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Production, gene structure and characterization of two orthologs of leptin and a leptin receptor in tilapia

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ABSTRACT

Full-length cDNA encoding two leptin sequences (tLepA and tLepB) and one leptin receptor sequence (tLepR) were identified in tilapia (*Oreochromis niloticus*). The full-length cDNA of tLepR was 3423 bp, encoding a protein of 1140 amino acid (aa) which contained all functionally important domains conserved among vertebrate leptin receptors. The cDNAs of tLepA and tLepB were 486 bp and 459 bp in length, encoding proteins of 161 aa and 152 aa, respectively. Modeling the three-dimensional structures of tLepA and tLepB predicted strong conservation of tertiary structure with that of human leptin, comprised of four helixes. Using synteny, the tLeps were found near common genes, such as *IMPDH1* and *LLRC4*. The cDNA for tLepA and tLepB was cloned and synthetic cDNA optimized for expression in *Escherichia coli* was prepared according to the cloned sequence. The tLepA- and tLepB-expressing plasmids were transformed into *E. coli* and expressed as recombinant proteins upon induction with nalidixic acid, found almost entirely in insoluble inclusion bodies (IBs). The proteins were solubilized, refolded and purified to homogeneity by anion-exchange chromatography. In the case of tLepA, the fraction eluted contained a mixture of monomers and dimers. The purified tLepA and tLepB monomers and tLepA dimer showed a single band of ~15 kDa on an SDS–polyacrylamide gel in the presence of reducing agent, whereas the tLepA dimer showed one band of ~30 kDa in the absence of reducing agent, indicating its formation by S–S bonds. The three tLeps were biologically active in promoting proliferation of BAF/3 cells stably transfected with the long form of human leptin receptor (hLepR), but their activity was four orders of magnitude lower than that of mammalian leptin. Furthermore, the three tLeps were biologically active in promoting STAT-LUC activation in COS7 cells transfected with the identified tLepR but not in cells transfected with hLepR. tLepA was more active than tLepB. Low or no activity likely resulted from low identity (9–22%) to mammalian leptins. In an *in vivo* experiment in which tilapia were fed *ad libitum* or fasted, there was no significant difference in the expressions of tLepA, tLepB or tLepR in the brain between the two groups examined both by real-time PCR and RNA next generation sequencing. In conclusion, in the present report we show novel, previously unknown sequences of tilapia leptin receptor and two leptins and prepare two biologically active recombinant leptin proteins.

1. Introduction

Leptin, a 16-kDa hormonal product of the obese (*ob*) gene, is a member of the class-I α-helical cytokines (Huising et al., 2006) secreted primarily by adipose tissue (Zhang et al., 1994). Leptin is a pleiotropic hormone that acts both centrally and peripherally. In mammals, it is known as an important hormone in the regulation of food intake and energy metabolism (Schwartz et al., 2000), reproductive function (Chehab et al., 1997; Ashworth et al., 2000) and immune responses (Gainsford et al., 1996; Peelman et al., 2004). However, it also has negative actions, such as enhancement of undesired immune responses in autoimmune diseases (Peelman et al., 2004), tumorigenesis (Gonzalez et al., 2006) and elevated blood pressure (Beltowski, 2006). In contrast, information regarding the role of leptin in nonmammalian animals, and in particular fish, is scarce and contradictory.

On the one hand, researchers have shown that mammalian leptin reduces food intake in goldfish (*Carassius auratus*) (de Pedro et al., 2006) and striped bass (*Morone saxatilis*) (Won et al., 2012), and that leptin affects the expression and release of pituitary hormones in the European sea bass (*Dicentrarchus labrax*)

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(Peyon et al., 2001), goldfish (*C. auratus*) (Volkoff et al., 2003), rainbow trout (*Onchorynchus mykiss*) (Weil et al., 2003) and bighead carp (*Aristichthys nobilis*) (Chowdhury et al., 2004). On the other, researchers have not observed any conclusive effects of leptin in coho salmon (*Oncorhynchus kisutch*) (Baker et al., 2000), green sunfish (*Lepomis cyanellus*) (Londraville and Duvall, 2002) or goldfish (*C. auratus*) (Tinoco et al., 2012). However, species-specific leptin experiments have shown a strong anorexic effect on food intake in grass carp (*Ctenopharyngodon idellus*) on the first day after intraperitoneal (IP) injection, but not on subsequent days. Moreover, in rainbow trout (*O. mykiss*), recombinant leptin suppresses food intake and leads to a transient reduction in Neuropeptide Y (NPY) mRNA levels (Murashita et al., 2008). Therefore, preparation of species-specific leptin is crucial to characterizing the function of leptin in certain fish species, in this study, tilapia.

The physiological actions of leptin are mediated by a glycoprotein consisting of a single-polypeptide-chain leptin receptor (LepR)—a single-membrane-spanning receptor of the class-I cytokine receptor family (Tartaglia, 1997). In human, there are at least six isoforms of leptin receptors (LepRa–LepRf) generated by alternative mRNA splicing and/or proteolytic processing of the protein products (Tartaglia et al., 1995). The long isoform, or LepRb, is the only one with clearly demonstrated signaling capability (Zabeau et al., 2003). Like other cytokine receptors, LepRb does not have intrinsic activity; rather, it affects its signal by activating the noncovalently associated tyrosine kinase Janus kinase 2 (JAK2) (Ghilardi and Skoda, 1997). After binding leptin, LepRb-associated JAK2 becomes activated by cross-phosphorylation and tyrosine phosphorylation in the cytoplasmic domain of the receptor (La Cava and Matarese, 2004). The phosphorylated JAK2s and tyrosine serve as docking sites for signal-transduction proteins bearing a phosphorylated binding domain called SH2, such as signal transducers and activators of transcription (STAT) factors, particularly STAT3 (Banks et al., 2000). At the hypothalamus, leptin activates mostly STAT3 (Vaisse et al., 1996), making it the major cellular-signal-transduction mediator of leptin in this tissue.

The Nile tilapia (*Oreochromis niloticus*), a euryhaline fish regarded as an important species in aquaculture, is an emerging model system for laboratory studies in many fields, including neuroendocrinology, physiology, genomic biology and molecular genetics (Golan and Levavi-Sivan, 2013). Its importance to research has grown further since the publication of the genome project for full genome of Nile tilapia in 2012 (Broad oreNil1.1/oreNil2). The present study was conducted to determine the full-length cDNA sequences coding for leptin (tLepA and tLepB) and leptin receptor (tLepR) genes in tilapia. Further, we produced both tilapia leptins as recombinant proteins and analyzed their biological activity. This study provides an initial step toward understanding the biological roles of leptin in teleost fish.

2. Materials and methods

2.1. Materials

Recombinant mouse leptin was prepared in our laboratory as described previously (Gertler et al., 1998; Niv-Spector et al., 2005). Restriction enzymes used in the molecular biology experiments were from Fermentas (Vilnius, Lithuania) and New England Biolabs (Beverly, MA). Highly pure DNA primers were ordered from Sigma (Rehovot, Israel; Table 1). RPMI-1640 medium, Bovine Serum Albumin (BSA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue, MTT) were purchased from Sigma; Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA and antibiotics were purchased from Biological Industries (Bet HaEmek, Israel); fetal bovine serum (FBS) for BAF/3 cell assay was from Bio-Lab Ltd. (Jerusalem, Israel); FBS for receptor transactivation assay was purchased from Invitrogen (RHENIUM Ltd., Modi'in, Israel); Superdex™ 75 HR 10/30 column and Q-Sepharose were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Antibiotic–antimycotic solution (5×10^4 U/ml penicillin, 50 mg/ml streptomycin, 0.125 mg/ml fungisone), NaCl and Tris-base were purchased from Bio-Lab Ltd. 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 BioRad (Hercules, CA).

2.2. Isolation of tilapia leptin ligands and receptor

Database sequence searches were performed with the BLAST package (NCBI, http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997) and BLAT (UCSC genome browser, http://genome.ucsc.edu) (Kent, 2002). We then designed specific primers to clone the putative tLep ligands and receptor (Table 1). The fragments were PCR-amplified from an adult tilapia pituitary cDNA library by Advantage 2 PCR System (Clontech, Mountain View, CA) and were cloned into the pCRII-TOPO vector (Invitrogen, Grand Island, NY).

2.3. Phylogenetic analysis and chromosomal synteny

Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 4 (Tamura et al., 2007). Phylogenetic analysis was generated with both neighbor-joining (ClustalW 2.1) and maximum-likelihood (Phylip 3.69, ProML) methods on the basis of alignments performed by both ClustalW and Muscle (3.8.31).

Synteny was observed using the Ensembl genome browser (http://www.ensembl.org/index.html).

Table 1
Primers used for cloning and real-time PCR.

Primer	Position	5′ to 3 sequence	Slope	R^2	Efficiency	Application
tLepA-F	1	ATGGACTACGGTCTGGTGCTCCTA				Cloning to pCRII-TOPO
tLepA-R	486	GCTTGGAAATACTGGTCCTG				
tLepB-F	1	ATGGGGGGGATAGAGGAGGAGAAC				
tLepB-R	523	TATTTGTCCCGATATATAGCAAGTCCTG				
tLepR-F	1	ATGACCGCTACAATGGTTCAGTCTG				
tLepR-R	3423	TCACAGCTGGGGTTCACGTT				
tLepR-F-pCDNA3.1		TGCTGGATATCTGCAGAATTCCACCACACTATGACCGCTACAATGGTTC				Cloning to pCDNA3.1
tLepR-R-pCDNA3.1		GGTTTAAACTTAAGCTTGGTACCGAGTCACAGCTGGGGTTCACG				
tLepA-236F	236	GCCTGATCCCGGACACCTTT	−3.025	0.979	104.2%	Real-time PCR
tLepA-414R	414	GATGCCCACGGTTTGAATGA				
tLepB-203F	203	AGGTTCCCACTCCTTCCATTGA	−3.044	0.996	103.1%	
tLepB-353R	353	TCCATTGATAAGGCGAAGGAGC				
tLepR-2818F	2818	CCACATCCAGGTAGCACCGA	−2.925	0.998	101.0%	
tLepR-2971R	2971	CCGAAATGTCAGAGTTGTTGGC				

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