. In most tissues sLepA2 generally had a lower expression than sLepA1 except for the gastrointestinal tract (stomach and mid-gut) and kidney. Only one leptin receptor ortholog was identified and it shares 24.2% aa sequence similarity with human LepR, with stretches of highest sequence similarity corresponding to domains considered important for LepR signaling. The sLepR was abundantly expressed in the ovary, and was also high in the brain, pituitary, eye, gill, skin, visceral adipose tissue, belly flap, red muscle, kidney, and testis. Fish reared on a rationed feeding regime (60% of satiation) for 10 months grew less than control (100%) and tended to have a lower sLepA1 mRNA expression in the fat-depositing tissues visceral adipose tissue ($p < 0.05$) and white muscle (n.s.). sLepA2 mRNA levels was very low in these tissues and feeding regime tended to affect its expression in an opposite manner. Expression in liver differed from that of the other tissues with a higher sLepA2 mRNA in the feed-rationed group ($p < 0.01$). Plasma levels of sLep did not differ between fish fed restricted and full feeding regimes. No difference in brain sLepR mRNA levels was observed between fish fed reduced and full feeding regimes. This study in part supports that sLepA1 is involved in signaling the energy status in fat-depositing tissues in line with the mammalian model, whereas sLepA2 may possibly play important roles in the digestive tract and liver. At present, data on Lep in teleosts are too scarce to allow generalization about how the Lep system is influenced by tissue-specific energy status and, in turn, may regulate functions related to feed intake, growth, and adiposity in fish. In tetraploid species like Atlantic salmon, different Lep paralogues seems to serve different physiological roles.

1. Introduction

Leptin (Lep), a 16-kDa protein hormone, is a member of the class-I helical cytokine that is produced primarily by adipose tissues in mammals. Following the first discovery in mouse (Zhang et al., 1994) mammalian Lep has been extensively explored and demonstrated to be a central link between adiposity, appetite, and energy homeostasis in several species (e.g. Altmann and Von Borell, 2007; Morris and Rui, 2009; Spady et al., 2009; Vishesh and Arora, 2008). In addition, Lep is involved in regulation of a wide range of processes in mammals, such as reproduction, hematopoiesis, immune response, and bone formation (Ahima and Flier, 2000; Friedman, 2002). In teleosts, Lep was first identified in pufferfish, *Takifugu rubripes* (Kurokawa et al., 2005). Phylogenetic analysis revealed that amino acid conservation with other vertebrate Lep orthologs was low, with only 13.2% sequence identity between pufferfish and human Lep (Kurokawa et al., 2005). Subsequent identification of Lep in other teleosts including common carp, *Cyprinus carpio*; grass carp, *Ctenopharyngodon idella*, medaka, *Oryzias latipes*; zebrafish, *Danio rerio*; rainbow trout, *Oncorhynchus mykiss*; Arctic charr, *Salvelinus alpinus* and Atlantic salmon, *Salmo salar* (the present study) confirms

Abbreviations: aa, amino acids; EF1α, elongation factor 1 alpha; LBD, leptin-binding domain; Lep, leptin; LepR, leptin receptor; ORF, open reading frame; RACE, rapid amplification of cDNA ends.

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low amino acid identity with mammalian Lep (Huising et al., 2006; Kurokawa et al., 2005, 2008; Frøiland et al., 2010; Li et al., 2010). However, the available data indicate that the three-dimensional structure of the predicted Lep protein is well conserved among mammals and teleosts.

The differences among mammalian and fish Lep orthologs have led researchers to raise questions whether Lep function is conserved or reflect differential roles in regulation of energy metabolism and/or other physiological functions, and also whether there is a fundamental difference between ectothermic and endothermic vertebrates. Studies have revealed that liver, rather than adipose tissue, is the major Lep-expressing tissue in pufferfish (Kurokawa et al., 2005), common carp (Huising et al., 2006), and rainbow trout (Murashita et al., 2008). In other non-amniotic vertebrates such as the tiger salamander, *Ambystoma tigrinum*, and the frog, *Xenopus laevis*, Lep mRNA is highly expressed in tissues such as skin and testis (Boswell et al., 2006; Crespi and Denver, 2006). Hepatic Lep expression in carp increases after feeding but does not change during long-term fasting which questions a possible link between Lep expression and energy status in teleosts (Huising et al., 2006). In contrast, injection of species specific recombinant Lep in rainbow trout has an anorexigenic effect (Murashita et al., 2008) in line with findings in post-metamorphic frog (Crespi and Denver, 2006). On the other hand, plasma Lep levels are elevated during fasting in rainbow trout (Kling et al., 2009). At present, the available information suggests multiple functions of Lep in ancestral vertebrates, although there is no clear understanding on the role of Lep in energy homeostasis comparable to that in humans.

The physiological actions of Lep are mediated by membrane-associated Lep receptors (LepR). Mammalian LepR is known to have at least six isoforms (Zabeau et al., 2003). The Lep effects on food intake, glucose metabolism, and weight gain are reportedly linked to binding of Lep to the extracellular domain of the long form of the LepR (Bates et al., 2005). In teleosts, the gene for LepR has been identified in marine medaka, *O. melastigma* (Wong et al., 2007) pufferfish (Kurokawa et al., 2008), but no information currently exists in salmonids.

The target species of the present study, the Atlantic salmon, is a major aquaculture species, and has been extensively investigated in order to understand processes associated with growth, feed intake, energy homeostasis, and adiposity. The endocrine regulation of these processes is known to be complex, involving multiple hormones such as growth hormone, insulin-like growth factor I, insulin, ghrelin, thyroid hormones, and androgens, but to date, limited information on the role of Lep is available for this species. Previous studies have indicated that Atlantic salmon has a lipostatic regulation of feed intake where adiposity has a regulatory role governing appetite (Johansen et al., 2001, 2002) although a comprehensive understanding of the endocrine control of lipid homeostasis is still lacking (Leaver et al., 2008). The main aim of the present study was therefore to obtain full-length mRNA sequences for Atlantic salmon orthologs of sLep and sLepR. The sequence information was then used to establish methods for quantifying mRNA levels, and elucidating the tissue distribution of mRNA of sLep and sLepR. As an initial step towards understanding the biological roles of Lep in Atlantic salmon, effects of feeding status on plasma levels of sLep, and mRNA levels of sLep and sLepR in selected organs were examined in two groups of fish which had been either fed to satiation (control group), or fed 60% ration (rationed group) for several months.

2. Materials and methods

2.1. Animals and tissue sampling

Except for assessment of restricted feeding, all materials for cDNA cloning and analysis of tissue distribution was based on Atlantic salmon (AquaGen breed http://www.aquagen.no/; juvenile post-smolts: 45–350 g) reared at the Bergen High-Technology Centre (Bergen, Norway) in 1 m^2 indoor tanks (500 L rearing volume) supplied with a continuous water flow (2.5 L min^{-1}, 8 °C) and fed a commercial pellet diet (Ewos, Bergen, Norway) in excess for 12 h during the photo phase. At sampling, fish were randomly collected by dipnet and killed with an overdose of MS-222 (3-aminobenzoic acid ethyl ester; Sigma, St. Louis, MO), and tissues were collected and stored in RNAlater (Ambion, Austin, TX, USA) at −20 °C until RNA isolations and further analyses.

For evaluation of long-term effects of rationed feeding, Atlantic salmon (AquaGen strain) was sub-sampled from a large-scale experiment ($N = 3200$) conducted at the Institute of Marine Research Aquaculture Research Station at Matre (80 km north of Bergen, Norway) from October 2006 until August 2007. The fish (average initial weight 849 ± 70 g) were kept in four sea cages ($5 \times 5 \times 5$ m). Two groups were established: a control group (100%) which was fed a commercial diet to satiation (Biomar Classic Diet; Biomar, Bergen, Norway) 7 days a week with feeding levels adjusted to fish size and temperature, and a rationed group which was fed 60% of the control group. This was obtained by feeding the same amount of food per day, but only 4 days a week (Mondays, Tuesdays, Thursdays, and Fridays).

At sampling, rationed and control fish were randomly dipnetted out of the cages and anesthetized (MS-222). Fish fork length and wet weight was recorded to nearest mm and g, before 4 ml of blood was drawn from the caudal vein using a 5 ml heparinized syringe fitted with a 21G needle. After centrifugation (3000*g*; 5 min) the obtained plasma was frozen at −80 °C. The gender was determined for each fish and only immature males ($n = 6$ from each group) were included in the present set of analysis. Tissue samples were collected and flash-frozen in liquid N_2 and stored at −80 °C until subsequent analysis of mRNA expression.

2.2. Leptin

2.2.1. Cloning of salmon leptin A1 (sLepA1)

Total RNA was prepared from liver of *ad libitum* fed post-smolt Atlantic salmon (350 g) according to Kurokawa et al. (2003), whereas genomic DNA was extracted using Tri reagent as outlined by Chomczynski (1993).

For amplification of 5′ ends of salmon sLepA1 cDNA, RACE PCR was performed according to Kurokawa et al. (2003). Because EST data (GenBank Accession No. BI468126) included a partial sequence of salmon sLepA1 mRNA; the primers for 5′ RACE were designed in the EST sequence (SMA5a, SMA5b, sLepA1 Rv1, and sLepA1 Rv2; Table 1). Based on the full cDNA sequences of sLepA1 (GenBank Accession No. FJ830677) obtained by RACE PCR, sLepA1 Fw1, and sLepA1 Rv3 primers were designed for subsequent cloning in order to obtain more information about the sLepA1 gene. A PCR (50 μl) consisting of 50 ng gDNA, 200 nM forward and reverse primers, 1.25 mM dNTPs, 1.5 mM $MgCl_2$, and 2 U/μl *Taq* polymerase (Promega, Madison, WI, USA) and thermal conditions of 3 min at 94 °C, then 35 cycles of 94 °C for 45 s, 60 °C for 30 s, 72 °C for 40 s and final extension at 72 °C for 10 min was performed. The PCR products were separated by 1% agarose gel electrophoresis, bands extracted using QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany) and PCR fragments cloned into a pCR4-TOPO sequencing vector (Invitrogen, Carlsbad, CA, USA) following the manufacture's instructions. Plasmids were transformed into One Shot TOP10 chemically competent *Escherichia coli* and grown on ampicillin LB-agar plates. Colonies containing inserts were cultured overnight, purified using QIAGEN Mini Plasmid Kit and sequenced in both directions using Big-Dye Ver. 3.1 and ABI 3700 automated sequencer (Applied Biosystems, Inc., Foster City, CA, USA) at the University of Bergen.