Identification and gene expression profile analysis of a major type of lipoprotein lipase in adult medaka *Oryzias latipes*

Lu Wang · Gen Kaneko · Shin-Ichiro Takahashi · Shugo Watabe · Hideki Ushio

Abstract Lipoprotein lipase (LPL) plays a critical role in the metabolism of circulating triacylglycerols (TAGs) packaged in lipoproteins. Fish have two LPL genes, encoding the LPL1 and LPL2 enzymes, but their functions are not yet clearly understood. To provide a basis for further investigation, we cloned a cDNA encoding LPL from the liver of adult medaka *Oryzias latipes*. The cloned LPL gene was found to share a high identity with LPL1 genes from several fish species and was denoted medaka LPL1. Screening against publicly available medaka expressed sequence tag (EST) databases revealed that LPL1 transcripts are abundant in ESTs from various developmental stages and tissues. The EST screening also identified a second LPL-like gene, denoted medaka LPL2, the transcripts of which were found exclusively in EST libraries from embryonic stages. The LPL1 cDNA obtained by a combination of 3′ and 5′ rapid amplification of cDNA ends was 2,259 bp, with an open reading frame of 1,551 bp encoding 516 amino acids. Quantitative real-time PCR further confirmed the ubiquitous distribution of LPL1 transcripts, with high levels in the liver and visceral adipose tissue, and the lowest level in the intestine. A relatively high expression of the LPL1 gene was also found in the brain, suggesting the potential function of LPL1 in this organ.

Introduction

Triacylglycerol (TAG) is the major lipid providing the energy required for metabolic processes in organisms. It is composed of one glycerol molecule esterified with three fatty acids. Due to its poor water solubility, TAG transport in the bloodstream can only occur through the packaging of circulating TAGs in lipoproteins, such as chylomicrons and very low-density lipoproteins [1, 2]. The outer layer of these lipoprotein particles is polar, containing free cholesterols, phospholipids and apolipoproteins, which enables these particles to be transported in the bloodstream [1, 3, 4]. However, due to the large size and water solubility of the lipoprotein particles, they cannot diffuse through plasma membranes. Consequently, partial degradation or receptor-mediated endocytosis is needed for the passage of these lipoproteins and, therefore, TAGs into cells.

Lipoprotein lipase (LPL) is one of the important enzymes involved in the catabolism of circulating lipoproteins. It is responsible for the hydrolysis of TAGs packaged in lipoproteins, providing non-esterified fatty acids to peripheral tissues for either energy storage by re-esterification or energy provision via β-oxidation. LPL is anchored to the luminal surface of blood vessels through its interaction with heparan sulfate proteoglycans and/or glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 [5–8]. In addition to its conventional hydrolytic function, LPL is also known to act as a ligand

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in the uptake of lipoprotein particles via receptor-mediated endocytosis [9, 10]. In their study with LPL knockout mice, Weinstock et al. noted that a null mutation in LPL resulted in hypertriglyceridemia and a low survival rate of newborn mice [11]. These results demonstrated that LPL plays a vital role in the catabolism of circulating TAG-rich lipoproteins.

cDNAs coding for LPL have been cloned and sequenced in a number of vertebrate animals, such as human, rat, mouse, bovine and fish [12–17]. LPL transcripts are widely distributed in mammalian tissues and organs, with relatively high levels in adipose tissues, heart and skeletal muscles, and small amounts in the kidney, lung and small intestine [18, 19]. Interestingly, LPL is expressed in the liver of newborn mammals, but not in adults [20, 21]. In contrast, LPL is expressed in the liver of adult fish, suggesting its functional diversification among vertebrates [16, 22–25]. Moreover, an LPL isoform, called LPL2, has been recently identified in two fish species, red seabream [24] and torafugu [25]. LPL2 transcripts have a widespread distribution in tissues of red seabream as those of LPL1, but the levels of the former are tenfold lower than those of LPL1 in some tissues, including adipose tissue, liver and muscle, suggesting that LPL2 plays a relatively minor role in these tissues.

Medaka *Oryzias latipes* is a small fish species with a body length of about 3 cm. Due to its short generation time of 2–3 months, short spawning cycle and high transparency of eggs, it has been widely used as a model vertebrate animal in molecular biological studies, especially in those focusing on transgenic research [26–28]. In the study reported here, we cloned the cDNA of LPL from the liver of adult medaka and investigated the expression pattern for further understanding of fish LPL function in circulating lipoprotein metabolism.

Materials and methods

Experimental fish

Medaka specimens (orange–red strain) were obtained from a local fish market (Ichigaya Fish Center, Tokyo, Japan) and reared in indoor tanks at a constant water temperature of 28 ± 0.5 °C. Fish were fed with commercial diets (Kamihata Fish Industry Group; Kyorin Co. Ltd., Himeji, Japan) twice a day. The body weight of fish used for cloning was approximately 200 mg, while specimens of 315–410 mg were used for the quantitative real-time PCR.

RNA isolation

Total RNAs were isolated from approximately 10 mg of liver tissue using an RNeasy Lipid Tissue Mini kit (Qiagen,

Hilden, Germany) and stored at -80 °C until further analysis. The quantity and purity of these total RNAs were determined by absorbance measurements at 260 and 280 nm using a nanophotometer (Implen GmbH, Munich, Germany). The integrity of RNA was assessed by 1 % agarose–formaldehyde denaturing gel electrophoresis.

cDNA cloning of medaka LPL

First-strand cDNA was synthesized using 1 μg of extracted total RNA as template and the GeneRacer Oligo $(dT)_{24}$ primer with SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

For amplification of a partial fragment of the LPL gene, including the start codon and 3′ untranslated region (UTR), we first performed alignment searches against medaka expressed sequence tag (EST) databases (http://www.shigen.nig.ac.jp/medaka/top/top.jsp) using the TBLASTN program. Amino acid (AA) sequences of *Siniperca chuatsi* LPL (GenBank accession no. ACZ55136), *Pagrus major* LPL1 and LPL2 (BAE95413 and BAB20997, respectively) and *Takifugu rubripes* LPL1 and LPL2 (AB735414 and AB735415, respectively) were used as reference sequences. A specific primer, LPL-F (Table 1), was designed based on the EST sequences of medaka LPL using Oligo version 7.37 software (MBI, Cascade, CO).

PCR assays were then carried out, each in a 20-μl volume reaction mixture consisting of approximately 80 ng first-strand cDNA, 0.12 μM LPL-F and GeneRacer 3′ primers and 0.4 U of KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan). The cycling parameters for amplification were determined as recommended by the manufacturer. Nested PCR was carried out by the same method except that tenfold-diluted products of the first PCR were used as a template and the GeneRacer 3′ primer was replaced by the GeneRacer 3′ nested primer.

Amplified DNA fragments were gel purified using a QIAquick Gel Extraction kit (Qiagen) and cloned into the pCR-Blunt II-TOPO vector (Invitrogen) according to the manufacturer's instructions, followed by the transformation of JM109 competent cells. Purified plasmids were sequenced by an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

Rapid amplification of the 5′ end

To obtain the 5′ end, first-strand cDNA was synthesized with the 5′-rapid amplification of cDNA ends (RACE) CDS primer (Table 1) according to the manufacturer's instructions (SMART™ RACE cDNA Amplification kit; Clontech, BD Biosciences, Palo Alto, CA). The gene-specific primers for amplification of the 5′ end of the cDNA were designed based

Table 1 Primers used for molecular cloning and expression analysis of medaka lipoprotein lipase

Primer name	Sequence 5′ → 3′
3′-RACE	
GeneRacer Oligo dT	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)$_{24}$
LPL-F	ATGGGAAAGGAACTCCTCTGC
GeneRacer3′	GCTGTCAACGATACGCTACGTAACG
GeneRacer3′ nested	CGCTACGTAACGGCATGACAGTG
5′-RACE	
SMART II A oligonucleotide	AAGCAGTGGTATCAACGCAGAGTACGCGGG
5′-RACE CDS A	(T)$_{25}$V N (N = A, C, G or T; V = A, G or C)
LPL-5R	GCTCTCAAACATCCCCGTTACCGTCCAG
LPL-5RN	AGCCGTGTATGACGATGAAGGTCTGAG
Nested universal	AAGCAGTGGTATCAACGCAGAGT
Real-time PCR	
LPL_F	TCCACCTGTTCATCGACT
LPL_R	AGCTTGTTGCAGCGGTTC
LPL_probe	(VIC)-TTGCAGCGGTAGGCCATGCTC-(TAMRA)
RPL-7_F	AGCTCTCGGCAAATATGGCATC
RPL-7_R	GTGTGGTCTTCTTGTTCATTCCTC
RPL-7_probe	(FAM)-TTCCTGTGGCCCTTCAAGCTGTCGT-(TAMRA)

3′, 5′ RACE 3, 5′ Rapid amplification of cDNA ends, respectively; *LPL* lipoprotein lipase; *RPL-7* ribosomal protein L-7

on the obtained partial sequence of the LPL gene (Table 1). Each PCR assay was carried out a 20-µl volume reaction mixture consisting of approximately 100 ng first-strand cDNA, 0.4 µM LPL-5R primer and nested universal primers (Clontech, BD Biosciences) and 0.5 U Ex Taq DNA polymerase (Takara, Otsu, Japan). After an initial denaturation at 95 °C for 2 min, 35 cycles of PCR were carried out with denaturation at 94 °C for 30 s, annealing at 68 °C for 30 s and extension at 72 °C for 2 min; the final extension step was performed at 72 °C for 7 min. The tenfold-diluted PCR products were used as a template for the second PCR that was carried out under the same conditions except for the primer LPL-5RN. PCR products were cloned into the pCR4-TOPO vector (Invitrogen) and sequenced as described above.

Sequence analyses

The sequence of medaka LPL was obtained through the assembly of the above sequences using SeqMan II software (DNASTAR, Inc., Madison, WI). A multiple alignment of AA sequences was made using Clustal W [29]. The presence of the signal peptide and of potential N-linked glycosylation sites were predicted with SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and NetNGlyc version 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively. The conserved domains of the LPLs were identified by conserved domain database search (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The phylogenetic tree based on LPL AA sequences was constructed by the maximum likelihood (ML) method of MEGA software version 5.10 [30] with the JTT + G model, which was

determined by the evaluation of best-fit substitution. Bootstrap analysis (10,000 replicates) was used to estimate the branches of the tree.

Real-time PCR assay for LPL transcripts

The expression of LPL at the mRNA level was determined in various tissues of medaka by quantitative real-time PCR. The ribosomal protein L-7 (RPL-7) gene was used as the internal control because of its relatively constant expression in various tissues of medaka [31]. Gene-specific primers and TaqMan probes were designed based on the LPL nucleotide sequence identified in this study, and the sequence of RPL-7 obtained from the GenBank database (DQ118296). Total RNAs were extracted from visceral adipose tissue, brain, gill, heart, intestine, liver and muscle of medaka. First-strand cDNA was synthesized from each tissue sample ($n = 10$), as described above, except for the GeneRacer Oligo (dT)$_{24}$ primer substituted by a blend of oligo (dT)$_{18}$ and random hexamer. The reaction system for real-time PCR was prepared according to the manufacturer's protocol of the TaqMan Fast Advanced Master Mix kit (Applied Biosystems), followed by running on the ABI 7300 Real Time PCR System (Applied Biosystems). The relative LPL mRNA levels were determined by the comparative C_T method.

Statistical analysis

Data were analyzed by one-way analysis of variance, followed by the Tukey–Kramer HSD test using the statistical program JMP version 9 (SAS Institute Inc., Cary, NC).

Table 2 The identities of deduced amino acid sequences of medaka expressed sequence tags compared to those of medaka LPL1, torafugu and red seabream LPLs 1 and 2 and mandarin fish LPL

Medaka ESTs	Accession number	Tissue	Developmental stage	Reference sequence	Identities (%)
olea56p20	DK064272	Embryo	Stage 22	Medaka	50.7
				Mandarin fish	52.8
				Red seabream LPL1	52.8
				Red seabream LPL2	78.9
				Torafugu LPL1	57.6
				Torafugu LPL2	73.7
roleb56p14	DK132162	Embryo	Stage 35	Medaka	45.4
				Mandarin fish	46.8
				Red seabream LPL1	45.9
				Red seabream LPL2	79.7
				Torafugu LPL1	43.7
				Torafugu LPL2	74.0
MF015DA031e09	BJ736075	Embryo library	Stage 35 whole embryos mRNA	Medaka	100.0
				Mandarin fish	89.7
				Red seabream LPL1	87.2
				Red seabream LPL2	52.1
				Torafugu LPL1	85.0
				Torafugu LPL2	53.6
MF01FSA036n23	BJ500216	Fry library	Stage 40 whole embryos mRNA	Medaka	99.3
				Mandarin fish	80.1
				Red seabream LPL1	80.1
				Red seabream LPL2	35.3
				Torafugu LPL1	73.9
				Torafugu LPL2	30.9
olvl25a13	DK234942	Male liver	Adult males	Medaka	100.0
				Mandarin fish	78.4
				Red seabream LPL1	73.2
				Red seabream LPL2	50.0
				Torafugu LPL1	71.8
				Torafugu LPL2	57.0
olli49h22	–	Female liver	Adult females	Medaka	100.0
				Mandarin fish	78.3
				Red seabream LPL1	73.1
				Red seabream LPL2	50.0
				Torafugu LPL1	71.7
				Torafugu LPL2	57.0
olsp54e15	–	Spleen	Adult males and females	Medaka	99.5
				Mandarin fish	78.0
				Red seabream LPL1	72.7
				Red seabream LPL2	49.8
				Torafugu LPL1	71.3
				Torafugu LPL2	56.7
olbr63h04	DK021694	Brain	Adult males and females	Medaka	100.0
				Mandarin fish	77.2
				Red seabream LPL1	72.1
				Red seabream LPL2	47.3
				Torafugu LPL1	70.1
				Torafugu LPL2	54.2

Table 2 continued

Medaka ESTs	Accession number	Tissue	Developmental stage	Reference sequence	Identities (%)
olova59e14	DK202485	Ovary	Adult females	Medaka	100.0
				Mandarin fish	77.0
				Red seabream LPL1	71.9
				Red seabream LPL2	47.3
				Torafugu LPL1	70.3
				Torafugu LPL2	54.2
olte29l06	FS518889	Testis	Adult males	Medaka	100.0
				Mandarin fish	77.1
				Red seabream LPL1	72.4
				Red seabream LPL2	48.1
				Torafugu LPL1	70.3
				Torafugu LPL2	55.3
olgi31a18	–	Gill	Adult males and females	Medaka	35.8
				Mandarin fish	33.6
				Red seabream LPL1	34.3
				Red seabream LPL2	41.5
				Torafugu LPL1	37.5
				Torafugu LPL2	40.0

EST expressed sequence tag

Results

Identification of medaka LPL isoforms from ESTs

The medaka EST database search revealed several ESTs that shared high identities with the LPL1 and LPL2 genes from other fish species (Table 2). We used the conserved sequence of olvl25a13 and olli49h22 for primer design because these ESTs were obtained from the adult liver, whereas the other ESTs were from fry, embryo and other tissues. However, our LPL sequence was later revealed to be almost identical (>99 %) with that of other ESTs (MF015DA031e09, MF01FSA036n23, olsp54e15, olbr63h04, olova59e14 and olte29l06), suggesting that the LPL gene cloned in this study is the single and major form expressed during various developmental stages and in various tissues of medaka. Taken together with the results of the alignment and phylogenetic analyses (see below), we denoted this gene medaka LPL1. In comparison, ESTs sharing high identities with LPL2 genes were obtained only from embryonic EST databases (olea56p20 and roleb56p14). It is thus likely that medaka has a second LPL gene (LPL2) which may be mainly expressed in embryonic tissues.

cDNA cloning of medaka LPL1

The PCR assay using the LPL-F primer and GeneRacer 3′ nested primer resulted in specific amplification of a 2,157-bp product containing the whole coding and 3′ UTR regions. 5′ RACE also amplified a single PCR product of 479 bp. These sequences shared a 304-bp overlapping region in which no sequence mismatch was found. 5′ RACE using another gene-specific primer yielded a PCR product with a 1,016-bp overlap identical with the 3′ RACE product (data not shown). Because of the low nucleotide identity between the LPL1 and LPL2 genes and the absence of other LPL isoform genes in the EST- and PCR-amplified fragments, we assembled these sequences to obtain the long medaka LPL1 cDNA sequence that extended from the 5′ UTR to the 3′ UTR. This sequence was 2,259 bp in length [excluding the poly(A) tail] with an open reading frame of 1,551 bp encoding a 516-AA protein, a 145-bp 5′ UTR and a 563-bp 3′ UTR. One AAAATA motif representing the putative polyadenylation signal was found downstream of the 3′ region. This cDNA sequence was deposited in the DDBJ/EMBL/GenBank databases under accession number AB698560.

AA sequence analysis of medaka LPL1

A theoretical molecular mass of 57.98 kDa and isoelectric point (pI) of 7.26 for medaka LPL1 protein were obtained using ExPASy web tools. Based on the conserved domain database search, we determined that medaka LPL1 consists of two structural regions corresponding to LPLs from other species (Fig. 1), i.e., the N-terminal (24–363 residues) and C-terminal domains (364–516 residues). A short polypeptide of 23 AAs, considered to be a putative signal peptide, was

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Oryzias latipes            ----MGKELL CFFTLWITLG NISATFSSDL ES-------- -NTTSLFVNS TTTAAPLPTT TEWMTDYADI LSKFSLRTAD   67
Pagrus major LPL1          ----...NI S.L.V..I.. K.F......P .P--TTTSSS SSSSTV...T .I..T..... ...I...T.. V.........   74
Pagrus major LPL2          -MKAWRVVF. Y.LV.NAVVQ HVTSLEEELS D.-------- -------IF GNFLD..KDL I.HKD.ANQT VA.....KPS   63
Takifugu rubripes LPL1     ----..IKNI F......I.. KFC...A.EP DT-------- -G.DAE...T SLPGT..... AD.INNFT.. V........E   67
Takifugu rubripes LPL2     -MKAWKSGVV FILV.NAAVC HVTTLEGELA D.-------- -------I. GNFLD.IKDL LNDRD.SNQT VAR....KPS   63
Siniperca chuatsi          ----..NI .L.V..I.. K.F......P .P------T T---TV...T .V..T..L.. A..I..T... V........G.   66
Danio rerio                MMFNK.RVSS A.LIS.MYFV Y..SGLETTI DP-------- ------TAE. I.LSDIIGNA ....M.FT.. E....F..LE   66
Sparus aurata              ----...NI S.L.V..I.. K.F......P .PNTTTTSSS SSSSTV...T .I..TA.... A..I...T.. V.........   76
Oncorhynchus mykiss        ----..NT FLV.V..I.A ..CVS...TP .Q-------- ----T..G.. --------NS ..LE..T... V........E   56
Dicentrarchus labrax       ----...NI S.L.V..I.. K.F......P .P------T TSNSTV...T .V..T..... A..I..FT.. V........SE   69
Mus musculus               ----.ESKA. LLVV.GVW.Q SLT.FRGG-- ---------- ---------- -------VAA ADAGR.FS.. E...A...PE   47
Homo Sapiens               ----.ESKA. LVL..AVW.Q SLT.SRGG-- ---------- ---------- -------VAA ADQRR.FI.. E...A...PE   47
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Oryzias latipes            IPDEDMCYIQ AGHPETIKDC QFNAEAQTFI VIHGWTVTGM FESWVPKLVS ALYERVPNAN VIVVDWLTRA SQHYPTSASY  147
Pagrus major LPL1          ...D.....V ..S.D..E.. T..E..P.T. ..........L ......LS.. .........A .........E.S. ..........N.......A.  154
Pagrus major LPL2          H..D.L...V P.K.DSLAA. T..SSSK..L ......LS.. .........A .........EQT. .........S. QN..VVA.QN  143
Takifugu rubripes LPL1     ...D.....V P.R....SE. H..PDT...V ......... .........E.S. ..........N....M..AF  147
Takifugu rubripes LPL2     H..A.Q...V P.RAD.LAA. T..RTSK..M ......LS.. .........S..... ........EHG. .........L. QN..VLA.QK  143
Siniperca chuatsi          ...D.....V ..R.....E. E..S.T.... .......... .........E.S. ..........N.......A.  146
Danio rerio                E.ED.L...V P.Q.QS.... N..T.TK... .......... .........T ......E.S. .........S.. Q........  146
Sparus aurata              ...D.....V P.K.D...E. E..N.T.S.. .......... .........E.S. ..........N.......A.  156
Oncorhynchus mykiss        ...D.L...V P.Q.S..PK. E..PGYK..V .........L ......T ...K.E.K. ..........Q...L..AN  136
Dicentrarchus labrax       ...D.....V ..R.....E. E..SAT.... .......... .........E.S. ..........N.......A.  149
Mus musculus               DTA..T.HLI P.LADSVSN. H..HSSK..V .........Y .........A ...K.E.DS. .........Y.. Q....V..G.  127
Homo Sapiens               DTA..T.HLI P.VA.SVAT. H..HSSK..M .........Y .........A ...K.E.DS. .........S.. QE...V..G.  127
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Oryzias latipes            TKLVGRDVAK FVTWLQNELQ LPWDRIHVLG YSLGAHVAGV AGHLTDNKIS RITGLDPAGP SFEHADDQST LSRDDGQFVD  227
Pagrus major LPL1          .......... .....I.K..H .......... .........I ..D..EH... .......... T...N..N.. ..P..A...  234
Pagrus major LPL2          ..A..QEI.R .ID.IEETTN M.LEN..LI. .........F ..SHAT..VG .......... D..GMHAHRR ..P..AH...  223
Takifugu rubripes LPL1     .......... .....S.I...N ..E...L.. .........I ..D..H.... .......... T...N..N.. ..P..AK...  226
Takifugu rubripes LPL2     ..A..QEI.R .ID.IEESTN A.VEN..LI. .........F ..SHVT..VG .......... D..GMHAHRR ..P..AH...  223
Siniperca chuatsi          .......... .....I.K.. ..E...L... .........I ..D..H.... .......... T.....N.D. ..K..A...  226
Danio rerio                ......K.... ..N..A.ID Y..EKL.L.. .........I ..L..KH.VN ......M.... T..Y..SL... ..P..AN...  226
Sparus aurata              .......... .....I.K.. ..E...L... .........I ..D..ER... .......... T...N..N.. ..K..A...  236
Oncorhynchus mykiss        ......K.... ..N...KT.D Y..EK..L.. .........I ..L..NH.V. .......... T..F..A... ..P..AL...  216
Dicentrarchus labrax       .......... ....E.V.L. .......... .........D ..H....... .......... T.....N.N. .....A...  229
Mus musculus               ......N...R .IN.MEE.FN Y.L.NV.L.. ......A... ..S..NK.VN .......... N..Y.EAP.R ..P..AD...  207
Homo Sapiens               ......Q...R .IN.MEE.FN Y.L.NV.L.. ......A..I ..S..NK.VN .......... N..Y.EAP.R ..P..AD...  207
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Oryzias latipes            VLHTNTRGSP DRSIGIQRPV GHIDIYPNGG TFQPGCDIQN TLLGIASAGI KGLQNMDQLV KCSHERSIHL FIDSLVN-TQ  306
Pagrus major LPL1          .......... .......... ..........V ........S ...LE.. .......... .......... .....L.-.E  313
Pagrus major LPL2          ....F....L GL.....Q.. .V....... S....NLRG A.EK..NF.. F---AITDA. ..E....... .....L.-E.  299
Takifugu rubripes LPL1     .......... .....N..... ..V....... ......... ...AE..... H........ .......... .....I.-.E  305
Takifugu rubripes LPL2     ....F....L GL.....Q.. .V....... S....NLRG ..EK..NF.. F---AITDA. ..E....V.. .....L.-E.  299
Siniperca chuatsi          .......... .......... .......... ......... ...LK.. .......... .......... .....L.-..  305
Danio rerio                .......... .......... .......... ......L.. .M.MV.TT.L R---....I. ..........  ......-QD  302
Sparus aurata              .......... .........D .......... ......... ...LE.. .......... .......... .....L.-IE  315
Oncorhynchus mykiss        .......... ....E.V.L. ..V....... ......L.. .MMM..TT.. R---.... ..........  .....AAE  293
Dicentrarchus labrax       .......... .......... .......... ......... ...LE.. .......... .......... .....L.-..  308
Mus musculus               ....F..... G.......K. ..V....... .....N.GE AIRV..ER.L G---DV... ..........  .....L.-EE  283
Homo Sapiens               ....F..... G.......K. ..V....... .....N.GE AIRV..ER.L G---DV... ..........  .....L.-EE  283
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                                   #                    #   #     #
Oryzias latipes            HQSMAYRCNS KEAFNKGVCL SCRKNRCNKL GYNINKVRTA RSTKMYLKTR GMMPYKVFHY QVKAHFFSKS EQSFTEQPMK  386
Pagrus major LPL1          Q.........  ......L... ........... ........RT ..........E ..........V...D PL...D....  393
Pagrus major LPL2          EAAK....G. SDM..R.M.. ....G...TV ..D.S..K..NVQ...T.. AS..FR.Y.. .L.I..S..V NR.EM.PSLT  379
Takifugu rubripes LPL1     Q..V.......  ......L... ........... ..V.Q..T.G.L.... E.......... ...V....ET Q.........  385
Takifugu rubripes LPL2     DAAQ....S. SQT..R.M.. ....S...TV ..D.S..K..NVQ...T.. AT..FR.Y.. .L.I..S..V NR.EM.PSLT  379
Siniperca chuatsi          Q.........  ......L... ........... ........R ..........E ..........V....D QL.......  385
Danio rerio                .E...F..S. RDS....M.. ........V ..AV..R..S...M.. E.......... ...V..G.T QL.Y.D....  382
Sparus aurata              Q..L...... ......L... ........... ........RT ..........E ..........V....D PL......  395
Oncorhynchus mykiss        ..T.....S. ..M..M.. N.........V ..GV...LP.N... E...F.L.. ...V....SE KLAY......  373
Dicentrarchus labrax       Q.........D....L... ........T. .........M. D.......... ...V.I..D SL......  388
Mus musculus               NP.K...... ....E..L.. ........N. ..E.........AK..S... SQ......... ...I..SGTE NGKQHN.AFE  363
Homo Sapiens               NP.K....S. ....E..L.. ........N. ..E.........AK..S... SQ......... ...I..SGTE SETH.N.AFE  363
                                                                     N-Terminal ◄---------  ---------► C-Terminal
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                                                      NXT
Oryzias latipes            ISLFGTHGET EDIAFVLPE- LSCNNTVSFL ITTDVDIGEL MIVKLRWEKD A-------IL SWSDWWGSS- ---KFHIRKL  454
Pagrus major LPL1          ...K.. ..PH...V- MKG.T.L... ........D. .........T-------I ........- ---.......  461
Pagrus major LPL2          V..Y..N..A .NLELK.K.K IAT.K.H... LV.EK..D. LML.FK..ET NGWSTSNMLK MV.S..SGDS DGANMEVH.I  459
Takifugu rubripes LPL1     ...K.. ..P....- .NS.T..... L.....L.D. .........T-------I ........S ---RV....  454
Takifugu rubripes LPL2     V..Y..K..A .NLELK.K.K IAV.K.H... LV.EK.V.D. LMLTFK..ET NSWSSSRLLK MV.S..GDS GGADVQVH.I  459
Siniperca chuatsi          ...Y..Q..K ..S....YG ..NG.T.L... L.....D. .--------I ........- ---........  453
Danio rerio                ...Y.I...K .N.PYIM.T- .NT.S..... L...A.... LM...L.... T-------LI ...P-...N.D- ---T......  449
Sparus aurata              ...Y......K ..P....V- .KG.T.M... ........D. .........T-------I ........- ---...V...  463
Oncorhynchus mykiss        ...Y..D.K I..PYTM.F- .NT.S..... L...V... LM......... .-------YF ...I..NK- ---N.....M  441
Dicentrarchus labrax       ...Y...K ...P...D- .TG.T.L... ......F.. .........T-------I ...N..- ---......  456
Mus musculus               ...Y..VA.S .N.P.T...- V.T.K.Y... .Y.E..... LMM..K.MS. S-------YF ...P...S.P- ---S.V.ERI  431
Homo Sapiens               ...Y..VA.S .N.P.T...- V.T.K.Y... .Y.E..... LML..K.KS. S-------YF ......S.P- ---G.A.Q.I  431
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Oryzias latipes            RIKSGETQSK VIFNAKEGEF ADLVRGGEDA VFVKSKEDNI SRKERRMHRL KTEGSLFGKS DA------- 516
Pagrus major LPL1          .......... .....S..... VY.......G .......... L ....KL..K. .KQ.....Q. .-------  523
Pagrus major LPL2          ..RA....Q. MV.CV.DP.S QK.TQ---EV T...C.DAWR TNSKQTPK.V TL.KH---- ---------  511
Takifugu rubripes LPL1     ......S... ....G.D..Y VH.......G ...L...PM ...KL..K. ..Q....--- ---------  511
Takifugu rubripes LPL2     .VRA....Q. MV.CV.NPDV QN.M.---EV T...RC.DAWR TSGK----.V TL.NHRP--- ---------  509
Siniperca chuatsi          .......... .....S.... .....M ....KL.K. RMQ.....QN S.-------  515
Danio rerio                .......... I..S....S.. SY.S....A. ....D..AQS ...NQ.L.KM .MH..S.KQN NE-------  511
Sparus aurata              .......... .....S.... VY.......G E.........L ....KL..K. ..Q.T....Q. .-------  525
Oncorhynchus mykiss        .V.A.....R ..S...D..Y .Y.I..KD.V .........M ...KT..... .MQ..H.KNN I.-------  503
Dicentrarchus labrax       .......... .....S.... .Y.......... .......R ...KL..K. .VQ......QN .--------  518
Mus musculus               .V.A.....K. ..C.R.-KV SH.QK.KDS. ....CHDKSL KK-SG----- ---------- --------- 474
Homo Sapiens               .V.A.....K. ...CSR.-KV SH.QK.KAP. ....CHDKSL NK.SG----- ---------- --------- 475
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◀ **Fig. 1** Multiple alignment of the deduced amino acid sequence of medaka lipoprotein lipase 1 (LPL1) with LPL isoforms (LPL1, LPL2) from other animals. *Dots* Amino acids identical to those of medaka (*Oryzias latipes*, *top line*), *dashes* alignment gaps, *bold underlining* the signal peptide, *hatch marks* conserved cysteine residues, *inverted filled triangle* active site residues, *single underline* putative heparin binding domains, *bold letters* in putative heparin binding domains conserved residues in all species examined, *NXT* a potential conserved N-linked glycosylation site, *box* a putative lipid binding site

Table 3 Amino acid sequences of LPL genes used in alignments and their identities (%) with that of medaka

Source	Species	Accession number	Identity (%)
Red seabream LPL1	*Pagrus major*	BAE95413	83.9
Red seabream LPL2	*Pagrus major*	BAB20997	46.8
Torafugu LPL1	*Takifugu rubripes*	BAM76378	78.3
Torafugu LPL2	*Takifugu rubripes*	BAM76379	45.0
Mandarin fish	*Siniperca chuatsi*	ACZ55136	84.7
Zebrafish	*Danio rerio*	NP571202	66.5
Rainbow trout	*Oncorhynchus mykiss*	NP001118076	71.0
Gilthead seabream	*Sparus aurata*	AAS75120	83.3
European seabass	*Dicentrarchus labrax*	CAL69901	84.3
Human	*Homo sapiens*	EAW63764	59.6
House mouse	*Mus musculus*	CAJ18552	58.4

identified in front of the N-terminal region. Comparison of AA sequences indicated that the N-terminal region of medaka LPL1 contains several conserved functional sites of vertebrate LPLs that are essential for the structure and functions of LPL [32–36], including one catalytic triad (Ser179, Asp203 and His291), one heparin-binding site (329–332 residues) and a putative polypeptide 'lid' (residues 263–289) that covers the active site and contributes to the preference for lipid substrate specificity [33, 37]. We also identified eight conserved cysteine residues in the N-terminal region of medaka LPL1 that play a role in the structural stability of LPL proteins and two conserved motifs in the C-terminal region, i.e., one lipid-binding site (Trp440, Trp443 and Trp444) and one putative *N*-linked glycosylation site (Asn409). Interestingly, one unpaired cysteine residue (Cys408) was found in the C-terminal domain of medaka LPL1 next to the putative N-linked glycosylation site. The deduced AA sequence of medaka LPL1 showed 78.3–84.7 % identities to LPL1 sequences from mandarin fish, gilthead seabream, European seabass, red seabream and torafugu, while only 45.0 and 46.8 % identities were found with torafugu and red seabream LPL2 sequences, respectively (Table 3).

Phylogenetic analysis

A phylogenetic tree was constructed by the ML method using LPL AA sequences from 16 vertebrate species (Fig. 2). All LPL1 sequences from fish were grouped to one clade, with a separate clade for LPL2 sequences from red seabream and torafugu. Mammalian LPL sequences were in the same clade with the LPL1 sequences from fish.

Expression profile of the medaka LPL1 gene

Quantitative real-time PCR was performed to determine the expression levels of LPL1 transcripts in various tissues of medaka. Overall, LPL1 transcripts were ubiquitously expressed in all tissues examined (Fig. 3). The highest expression was found in liver, followed by visceral adipose tissue. The LPL1 transcript levels in these tissues were significantly higher than those in other tissues (*P* < 0.05). Relatively high mRNA levels of LPL1 were detected in the brain and muscle, and the lowest level of LPL1 transcript expression was found in the intestine.

Discussion

In this study, we cloned the 2,259-bp cDNA encoding medaka LPL isoform 1 from the liver of adult medaka using the RACE approach. The deduced AA sequence of 516 residues was similar to those of LPL1s isolated from other fish species and LPLs from mammals and differed from the AA sequence of LPL2s from red seabream and torafugu. These results, together with those of the phylogenetic analysis, suggest that some conservative structural features are present in fish LPL1s and mammalian LPLs while LPL2 diverged earlier and probably acquired functions different from those of LPL1.

The three-dimensional model of human LPL based on human pancreatic lipase [34] aids our understanding of the functional property of vertebrate LPLs. All LPLs examined to date consist of two domains, i.e., a larger N-terminal domain and a smaller C-terminal domain, separated by Val363 and Phe364 (numbers for medaka LPL1). The N-terminal domain is considered to be a catalytic domain [36, 38], containing a highly conserved Ser-Asp-His catalytic triad on the active site. Two heparin-binding regions (306–309 and 321–327 residues in human LPL) that play a vital role in LPL catalytic function [39, 40] have also been located in the N-terminal domain. These regions are the same as the heparin-binding consensus sequences determined as [B-B-X-B] and [B-X-B-B-X-X-B], where B and X represent basic and hydropathic residues [32, 34], respectively. In all LPLs shown in Fig. 1, three basic residues (Arg306, Lys307 and Arg309, number for human LPL) of the first heparin-binding region are strictly conserved. However, in the second heparin-binding region, only two of the four basic residues (Arg321 and Arg324, number for human LPL) are fully conserved—Lys327 was conserved

in fish LPL1s and mammalian LPLs, while Lys323 is substituted by various residues in fish LPLs. Interestingly, we found that the second heparin-binding region in zebrafish LPL exactly matched the consensus sequence (B-X-B-B-X-X-B), with Lys323 (number for human LPL) substituted by Arg342 (number for zebrafish LPL). These findings suggest that the function of the first heparin-binding region is more critical than the second region in vertebrate LPLs.
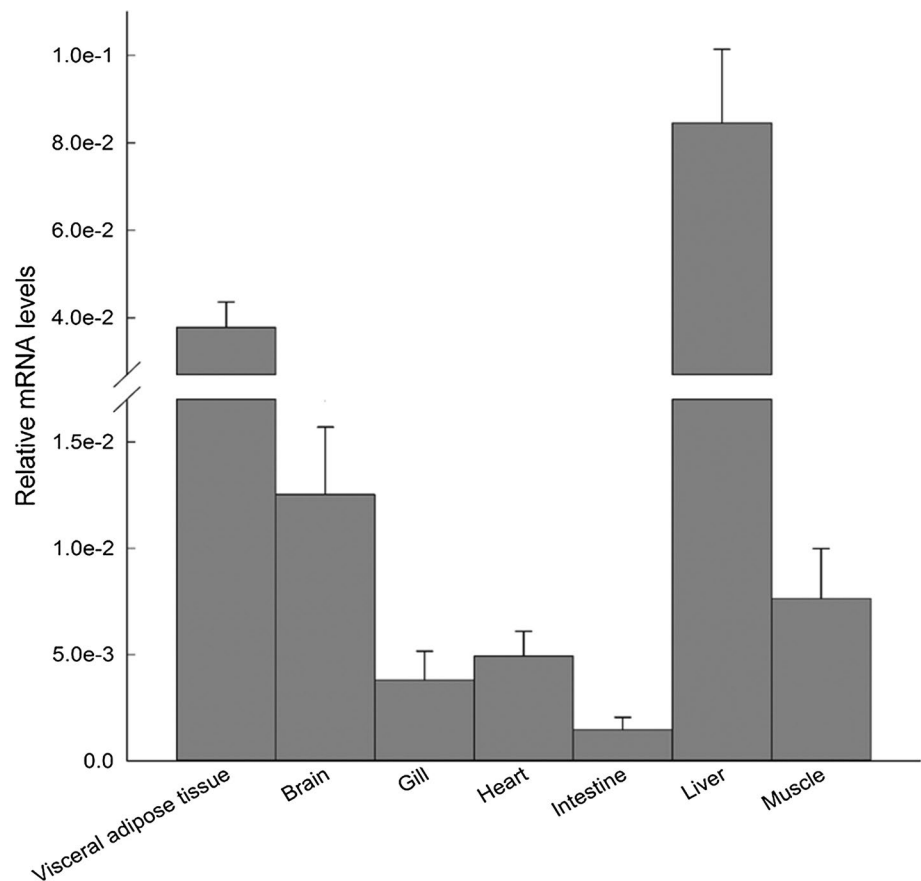
In contrast to the N-terminal domain, the C-terminal domain has been considered to play a role in substrate binding and specificity [36, 38]. Previous mutagenesis studies for Trp417, Trp420 and Trp421 (numbers for human LPL) by Williams et al. [41] and Krapp et al. [42] revealed that these residues play a vital function in the binding of LPL to lipoproteins. In our study, we found that these residues were generally retained in the LPLs examined but that Trp417 was substituted by Val in LPL2s from red seabream and torafugu and Trp420 was substituted by Ile in rainbow trout LPL. These LPL proteins might have different substrate specificity compared to other LPLs.

Disulfide bridges have been shown to play an important role in the stability of LPL structure [16, 43, 44]. Human LPL has been reported to contain five disulfide bridges (Cys54/67, Cys243/266, Cys291/310, Cys302/305 and Cys445/465 in numbering for humans) [45]. The fish LPL1s examined in our study shared eight of the ten cysteine residues, which are all located in the N-terminal domain. Notably, the fish LPL2s retained all cysteine residues, including Cys445 and Cys465 (human numbering), in the C-terminal domain. The C-terminal domain and associated functions of fish LPL2s may thus differ from those of fish LPL1s and mammalian LPLs.

The homology analysis of the domains [Electronic Supplementary Material (ESM) Tables 1 and 2] also supported the notion of functional conservation in both domains of LPLs examined. Due to the several highly conserved

Fig. 3 Relative mRNA levels of medaka LPL in various tissues determined by quantitative real-time PCR. Ribosomal protein L-7 (*RPL-7*) was used as the internal control. *Bars*: Mean + standard deviation (*n* = 10)

functional sites, the N-terminal domain of fish LPL1s and mammalian LPLs shared a higher homology (54.0–62.6 %) with fish LPL2s than did the C-terminal domain (28.1–44.8 %). It is possible that these differences in the C-terminal domain indicate that LPL2s interact differently than LPL1s with lipoproteins.

The RPL-7 gene and two other housekeeping genes [encoding elongation factor 1α (EF1α) and β-actin] were used in quantitative real-time PCR assay to increase the accuracy of the analysis (ESM Fig. 1). In general, the gene expression patterns of medaka LPL1 normalized to the three internal control genes were similar to each other. The highest level of LPL1 transcripts were found in the liver followed by visceral adipose tissue, and the lowest levels were found in the heart, gill and intestine. In muscle and brain, there were minor inconsistencies in LPL transcript levels normalized to those of RPL-7, EF1α and β-actin. Our comparison of cycle threshold (C_T) values of these housekeeping genes revealed that these inconsistencies were caused by relatively low expressions of the EF1α and RPL-7 transcripts in muscle and brain, respectively (ESM Fig. 2). However, compared with the transcript level of EF1α and β-actin, those of the RPL-7 gene showed less variation among the tissues tested; this result is similar to that reported by Zhang et al. [31]. Thus, among the three

housekeeping genes used in our analysis, the RPL-7 gene was the most suitable internal control gene to determine the tissue distribution of LPL transcripts.

Another characteristic of medaka LPL1 was its relatively high expression in the brain. To date, mammalian LPL has been reported to be present in neurons and vascular endothelial cells of various brain regions [46–49]. Although only trace amounts of TAG have been detected in human cerebrospinal fluid due to the blood–brain barrier (BBB) [50], LPL plays a role in the catabolism of TAG-rich lipoproteins in both endothelial cells [51] and neurons of certain brain regions lacking the BBB [52]. Furthermore, the results of several studies suggest that neuronal LPL contributes to the uptake of lipoproteins and vitamin E [53, 54], as well as cell regeneration [55–57], by either conventional or unconventional mechanisms. This has led to the in vivo functions of brain LPL in mammals being the subject of intense investigations. To our knowledge, however, this is the first report to indicate LPL gene expression in the fish brain. Given the unique nature of fish BBB [58], it is therefore interesting to investigate the function of brain LPL in vivo using the transgenic system of medaka.

The results from our EST screening also supported a wide distribution of LPL1 transcripts in medaka tissues. We found that the cloned medaka LPL1 gene shared

high homology with ESTs from fry and various tissues of adult medaka other than gill, while the ESTs sharing high homology with LPL2s were found only in embryonic databases. These findings suggest that LPL1 is the major type LPL isoform in adult medaka and that it functions in various developmental stages and tissues, whereas LPL2 may play a role during embryonic developmental stages. It was noted that ESTs cloned from medaka gill shared higher homology with LPL2 than with LPL1 (see Table 2 EST olgi31a18), suggesting that LPL2 is expressed at higher levels than LPL1 in medaka gill. This hypothesis is consistent with the tissue distribution of red seabream LPL isoform genes reported by Oku et al. [24].

In conclusion, we have identified LPL1 as the major LPL isoform in adult medaka. High expressions of LPL1 transcripts were found in the liver and visceral adipose tissue as well as the brain. To obtain insight into the potential functions of LPL1, the transgenic technique will be used in future studies. LPL2 may also be present in medaka and play an important role during embryonic developmental stages. Therefore, the function of this isoform should be investigated as well.

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