Alternative Splice Variants of the Rainbow Trout Leptin Receptor Encode Multiple Circulating Leptin-Binding Proteins

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In mammals, leptin (Lep) binding proteins (LepBPs) derived from Lep receptor (LepR) gene or protein bind most of the circulating Lep, but to date, information on LepBPs in nonmammalian vertebrate classes is lacking. This study details the characterization of multiple LepBPs in rainbow trout (*Oncorhynchus mykiss*), an early poikilothermic vertebrate, and presents the complete coding sequences for 3 of them. Size-exclusion chromatography and cross-linking assay identified plasma proteins bound to Lep ranging from 70 to 100 kDa. LepBPs were isolated from plasma by affinity chromatography, and their binding specificity was assessed by a competitive binding assay. A RIA for LepBPs indicates that plasma LepBP levels decline after fasting for 3 weeks. Immunoblotting of LepBPs using antibodies against different LepR epitopes shows that the LepBPs are indeed LepR isoforms. The alternatively spliced *LepR* transcripts ($LepR_{S1-3}$) that include only the extracellular segment transcribe the 90-kDa LepBP1, the 80-kDa LepBP2, and the 70-kDa LepBP3, respectively. $LepR_{S1}$ generally has lower expression than the long-form *LepR* in most tissues. $LepR_{S2}$ is primarily expressed in adipose tissue, whereas $LepR_{S3}$ is expressed abundantly in brain and spleen, and moderately in liver and gills. The mRNA levels of hepatic $LepR_{S3}$ increase after 2 weeks of fasting. This study demonstrates a mechanism in fish for the generation of LepBPs that differs from that seen in mammals and indicates that the physiologic action of Lep in these poikilothermic vertebrates can be modulated, both centrally and peripherally, by the differentiated, tissue-specific expression of multiple LepBPs. **(*Endocrinology* 154: 2331–2340, 2013)**

The hormone leptin (Lep) belongs to the class-I cytokine family and is primarily expressed in adipose tissues in mammals (1). Research has demonstrated the key importance of Lep in regulation of fat metabolism and energy balance in mammals (2–4).

The *lep* gene has been cloned from many fish species in the last decade, including pufferfish *Takifugu rubripes* (5), medaka *Oryzias latipes* (6), zebrafish *Danio rerio* (7), rainbow trout *Oncorhynchus mykiss* (8), Atlantic salmon *Salmo salar* (9) and Arctic char *Salvelinus alpinus* (10), catfish *Ictalurus punctatus* (11) and *Pelteobagrus fulvidraco* (12), goldfish *Carassius auratus* (13), grouper *Epinephelus coioides* (14), striped bass *Morone saxatilis* (15), and carps *Cyprinus carpio*, *Ctenopharyngodon idellus*, and *Hypophthalmichthys molitrix* (16, 17). Rainbow trout Lep has high amino acid identity with Atlantic salmon Lep (95%) and Arctic char Lep (78%), but lower identity with Lep from other fish species (15%–30%) and mammals (20%). In fish, *lep* appears to be primarily expressed in liver, whereas lower *lep* expression has been detected in the central nervous system, adipose tissues, and reproductive organs. Despite considerable differences in the primary sequences among species, mammalian and fish Leps have maintained similar secondary and tertiary structures, including 4 conserved helixes and a disulfide bridge (8, 9, 14, 16, 17).

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Abbreviations: GST, glutathione-*S*-transferase; Lep, leptin; LepR, leptin receptor; LepBP, leptin binding protein; $LepR_L$, long-form LepR; $LepR_S$, short-form LepR; RACE, rapid-amplification of cDNA ends; SEC, size-exclusion chromatography.

Recent fish studies using homologous Lep research tools indicate that, at least in salmonids, Lep has an anorexigenic function similar to that in mammals. Short-term Lep treatment in rainbow trout decreases food intake (8), and growth rate in Atlantic salmon decreased after 20-day Lep administration (18). These effects appear to be mediated through activation of anorexigenic pathways in the central nervous system (18). On the other hand, plasma Lep levels increase in fish during fasting or feed restriction (19–22), which is opposite to that in mammals where Lep levels decrease during fasting (23, 24). This may indicate some fundamental differences in Lep physiology between endothermic and poikilothermic vertebrates. It has been suggested that this may be linked to a passive survival strategy of fish species that may naturally experience prolonged periods of food shortage, through lowering appetite and thus limiting energy-wasting foraging activity (21).

The long-form Lep receptor ($LepR_L$) is a membrane-bound receptor, and ligand stimulation results in signaling transduction (25). Shorter forms of LepR, missing the intra- and transcellular segments, but maintaining the ligand-binding properties of the extracellular segment, are produced as soluble binding proteins for Lep (LepBPs). In mammals, LepBPs bind a large proportion (60%–98%) of circulating Lep (4, 26–28). The binding of Lep to the LepBPs decreases the availability of the circulating hormone to the $LepR_L$. Thus, LepBPs are suggested to be important regulators of Lep action (29–31). LepBPs can be generated through proteolytic cleavage or ectodomain shedding of the $LepR_L$ (28, 32). Alternatively, an alternative splicing *LepR* variant (Ob-Re gene) in rodents encodes only the extracellular segment and generates LepBPs (29, 33). Irrespective of the mechanisms through which these LepBPs are generated, they maintain the same binding specificity as the $LepR_L$ and usually bind to Lep at a 1:1 ratio (31, 32, 34).

The $LepR_L$ gene has been cloned from several fish species (6, 9, 12–14, 35), and several *LepR* variants have been found in Atlantic salmon (9) and carp (35). The shorter LepR isoforms contain the extracellular segment (9) or the extracellular segment with partial transmembrane segment (35), but all of them lack the functional intracellular domain. Their physiologic relevance remains to be defined.

In the present study, LepBPs were characterized in rainbow trout in order to elucidate their potential roles in Lep endocrinology in an earlier vertebrate. In order to detect, isolate, and characterize LepBPs in the circulation, Lep affinity chromatography and immunoblotting assays were applied. The binding capacity of the isolated LepBPs was assessed by a competitive binding assay, size exclusion chromatography, and cross-linking assay. To identify the genes encoding LepBPs, rapid amplification of cDNA ends (RACE) PCRs were applied for amplifying *LepR* variants. A RIA was established to measure plasma LepBP levels in rainbow trout during a 3-week fasting in order to explore the involvement of LepBPs in regulation of energy balance and to correlate circulating LepBP levels with their gene expression levels in various tissues.

Materials and Methods

Animals and fasting experiment

Juvenile rainbow trout (~250 g) were purchased from a local hatchery (Laxodling AB, Alingsås, Sweden), distributed among 4 experimental tanks, and acclimated to an ad libitum feeding regimen in recirculating aerated freshwater at 11°C for 1 week. Then, fish in 2 of the tanks were fasted for 3 weeks while the fish in 2 tanks were fed ad libitum. Eight fish from each tank were sampled after 1, 2, and 3 weeks, without regard to sex, which was not considered as a factor in the statistical analysis of the data. Liver and hypothalamus were sampled and preserved in liquid nitrogen. Blood was collected from the caudal vein, and plasma was obtained by centrifugation and stored at −80°C. The experiment was approved by the Ethical Committee for Animal Research in Gothenburg (license 46-2009) and complies with current Swedish legislation.

Partial purification of rainbow trout LepBPs

In order to establish Lep affinity chromatography, recombinant rainbow trout Lep was produced by the method described by Murashita et al. (8). The bacteria transformed with the mature *lep* were generously provided by Professor Rønnestad, University of Bergen, Norway. Lep (0.5 mg) was coupled to NHS-activated sepharose 4 fast-flow beads as the ligand for rainbow trout LepBPs (GE Healthcare, Buckinghamshire, United Kingdom). One milliliter of the prepared sepharose beads was mixed with 10 mL of rainbow trout plasma and incubated at 4°C overnight with gentle shaking on a table orbital shaker. After a thorough wash with 50 mM Tris-HCl buffer (pH 7.4), the bound proteins were eluted from the sepharose beads with 2 mL of 100 mM ethylene glycol (pH 11.6) and instantly neutralized with 1 M NaH_2PO_4. This elution procedure was repeated 4 times, and the solutions were pooled and dialyzed against PBS (pH 7.3) with snakeskin-pleated dialysis tubing (3500 molecular weight cut-off; Thermo Scientific, Rockford, Illinois). LepBPs in PBS were concentrated by ultrafiltration using centrifugal ultracel-3k filters (Millipore Corp, Cork, Ireland). The concentrated LepBP solution was used for [^{125}I]Lep binding assays, size exclusion chromatography, cross-linking assay, and immunoblotting.

[^{125}I]Lep binding assay

Lep was iodinated by the method described by Kling et al (19). The prepared [^{125}I]Lep (~300 000 cpm) was incubated with 50 μg of the affinity-purified LepBPs in the absence or presence of cold unlabeled Lep in different concentrations. The incubation was carried out overnight at 4°C, followed by size-exclusion chromatography.

Size-exclusion chromatography (SEC)

SEC experiments were performed using a sepharose CL-6B column (1.6 × 70 cm) (Sigma-Aldrich, Steinheim, Germany). The column was equilibrated with 50 mM Tris-buffered saline, 150 mM NaCl (pH 7.5) at 4°C at a flow rate of 7 mL h^{-1}. The applied sample volumes were 0.25 mL. Fractions of 0.9 mL were collected. The void volume was determined with 2 mg mL^{-1} blue dextran. The column was calibrated with gel filtration molecular mass markers through measuring the absorbance of thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), and carbonic anhydrase (29 kDa) (Sigma-Aldrich, St Louis, Missouri). The radioactivity in the collected fractions was measured in a γ-counter (PerkinElmer, Waltham, Massachusetts). The fractions that contained LepBPs bound to iodinated Lep were pooled and concentrated by ultrafiltration with ultracel-3k centrifugal filters.

Cross-linking assay

Samples were incubated with [^{125}I]Lep tracer (~200 000 cpm) overnight at 4°C, including plasma, the affinity-purified LepBPs, and the pooled fractions from SEC. Disuccinimidyl suberate (Sigma-Aldrich, Steinheim, Germany) dissolved in dimethylsulfoxide was used as cross-linker at the final concentration of 1 mM. Cross-linking was performed for 1.5 hours on ice. The reaction was quenched by adding Tris buffer (50 mM) for 15 minutes. The prepared samples were heated to 95°C for 10 minutes in loading buffer and subsequently separated by 10% SDS-PAGE. The gel was enveloped in a plastic bag and exposed to a high performance autoradiography film for 5 days at −80°C.

Antibody production

The polyclonal LepR-Ab1 antibody was raised against the recombinant Lep binding domain (LepR395/604) composed of amino acids 395–604 of rainbow trout LepR. The gene encoding LepR395/604 was inserted into the expression vector pET28b (Novagen, Madison, Wisconsin). The poly-histidine tagged LepR395/604 was produced by transformed *Escherichia coli* and purified with his-select nickel affinity gel (Sigma-Aldrich). LepR395/604-his protein dissolved in 4 M urea was used to immunize rabbits (Agrisera, Vännäs, Sweden). LepR-Ab1 was purified from the collected antiserum through rProtein A sepharose fast-flow affinity chromatography (GE Healthcare, Uppsala, Sweden).

The polyclonal LepR-Ab2 antibody were raised in rabbits against 3 synthesized polypeptides, RPAQLHNRGKEEDRC, KNHQDRPDQTSESRER, and PDQTSESRERWTRFPPK, corresponding to amino acid residues 51–64, 747–761, and 752–767 of Atlantic salmon LepR (GenBank BAI23197.1), respectively (PinkCell Laboratories Amsterdam, The Netherlands). LepR-Ab2 can also recognize rainbow trout LepR within the epitopes between amino acids 746–760 and 751–766 because of amino acid identity in the third FNIII domain (Supplemental Figure 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

The polyclonal LepR-Ab3 antibody was raised against the recombinant third FNIII domain (LepR703/802) composed of amino acids 703–802 of rainbow trout LepR. The gene was inserted into the expression vector pGEX-5 × 1 (GE Healthcare). The glutathione-*S*-transferase (GST)-tagged protein LepR703/802-GST was produced by the transformed *E. coli* and purified with Glutathione sepharose 4B (GE Healthcare). LepR703/802-GST in PBS was used to immunize rabbits (Agrisera, Vännäs, Sweden). LepR-Ab3 was subsequently affinity purified from the antiserum. A summary table of the antibodies used is provided in Supplemental Table 1.

Immunoblotting

Affinity-purified LepBPs and rainbow trout plasma were subjected to SDS-PAGE (12%) and then transferred to nitrocellulose blotting membrane (Bio-Rad, Munich, Germany). The membrane was blocked with 5% skimmed milk in PBS (pH 7.3). LepR-Ab1, LepR-Ab2, or LepR-Ab3 was used as the primary antibody. Donkey antirabbit IgG horseradish peroxidase-linked whole antibody (GE Healthcare, Buckinghamshire, United Kingdom) was used as a second antibody. The signal was detected by ECL plus Western-blotting detection system and high performance chemiluminescence film (GE Healthcare).

Rainbow trout LepBP RIA

A noncompetitive RIA was established to measure LepBP levels in rainbow trout plasma. The gene encoding LepR395/604 was inserted in pGEX-5 × 1 vector and expressed by *E. coli* in a soluble form. The recombinant GST-tagged LepR395/604 protein was subsequently purified with glutathione sepharose 4B to be used for iodination and standards. The recombinant protein (7 μg) was iodinated (^{125}I) using 600 μg chloramine T to catalyze the reaction, which was stopped after 75 seconds with sodium metabisulfide. The radiolabeled protein was separated from free iodine with size exclusion chromatography (Sephadex G75, GE Healthcare) in a 0.7 × 20 cm column (Bio-Rad, Sundbyberg, Sweden) using 0.1 M Tris-HCl with 0.1% Triton X-100 as elution buffer. Fractions (500 μL) were collected into 50 μL elution buffer containing 2% BSA. The assay buffer used in all subsequent steps was 30 mM phosphate buffer (pH 7.5), containing 10 mM EDTA, 0.05% Tween 20, 0.15 M NaCl, 0.025% NaN_3, and 1% BSA. Recombinant LepR395/604-GST was serially diluted and used as standards (7.8 to 2000 ng ml^{-1}). Plasma (100 μL) and standards (in duplicate) were incubated with 50 μL LepR-Ab1 (diluted 1:10 000) overnight. After 18–24 hours, a fraction of the radiolabeled antigen was diluted, and 50 μL (4000 cpm) were added to each vial. After 18–24 hours, 25 μL 1% normal rabbit serum was added to each tube, and the antigen-antibody complex was precipitated with 50 μL secondary antirabbit antibody (1:30; R0881, Sigma-Aldrich) and with 1 mL ice-cold 3% polyethylene glycol 1 hour later. The complex was separated from free antigens 30 minutes after the addition of the polyethylene glycol, by centrifugation at 3270 × *g* at 4°C for 1 hour. After aspiration of the supernatant, the pellet was counted in a γ-counter. Standard curves were plotted and LepBP concentrations of unknown samples were calculated using the AssayZap (BioSoft, Ferguson, California) software.

Cloning of rainbow trout LepR variants

The sequences of *LepR* variants were obtained from 3′- and 5′-rapid amplification of cDNA ends (RACE) PCRs. Total RNA was isolated from rainbow trout pituitary gland with RNeasy Mini kit (Qiagen, Hilden, Germany). RACE-ready cDNAs were synthesized from the total RNA using SMARTer RACE cDNA amplification kit (CLONTECH Laboratories, Inc, Palo Alto, California). The primers are listed in Table 1. Advantage 2 polymerase mix kit

Table 1. Primer Nucleotide Sequences Used in RACE PCR and RT/Quantitative Real-Time PCR

Name	Sequences (5′-3′)	Use
5′-RACE-1	GGGCCTTGCCAGTTGAGAGG GCATAGCG	*LepR* 5′-RACE
5′-RACE-2	CCACCCTCTCCGCCTCCTCCATGACG	*LepR* 5′-RACE, nested
3′-RACE-1	GGAGGCCAAGACGGACAACAGCACGAGG	*LepR* 3′ RACE
3′-RACE-2	CGCTATGCCCTCTCAACTGGCAAGGCCC	*LepR* 3′-RACE, nested
LepR-f	GTTATCTCACTCACAGAGGCCATG	Long PCR forward primer
$LepR_{S1}$-f	CTACTGACAAGCTCCTCTACC	$LepR_{S1}$ PCR forward primer
$LepR_{S1}$-r	TGTAAATGCAGCCATTGAGAC	$LepR_{S1}$ PCR reverse primer Long PCR reverse primer
$LepR_{S2}$-f	TAGACACCCCAAACGTCAG	$LepR_{S2}$ PCR forward primer
$LepR_{S2}$-r	TCATGTGCTTATGCTGTACAGTC	$LepR_{S2}$ PCR reverse primer Long PCR reverse primer
$LepR_{S3}$-f	ACTAGGAACACCCACATGAC	$LepR_{S3}$ PCR forward primer
$LepR_{S3}$-r	ACAATCCTTCAGCCCTCTAC	$LepR_{S3}$ PCR reverse primer Long PCR reverse primer
Lep-f	GGTGATTAGGATCAAAAAGCTGGA	*Lep* PCR forward primer
Lep-r	GACGAGCAGTAGGTCCTGGTAGAA	*Lep* PCR reverse primer
GADPH-f	CCACTCCATCTCCGTATTCC	*GADPH* PCR forward primer
GADPH-r	ACTTGTCTTCGTTGACTCCC	*GADPH* PCR reverse primer

f, forward; GADPH, glyceraldehydes-3-phosphate dehydrogenase; r, reverse.

(CLONTECH Laboratories Inc) was used for RACE PCR. The RACE products were cloned into pGEM-T easy vector (Promega Corp, Madison, Wisconsin) and sequenced by Eurofins MWG Operon Company (Ebersberg, Germany). The full length of LepR variants was amplified by long PCR using the primers listed in Table 1 and Platinum *Taq* DNA polymerase high-fidelity PCR reagents (Invitrogen, Carlsbad, California). Gene sequences were obtained by DNA sequencing.

RT-PCR and quantitative real-time PCR

The primers for RT-PCR and quantitative real-time PCR are listed in Table 1. Total RNA of tissues were extracted with RNeasy Mini kit (Qiagen) and treated by DNase I (Invitrogen) to remove genomic DNA residue. The cDNA templates were synthesized from the total RNA by SuperScript III first-strand synthesis system (Invitrogen). RT-PCR reagents were from Sigma-Aldrich. Real-time PCRs were prepared with triplicate 25 μL samples including 300 nM primers, cDNA template, and iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, California). Data from the real-time PCR runs were collected with BioRad iCycler iQ Optical System. Data were analyzed by 1-way ANOVA (n = 3). Differences were considered significant at $P < .05$.

Results

Immunologic identification of LepBPs in rainbow trout plasma and affinity-purified sample

SDS-PAGE analysis of affinity-purified LepBPs exhibited at least 5 protein bands between 28 and 90 kDa stained by Coomassie blue (Figure 1A). Immunoblotting

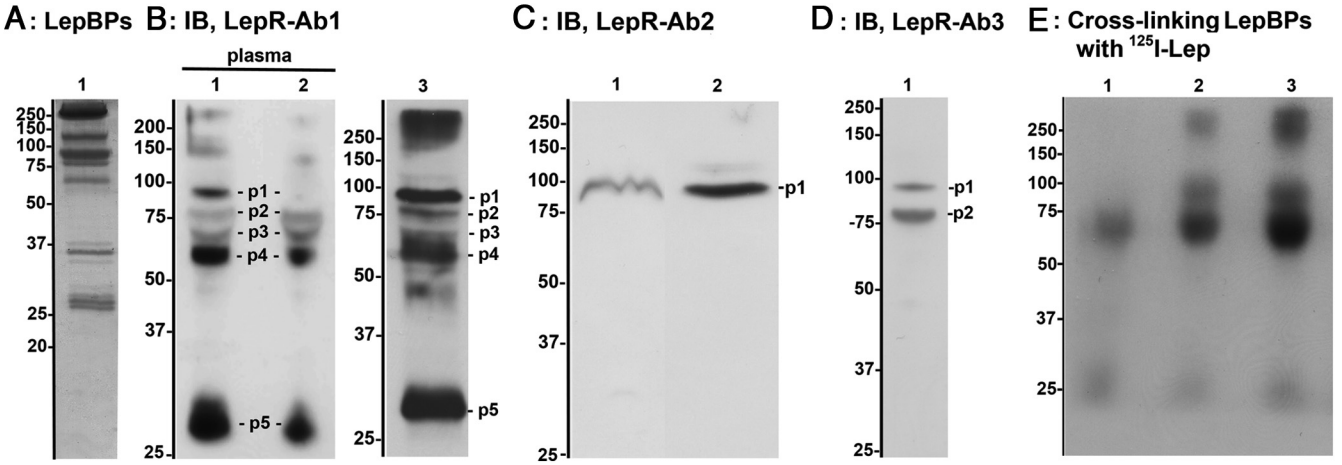

Figure 1. Characterization of LepBPs in Rainbow Trout Plasma. A, The affinity-purified LepBPs (lane 1) were stained by Coomassie brilliant blue. B, LepR-Ab1 immunoblotting (IB) of plasma (1 μL in lane 1; 0.5 μL in lane 2) and affinity-purified LepBPs (lane 3); 5 protein bands were detected (p1–5). C, LepR-Ab2 immunoblotting of plasma (lane 1) and affinity-purified LepBPs (lane 2); D, LepR-Ab3 immunoblotting of plasma (lane 1). E, Cross-linking assays of 1) plasma, 2) affinity-purified LepBPs, and 3) the pooled fractions 90–115 from the SEC; autoradiographic identification of [^{125}I]Lep bound proteins. Protein markers, kDa.

of plasma and the isolated LepBPs with different antibodies against LepR revealed different types of LepBP. The LepR-Ab1 positively recognized 5 bands near 90 kDa (LepBP1, Figure 1B), 80 kDa (LepBP2), 70 kDa (LepBP3), 60 kDa (LepBP4), and 30 kDa (LepBP5). LepR-Ab2 recognized only LepBP1 (Figure 1C). LepR-Ab3 detected LepBP1 and LepBP2 (Figure 1D).

Dynamic binding of [^{125}I]Lep tracer to rainbow trout LepBPs in SEC

Affinity-purified LepBPs bound to [^{125}I]Lep were subjected to SEC. The radioactive fractions from SEC formed a broad elution profile between 30 and 200 kDa (Figure 2A). This includes 2 peaks at 70 kDa and 100 kDa. Cold unlabeled Lep was added as a competitor to [^{125}I]Lep. The radioactivity in the peaks decreased when more Lep was added, and the reduction approached a plateau after 10 nM Lep (Figure 2, A and B). Using the Cheng and Prussoff equation $K_d = K_i = IC_{50} - [\text{Radio-ligand}]$ (36), the average dissociation constant of LepBPs with Lep was calculated to be 2 nM (Figure 2B). Lep used in the assay was a recombinant protein with proven bioactivity (8), and the purity was about 60% determined by SDS-PAGE analysis.

Identification of [^{125}I]Lep bound proteins by cross-linking assay

Plasma, affinity-purified LepBPs, and the pooled radioactive SEC fractions were subjected to cross-linking assays. The radiography film revealed dense bands ranging from 70 to 100 kDa (Figure 1E). A band (>250 kDa) was observed at the top of separating gel, possibly due to protein aggregation. The band near 70 kDa was shown in all of the assays. The band near 100 kDa was barely detected in the assay with plasma sample, but was shown in the analysis of concentrated LepBP samples.

Sequences of rainbow trout *LepR* variants

The long-form rainbow trout $LepR_L$ (GenBank JX878485) shares 90% amino acid similarity to Atlantic salmon *LepR* (BAI23197.1). It contains the 3 essential segments for full function (Figure 3). The extracellular segment contains 3 fibronectin type 3 (FNIII) domains and one Ig-like C2-type domain. The intracellular segment includes two Janus kinase motifs and a signal transducer STAT motif.

Three shorter *LepR* variants (JX878487; KC628721; JX878486) were obtained in RACE PCR and termed $LepR_{S1}$, $LepR_{S2}$, and $LepR_{S3}$, respectively. Different splice sites are selected in the generation of $LepR_S$ transcripts, which encode only the extracellular segment. They have differences in the 3′-end sequence, which contains

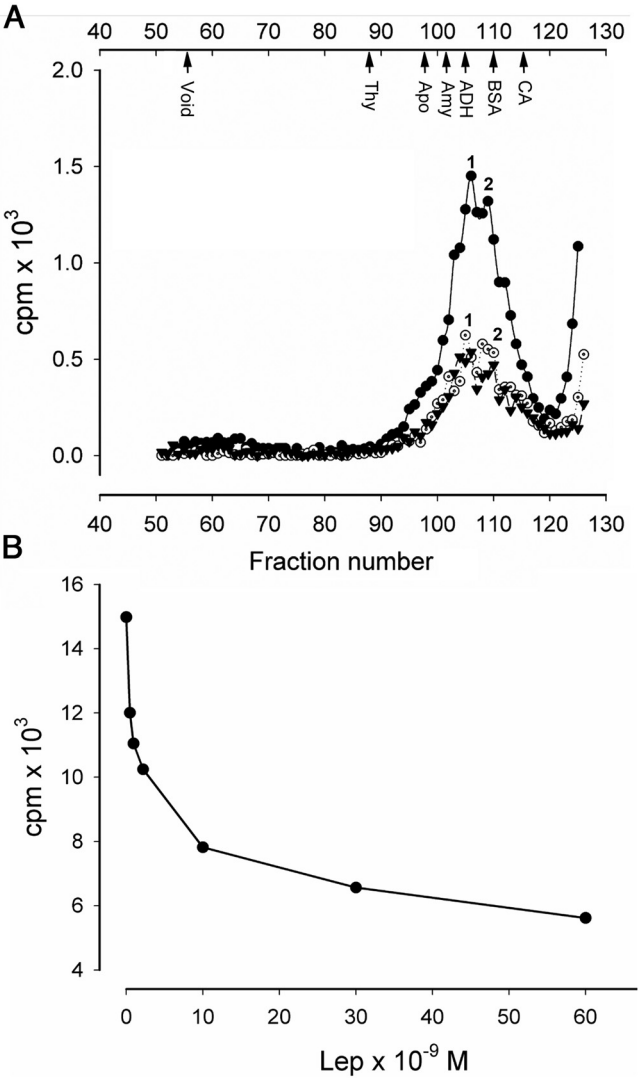

Figure 2. Analysis of Dynamic Binding between Rainbow Trout LepBPs and [^{125}I]Lep by SEC and Competitive Binding Assay. A, A broad elution profile was formed from 443–29 kDa, with peak 1 at 100 kDa and peak 2 at 70 kDa. The SEC column was calibrated with gel filtration markers, thyroglobulin (Thy), apoferritin (Apo), β-amylase (Amy), alcohol dehydrogenase (ADH), BSA, and carbonic anhydrase CA. Free iodinated Lep eluded after CA. Competitive binding assay: no unlabeled Lep added (black circle), addition of cold unlabeled Lep in concentrations of 30×10^{-9} M (empty circle), and 60×10^{-9} M (triangle). B, The radioactivities (cpm) of the pooled SEC fractions in the competitive binding assays after the additions of unlabeled Lep in different concentrations. The curve approaches a plateau equal to nonspecific binding. The IC_{50} (inhibitory concentration 50%) was calculated to be 2.2×10^{-9} M from the curve.

a stop codon and a polyadenylation signal (Figure 3). Amplification and sequencing of the full-length gene variants showed the same 5′-sequence and no intron insertion (Supplemental Figure 2 and Figure 3). Thus alternative 3′-splicing junction is the only event found in the generation of LepR variants. $LepR_{S1}$ encodes all functional domains in the extracellular region. $LepR_{S2}$ and $LepR_{S3}$ transcripts contain fewer exons. $LepR_{S2}$

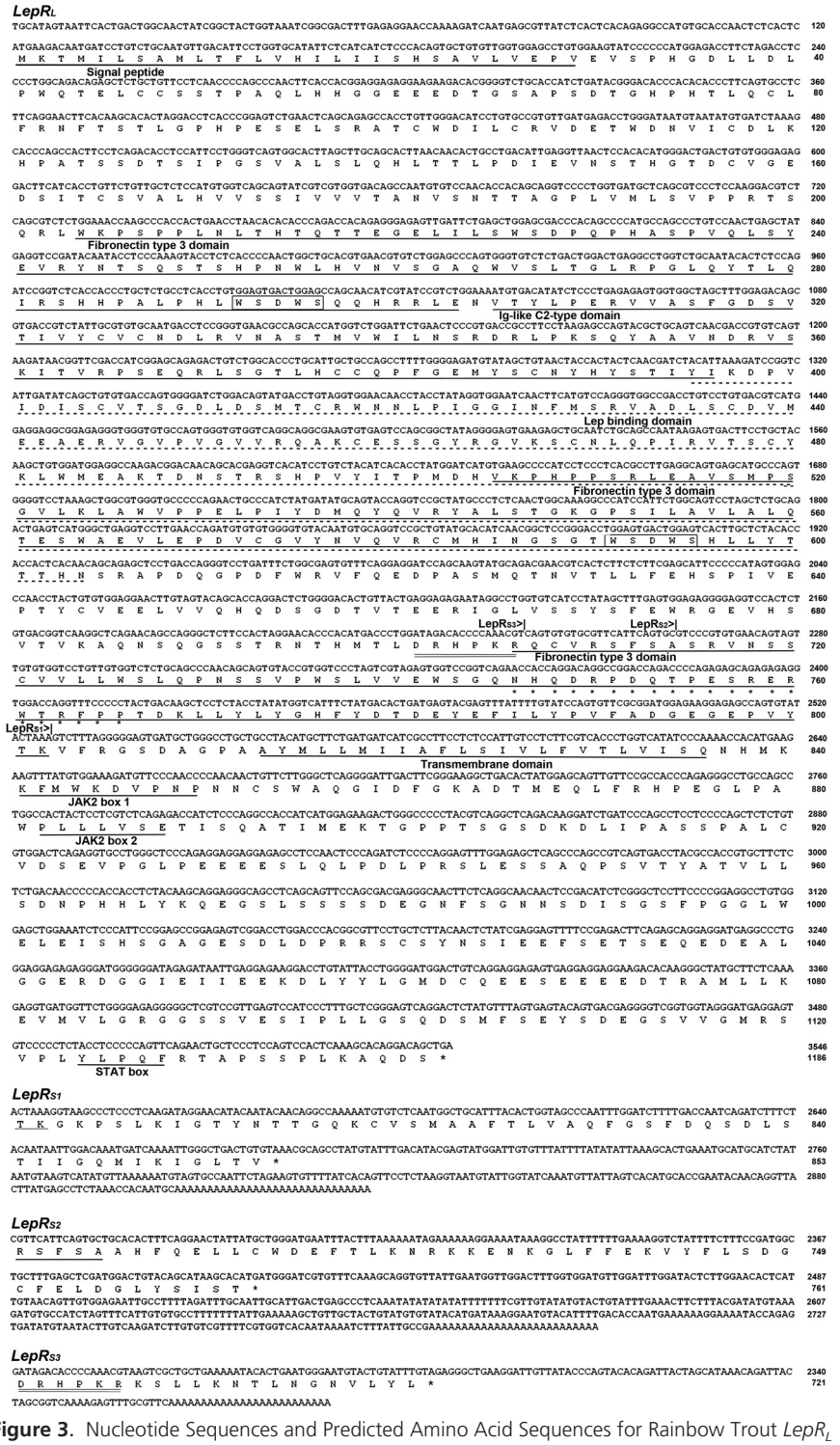

Figure 3. Nucleotide Sequences and Predicted Amino Acid Sequences for Rainbow Trout $LepR_L$ (GenBank JX878485), $LepR_{S1}$ (JX878487), $LepR_{S2}$ (KC628721), and $LepR_{S3}$ (JX878486). Signal peptide was estimated using the SignalP Ver. 4.0 program. Underlined amino acid sequences denote conserved domains. The conserved WSXWS repeated tryptophan/serine motifs for LepR are boxed. The sequence of the recombinant LepR395/604 in Lep binding domain is marked by a broken line. The polypeptide against LepR-Ab2 is marked by asterisks. The alternative 3′-splice sites are indicated by >.

isoform includes the first 15 amino acids of the third FNIII domain, whereas $LepR_{S3}$ isoform excludes the third FNIII domain.

Distribution of rainbow trout *LepR* variants in tissues

$LepR_L$ had wide expression in the examined tissues, significantly in brain cerebellum, pituitary gland, hypothalamus, liver, belly-flap, adipose, gill, kidney, and spleen (Figure 4). $LepR_{S1}$ was lowly expressed in pituitary gland, hypothalamus, liver, belly-flap, adipose, stomach, and midgut (Figure 4). $LepR_{S2}$ was primarily expressed in adipose (Figure 4). $LepR_{S3}$ was strongly expressed in cerebellum and spleen, and moderately expressed in gill and liver (Figure 4).

Expression of rainbow trout $LepR_{S1}$ and $LepR_{S3}$ during 3-week fasting

Expression of $LepR_{S1}$ and $LepR_{S3}$ in hypothalamus and liver was quantified by real-time PCR, and the reaction efficiency was higher than 98%. $LepR_{S1}$ and $LepR_{S3}$ expression was stable in fed rainbow trout, whereas $LepR_S$ expression varied in fasted fish (Figure 5). Hepatic $LepR_{S3}$ expression increased after 2 weeks, and hypothalamic $LepR_{S1}$ expression showed a similar tendency. After 3-week fasting, expression of hypothalamic $LepR_{S1}$ and hepatic $LepR_{S3}$ seemed to decrease. No significant change was found in hypothalamic $LepR_{S3}$ expression in fasted fish. Hepatic $LepR_{S1}$ expression was too low to be accurately quantified.

Plasma LepBP levels in fed and fasted rainbow trout

The logit-log standard curve was linear between 65.5 and 1000 ng mL^{-1}, and dilution series of Atlantic salmon and rainbow trout plasma showed good parallelism with the standard series (Figure 6). This indicates that the RIA is valid for plasma LepBP assessment for both species. ED_{80}, ED_{50}, and ED_{20} were 15.5, 144, and 2200 ng mL^{-1}, respectively. Non-specific binding was less than 3% and specific binding was 25%–30%. Intra- and interassay variability (CV%) was

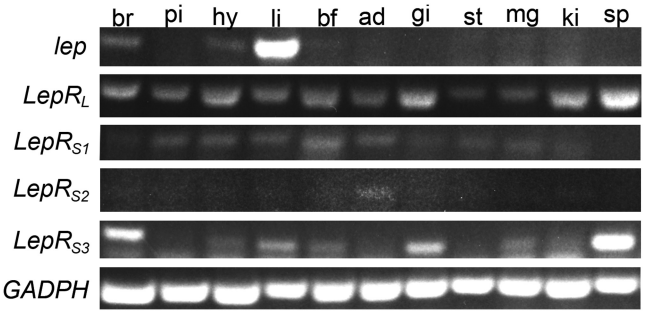

Figure 4. Amplification of *lep*, $LepR_L$, $LepR_{S1}$, $LepR_{S2}$, and $LepR_{S3}$ from Brain Cerebellum (br), Pituitary Gland (pi), Hypothalamus (hy), Liver (li), Belly Flap (bf), Adipose (ad), Gill (gi), Stomach (st), Midgut (mg), Kidney (ki), and Spleen (sp) of Rainbow Trout. Glyceraldehyde 3-phosphate (GADPH) was used as the control.

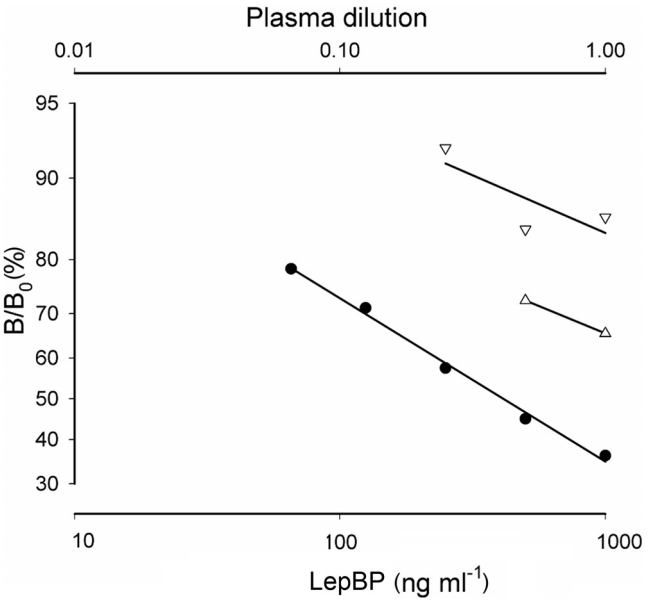

Figure 6. LepBP RIA validation for rainbow trout (Δ) and Atlantic salmon (▽), with plasma dilutions parallel to the standard curve; ranging from 65.5 to 1000 ng mL^{-1}.

8.4% and 12.8%, respectively. Serial addition of antigen to plasma was correctly measured, indicating no interference in the RIA by unknown plasma components.

Plasma LepBP levels did not differ between the fed and fasted rainbow trout during the first 2 weeks. However, after 3-week fasting, the plasma LepBP levels diverged significantly ($P < .05$) with the fed fish having higher (56.6 ± 7.0 ng mL^{-1}; n = 7) levels than the fasted fish (30.4 ± 3.4 ng mL^{-1}; n = 8) (Figure 5D). After 3 weeks, the fasted fish weighted significantly less (79%) than the fed fish ($P < .0001$).

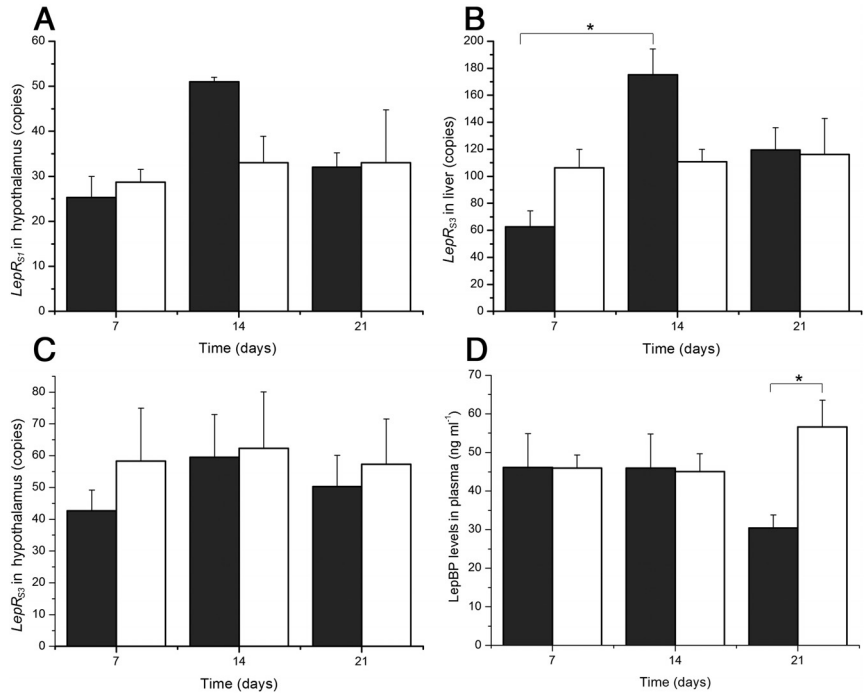

Figure 5. $LepR_S$ Gene Expression (A–C) and Plasma LepBP Levels (D) in Rainbow Trout during a 21-Day Fast. Gene copies (n = 100) present in 150 μg cDNA. White bars represent fed fish and black bars represent fasted fish. An asterisk denote significant differences ($P < .05$).

Discussion

The present study details the characterization of multiple plasma LepBPs in rainbow trout, an early poikilothermic vertebrate, and presents the complete coding sequences for 3 of the LepBPs. Affinity chromatography was used to identify 5 proteins (30–90 kDa) with immunoreactivity to LepR-Ab1 raised against the recombinant Lep binding domain of rainbow trout LepR. This indicates that these LepBPs are LepR isoforms. The 90-kDa LepBP1 and the 80-kDa LepBP2 have immunoreactivity to LepR-Ab3 raised against the recombinant third FNIII domain, indicating that these proteins contain the extracellular segment and should therefore have similar Lep binding capability as the $LepR_L$. Thus, the protein-Lep complexes identified at around 100 kDa represent LepBP1 and LepBP2 bound to Lep (14 kDa) at a 1:1 ratio, similar to the Lep ligand-receptor binding in mammals (34). Similarly, the protein-Lep complexes identified in the range of 70–80 kDa are mostly composed of the smaller LepBPs, such as the 70-kDa LepBP3 and the 60-kDa LepBP4. However, the 30-kDa LepBP5 bound to rtLep at 1:1 ratio is not within the deter-

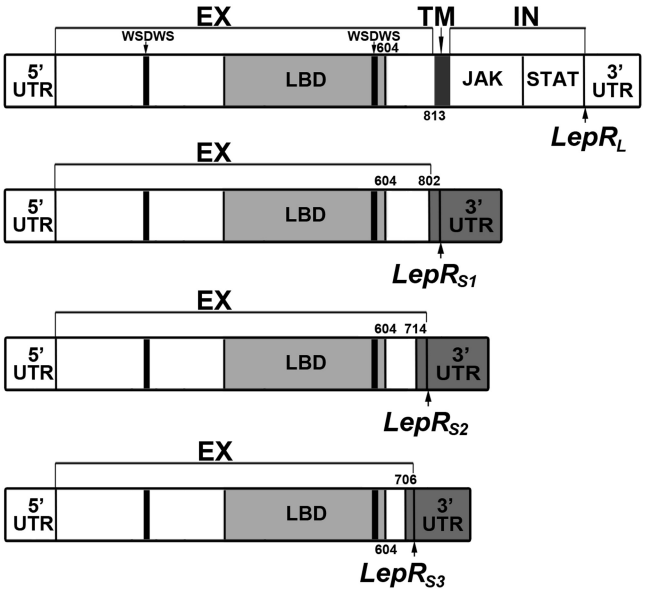

Figure 7. Schematic Description of the Sequences of the Long-Form $LepR_L$ and the Shorter Variants ($LepR_{S1}$, $LepR_{S2}$, and $LepR_{S3}$). $LepR_L$ includes the 5′-untranslated region (UTR), the extracellular segment (EX), the transmembrane segment (TM), the intracellular segment (IN), and 3′-UTR. IN includes two Janus kinase boxes and one signal transducer and activator of transcription box. All the genes include the Lep-binding domain (LBD, in gray) within EX. WSDWS refers to the conserved WSXWS repeated tryptophan/serine motif; vertical arrows mark stop codons. The shadowed region at the 3′ denotes the differentiated 3′-end sequence of $LepR_S$. Arabic numerals refer to the amino acid sequences.

mined mass range and thus possibly has a different stoichiometry. The calculated dissociation constant (2 nM) is close to that reported for Lep binding affinity in human serum (750 pM; Ref. 29), indicating that rainbow trout LepBPs have similar binding affinity to Lep.

The characterized LepBPs (30–90 kDa) have lower molecular mass than the LepBPs in mouse and human (110–150 kDa; Refs 29 and 31). Mouse Ob-Re, which is the smallest LepR isoform, contains no transmemebrane or cytoplasmic segments and functions as a LepBP in the circulation (31, 33, 38). No Ob-Re transcript has been found to exist in humans. Instead, human LepBPs are generated by proteolytic cleavage and ectodomain shedding of membrane-spanning receptors (28, 32). In fish, alternative 3′-splicing junction events have been found to produce more than 1 transcript that contains only the extracellular domains (9, 35). In rainbow trout, alternatively spliced *LepR* transcripts are the major source of LepBPs.

Three short-form *LepR* ($LepR_{S1}$ through $LepR_{S3}$) transcripts identified in the present study differ from the $LepR_L$ in that they skip the transmembrane and cytoplasmic segments and thus mostly produce soluble proteins (Figure 7). $LepR_{S1}$ transcript contains all the extracellular domains and can synthesize a protein of 91 kDa, which includes the epitopes to all 3 LepR antibodies generated. This suggests that $LepR_{S1}$ isoform corresponds to LepBP1. The shorter $LepR_{S2}$ encodes the first 714 amino acids of LepR, and the first 15 amino acids of the third FNIII domain are included, which can be identified by LepR-Ab3. Thus $LepR_{S2}$ isoform with the predicted molecular mass of 81 kDa has immunoreactivity to both LepR-Ab1 and LepR-Ab3, but not to LepR-Ab2. These characteristics correspond to the 80-kDa LepBP2, indicating that $LepR_{S2}$ codes for LepBP2. The shortest variant, $LepR_{S3}$, encodes the Lep binding domain, but excludes the sequence for the third FNIII domain. Thus the isoform expressed by $LepR_{S3}$ has no epitopes recognized by LepR-Ab2 and LepR-Ab3. The protein from the open reading frame has the molecular mass closest to LepBP3. These data suggest that $LepR_{S3}$ transcript generates LepBP3. No variant shorter than $LepR_{S3}$ was found in the RACE PCR. It is thus currently unclear how the 60-kDa LepBP4 is generated. It may be a LepR isoform translated from $LepR_{S3}$, but modified after transcription. Alternatively, the smaller LepBPs may be generated through ectodomain shedding or proteolytic cleavage of the membrane-spanning LepR isoform (28, 32), or by LepR-overlapping transcript (12, 37).

LepBPs usually have different physiologic functions from the full-functional LepR, such as to bind Lep in the circulation, to transport Lep through the blood-brain barrier, and to regulate LepR signaling activation negatively (29–31, 38). The present study demonstrates that the 4 *LepR* variants have different expression patterns in tissues, indicating that they may have different physiologic functions. The full-functional $LepR_L$ is extensively expressed in all of the examined tissues, suggesting a broad effect of Lep in a wide variety of tissues, as earlier indicated for the LepR in Atlantic salmon (9). $LepR_{S1}$ is widely expressed, but the mRNA abundance is lower than $LepR_L$, indicating $LepR_{S1}$ isoform may be an important negative regulator for $LepR_L$ signaling in rainbow trout tissues. $LepR_{S2}$ has trace expression in most of the examined tissues, except in adipose tissue, where it is higher. This reveals a possible regulation of $LepR_{S2}$ in lipid metabolism. *lep* is strongly expressed in liver and moderately expressed in cerebellum. The abundance of $LepR_{S3}$ mRNA in cerebellum and liver may be correlated to Lep production in the tissues.

Because the antibody LepR-Ab1 used in the LepBP RIA recognizes the leptin binding domain of the LepR, the assay recognizes collectively all LepBPs derived from the LepR without being able to distinguish between them. Plasma LepBP levels in the rainbow trout were found to be in the same range (10–100 ng mL^{-1}) as LepBPs in human plasma (39). After fasting for 3 weeks, the plasma LepBP levels declined, concomitant with elevated plasma Lep lev-

els (data not shown). The elevation of plasma Lep levels was similar as in a previous study on fasting rainbow trout in which plasma Lep levels were elevated already after 1 week, and remained high for 3 weeks (19). Thus, in rainbow trout, the endocrine Lep regulation during fasting appears to be geared toward increasing the plasma levels of free Lep. The increasing availability of active Lep would strengthen the anorexigenic effects of Lep on the fish and prevent energy expenditure during food deficiency (21, 40). Although converse, with high Lep levels and low LepBP levels, such an inverse relationship between plasma Lep and LepBP levels is also seen in humans during fasting (39, 41) and thought to be advantageous to restrict the availability of free Lep during nutritional deprivation (42). This strengthens the notion of some fundamental differences in Lep-regulatory mechanisms between endothermic and poikilothermic vertebrates.

The expression of $LepR_{S1}$ in hypothalamus and $LepR_{S3}$ in liver was clearly affected by the nutritional state of the fish, indicating that expression of these genes may influence Lep action, both centrally and peripherally, depending on the physiologic state of the fish.

In conclusion, it appears that the action of Lep in rainbow trout can be regulated by a series of LepR isoforms derived from alternative splicing. This mechanism, which is different from that found in mammals, involves tissue-specific and differentiated gene expression and results in at least 3 LepBPs. The expression of short-form LepR variants, as well as the circulating LepBP levels, is affected by the physiologic state of the fish, indicating the existence of complex regulatory mechanisms of Lep action, both at the central and the peripheral level.

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