

Zebrafish and chicken lipocalin-type prostaglandin D synthase homologues: Conservation of mammalian gene structure and binding ability for lipophilic molecules, and difference in expression profile and enzyme activity[☆]

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Abstract

Lipocalin-type prostaglandin (PG) D synthase (L-PGDS) is a bifunctional protein possessing both the ability to synthesize PGD₂ and to serve as a carrier protein for lipophilic molecules. L-PGDS has been extensively studied in mammalian species, whereas little is known about non-mammalian forms. Here, we identified and characterized the L-PGDS homologues from non-mammals such as zebrafish and chicken. Phylogenetic analysis revealed that L-PGDSs of mammalian and non-mammalian organisms form a “L-PGDS sub-family” that has been evolutionally separated from other lipocalin gene family proteins. The genes for zebrafish and chicken L-PGDS homologues consisted of 6 exons, and all of the exon/intron boundaries were completely identical to those of mammalian L-PGDS genes. Zebrafish and chicken L-PGDS genes were clustered with several lipocalin genes in the chromosome, as in the case of mouse and human genes. Gene expression profiles were different among chicken, mouse, human, except for conservation of abundant expression in the brain and heart. The chicken L-PGDS homologue carried weak PGDS activity, whereas the zebrafish protein did not show any of the activity. However, when the amino-terminal region of the zebrafish L-PGDS homologue was exchanged for that of mouse L-PGDS carrying the Cys residue essential for PGDS activity, this chimeric protein showed weak PGDS activity. Both zebrafish and chicken L-PGDS homologues bound thyroxine and all-*trans* retinoic acid, like mammalian L-PGDSs and other lipocalin gene family proteins. These results indicate that non-mammalian and mammalian L-PGDS genes evolved from the same ancestral gene and that the non-mammalian L-PGDS homologue was the primordial form of L-PGDS but whose major function was and is to serve as a carrier protein for lipophilic molecules. During molecular evolution, the mammalian L-PGDS protein might have acquired effective PGDS activity through substitution of several amino acid residues, especially in the amino-terminal region including the Cys residue, which is essential for PGDS activity.

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1. Introduction

Lipocalin-type prostaglandin (PG) D synthase (L-PGDS; EC 5.3.99.2) was isolated from the rat brain and identified as the enzyme (Urade et al., 1985) catalyzing the production of PGD₂, from PGH₂, which is a common precursor of all prostanoids (Urade and Hayaishi, 2000; Urade et al., 2006). L-PGDS is known to be identical to β -trace protein, the second most abundant protein in the cerebrospinal fluid (Urade and Hayaishi, 2000). PGD₂ is a major PG in the brain and has various physiological functions such as the regulation of sleep (Hayaishi and Urade, 2002) and an allergy mediation

Abbreviations: GST, glutathione *S*-transferase; *K*_d, dissociation constant; L-PGDS, lipocalin-type PGD synthase; PG, prostaglandin; RT, reverse transcription; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; SCR, structurally conserved region; COX, cyclooxygenase; C8 γ , complement component 8 γ chain; ERABP, epididymal retinoic acid-binding protein.

[☆] The nucleotide sequence data reported will appear in the DDBJ/EMBL/GenBank under accession numbers, AB071601 (zebrafish cDNA), AB071602 (zebrafish gene), AB072378 (chicken cDNA), and AB077952 (chicken gene).

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(Matsuoka et al., 2000). Alternatively, L-PGDS proteins were found to belong to the lipocalin gene family by the homologies of their primary structures to lipocalin (Tanaka et al., 1997), which family consists of small secretory proteins that carry lipophilic molecules (Flower, 2000). L-PGDS has the ability to bind retinal and retinoic acid ($K_d=70\text{--}80\text{ nM}$) (Tanaka et al., 1997), as well as biliverdin and bilirubin ($K_d=33\text{--}37\text{ nM}$) (Beuckmann et al., 1999; Inui et al., 1999). Therefore, L-PGDS is a unique dual-function protein, acting as a PGD₂-producing enzyme and a carrier protein for lipophilic molecules.

L-PGDS has been well characterized in mammals (Urade and Hayaishi, 2000). Their mRNAs are dominantly expressed in the central nervous system (Urade et al., 1993; Taniike et al., 2002), heart (Eguchi et al., 1997), and male genital organ (Tokugawa et al., 1998). L-PGDS gene expression is regulated through some nuclear receptors such as thyroid hormone receptor (White et al., 1997; García-Fernández et al., 1998), glucocorticoid receptor (García-Fernández et al., 2000), and estrogen receptors (Otsuki et al., 2003). Moreover, its expression is regulated by Notch-Hes signal, interleukin-1 β , and protein kinase C in brain-derived cells (Fujimori et al., 2003, 2005), and is enhanced in vascular endothelial cells via the action of activator protein-1 (Miyagi et al., 2005).

Functional studies using gene-manipulated mice such as those overexpressing human L-PGDS cDNA and those deficient in the L-PGDS gene demonstrated that L-PGDS is involved in the regulation of non-rapid eye movement sleep (Hayaishi and Urade, 2002), control of tactile pain responses (Eguchi et al., 1999), allergic reactions in the bronchial asthma (Fujitani et al., 2002), and is associated with glucose intolerance, nephropathy, and atherosclerosis (Ragolia et al., 2005). The X-ray crystallography of mouse L-PGDS showed that L-PGDS possesses a β -barrel structure consisting of eight β -strands and one α -helix, as is highly conserved among the proteins of the lipocalin gene family (Flower, 2000). On the contrary, little is known about non-mammalian L-PGDS, although an amphibian L-PGDS homologue was identified as a lipocalin protein (Achen et al., 1992; Lepperdinger et al., 1997).

In this study, we report the biochemical and molecular biological characterization of zebrafish and chicken non-mammalian homologues of mammalian L-PGDS. The exon/intron junctions were conserved in all mammalian and non-mammalian L-PGDS genes. Zebrafish and chicken L-PGDS homologues bound lipophilic molecules like other lipocalin gene family proteins. The expression patterns of the non-mammalian L-PGDS genes were divergent, and the non-mammalian L-PGDS proteins showed weak or no PGDS activity. Thus, mammalian and non-mammalian L-PGDSs evolved from the same ancestral gene, and non-mammalian L-PGDS was the primordial form of L-PGDS to appear, whose major function was and still is to serve as a carrier protein for lipophilic molecules.

2. Materials and methods

2.1. Identification of cDNA and gene for zebrafish L-PGDS homologue

Through a translated BLAST search of the NCBI nucleotide sequence database using the primary structure of rat L-PGDS

(Urade et al., 1989), we identified zebrafish (*Danio rerio*) EST clones. One EST clone (fa96h08.y1), which was the longest, was obtained from Research Genetics (Huntsville, AL, USA).

Zebrafish genomic DNA fragment containing the L-PGDS homologue was isolated by PCR with 5'-GAGAGAAGCAA-CACCTTCTG-3' (corresponding to nucleotides from -24 to -6; A of ATG translational initiation codon of cDNA was defined as +1) and 5'-GACTTTTGTAACCAATTTAATC-3' (corresponding to nucleotides from +965 to +987 of cDNA) as the primers, genomic DNA of *D. rerio* AB strain as the template, and LA Taq DNA polymerase (Takara Shuzo, Kyoto, Japan). After an initial denaturation step at 96 °C for 5 min, 30 cycles were performed, which consisted of denaturation at 96 °C for 20 s, annealing at 53 °C for 20 s, and elongation at 72 °C for 3 min. The resultant PCR fragments were separated by agarose gel electrophoresis, cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and then sequenced.

2.2. Isolation of chicken L-PGDS cDNA and gene

The database search using the rat L-PGDS protein sequence provided us with 10 chicken EST clones showing homology to mammalian L-PGDS sequences. The EST clone (GenBank Accession No. AL588422) carrying the longest cDNA was amplified by rapid amplification of the cDNA end method using the primer designed from the EST sequence. The reverse transcription (RT) reaction was performed as described previously (Fujimori et al., 2000, 2002) with chicken brain total RNA and Oligo dT Adaptor Primer (Takara Shuzo). PCR was conducted with C6 primer (5'-TCCCGCTCAGCTCAGCTCAGCTC-3', corresponding to nucleotides from -38 to -16) and M13 M4 primer (Takara Shuzo) as the primers and LA Taq DNA polymerase under the following conditions: initial denaturation at 96 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 74 °C for 1 min. PCR fragments were directly cloned into the pGEM-T Easy vector, and sequenced.

The genomic fragment carrying the L-PGDS gene was amplified by using the above C6 primer and C8 primer (5'-GTGGTTTATATGGGCTTTAATAGGG-3', corresponding to nucleotides from +712 to +699 of cDNA) as the primers with the chicken kidney DNA as the template. The following PCR conditions were used: initial denaturation at 96 °C for 5 min, followed by 28 cycles of 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min. The resultant PCR products were cloned into the pGEM-T Easy vector, and sequenced.

2.3. Chromosomal mapping

The LN54 radiation hybrid panel, obtained from Dr. Marc Ekker (Ottawa Health Research Institute, Ottawa, Canada), was utilized to map the zebrafish L-PGDS gene as described earlier (Hukriede et al., 1999). This experiment was performed three times, and the linkage group number was determined by using the program available on the web site (<http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi>).

2.4. RNA isolation and RT-PCR analysis

Preparation of total RNA and RT-PCR were performed as described previously (Fujimori et al., 2000, 2002). First-strand cDNAs were synthesized from one μ g of total RNA, and subsequent PCR amplification was conducted under the condition as described above by using the following primers: Ch-F, 5'-CGG-AATTCCACGCGCAGAACAGCATTCTG-3' (corresponding to nucleotides from +52 to +73 of chicken L-PGDS cDNA), and Ch-R, 5'-CGCTCGAGCTAGGCAGCATCTGCCATACAC-3' (from +558 to +537 of chicken L-PGDS cDNA), for the chicken L-PGDS; Mu-F, 5'-CGGAATTCAGGGCCATGACACAGTG-CAGC-3' (from +73 to +94 of mouse L-PGDS cDNA) and Mu-R, 5'-CGCTCGAGCTCTTGAATGCACTTATCCGG-3' (from +567 to +547 of cDNA), for the mouse L-PGDS; and Hu-F, 5'-GCACCCGAGGCCAGGTCTCCG-3' (from +67 to +88 of human L-PGDS cDNA), and Hu-R, 5'-AAGTGCATGACGGAA-CAATAG-3' (from +573 to +553 of cDNA), for human L-PGDS. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was amplified as the internal control with 5'-ACACGGACACTTCAAGGG-CAC-3' (from +150 to +170 of chicken G3PDH cDNA) (Panabieres et al., 1984) and 5'-GGCAGTTGGTGGTGAC-GATGC-3' (from +463 to +443 of cDNA), and 5'-ACCA-CAGTCCATGCCATCAC-3' (from +526 to +545 of human and mouse G3PDH cDNA) (Tokunaga et al., 1987) and 5'-TCCAC-CACCCTGTTGCTGTA-3' (from +959 to +977 of cDNA) as the primers. The resultant PCR products were analyzed by agarose gel electrophoresis, purified, and verified by DNA sequencing.

2.5. Molecular phylogenetic analysis

The primary structures of L-PGDSs were aligned by using the CLUSTAL W program (Thompson et al., 1994). A phylogenetic tree was constructed by using the PHYLIP program, ver. 3.5c (Felsenstein, 1996). Alignment positions with gaps were excluded. The accession numbers for sequences used in the molecular phylogenetic analysis were as follows: L-PGDS for human (M61901), mouse (X89222), rat (J04488), sheep (AJ133642), and stallion (AJ133469), human complement component 8 γ chain (C8 γ ; NP_000597), human α 1 microglobulin (CAA38585), rat von Ebner's gland protein (P20289), rat odorant binding protein II (A40464), chicken quiescence specific polypeptide 20 K (A30230), rat α 2-urinary globulin (P02761), rat probasin (NP_061998), rat odorant binding protein (AAA41736), hamster aphrodisin (P09465), human placental protein 14 (J04129), ovine β -lactoglobulin (AAA31510), human α 1 acid glycoprotein (CAA26397), rat retinol-binding protein (NP_036385), human apolipoprotein D (XP_003067), butterfly bilin binding protein (S41410), and mouse 24p3 (P11672).

2.6. Expression and purification of recombinant proteins

Primary structures of mammalian L-PGDSs carry the secretion signal sequences at their amino-terminal portions, and the cleavage site for the secretion signal sequence of mouse L-PGDS has been identified (Hoffmann et al., 1996). The cleavage sites of the secretion signal sequences for zebrafish and chicken L-PGDS

homologues were predicted by hydropathy analysis (Kyte and Doolittle, 1982). The coding regions with truncated secretion signal sequences for zebrafish, chicken, and mouse L-PGDS homologues were amplified by PCR using the following gene-specific primer sets: Ze-F, 5'-CGGAATTCCTTTGCTTCGGCT-GATGTCATG-3' (corresponding to nucleotides from +49 to +69 of zebrafish L-PGDS cDNA), and Ze-R, 5'-CGCT-CGAGTGCTGCAACTGAGCATTCTC-3' (from +552 to +533 of cDNA), for zebrafish L-PGDS; Ch-F and Ch-R for chicken and Mu-F and Mu-R for mouse L-PGDSs with *Eco*RI and *Xho*I sites (underlined) at their respective 5'-ends. A chimeric protein construct carrying the mouse amino-terminal portion (from Asp25 to Leu84, corresponding to exons 1 and 2 of mouse L-PGDS) and zebrafish carboxyl-terminal portion (from Lys79 to Ala184, corresponding to exons 3 to 6 of zebrafish L-PGDS), called MZ chimeric protein, was made by overlap-PCR as follows: Firstly, the amino-terminal portion of mouse L-PGDS was amplified by PCR with Mu-F and Mz (5'-CTCACCTC-TACCTTCCTCAAGAGTGATGGTTCCTGC-3') as the primers; and the carboxyl-terminal portion of the zebrafish L-PGDS homologue, by PCR with zM (5'-GCAGGAACCATCACTCTT-GAGGAAGGTAGAGGTGAG-3') and Mu-R as the primers. Both PCR products were separated by agarose gel electrophoresis, and purified with a Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany). Secondly, each purified fragment was mixed and used as the template for subsequent PCR with mouse Mu-F and zebrafish Ze-R as the primers. The chimeric construct for the amino-terminal portion of zebrafish (from Phe27 to Leu78, corresponding to exons 1 and 2 of zebrafish L-PGDS) and the carboxyl-terminal portion of mouse (from Arg85 to Glu189, corresponding to exons 3 to 6 of mouse L-PGDS), called ZM chimeric protein, was constructed in the same manner by using Zm (5'-TACCTCGCCAAGAAAACCGGGGCTCCTGGACAC-TAC-3') and mZ (5'-GTAGTGTCCAGGAG-CCCCGGTTTTC-TTGGCGAGGTA-3') and zebrafish Ze-F and mouse Mu-R as the primers. Their resultant PCR products were then purified as described above and digested with *Eco*RI and *Xho*I, followed by cloning into the corresponding sites of pGEX-6P-1 vector (Amersham Biosciences, Piscataway, NJ, USA). Site-directed mutagenesis was introduced by PCR with mutated primers as described previously (Fujimori et al., 2000). Each construct was sequenced to verify the correct sequence.

Each of the constructs was utilized to transform *E. coli* JM109 or BL21 (DE3) strains. The production of recombinant proteins was induced in the late exponential phase by the addition of IPTG (final concentration, 0.3 mM), and the cultivation was continued for a further 3 h. The cells were then harvested and disrupted by sonication. Lysates from the induced cells were incubated with Glutathione-Sepharose 4B (Amersham Bioscience) to purify the fusion protein, and then the proteins of interest were separated from GST by digestion with PreScission Protease (Amersham Bioscience) according to the instructions from the manufacturer. Proteins were analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. Protein concentrations were measured by using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

2.7. Enzyme assay for PGDS

PGDS activity was measured by incubating the enzyme at 25 °C for 1 min with [^{14} C]PGH₂ (final concentration of 40 μM) in 50 μl of 100 mM Tris–Cl, pH

8.0, containing 1 mg/ml IgG and 1 mM dithiothreitol (Urade et al., 1989). [^{14} C]PGH₂ was prepared from [^{14} C]arachidonic acid (2.20 GBq/mmol, PerkinElmer, Boston, MA, USA) as described previously (Urade et al., 1985).

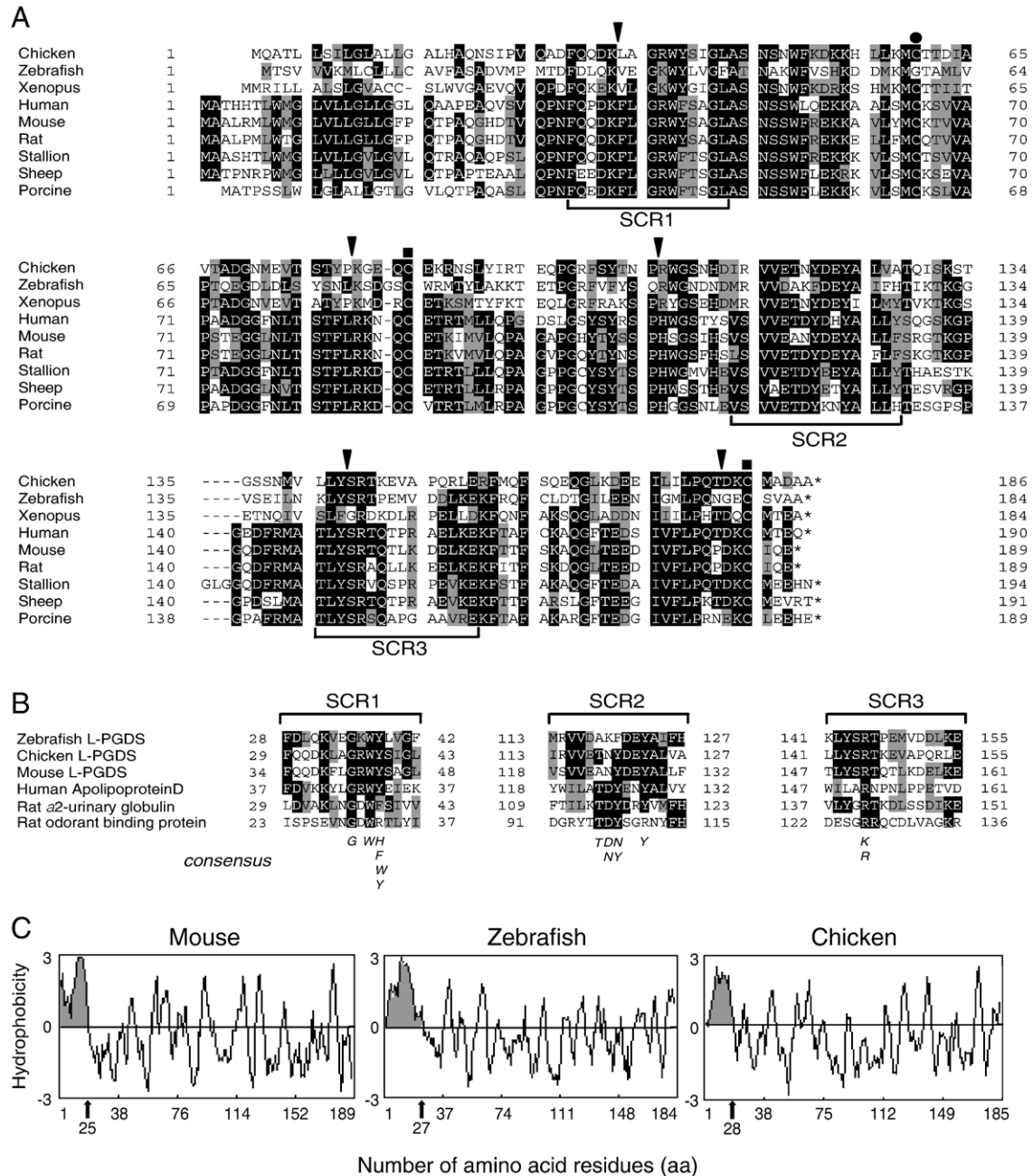


Fig. 1. Alignment of the amino acid sequences predicted from zebrafish and chicken L-PGDS cDNAs and the corresponding mammalian L-PGDS cDNAs. A, Amino acid sequences predicted from zebrafish and chicken L-PGDS cDNAs were compared with those of known mammalian L-PGDS cDNAs. Dashes indicate the gap inserted to maximize the similarity. Identical or similar amino acid residues in at least five of nine sequences are indicated by a *black* or a *gray* background, respectively. The Cys residues essential for PGDS activity in mammals are shown by a *closed circle*, and the two Cys residues required for the disulfide-bond formation are indicated by *closed squares* above the alignment. Three SCRs are indicated below the alignment. Positions of intron insertion sites were indicated by an *arrowhead*. B, Amino acid sequences of the three SCRs of zebrafish, chicken, and mouse L-PGDSs, and those of three lipocalin gene family proteins, human apolipoprotein D, rat α2-urinary globulin, and rat odorant binding protein in the lipocalin gene family, are aligned. Identical or similar amino acid residues in at least three of six sequences are indicated by a *black* or a *gray* background, respectively. Amino acid residues conserved in the amino acid sequences of the lipocalin gene family proteins are indicated below the alignment. C, Hydropathy analysis of zebrafish, chicken, and mouse L-PGDS proteins was performed by the method of Kyte and Doolittle. The hydrophobic regions of the putative secretion signal sequence are shown in *gray*. Putative amino-terminal ends of mature proteins are indicated by an *arrowhead*.

Table 1
Comparison of whole sequence, amino-terminal portion (in parentheses), and carboxyl-terminal segment (in brackets) of zebrafish, chicken, and mouse L-PGDS proteins

Identity (%)			
	Zebrafish	Chicken	Mouse
Zebrafish		31.5 (25.6) [35.8]	29.1 (23.8) [33.3]
Chicken	46.2 (44.9) [47.2]		42.3 (45.2) [40.0]
Mouse	38.1 (32.1) [42.9]	52.9 (51.2) [54.3]	
Similarity			

2.8. Fluorescence quenching analysis

A binding study using all-*trans* retinoic acid and thyroxine was performed by internal Trp fluorescence quenching analysis as described previously (Inui et al., 2003). All-*trans* retinoic acid and thyroxine were dissolved in ethanol and dimethyl sulfoxide, respectively. The concentrations were determined spectrophotometrically from their respective molar absorption coefficients of ϵ_{336} in ethanol ($45,000\text{ M}^{-1}\text{ cm}^{-1}$) for all-*trans* retinoic acid (Cogan et al., 1976) and ϵ_{325} at pH 11 ($6180\text{ M}^{-1}\text{ cm}^{-1}$) for thyroxine (Cogan et al., 1976). Each lipophilic ligand at various concentrations was added to the recombinant proteins in 990 μl of

5 mM Tris–Cl, pH 8.0. After incubation at 20 °C for 60 min, the internal Trp fluorescence was measured by an FP-750 spectrofluorometer (Jasco, Tokyo, Japan) with an excitation wavelength at 282 nm and an emission wavelength at 338 nm. The quenching of Trp fluorescence caused by nonspecific interactions with various lipophilic molecules was corrected by use of 1.5 μM *N*-acetyl-*L*-tryptophanamide. The dissociation constant (K_d) values for binding between various kinds of lipophilic molecules and the recombinant proteins were calculated by the method described previously (Edelhoch and Lippoldt, 1962).

3. Results

3.1. Molecular cloning of cDNAs for zebrafish and chicken L-PGDS homologues

Through the BLAST search of the NCBI database using the rat L-PGDS protein sequence, we found EST clones for zebrafish and chicken.

The zebrafish EST clone (fa96h08.y1) was obtained from Research Genetics, and its insert DNA was fully sequenced. This clone contained an ORF of 552 bp encoding a polypeptide of 184 amino acids with a calculated molecular weight of 20.8-kDa (Fig. 1A).

We found 10 chicken EST clones, but none of them contained the full-length ORF. To isolate the full-length chicken cDNA, we amplified the DNA fragment containing the full-length ORF by PCR with the specific primers designed from the sequence information on the chicken EST clones. Our isolated chicken cDNA contained an ORF of 555 bp encoding a protein of 185 amino acids with a calculated molecular weight of 20.8-kDa (Fig. 1A). The molecular weight of both zebrafish and chicken L-PGDS homologues was in good agreement with that of mammalian L-PGDSs (approximately 21-kDa) (Urade and Hayaishi, 2000).

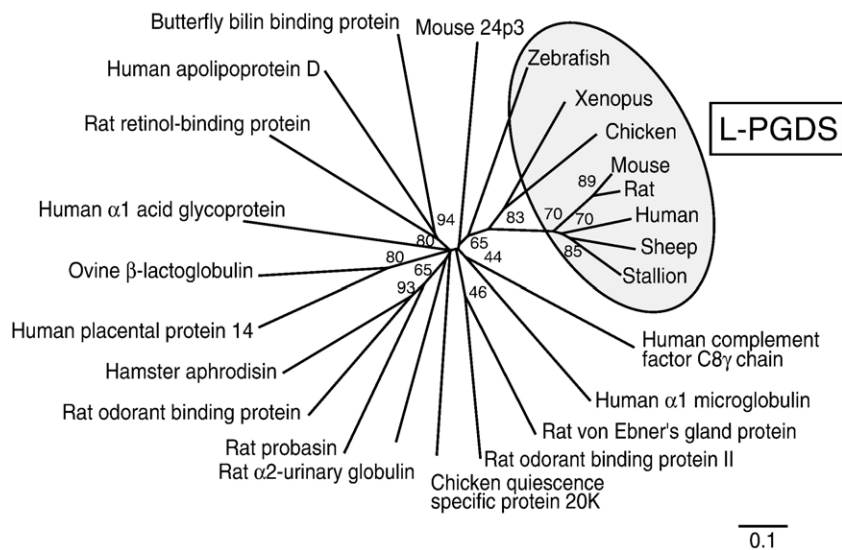


Fig. 2. Evolutional relationship between non-mammalian and mammalian L-PGDSs in the lipocalin gene family. Amino acid sequences of 8 L-PGDSs and 16 selected lipocalin gene family proteins were used for the phylogenetic analysis. Phylogenetic tree was constructed by the neighbor-joining method with PHYLIP program. The tree branches were labeled with the 100 bootstrap values. The scale bar represents 0.1 substitution/site.

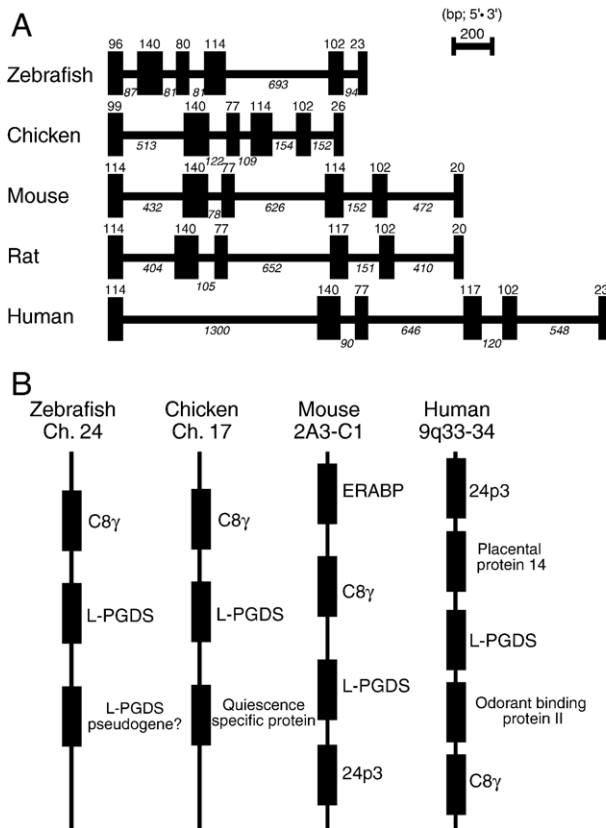


Fig. 3. Genomic structure and chromosomal location of non-mammalian and mammalian L-PGDS genes. A, Genomic structures in the coding regions for zebrafish, chicken, mouse, rat, and human L-PGDSs were compared. Exons are shown as boxes; and introns, as lines. The numbers above and below the boxes or lines indicate sizes of exons or introns, respectively. The scale bar represents 200 bp. B, Schematic maps of zebrafish, chicken, mouse, and human chromosomes with lipocalin genes and L-PGDS gene were shown. Assignments of mouse and human lipocalin genes are taken from previous results (Salier, 2000).

The primary structures predicted from cDNAs for zebrafish and chicken L-PGDS homologues were compared with all homologous protein sequences deposited in the NCBI databases. The primary structures of the non-mammalian L-PGDS homologues most resembled those of mammalian L-PGDSs in all proteins included in the lipocalin gene family, as assessed by the Clustal W program (Fig. 1A). The primary structure of zebrafish L-PGDS homologue showed low identities to those structures of mouse L-PGDS (29.1%) and chicken L-PGDS homologue (31.5%). The predicted amino acid sequence of chicken L-PGDS homologue revealed 42.3% identity to that of mouse L-PGDS. On the contrary, the zebrafish L-PGDS homologue shared moderate similarities to mouse and chicken L-PGDSs, i.e., 42.9% to mouse L-PGDS and 47.2% to chicken L-PGDS. Moreover, the chicken L-PGDS homologue showed 54.3% similarity to mouse L-PGDS (Table 1). Next, both the amino-terminal region corresponding to exons 1 and 2, and the carboxyl-terminal portion encoded by exons 3 to 6, were compared among zebrafish, chicken, and mouse L-PGDSs. Identities and similarities of the amino-terminal segments between zebrafish and chicken (25.6%/44.9% of identity/similarity) or mouse (23.8%/32.1%) were lower

than those between chicken and mouse (45.2%/51.2%). Moreover, identities and similarities of their carboxyl-terminal portions were almost the same (35.8–40%/42.9–54.3% of identity/similarity) among zebrafish, chicken, and mouse. Thus, these results indicate that the amino-terminal segment of the zebrafish L-PGDS homologue was more diversified than the carboxyl-terminal portions.

Mammalian L-PGDSs contain three conserved Cys residues in their primary structures. The Cys residue at 65 in the rat protein (closed circle in Fig. 1A) was identified as the essential amino acid residue for the catalytic activity of PGDS (Urade et al., 1995), being conserved in all mammalian L-PGDSs and unique to L-PGDS in the lipocalin gene family (Urade and Hayaishi, 2000). The other two Cys residues (closed squares in Fig. 1A) are essential for disulfide bond formation, and these residues were conserved in all L-PGDSs including the non-mammalian homologues. The chicken L-PGDS homologue contained a Cys residue at position 60, corresponding to the essential amino acid residue for PGDS activity in mammalian, whereas the zebrafish homologue had no corresponding Cys residue.

Lipocalin gene family proteins show conserved folding patterns and carry three structurally conserved regions (SCRs) (Flower et al., 1993). Proteins carrying these SCRs are defined as “lipocalin,” which regions are thought to play an important role in receptor binding (Ganfornina et al., 2000). Primary structures of zebrafish and chicken L-PGDS homologues carried SCRs (Fig. 1A) showing significant homologies to those of mammalian L-PGDSs as well as other lipocalin gene family proteins such as human apolipoprotein D, rat α 2-urinary globulin, and rat odorant binding protein (Fig. 1B). These results indicate that zebrafish and chicken L-PGDS homologues are to be considered members of the lipocalin gene family.

Mammalian L-PGDSs carry a secretion signal sequence at their amino-terminal portion (Urade and Hayaishi, 2000), and the amino-terminal end of the mature (secreted) protein of mouse L-PGDS was determined as Asp25 (Hoffmann et al., 1996). Hydropathy analysis revealed the existence of a

Table 2

Exon–intron boundaries of zebrafish, chicken, and mouse L-PGDS genes; exon sequences are indicated by capital letters, and intron sequences, by lower case letters

Zebrafish	CTG CAA AAG gtaaattcc ... (intron 1) ...tttttcag GTG GAA GGA
Chicken	CAG GAC AAG gtgatcaag ... (intron 1) ...tgtgtcag CTT GCG GGG
Mouse	CAA GAC AAG gtgagcagg ... (intron 1) ...tggtgcag TTC CTG GGG
Zebrafish	T AAC TAA AA gtaagccta ... (intron 2) ...gtttgtcag G AGT GAT GG
Chicken	C TAC CCC AA gtacgggtg ... (intron 2) ...ttctcagag G GGT GAA CA
Mouse	C TTC CTC AG gtgagacaa ... (intron 2) ...taccacag G AAA AAC CA
Zebrafish	AC AGC CAG C gtaaggagt ... (intron 3) ...atttttag GT TGG GGG A
Chicken	CC AAC CCA C gtgagcctc ... (intron 3) ...ttctcagag GC TGG GGC A
Mouse	GC AGC CCC C gtgagtgag ... (intron 3) ...gggtccag AC TCG GGC A
Zebrafish	AA CTC TAC A gtaaggaca ... (intron 4) ...gtgtcag GT CGC ACT C
Chicken	TG CTC TAC A gtacagtcc ... (intron 4) ...tttccgag GC CGC ACC A
Mouse	CC CTC TAC A gtaggtatc ... (intron 4) ...atctaccag GC AGA ACC C
Zebrafish	CA CAA AAT G gtaattttt ... (intron 5) ...gtttaccg GA GAA TGC T
Chicken	CC CAG ACG G gtaaggggtg ... (intron 5) ...tttctcgg AC AAG TGT A
Mouse	CC CAA CCG G gtgaggggg ... (intron 5) ...tcttgcag AT AAG TGC A

Triplet nucleotides correspond to an amino acid.

hydrophobic region in the amino-terminal portion of zebrafish and chicken L-PGDSs like that in mouse L-PGDS, and the amino-terminal ends for the zebrafish and chicken proteins were predicted to be Phe27 and His28, respectively (Fig. 1C).

To understand the evolutionary relationship between mammalian and non-mammalian L-PGDSs in the lipocalin gene family, we carried out the phylogenetic analysis shown in Fig. 2. Both zebrafish and chicken L-PGDS homologues were separated from other lipocalin gene family proteins, and assigned to the same group with other L-PGDSs. Therefore, non-mammalian and mammalian L-PGDSs formed an “L-PGDS sub-family” in the lipocalin gene family.

3.2. Isolation and characterization of the genes for zebrafish and chicken L-PGDS homologues

The zebrafish L-PGDS gene was isolated by PCR using gene-specific primers designed from the zebrafish cDNA sequence. The resultant PCR products were cloned and sequenced. Three independent clones were picked up, and each insert of these clones was completely sequenced (Fig. 3A).

The chicken gene for the L-PGDS homologue was also cloned by PCR with gene-specific primers. The nucleotide sequences of three independent genomic clones for chicken L-PGDS homologue were determined (Fig. 3A), and the results of the database search indicated the chicken L-PGDS gene to be located on chromosome 17 (GenBank Accession No. NW_060632).

Comparison of the genomic sequences with those of their cDNAs revealed the existence of 6 exons in the coding region of the chicken and zebrafish L-PGDS genes (Fig. 3A, Table 2). The

genomic structures of zebrafish and chicken L-PGDS genes were compared with those of mammalian L-PGDS genes. Mouse, rat, and human L-PGDS genes also consist of 6 exons in their coding region. The insertion positions of all introns in the coding region were completely conserved, as judged from the multiple alignment of the amino acid sequences of both non-mammalian and mammalian L-PGDSs (Fig. 1A and Table 2). These results reveal that the non-mammalian and mammalian L-PGDS genes have maintained the exon/intron structure of the ancestral gene during their molecular evolution.

To conduct the chromosomal mapping of the zebrafish L-PGDS gene, we screened the LN54 radiation hybrid panel (Hukriede et al., 1999). As a result, the zebrafish L-PGDS gene was mapped to a position 2.43 centirays from the fi16f03 marker gene (GenBank Accession No. AW134128) on chromosome 24. We performed a BLAST search against the database for The *D. rerio* Sequencing Project on The Wellcome Trust Sanger Institute (http://www.ensembl.org/Danio_rerio/index.html), and found that C8 γ and a putative L-PGDS pseudogene were located as neighbors of the L-PGDS homologue in the zebrafish chromosome 24 (Fig. 3B). Chicken genes for C8 γ , L-PGDS, and quiescence specific protein were clustered on chromosome 17. Likewise, in the mouse, epididymal retinoic acid-binding protein (ERABP), C8 γ , L-PGDS, and 24p3 genes were clustered on chromosome 2A3-C1 (Salier, 2000). In the case of humans, a cluster of lipocalin genes such as 24p3, placental protein 14, L-PGDS, odorant binding protein II, and C8 γ was found on chromosome 9q33-34 (Salier, 2000). Thus, the clustering feature of the lipocalin genes including the L-PGDS gene has been conserved in the chromosomes of human and

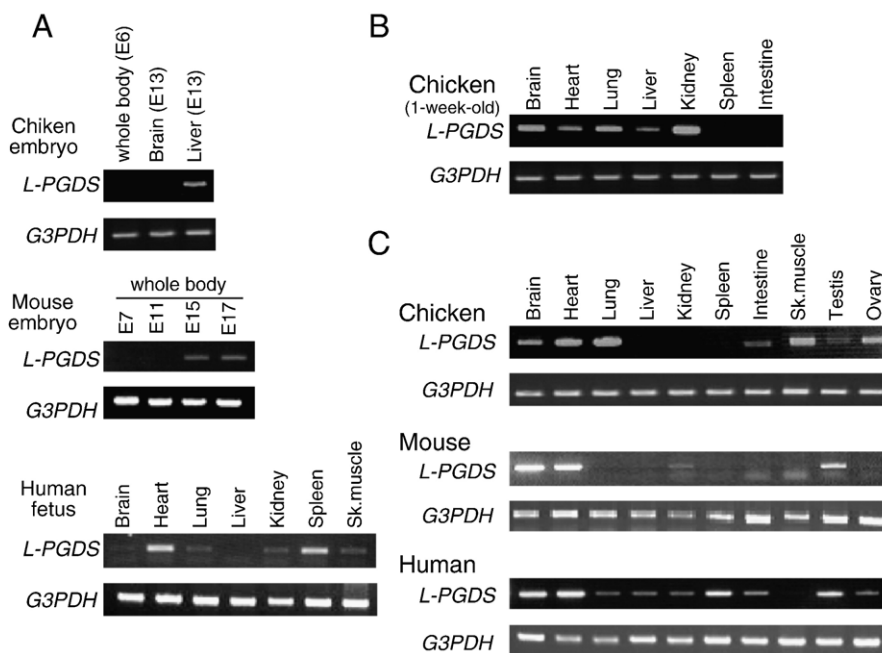


Fig. 4. Tissue distribution of chicken, mouse, and human L-PGDS transcripts. A, Expression of L-PGDS mRNAs in embryos. B, Expression of L-PGDS mRNA in the various tissues of 1-week-old chicken. C, Comparison of L-PGDS mRNA expression pattern in tissues among chicken (1-year-old), adult mouse, and adult human. In A–C, 1 μ g of total RNA was used for synthesis of cDNAs. PCR analyses were carried out by 30 cycles (for L-PGDS) or 25 cycles (for G3PDH) as described in Materials and methods. Chicken, mouse and human G3PDH, as an internal control, were amplified together with each cDNA for L-PGDS. Sk. muscle means skeletal muscle.

mouse as well as in those of non-mammals such as zebrafish and chicken.

3.3. Expression profiles of chicken, mouse, and human *L-PGDS* genes

Total RNA was prepared from various tissues of chicken (embryo, 1-week-old chicken, and 1-year-old adult), mouse (embryo and adult), and human (fetus and adult, RNAs were purchased from Clontech, Palo Alto, CA, USA). RT-PCR analysis was performed by using of one μ g of total RNA prepared from various tissues in each developmental stage. The G3PDH mRNA for mouse, human, and chicken, as an internal control, was amplified together with the each cDNA (Fig. 4A–C).

The *L-PGDS* mRNA was not detected in the 6-day-old chicken embryo (E6) or in the 7- and 11-day-old mouse embryo (E7 and E11, respectively) as early embryonic stages (Fig. 4A). However, in the late embryo stage, the gene was expressed in the liver of the 13-day-old chicken embryo (E13) and 15- and 17-day-old mouse embryos (E15 and E17, respectively). These results indicate that

the *L-PGDS* gene was expressed in the late embryonic stage in each animal species, and are consistent with previous findings showing that its mRNA was initially detected at 14.5 days in the mouse embryo (Hoffmann et al., 1996). In 1-week-old chickens, the mRNA was detected highly in the brain, lung, and kidney, and moderately in the heart and liver, but not in the spleen and intestine (Fig. 4B).

Next, we compared the tissue distribution of *L-PGDS* mRNAs of adult chicken (1-year-old), adult mouse and adult human (Fig. 4C). *L-PGDS* mRNA was expressed abundantly in the brain and heart of chicken, human, and mouse, although their mRNAs were not detected in the brain of their embryos. In other tissues, the chicken *L-PGDS* mRNA level was high in the lung, skeletal muscle, and ovary, and lower in the intestine and testis. The mouse *L-PGDS* gene was slightly expressed in the kidney and testis. The human *L-PGDS* mRNA was detected at a lower level in the lung, liver, kidney, spleen, testis and ovary. Taken together, these results suggest that *L-PGDS* gene expression is differentially regulated in each animal species but that high-level expression is conserved in the brain and heart in all adult animals.

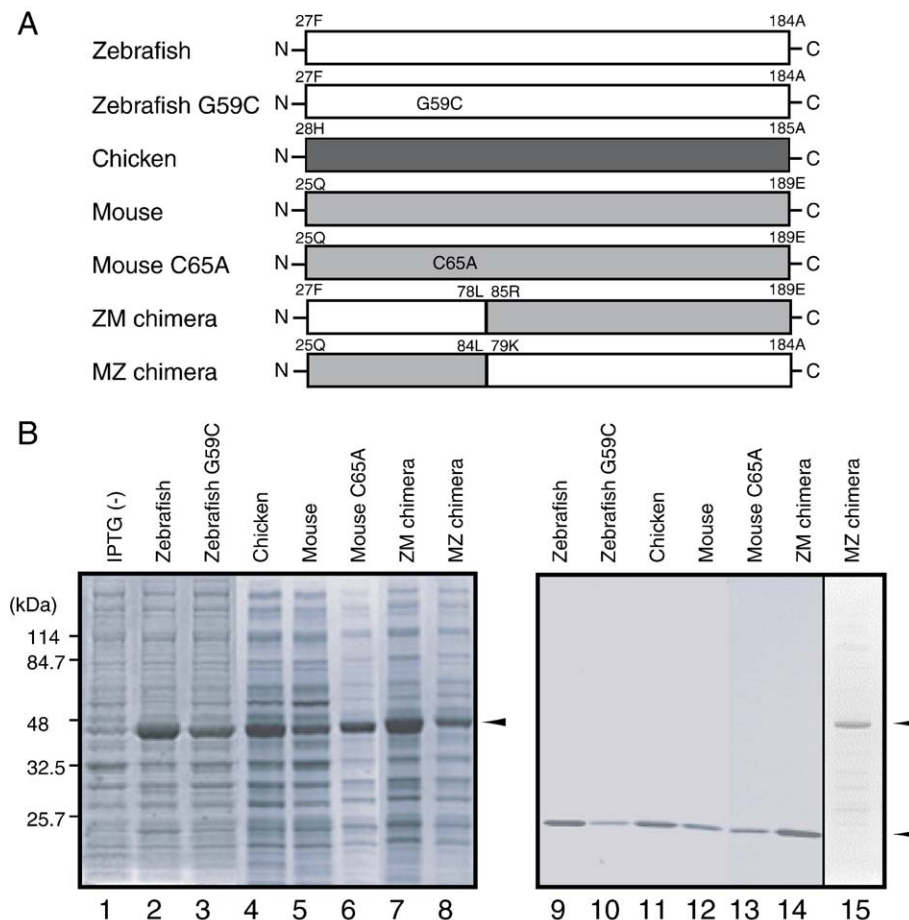


Fig. 5. Expression and purification of recombinant *L-PGDS*s. A, Structure of the fragments used for the expression of recombinant proteins. B, Recombinant zebrafish, zebrafish G59C mutant, chicken, mouse, and mouse C65A mutant *L-PGDS*, and MZ and ZM chimeric proteins were heterologously expressed in *E. coli*. Extracts of transformants before (IPTG(-)) and after induction with IPTG were separated by SDS-PAGE. The recombinant proteins were affinity-purified and subsequently digested enzymatically to separate them from GST, except for the MZ chimeric protein, which was purified as the fusion protein with GST. Proteins were separated by SDS-PAGE and then stained with Coomassie Brilliant Blue. Molecular sizes of the markers are shown on the left.

Table 3
Enzymatic property of zebrafish, chicken, and mouse L-PGDS proteins

Specific activity for PGDS (nmol/min/mg protein)	
Zebrafish	n.d.
Zebrafish G59C mutant	n.d.
Chicken	11.7±2.3
Mouse	434.2±103.3
Mouse C65A mutant	n.d.
ZM	n.d.
MZ*	14.9±1.5
Chicken (boiled)	n.d.
MZ* (boiled)	n.d.

*MZ chimeric protein is fused with GST. n.d.; not detected (<0.1 nmol/min/mg protein). Data are expressed as mean ± S.D.

3.4. Characterization of heterologously expressed recombinant enzymes

We amplified the secretion signal sequence-truncated coding regions of zebrafish, chicken, and mouse L-PGDS cDNAs, as well as ZM or MZ chimeric fragments by PCR (Fig. 5A). Each insert was cloned into the pGEX-6P-1 vector. By using site-directed mutagenesis, we also constructed plasmids for mutant zebrafish protein substituted Gly59 by Cys (G59C), that is essential for PGDS activity in mammals and mouse L-PGDS with Cys65 changed to Ala (C65A). Each of these expression vectors was used to transform *E. coli*. Recombinant GST fusion proteins were efficiently produced by the induction with IPTG (Fig. 5B, lanes 2–8) as compared with the production obtained with no induction by IPTG (lane 1; IPTG(–)). Each L-PGDS protein except for the MZ chimeric protein was separated from GST by digestion with specific protease. Only the MZ chimeric protein was used as the fusion protein with GST for further study, because this protein separated from GST was unstable. Each purified protein showed >90% homogeneity on SDS-PAGE followed by staining with Coomassie Brilliant Blue (Fig. 5B, lanes 9–15).

PGDS activities of the purified recombinant proteins were measured (Table 3). Mouse L-PGDS showed efficient PGDS

activity (434.2±103.3 nmol/min/mg protein), whereas the mouse L-PGDS C65A mutant lacking the essential Cys residue for PGDS activity, did not show any PGDS activity. As for the non-mammalian L-PGDS homologues, the chicken L-PGDS homologue showed weak PGDS activity (11.7±2.3 nmol/min/mg protein), whose PGDS activity disappeared upon heating (100 °C for 30 min) the protein. On the other hand, no PGDS activity was detected in the zebrafish L-PGDS homologue, whose protein sequence does not contain the corresponding Cys residue essential for PGDS activity. When the Gly residue at position 59 of the zebrafish protein, which position corresponds to the essential Cys residue for the PGDS activity in mammals and chickens, was substituted by a Cys residue by site-directed mutagenesis, the zebrafish L-PGDS G59C mutant unexpectedly showed no PGDS activity. We thus suspect that not only the essential Cys residue but also other several amino acid substitutions are required for PGDS activity. Next, we generated the chimeric protein between mouse and zebrafish. The ZM chimeric protein carrying the amino-terminal portion of zebrafish protein and the carboxyl-terminal segment of the mouse protein showed no PGDS activity. On the contrary, the MZ chimeric protein containing the amino-terminal portion of mouse protein carrying the corresponding Cys residue and the carboxyl-terminal segment of zebrafish protein showed weak PGDS activity (14.9±1.5 nmol/min/mg protein), although GST showed no PGDS activity (data not shown). In addition, the PGDS activity of the MZ chimeric protein was completely lost upon heat treatment (100 °C for 30 min) of the protein. These results indicate that non-mammalian L-PGDSs carry no or weak PGDS activity. Residues other than Cys65 would also be involved in the catalytic action as L-PGDS.

3.5. Binding of lipophilic molecules to L-PGDS homologues

It is known that the lipocalin gene family proteins including the mammalian L-PGDSs bind lipophilic molecules such as retinoic acid, retinal, and others (Tanaka et al., 1997; Flower, 2000).

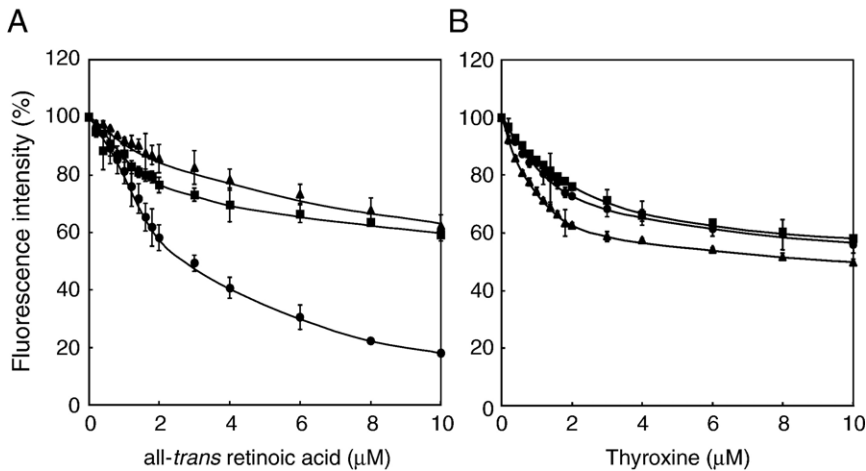


Fig. 6. Fluorescence quenching of intrinsic Trp of zebrafish, chicken, and mouse L-PGDSs by lipophilic molecules. The relative fluorescence intensities of L-PGDSs for zebrafish (▲), chicken (■), and mouse (●) were measured in the presence of various concentrations of all-trans retinoic acid (A) or thyroxine (B). Data are expressed as mean±S.E. of three independent experiments.

Table 4
Ability of L-PGDS proteins to bind lipophilic molecules

	K_d (μ M)	
	All- <i>trans</i> retinoic acid	Thyroxine
Zebrafish	1.01	0.43
Chicken	0.74	0.60
Mouse	0.34	0.64

To elucidate whether the non-mammalian L-PGDS homologues could bind lipophilic molecules, we measured the fluorescence quenching of the intrinsic Trp residue of L-PGDS homologues by using the recombinant proteins of zebrafish, chicken, and mouse L-PGDSs. The zebrafish, chicken, and mouse L-PGDSs showed fluorescence quenching in a concentration-dependent manner by the addition of all-*trans* retinoic acid and thyroxine (Fig. 6). The fluorescence intensity of chicken and zebrafish L-PGDS homologues decreased to approximately 60% of that in the absence of ligand, and that of mouse L-PGDS, to below 20%, in the presence of 10 μ M all-*trans* retinoic acid. On the other hand, when each recombinant protein was incubated with 10 μ M thyroxine, the fluorescence intensity of chicken and mouse L-PGDS homologues decreased to below 60%, and that of zebrafish L-PGDS homologue decreased to below 50%. The calculated K_d values for the zebrafish, chicken, and mouse L-PGDSs were 1.01, 0.70, and 0.34 μ M, respectively, for all-*trans* retinoic acid, and 0.43, 0.60, and 0.64 μ M, respectively, for thyroxine (Table 4). These results reveal that non-mammalian L-PGDSs have conserved the ability to bind to all-*trans* retinoic acid and thyroxine with slightly lower or higher affinities than those of mammalian L-PGDSs.

4. Discussion

Several reports characterizing the proteins involved in the arachidonate cascade in zebrafish and chickens have been previously published. Phospholipase A₂ (Nalefski et al., 1994), cyclooxygenases (COXs) (Grosser et al., 2002), PGE synthase (Pini et al., 2005), and several EST clones showing homologies to mammalian PG receptors have been identified in zebrafish. Chicken phospholipase A₂ (Nalefski et al., 1994), COXs (Xie et al., 1993), hematopoietic PGDS (Thomson et al., 1998), and PGD₂ receptor homologue (Fujimori, K and Urade, Y, unpublished data) have also been characterized. Thus, PG biosynthetic pathways are considered to present in zebrafish and chickens.

The zebrafish L-PGDS homologue did not show PGDS activity, and its primary structure did not include the essential Cys residue for PGDS activity found in mammalian L-PGDS (Urade et al., 1995). In addition, a partial sequence of the rainbow trout L-PGDS homologue also does not contain this corresponding Cys residue (Bayne et al., 2001). Thus, fish L-PGDS homologues do not have the ability to produce PGD₂, which is consistent with a previous report showing that PGE₂ is a major prostanoid and other PGs are negligible in zebrafish (Grosser et al., 2002). We suspect that substitution of several amino acid residues including the Cys

residue essential for PGDS activity in mammalian L-PGDS are needed to gain the enzymatic activity. In fact, the amino-terminal portion of zebrafish L-PGDS was not well conserved (23.8–25.6%), whereas the carboxyl-terminal segments were better preserved among zebrafish, chicken, and mouse (33.3–40%; Table 1). The chimeric protein study demonstrated the importance of the amino-terminal portion for acquiring the PGDS activity, which is consistent with a result that the several amino acid residues around the Cys65 are also important for PGDS activity (Irikura, D. and Urade, Y., unpublished data).

The structures of zebrafish and chicken L-PGDS genes were also well conserved with respect to those of mammalian genes, as there was complete conservation of exon–intron boundaries and their phases (Fig. 2, Table 2). These non-mammalian L-PGDS genes were smaller in size (–2 kb) than mammalian L-PGDS genes (approximately 3-kb) (Eguchi et al., 1999; Igarashi et al., 1992; White et al., 1992) and other lipocalin family genes (3–6 kb) (Salier, 2000). These differences are derived from variable intron sizes. The chromosomal mapping of zebrafish and chicken L-PGDS genes indicated that zebrafish and chicken genes were mapped to chromosome 24 and 17, respectively. Some zebrafish genes involved in the arachidonate cascade have been mapped to chromosomes 5, 2, 5, and 23 for COX-1 (Grosser et al., 2002), COX-2 (Grosser et al., 2002), microsomal PGES-1 (Pini et al., 2005), and cytosolic PGES (Pini et al., 2005), respectively. In addition, chicken genes for COX-1 (Xie et al., 1993), COX-2 (Xie et al., 1993), and hematopoietic PGDS (Thomson et al., 1998) were found on chromosome 17, 8, and 4, respectively. The chromosomal locations of the genes involved in the arachidonate cascade are thus divergent in each animal species. In contrast, some lipocalin genes were found to be clustered in human and mouse chromosomes (Salier, 2000). Recently, Pagano et al. (2004) reported that chicken lipocalin genes were also clustered. Mammalian and non-mammalian lipocalin genes including the L-PGDS gene are thus clustered, and the C8 γ gene is a neighbor of the L-PGDS gene in all mammalian and non-mammalian species identified up to now. Zebrafish and chicken lipocalin genes also seem to have evolved through a gene-duplication event, as in the case of human and mouse lipocalin genes (Salier, 2000).

Expression profiles of chicken, mouse, and human genes were not preserved except for high-level expression in the heart and brain. The present results are in good agreement with previous studies demonstrating the high-level expression of mammalian L-PGDS genes in the brain and heart (Urade and Hayaishi, 2000). On the contrary, in other tissues except for the brain and heart, the expression profiles are different among animal species. Thus, the transcriptional regulatory mechanisms of the L-PGDS gene in the genital organs are different between non-mammals and mammals.

Recently, differential expression analysis of the regeneration of the zebrafish caudal fin demonstrated that zebrafish L-PGDS gene expression was induced 4 days postamputation (Padhi et al., 2004). Moreover, the zebrafish L-PGDS gene was detected in embryos by in situ hybridization (Padhi et al., 2004), which is consistent with our findings that chicken, mouse, and human L-PGDS genes were also expressed during embryonic development. Thus, the expression of L-PGDS genes in the embryo has

been conserved in mammalian species as well as in non-mammalian ones. As retinoic acid and thyroid hormone are critical in embryogenesis (Darras et al., 1999; Niederreither et al., 1999), L-PGDS might act as the carrier protein for these lipophilic molecules in embryonic development. As a further study, L-PGDS functions during the embryo need to be clarified.

Several members of the lipocalin gene family, such as epididymal retinoic acid-binding protein, β -lactoglobulin, and plasma retinol-binding protein have the ability to bind their respective ligands (Åkerström et al., 2000). Zebrafish and chicken L-PGDS have retained almost the same ability to bind lipophilic molecules such as retinoic acid and thyroxine as the mouse protein (Tanaka et al., 1997). Thus, the binding ability with respect to lipophilic molecules has been conserved in both non-mammalian and mammalian L-PGDS proteins throughout the molecular evolution.

L-PGDS homologues in more ancestral organisms such as ascidians, *Caenorhabditis elegans*, and *Drosophila melanogaster* were not found in their databases. Thus, zebrafish and chicken L-PGDSs are useful for complementary studies in mammals for emergence of the L-PGDS function of lipocalin proteins. Our present study also provides an opportunity to elucidate the comparable transcriptional regulatory mechanism involved in the tissue- or stage-specific expression, especially in the brain and heart, during embryogenesis.

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