General and Comparative Endocrinology 276 (2019) 77–85

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

A novel type of prolactin expressed in the bullfrog pituitary specifically during the larval period

Reiko Okada[a,*], Masakazu Suzuki[a], Nozomi Ito[a], Susumu Hyodo[b], Sakae Kikuyama[c]

[a] Department of Biological Science, Faculty of Science, Shizuoka University, Shizuoka 422-8529, Japan
[b] Laboratory of Physiology, Atmosphere and Ocean Research Institute, The University of Tokyo, Chiba 277-8564, Japan
[c] Department of Biology, Faculty of Education and Integrated Sciences, Center for Advanced Biomedical Sciences, Waseda University, Tokyo 162-8480, Japan

ARTICLE INFO

Keywords:
PRL variant
PRL1A
PRL1B
Laval bullfrog
Pituitary

ABSTRACT

Prolactin (PRL) is one of the major hormones that control amphibian metamorphosis. Recently, a PRL (PRL1B) gene that is different from the known PRL (PRL1A) gene has been found in the genomes of several amphibian species. In order to ascertain whether the PRL1B gene is expressed in the bullfrog (*Rana catesbeiana*) pituitary, cloning of cDNA encoding PRL1B in the pituitary of the premetamorphic bullfrog tadpole was attempted. The bullfrog PRL1B amino acid sequence predicted from the obtained cDNA showed 62% identity with those of *Xenopus* PRL1Bs that have been presumed from the genome sequences, whereas the sequence identity between bullfrog PRL1A and PRL1B was 48%. A molecular phylogenetic tree showed that bullfrog PRL1B is most appropriately grouped with amphibian PRL1Bs. Quantitative PCR analysis revealed that the mRNA expression levels of bullfrog PRL1B in the pituitary were high during pre- and prometamorphosis, sharply declined at metamorphic climax and became undetectable after metamorphosis. In contrast, PRL1A mRNA levels were relatively low during pre- and prometamorphosis, rose at climax and remained high after metamorphosis. Immunohistochemical study using antibodies against partial peptides of PRL1A and PRL1B revealed that most of the PRL1A- and PRL1B-immunoreactive cells in the larval pituitary were distributed separately, but that some of the cells immunoreactive with both antibodies were also present. Western blot analysis with the larval pituitary extract indicated that PRL1B-immunoreactive band appeared at the position of molecular weight ca. 22.1 kDa and PRL1A-immunoreactive band at the position of ca. 22.8 kDa. The results obtained in this experiment suggest the possibility that PRL1B plays as-yet-unknown role(s) during the pre-climactic period of metamorphosis. This is the first report on the existence of PRL1B as a protein in the amphibian larval pituitary.

1. Introduction

Prolactin (PRL) is an adenohypophyseal hormone, which was first identified from ovine pituitary as a lactation-stimulating factor in rabbit (Stricker and Grueter, 1928). Today PRL is known as the most versatile hormone; more than 300 biological functions have been reported in mammalian and non-mammalian vertebrates (Bole-Feysot et al., 1998; Goffin et al., 2002). In amphibians, PRL plays important roles in metamorphosis (Kikuyama et al., 1993), osmoregulation (Takada and Hokari, 2007), reproduction (Kikuyama et al., 2000; Toyoda et al., 2005), and body color change (Tang et al., 2014).

Synteny analyses performed by Yamaguchi et al. (2015) revealed that the gene order around the PRL1 (conventional PRL) gene is completely different between fishes and tetrapods. Moreover, they indicated that two types of PRL1 genes are found exclusively in the genome of amphibian species. These PRL genes were designated PRL1A gene and PRL1B gene. The *Xenopus tropicalis* PRL1B gene shows a conserved synteny with fish PRL1 genes, while conservation of gene order has been identified between the regions containing the *X. tropicalis* PRL1A gene and amniote PRL1 genes. On the basis of observed gene synteny patterns, together with molecular phylogenetic trees, these investigators consider that the ancestral PRL1 gene duplicated in an early amphibian to generate these two PRL1 genes and that after the duplication of the PRL1 gene in amphibians, the original PRL1B gene was lost and the newly arisen PRL1A gene was inherited by amniotes.

Amphibian PRL was first purified from bullfrog (*Rana catesbeiana*) pituitaries (Yamamoto and Kikuyama, 1981), and its amino acid sequence was predicted from its cDNA (Takahashi et al., 1990) and also determined by protein sequencing (Yasuda et al., 1991). The primary structure of the bullfrog PRL was used for molecular phylogeny analysis

* Corresponding author at: Department of Biological Science, Faculty of Science, Shizuoka University, 836 Oya, Suruga-ku, Shizuoka 422-8529, Japan.
E-mail address: okada.reiko@shizuoka.ac.jp (R. Okada).

https://doi.org/10.1016/j.ygcen.2019.02.006
Received 12 October 2018; Received in revised form 2 February 2019; Accepted 4 February 2019
Available online 05 February 2019

and was classified as PRL1A (Yamaguchi et al., 2015). Likewise, only PRL1A protein or mRNA expression has been demonstrated experimentally in other amphibian species, such as Japanese toad, *Bufo japonicus* (Takahashi et al., 1993; Yamamoto et al., 1986), *Xenopus laevis* (Buckbinder and Brown, 1993; Yamashita et al., 1993), Japanese red-bellied newt, *Cynops pyrrhogaster* (Matsuda et al., 1990), and Chinese giant salamander, *Andrias davidianus* (Yang et al., 2010). To date, however, neither the expression of mRNA nor the existence of protein of PRL1B has been demonstrated in the pituitary of any amphibian species.

In the present experiment we cloned cDNA encoding bullfrog PRL1B from the pituitaries of premetamorphic larvae. Using the PRL1B cDNA thus obtained and PRL1A cDNA obtained previously by our group (Takahashi et al., 1990), we examined the expression of PRL1A and PRL1B mRNAs in larval and adult bullfrog pituitaries. Immunohistochemical study was also conducted to demonstrate the expression and localization of PRL1A and PRL1B proteins in the pituitary using specific antibodies against PRL1A and PRL1B, respectively.

2. Materials and methods

2.1. Animals

Adult and larval bullfrogs harvested from fields in the vicinity of Tokyo, Japan, were supplied by Oh-uchi Aquatic Animal Supply (Saitama, Japan). They were housed under laboratory conditions and fed bovine liver twice per week for adults or boiled spinach every day for tadpoles. All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Shizuoka University and were approved by Institutional Animal Care and Use Committees of Shizuoka University (2018F–5). Experiments using the bullfrog, a designated alien species in Japan, were performed in accordance with Invasive Alien Species Act of Japan and were authorized by Ministry of the Environment of Japan (13000084).

2.2. Molecular cloning of bullfrog PRL1B cDNA

Sequences of all PCR primers used in this study are listed in Table 1. Total RNA was extracted from the pituitaries of bullfrog tadpoles at stages VII–X (Taylor and Kollros, 1946) using an RNeasy plus universal mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One microgram of the total RNA was reverse transcribed using Moloney-murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo-deoxythymidine $(dT)_{12–18}$ primer. Bullfrog PRL1B cDNA fragment was obtained by reverse-transcription (RT)-PCR using primers XtPRL1B F1 and XtPRL1B R1 designed on the basis of a putative sequence of mRNA for *X. tropicalis* PRL1B that was predicted from its genome sequence (XM_018095782). PCR amplification was performed with TaKaRa Ex Taq polymerase (Takara Bio, Shiga, Japan) under the following conditions: denaturation at 94 °C for 1 min, followed by 35 cycles at 94 °C for 30 s, 40 °C for 30 s, and 72 °C for 1 min. PCR product of the expected size was excised after agarose gel electrophoresis, purified, subcloned into pMD20 T-vector (Takara Bio), and sequenced.

Table 1
Primers used for cDNA cloning, RT-PCR, and qPCR.

Name	Sequence (5′ to 3′)
Cloning of bullfrog PRL1B cDNA	
XtPRL1B F1	ACTGTTGATGTCTGATGGGTTC
XtPRL1B R1	CTCTCCGAATCTCCTTTTGGCATC
fPRL1B R1	AACTGTAATGCCTGTTCCTTGTC
fPRL1B R2	AAGAGTGGAAGTGTGACAG
fPRL1B R3	TCCCAGTAAATGCCTTCCCTG
Adaptor-oligo(dT)	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT
Adaptor	GGCCACGCGTCGACTAGTAC
fPRL1B F1	TCCATTTACTCTCCACTG
fPRL1B F2	TGACCAACAGTATTCTCAG
RT-PCR and qPCR	
fPRL1A F1	TCTCCTCGAAGGAATGGAAAG
fPRL1A R1	CTCCTGGTATTGAAGCTGGG
fPRL1B F3	ATTGCCAGGTTCTGCTCAGTG
fPRL1B R3	TCCCAGTAAATGCCTTCCCTG
frpL8 F1	AAGGCATGGCTACATCAAGG
frpL8 R1	CGGAAGGCAACTTTAGCAAG

The unknown sequences including 5′- and 3′-untranslated regions of the bullfrog PRL1B cDNA was analyzed by 5′- and 3′-rapid amplification of cDNA ends (RACE) methods. For 5′-RACE, the total RNA obtained from the larval pituitary was reverse transcribed with fPRL1B R1 primer, and single-strand cDNA was subsequently obtained following the application of RNase H (Takara Bio). Poly(A) was added to the 3′ terminal of the single-strand cDNA by terminal deoxynucleotidyl transferase (Promega, Madison, MI, USA), and the resultant poly(A)-tailed single-strand cDNA was used as a template in the first-round PCR with adaptor-oligo(dT) and PRL1B R2 primers. The second-round PCR was performed with adaptor and PRL1B R3 primers, using the diluted first-round PCR reaction solution as a template. The first- and second-round PCRs consisted of 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 2 min at 72 °C in a 25 μL reaction mixture. For 3′-RACE, the first-strand cDNA was synthesized using the adaptor-oligo(dT) primer, and the first-round PCR was performed with adaptor and fPRL1B F1 primers. The second-round PCR was performed with adaptor and fPRL1B F2 primers using the diluted first-round PCR reaction solution as a template. The first- and second-round PCRs consisted of 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 2 min at 72 °C in a 25 μL reaction mixture. The RACE products of the expected sizes were excised after agarose gel electrophoresis, purified, subcloned into pMD20 T-vector (Takara Bio), and sequenced. Putative phosphorylation site, signal peptide cleavage site, and receptor binding interfaces of bullfrog PRL1B were predicted using NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos/, Blom et al., 1999), SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/, Petersen et al., 2011), and NCBI Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, Marchler-Bauer et al., 2017), respectively.

2.3. RT-PCR

Total RNAs were extracted from various organs of the adult and larval (TK stage VI–IX) bullfrogs, including the anterior pituitary, brain, heart, intestine, kidney, and skeletal muscle. The total RNAs were reverse transcribed as described above. Ribosomal protein L8 (rpL8) mRNA was used as an internal control. Specific primer pairs for PRL1A (fPRL1A F1 and fPRL1A R1), PRL1B (fPRL1B F3 and fPRL1B R3), and rpL8 (frpL8 F1 and frpL8 R1) were used in PCRs to amplify the 101-, 128-, and 84-bp products, respectively. The PCRs consisted of 1 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C in 10 μL reaction mixtures. The amplified products were visualized on a 3% agarose gel containing ethidium bromide. Authenticity of the products was confirmed by sequencing.

2.4. Quantitative real-time PCR (qPCR)

The qPCR assay was performed on a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) with 10 μL buffer containing 4 μL diluted cDNA, 0.5 μL of each of the primers (final concentration 0.4 μM), and 5 μL FastStart Essential DNA Green Master (Roche Diagnostics) in each well of a 384-well plate. The PCR program consisted of denaturation at 95 °C for 5 min, and 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 10 s. Following cyclic amplification, melting curve analysis was conducted from 65 °C to 97 °C to assess the specificity of PCR amplification. The qPCR assay was carried out in duplicate. The expression levels of

PRL1A and PRL1B mRNA were calculated according to a standard curve and normalized to rpL8 mRNA using the LightCycler 480 multiple plate analysis software (Roche Diagnostics). PCR efficiencies (means of triplicate tests ± SEM) of PRL1A, PRL1B, and rpL8 were 1.948 ± 0.0189, 1.967 ± 0.0176, and 1.990 ± 0.0178, respectively. Total RNA without RT was used as a negative control to check for contamination of genomic DNA, and water was used to check for primer–dimer formation and reagent contamination.

2.5. Antibodies against bullfrog PRL1A and PRL1B

Peptides corresponding to $PRL1A_{114–127}$ and $PRL1B_{76–89}$ (Fig. 2) were synthesized, coupled to keyhole limpet hemocyanin, and used as antigens to immunize rabbits (Eurofins Genomics, Tokyo, Japan).

The obtained antisera were purified by affinity chromatography. The synthetic oligopeptides of PRL1A and PRL1B were separately coupled to a CNBr-activated Sepharose 4B (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The PRL1A antiserum was passed through the PRL1A oligopeptide-conjugated column five times. The column was washed with 10 mM phosphate buffered saline (PBS, pH 7.4), and bound protein was eluted with an elution buffer (0.2 M glycine–0.5 M NaCl, pH 2.7). The eluted solutions were immediately neutralized with a neutralizing buffer (2 M Tris-HCl, pH 8.0) and dialyzed against PBS. Protein concentrations were determined based on absorbance at 280 nm. PRL1B antiserum was first applied to the PRL1A oligopeptide-conjugated column, and then the flow-through was purified using the PRL1B oligopeptide-conjugated column as described above.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Pituitaries of adult and larval (TK stage IV–XVIII) bullfrogs were homogenized in a sample buffer [50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol] and were centrifuged 13,000 × *g* for 10 min to remove insoluble materials. The protein concentrations were determined using Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Bromophenol blue and β-mercaptoethanol were added to the extract at the final concentration of 0.01% (w/v) and 6% (v/v), respectively. The protein was denatured at 100 °C for 3 min and subjected to electrophoresis on a 18% polyacrylamide gel. The gel was divided into two: one for Coomassie brilliant blue (CBB) R-250 staining and another for Western blotting. For Western blotting, the proteins on the gel were transferred to an Immobilon-P membrane (Millipore, Billerica, MS, USA). The membrane was blocked in a Block Ace solution (KAC, Kyoto, Japan) for 2 h. The proteins on the membrane were reacted with anti-bullfrog PRL1A (200 ng mL^{-1}) or anti-bullfrog PRL1B (200 ng mL^{-1}) for 16 h, followed by 2-h-incubation with peroxidase-labeled goat anti-rabbit IgG (1:50,000; Jackson Immunoresearch, West Grove, PA, USA). Immunopositive bands were visualized using enhanced chemiluminescence Amersham ECL Western blotting detection reagents (GE Healthcare) and were detected using ImageQuant LAS 4000mini (GE Healthcare). To check the specificity of the immunoreaction, antibodies that had been preincubated with 10 μg mL^{-1} of the antigen peptides were used.

2.7. Immunohistochemistry

The pituitary from adult or larval (TK stage IX) bullfrog was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h, dehydrated, embedded in Paraplast plus (McCormick Scientific, St. Louis, MO, USA), and cut to a thickness of 4 μm. The sections were covered with anti-bullfrog PRL1A (1 μg mL^{-1}) antibody, anti-bullfrog PRL1B (1 μg mL^{-1}) antibody, or rabbit anti-bullfrog growth hormone (GH) serum (1:1000; Kobayashi and Kikuyama, 1991) for 16 h, followed by incubation with Cy3-labeled donkey anti-rabbit IgG (1:400; Jackson Immunoresearch) or Alexa Fluor 488-labeled donkey anti-rabbit IgG (1:400; Jackson Immunoresearch) for 2 h. For counter-staining of the nucleus, 4′,6-diamidino-2-phenylindole (DAPI) was added to the secondary antibody solution. The specimens were washed in PBS and mounted in PermaFluor aqueous mounting medium (Thermo Fisher Scientific). The preparations were observed under an Olympus BX61 microscope equipped with a BX-epifluorescence attachment (Olympus, Tokyo, Japan). The specificities of immunostaining were checked using the antibodies that had been pre-incubated with 10 μg mL^{-1} of the antigen peptide.

2.8. Molecular phylogenetic analysis

The amino acid sequences of PRL from fish to human were aligned using Clustal W (Thompson et al., 1994). An optimal unrooted tree was inferred by the neighbor-joining method (Saitou and Nei, 1987) in the MEGA7 program (Kumar et al., 2016). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000), and confidence in the neighbor-joining tree was assessed with 1000 bootstrap replications (Felsenstein, 1985).

2.9. Statistical analysis

The significance of difference between the values obtained in each experiment was assessed by Tukey's test. A p value of less than 0.05 was considered significant.

3. Results

3.1. Molecular cloning of bullfrog PRL1B cDNA

A cDNA encoding bullfrog PRL1B was cloned from the pituitaries of premetamorphic larvae at stages VII–X using the 5′- and 3′-RACE methods. The 1330-bp PRL1B cDNA included a 669-bp coding region, which encoded 222 amino acid residues (Fig. 1). Molecular weight of mature bullfrog PRL1B deduced from its amino acid sequence was ca. 22.1 kDa, and that of PRL1A was ca. 22.8 kDa. The nucleotide and amino acid sequence identities of bullfrog PRL1B with those of bullfrog PRL1A (mRNA, X16063; protein, CAA34199) were 36% and 48%, respectively. As with other vertebrate PRLs, bullfrog PRL1B contained six cysteine residues which may form three disulfide bonds, and a putative phosphorylation site at residue 202 which corresponds to human ser177/rat ser179 (position in the mature protein, Fig. 2).

The bullfrog PRL1B amino acid sequence showed 62% identity with the sequence of *X. tropicalis* (XP_017951271) or *X. laevis* (NP_001159915) PRL1B. The amino acid sequence of bullfrog PRL1B showed 28% identity with that of bullfrog GH (Fig. S1D). The bullfrog PRL1B, of which sequence was predicted from the cDNA obtained, was grouped on the molecular phylogenetic tree with other amphibian PRL1Bs (Fig. 3).

3.2. Expression of bullfrog PRL1A and PRL1B mRNAs

The tissue distribution of PRL1A and PRL1B mRNA expression in premetamorphic larvae and adults was examined by RT-PCR using specific primers (Fig. 4). In the tadpole, both PRL1A and PRL1B mRNA expression was recognized in the anterior pituitary. Besides the pituitary, a slight PRL1B mRNA expression was detected in the larval brain and intestine. In the adults, PRL1A mRNA expression was clearly observed in the anterior pituitary and marginally in the intestine, whereas only slight PRL1B mRNA expression was detected in the brain and intestine.

Subsequently, expression levels of PRL1A and PRL1B mRNA in the pituitary of tadpoles at various developmental stages and in the adult were analyzed by qPCR. PRL1A mRNA levels were relatively low during premetamorphosis and remained low during prometamorphosis. The

```
   1  AGGCTGATAATTCCAAATTAGATAGAAAGTCAACACAACCATGATTACCTCAAGAAAAAC    60
   1                                          M  I  T  S  R  K  T     7

  61  TTCATTTACAGGAAAGTTGTTCTTTGCACTTCTAGTCTCTAATTTGGTTTTGGTGAGAAA   120
   8    S  F  T  G  K  L  F  F  A  L  L  V  S  N  L  V  L  V  R  K    27

 121  ACTGGTCTTGTCCAACCCAATCTGCACCCCGGGAAGCCTCCATTGCCAGGTTCTGCTCAG   180
  28    L  V  L  S  N  P  I  C  T  P  G  S  L  H  C  Q  V  L  L  S    47

 181  TGATCTTTTTGACAGGGCAATCAGAATCTCACATTATATCCATTTACTCTCCACTGAAAT   240
  48    D  L  F  D  R  A  I  R  I  S  H  Y  I  H  L  L  S  T  E  I    67

 241  CTTTGAAGACTTTGACCAACAGTATTCTCAGGGAAGGCATTTACTGGGAAATGTTTTGAA   300
  68    F  E  D  F  D  Q  Q  Y  S  Q  G  R  H  L  L  G  N  V  L  N    87

 301  CAACTGTCACACTTCCACTCTTAATACCCCAGAAGACAAGGAACAGGCATTACAGTTGCA   360
  88    N  C  H  T  S  T  L  N  T  P  E  D  K  E  Q  A  L  Q  L  Q   107

 361  ACACAATGACTTGCTAAGCCTTGTACACCAGTTACTACGATCATGGAACCAACCACTACA   420
 108    H  N  D  L  L  S  L  V  H  Q  L  L  R  S  W  N  Q  P  L  Q   127

 421  GCACTTGACTACAGGGGCTCCAGATCACATGATCAAGAAGTTAAAAGAGGCTGAGGAACA   480
 128    H  L  T  T  G  A  P  D  H  M  I  K  K  L  K  E  A  E  E  H   147

 481  TACACAAGTGCTGCAAGGCGGAATAGACAGAATCTCTGGAAGGATGCTGACTGATCTGGA   540
 148    T  Q  V  L  Q  G  G  I  D  R  I  S  G  R  M  L  T  D  L  D   167

 541  TGACTTTTACCCACCATGGTTTGGTCCCATAGATGCTGCAGTGCCACTGAGAGAGTCTCA   600
 168    D  F  Y  P  P  W  F  G  P  I  D  A  A  V  P  L  R  E  S  Q   187

 601  GATGTTTGCTATCTATCATTTGCTGCACTGTTTCCGTAGAGACTCTCACAAGATTGACAA   660
 188    M  F  A  I  Y  H  L  L  H  C  F  R  R  D  S  H  K  I  D  N   207

 661  CTATCTGAAAATCCTGAGATGTCGCATGGTACATGCCAACAACTGCTAGGGAAAGTACTA   720
 208    Y  L  K  I  L  R  C  R  M  V  H  A  N  N  C  *               222

 721  TGCCTCTTATAGCTGAACCCTGGGATGAAACACAAAAATACCCTTGCAGTGGGACTTTTG   780
 781  CACTGCAAAAGTTAAATGTACATTATTTTTAGGAGGTAGTGGAAGATGATCTATTATGTA   840
 841  CCCTATTCCCCATTCCAGCAGGCCCCAGCGCTGTCCTCTCCCGGAAGCTCCCTGTGGAAG   900
 901  CCTGCTGGAGGGCAGAATAAGGTAAGTACTTCAGCTTTTTCCAGCACCTCTAGACATAAG   960
 961  GAACAGTGTGGGGGTGGGGGGGGAATACCACAGCAAGGGTTATTTTTTTTCCACTTGGAG  1020
1021  TTAAGCTTTAAGTTTTATGTGTCTGCTATTATCTCAACTATAGCTCAATTTACCATTATG  1080
1081  CCATGCTAGGAAAGAAAACAGCATGGGGCTCATAGTAACCTTGCTGTATAATAATGTGCT  1140
1141  AAATCTCACAATAATGAGTCAGCAGGCTTATTCTTTTTTTTTAAAGCAGATCATTTTTAT  1200
1201  TGAAAAAGAATTTTCTTTTTACTACAAACTTAACTTAATGTTGGGAAGACACAAGCTTAT  1260
1261  ACATTGGTATAACCAGCCAGTTCCAGTACAAAACAATATTCCATGTAAAATAAAGAAACA  1320
1321  TATTATAAAC                                                    1330
```

Fig. 1. Nucleotide and deduced amino acid sequences of bullfrog PRL1B (GenBank accession number LC424418). An asterisk indicates the termination codon. The positions of the specific primers utilized for RT-PCR and qPCR (fPRL1B F3 and fPRL1B R3) are underlined.

mRNA levels rose during metamorphic climax and the high levels were maintained into adulthood (Fig. 5A). On the other hand, PRL1B mRNA levels were high during premetamorphosis and prometamorphosis. During climax, the levels declined drastically. In the adult pituitaries, PRL1B mRNA expression became undetectable (Fig. 5B). The expression levels of PRL1A mRNA were higher than those of PRL1B mRNA at every developmental stage examined (Fig. 5).

3.3. Expression of bullfrog PRL1A and PRL1B proteins in the pituitary

Expression of PRL1A and PRL1B proteins in the bullfrog pituitary was examined by Western blot analysis. PRL1A (ca. 22.8 kDa) immunopositive band was detected in the extract of both larval (TK stage IV–XVIII) and adult pituitaries, whereas fPRL1B (ca. 22.1 kDa) band was stained only in the larval extract. Specificities of the antibodies were confirmed by preabsorption test; the immunopositive bands vanished when the antibodies were preincubated with the corresponding antigen peptides (Fig. 6A). CBB-staining following SDS-PAGE also

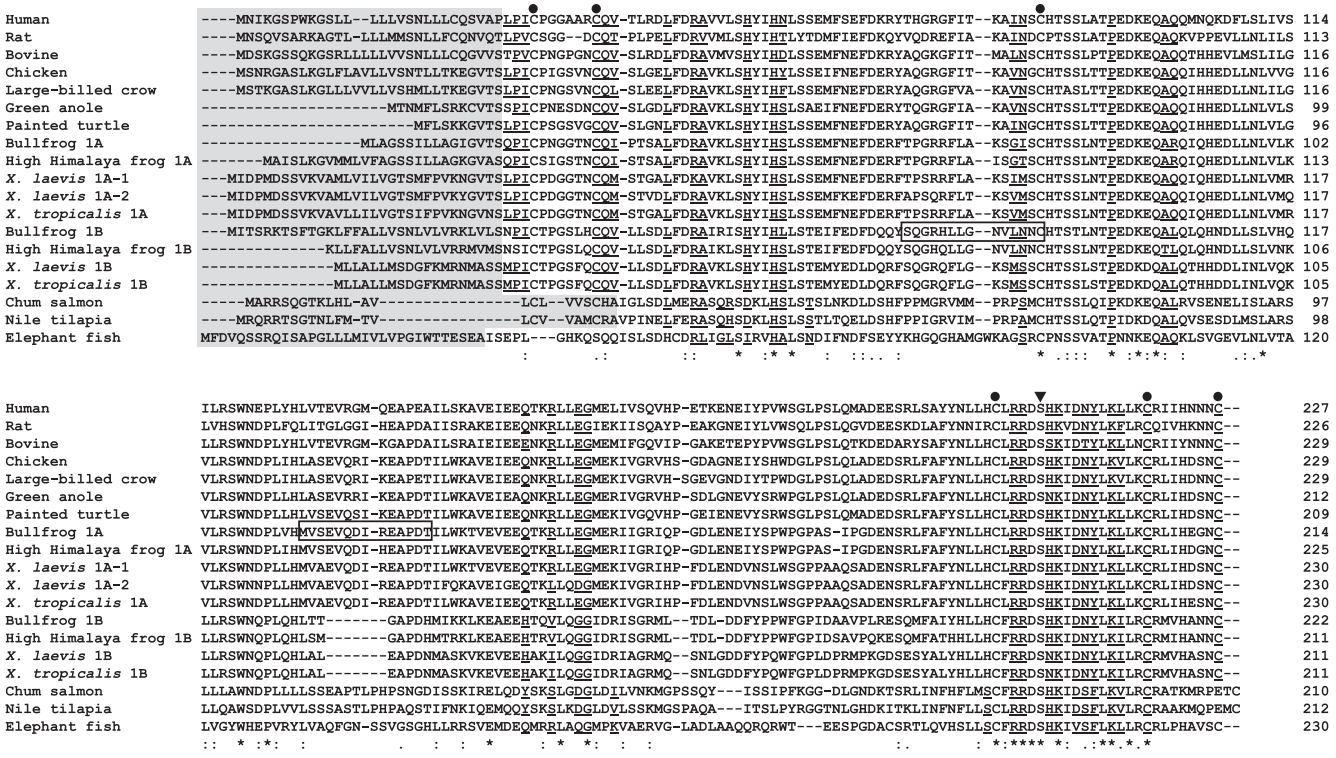

Fig. 2. Comparison of the amino acid sequences of PRL in various animals. Asterisks indicate fully conserved residues. Colons and dots indicate conservation between amino acids of strongly and weakly similar properties, respectively. Signal peptide regions have a tinted background. Putative receptor binding interfaces are underlined. The black inverted triangle indicates the serine residue that corresponds to human ser177/rat ser179 (position in the mature protein). Black circles indicate cysteine residues which form three or two disulfide bonds. Regions corresponding to the amino acid sequence of antigen peptides for bullfrog PRL1A and PRL1B antisera are boxed. The accession number of each sequence in this figure is noted in Fig. 3.

showed that the larval pituitary expressed PRL1B protein in addition to PRL1A protein, whereas the adult pituitary expressed only PRL1A protein. In the larval pituitary extract, protein content of PRL1B was slightly less than that of PRL1A (Fig. 6B).

Using mirror sections of the larval pituitary, immunofluorescence staining was performed to examine the expression and localization of PRL1A and PRL1B. PRL1A-immunoreactive signals were distributed widely in the anterior pituitary of premetamorphic larva (Fig. 7A). As for PRL1B, intense signals were detected in a large number of the cells of the larval pituitary (Fig. 7D). Most of the PRL1A- and PRL1B-immunoreactive signals in the pituitary cells did not overlap each other, but some of the cells were revealed to bear the fluorescent labeling for both PRL1A (Fig. 7C) and PRL1B (Fig. 7F) as seen in the merged image of mirror sections (Fig. 7I). When the antibodies against PRL1A and PRL1B preadsorbed with their respective antigens were used for immunostaining, no signals were detected (Fig. 7B, E).

Adjacent sections of the adult pituitary were stained with rabbit anti-bullfrog PRL1A IgG or anti-bullfrog PRL1B IgG. PRL1A-immunoreactive signals were observed widely in the anterior pituitary (Fig. 7J), whereas no PRL1B-immunopositive signal was detected in the pituitary (Fig. 7K). When the anti-bullfrog PRL1A IgG preabsorbed with antigen peptide was used for immunostaining, no signals was detected (Fig. 7L).

Mirror sections of larval and adult pituitary were also immunostained using anti-bullfrog GH and anti-bullfrog PRL1A or PRL1B. Most immunopositive signals of GH did not overlap with PRL1A- or PRL1B-signals, but non-negligible number of cells expressed both GH and PRL1A or PRL1B (Supplementary Fig. S1A–C).

4. Discussion

In the present experiment, a cDNA encoding bullfrog PRL1B was cloned from the larval pituitary and, using this, we successfully demonstrated the expression of PRL1B mRNA and the presence of PRL1B protein in the bullfrog. It is notable that neither mRNA for PRL1B nor PRL1B protein was detected in the pituitary when the animals had completed metamorphosis. This report is the first to verify that the PRL1B molecule exists in the amphibian pituitary.

The primary structure of the bullfrog PRL1B showed a high sequence similarity with the corresponding structures in other amphibians such as *X. laevis, X. tropicalis*, high Himalaya frog (*Nanorana parkeri*), and Mexican salamander (*Ambystoma mexicanum*), all of which had been predicted from their genome sequences. On the other hand, a relatively low sequence similarity was observed between the bullfrog PRL1B and PRL1A of which amino acid (Yasuda et al., 1991) and cDNA sequences (Takahashi et al., 1990) had been determined previously.

In mammals, physiological activity of PRL is generally altered by phosphorylation at serine 179 (human) or 177 (rat), and this is inferred to result from differential use of post-receptor signaling pathways (Binart, 2017). This residue is quite well conserved among vertebrates and is considered to be a phosphorylation site in most species (Walker, 2007). It was revealed that serine 202 of bullfrog PRL1B as well as serine 194 of bullfrog PRL1A corresponds to the mammalian serine 179/177. Tetrapod PRLs, including amphibian PRL1A, contain three disulfide bonds in homologous positions. Likewise, three disulfide bonds are expected to exist in the mature PRL1B of the bullfrog, according to the prediction of signal peptide cleavage site.

Analysis of mRNA expression of PRL1A and PRL1B the bullfrog pituitary revealed that adult pituitary expresses only PRL1A mRNA, while both PRL1A and PRL1B mRNAs are expressed in the pituitaries of pre-climactic tadpoles, suggesting that PRL1B is synthesized in the larval pituitary.

Quantitative PCR analysis of bullfrog PRL1A and PRL1B mRNAs indicated that their expression patterns during development are clearly

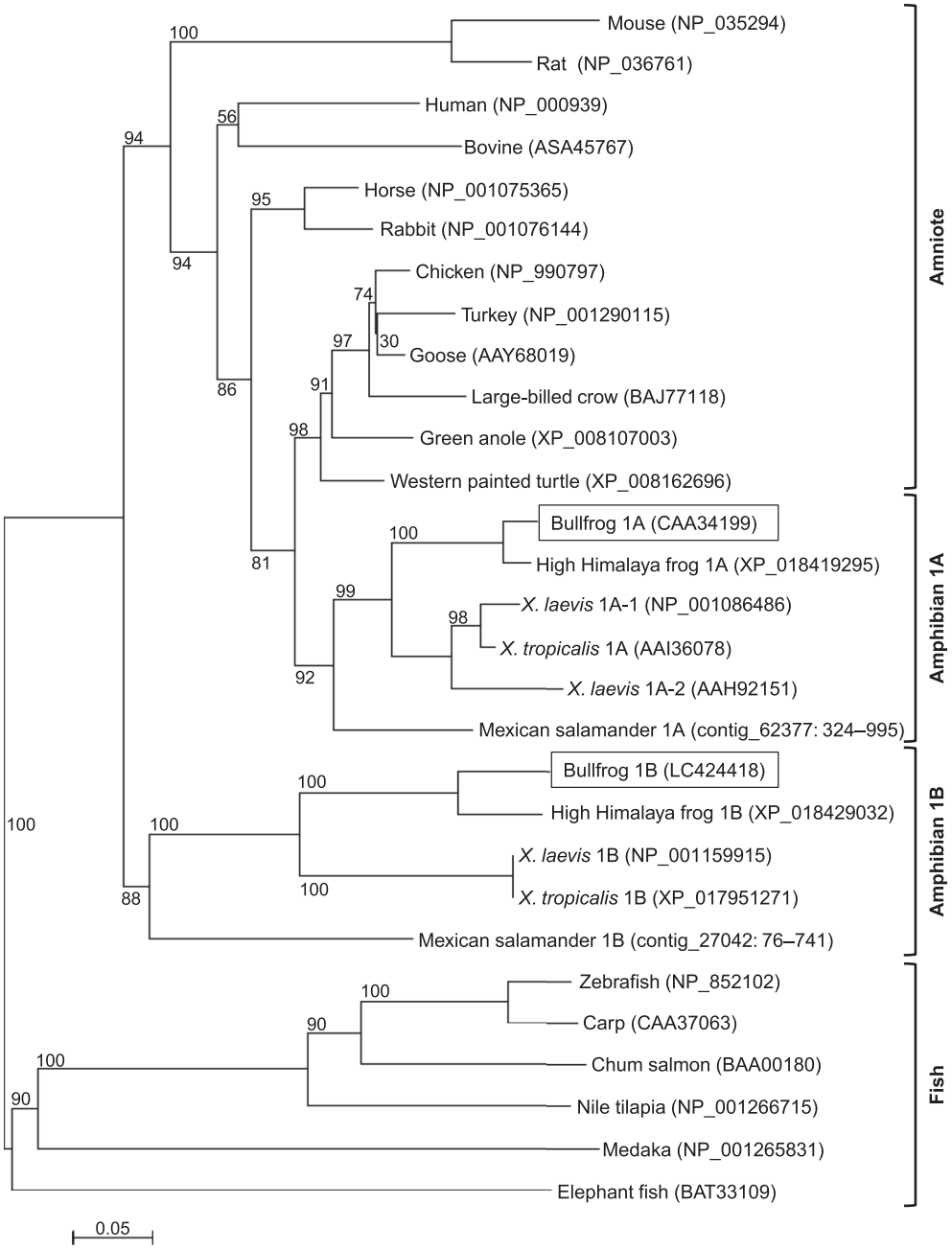

Fig. 3. Neighbor-joining unrooted tree of PRL proteins from fish to human. The length of each branch is proportional to the mean number of differences per residue, and the numbers around the interior branches are bootstrap probabilities (percent, 1000 replicates). The animal name and accession number are indicated for each PRL.

different. During premetamorphosis, the expression level of PRL1A mRNA was low, and still remained low during prometamorphosis. It was sharply elevated in the metamorphic climax stage, the maximum expression being approximately 7.8-fold higher than in the pre-metamorphic period. The pattern of PRL1A mRNA levels coincided well with the patterns previously reported in the bullfrog (Takahashi et al., 1990) and *X. laevis* (Buckbinder and Brown, 1993) larvae. On the other hand, the expression levels of PRL1B mRNA were high throughout premetamorphosis and prometamorphosis and sharply declined during metamorphic climax. However, it should be noted that the expression levels of PRL1B mRNA were relatively low as compared with those of PRL1A mRNA. Throughout the pre-climactic period, the mRNA expression levels of PRL1B were below one-fifth those of PRL1A. As for the protein levels of both type of PRLs, however, CBB-staining following SDS-PAGE showed that there is not much difference between PRL1A and PRL1B in the larval pituitary. Moreover, a considerable number of PRL1B-immunopositive cells were observed by immunohistochemistry. Accordingly, it is presumed that in the larval pituitary, both synthesizing and releasing activities for PRL1B are lower than those for PRLA. However, quantification of the pituitary contents and plasma concentrations of both PRL1A and PRL1B in the larvae will be necessary to reach a definite conclusion. Currently, development of specific immunoassay systems for PRL1A and PRL1B employing the antibodies that were used in this study is underway.

Immunoassay of plasma PRL concentrations during metamorphosis in the bullfrog had been performed using an antiserum against the full length of the PRL(1A) molecule (Yamamoto and Kikuyama, 1982). The pattern of changes of PRL levels was consistent with those of PRL1A

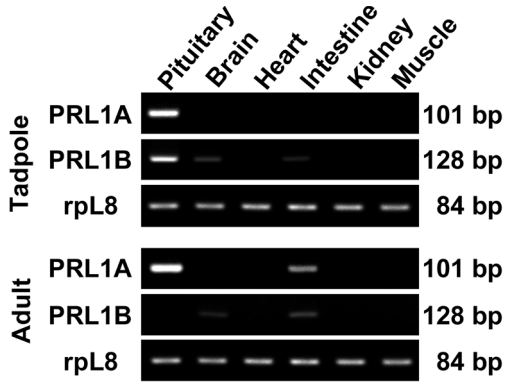

Fig. 4. Tissue distribution of PRL1A and PRL1B mRNAs in larval (TK stage VI–IX) and adult bullfrogs. PCR products were electrophoresed on an agarose gel and stained with ethidium bromide. Bullfrog rpL8 mRNA was used as an internal control.

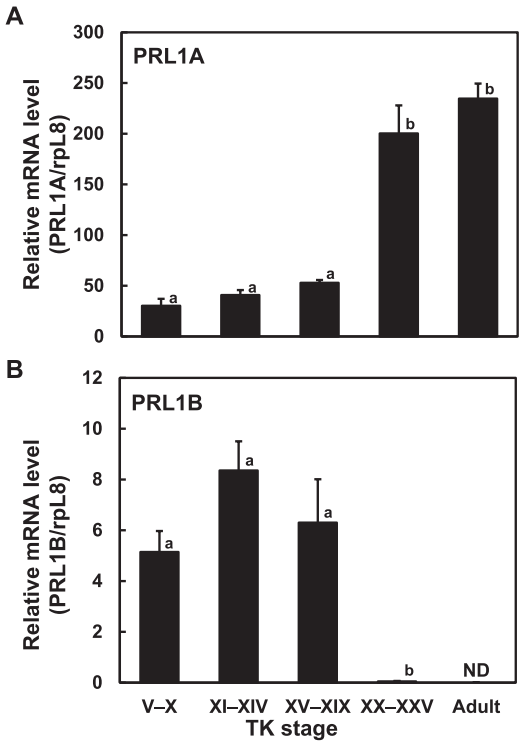

Fig. 5. Expression levels of mRNAs for PRL1A (A) and PRL1B (B) in the pituitaries of tadpoles at various stages and of adults. The relative values of mRNA expression were measured by qPCR according to the standard curve method and normalized to rpL8. Data are given as mean ± SEM (n = 9). Values with different letters are significantly different from each other at the 5% level (Tukey's test). TK stages V–X, XI–XIV, XV–XIX, and XX-XXV are classified to premetamorphosis, early prometamorphosis, late prometamorphosis, and climax, respectively. ND, not detected.

mRNA levels (Takahashi et al., 1990). Accordingly, the immunoassay data are considered to represent the PRL1A levels, although it is not clear whether the antiserum recognized PRL1B or not.

In this study, antibodies raised against partial peptides of bullfrog PRL1A and PRL1B were used for Western blot and immunohistochemical analyses. In the Western blot analysis, each of the anti-PRL1A and anti-PRL1B antibodies recognized a different band. The molecular weights of bullfrog PRL1A and PRL1B estimated by SDS-PAGE coincided well with those computed from the amino acid sequences of mature PRL1A and PRL1B.

It is known that PRL and GH belong to the same hormone family,

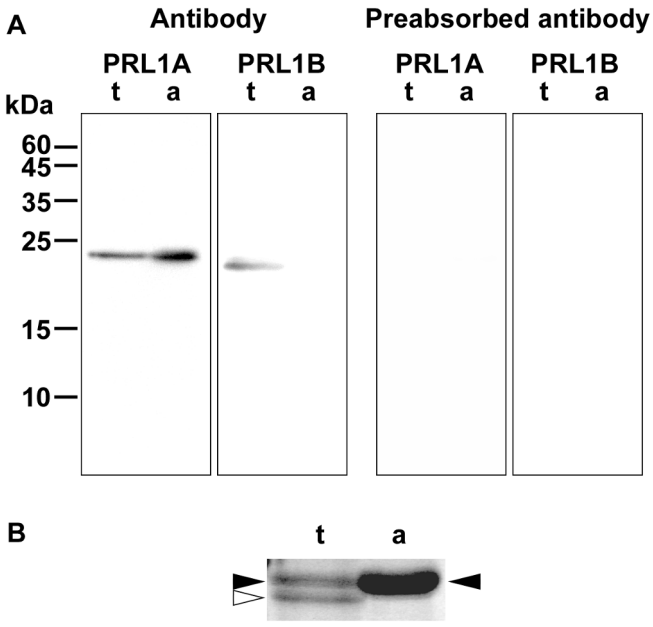

Fig. 6. Western blot analysis (A) and CBB-staining (B) of proteins extracted from larval (TK stage IV–XVIII) and adult pituitaries. (A) Proteins (2 μg/lane) were electrophoresed on a 18% polyacrylamide gel, transferred to Immobilon-P membrane, and reacted with rabbit anti-fPRL1A or anti-fPRL1B. Antibodies that had been preincubated with the antigen peptides were used as negative controls. (B) Proteins (20 μg/lane) were visualized in a CBB-R250 staining solution following SDS-PAGE. Filled and open arrowheads indicate fPRL1A (ca. 22.8 kDa) and fPRL1B (ca. 22.1 kDa) protein, respectively. t, tadpole; a, adult.

namely GH/PRL family. The antigen peptides for anti-PRL1A and anti-PRL1B antibodies showed quite low sequence similarities with the corresponding regions of GH (Fig. S1D). In fact, most of the PRL1A- or PRL1B-immunoreactive cells did not overlap with GH-immunoreactive ones. However, only a very few PRL1A- or PRL1B-immunoreactive cells were revealed to be also GH-immunopositive, when the antiserum against the full-length of GH was used. Thus, it is concluded that neither anti-PRL1A antibody nor anti-PRL1B antibody cross-reacted with GH and that a few PRL cells may contain GH or GH-like substance(s). The similar result has previously been obtained by an immunoelectron microscopic study with the larval bullfrog pituitary using antisera against full-length of GH and PRL(1A) (Kikuyama, 1994).

Western blot, SDS-PAGE, and immunohistochemical analyses performed in the present experiment revealed that the larval pituitary expresses both PRL1A and PRL1B proteins, whereas only PRL1A protein exists in the adult pituitary. Additionally, it was demonstrated that PRL1A and PRL1B colocalize in some of the cells of the larval pituitary.

It is known that thyrotropin-releasing hormone (TRH) induces the release of PRL from the adult bullfrog pituitary (Seki and Kikuyama, 1986) and that TRH is the major PRL-releasing factor in the bullfrog hypothalamus (Nakajima et al., 1993). Recently, it has been demonstrated that PRL cells in the bullfrog pituitary express type-3 TRH receptor mRNA (Nakano et al., 2018). Identification of the releasing factor and its receptor that are involved in the release of PRL1B is awaited for the understanding of the regulatory mechanisms of this newly found PRL.

The structures of PRL receptor were determined or predicted in several amphibian species (bullfrog, BAD14941, Hasunuma et al., 2004; *X. laevis*, BAA90400, Yamamoto et al., 2000; Japanese red-bellied newt, BAB61107, Yamamoto et al., 1998; Japanese toad, BAF75354; and high Himalaya frog, XP_018418958). In most of the amphibians, the presence of a single PRL receptor gene in the genome has been reported, except for *X. laevis* in which two PRL receptor genes, L (NM_001085616) and S (NM_001085736) homeologs were found. In

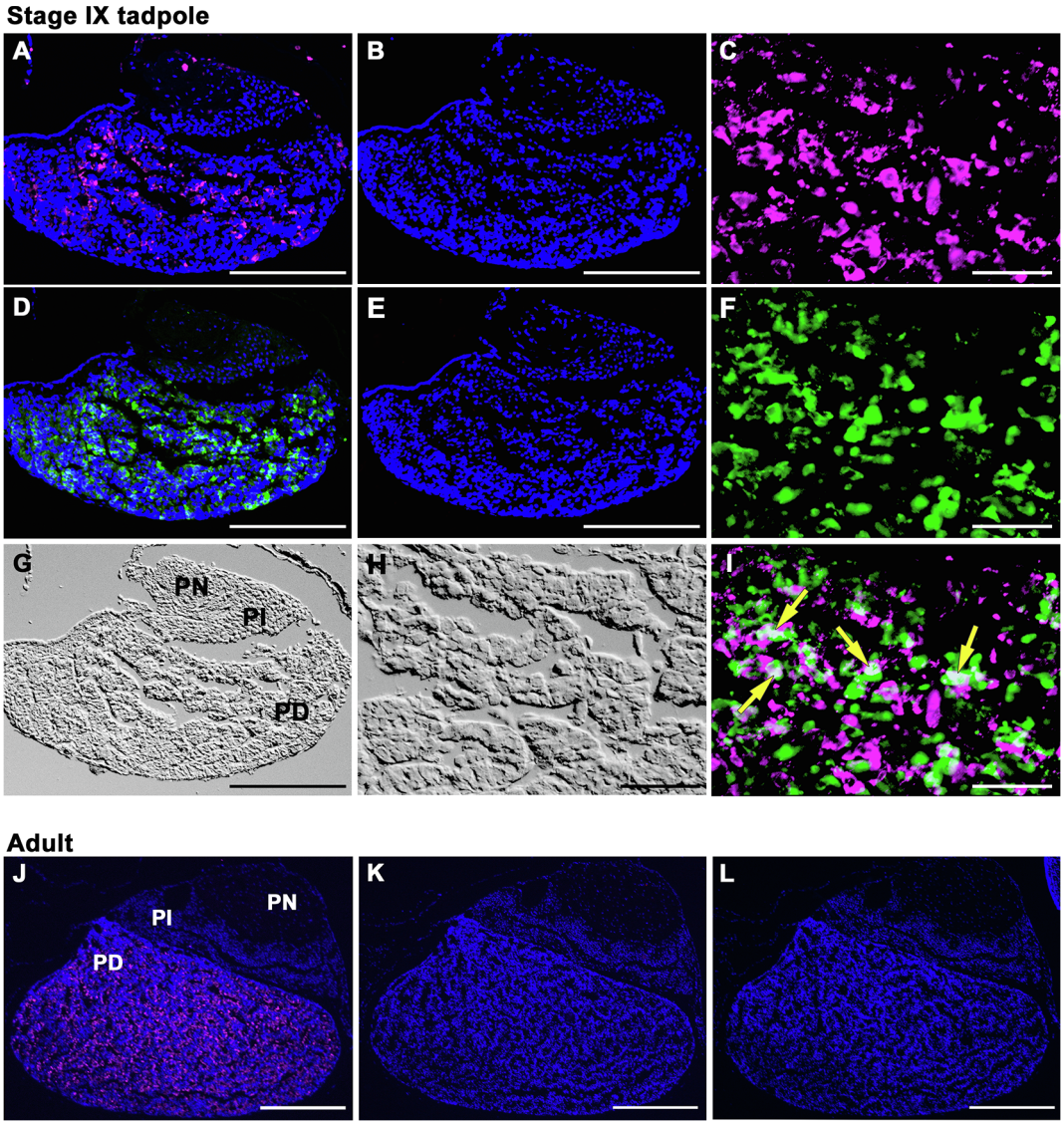

Fig. 7. Images of immunohistochemistry for PRL1A and PRL1B in the pituitaries of larval (TK stage IX) and adult bullfrogs. Mirror sections of the larval pituitary were stained with rabbit anti-bullfrog PRL1A IgG (A), anti-bullfrog PRL1A IgG preabsorbed with antigen peptide (B), anti-bullfrog PRL1B IgG (D), and anti-bullfrog PRL1B IgG preabsorbed with antigen peptide (E). Panels C and F are the magnified images of the anterior pituitaries in A and D, respectively, and panel I is a merged image of C and F. Arrows in panel I point to representative cells that expressed both PRL1A and PRL1B. Adjacent sections of the adult pituitary were stained with rabbit anti-bullfrog PRL1A IgG (J), anti-bullfrog PRL1B IgG (K), and anti-bullfrog PRL1A IgG preabsorbed with antigen peptide (L). Nomarski differential interference-contrast images are shown as the corresponding references: G to A, and H to C. Bar = 200 μm (A, B, D, E, and G), 50 μm (C, F, H, and I), and 500 μm (J–L). PD, pars distalis; PI, pars intermedia; PN, pars nervosa.

mammalian species, various isoforms of PRL receptor are known to be derived by differential splicing of the RNA transcribed from a single gene or by posttranslational modifications (Bole-Feysot et al., 1998; Kelly et al., 1991). Structural variations are observed in the extracellular and intercellular domains of PRL receptor and cause functional differences (Brooks, 2012). It remains to be clarified whether PRL1B binds to an as-yet-unidentified receptor or to the receptor common to that for PRL1A.

Finally, it should also be mentioned that the expression of PRL1B mRNA was noted in the brain and intestine of both the tadpole and adult, though not strongly. It has been reported that PRL mRNA and/or protein exist in the brain of several vertebrates, such as rat (Mejía et al., 1997), sheep (Roselli et al., 2008), turkey (Chaiseha et al., 2012), and elephant fish (Yamaguchi et al., 2015). The function of PRL in the brain has not been thoroughly clarified, but it is inferred to act as an antistress modulator and a neurotransmitter/neuromodulator (Marano and Ben-Jonathan, 2014). As for the intestine, the presence of immunoreactive PRL in the human small intestine has been recognized by Stevens and Shaw (1982). According to Urtishak et al. (2001), lymphocytes located between epithelial cells in the small intestine of the rat express PRL and PRL receptors. In the case of the bullfrog, further studies are needed to identify the PRL1B-expressing cells in the brain and intestine.

In conclusion, our study has demonstrated for the first time the existence of a novel PRL, namely, PRL1B exclusively in the larval bullfrog pituitary. The mode of secretion and physiological roles of PRL1B remain to be elucidated.

Funding

This work was supported in part by Grant-in-Aid for Scientific Research to R.O. and S.K. (JP16K07420) and Grant-in-Aid for Challenging Exploratory Research to R.O., M.S., and S.H. (JP16K14766).

Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.02.006.

References

Binart, N., 2017. Prolactin. In: Melmed, S. (Ed.), The Pituitary, fourth ed. Academic Press, New York, pp. 129–161. https://doi.org/10.1016/B978-0-12-804169-7.00005-2.

Blom, N., Gammeltoft, S., Brunak, S., 1999. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J. Mol. Biol. 294, 1351–1362. https://doi.org/10.1006/jmbi.1999.3310.

Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., Kelly, P.A., 1998. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr. Rev. 19, 225–268. https://doi.org/10.1210/edrv.19.3.0334.

Brooks, C.L., 2012. Molecular mechanisms of prolactin and its receptor. Endocr. Rev. 33, 504–525. https://doi.org/10.1210/er.2011-1040.

Buckbinder, L., Brown, D.D., 1993. Expression of the *Xenopus laevis* prolactin and thyrotropin genes during metamorphosis. Proc. Natl. Acad. Sci. 90, 3820–3824. https://doi.org/10.1073/pnas.90.9.3820.

Chaiseha, Y., Ngernsoungnern, P., Sartsoongnoen, N., Prakobsaeng, N., El Halawani, M.E., 2012. Presence of prolactin mRNA in extra-pituitary brain areas in the domestic turkey. Acta Histochem. 114, 116–121. https://doi.org/10.1016/j.acthis.2011.03.007.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791. https://doi.org/10.2307/2408678.

Goffin, V., Binart, N., Touraine, P., Kelly, P.A., 2002. Prolactin: the new biology of an old hormone. Annu. Rev. Physiol. 64, 47–67. https://doi.org/10.1146/annurev.physiol.64.081501.131049.

Hasunuma, I., Yamamoto, K., Kikuyama, S., 2004. Molecular cloning of bullfrog prolactin receptor cDNA: changes in prolactin receptor mRNA level during metamorphosis. Gen. Comp. Endocrinol. 138, 200–210. https://doi.org/10.1016/j.ygcen.2004.06.005.

Kelly, P.A., Djiane, J., Postel-Vinay, M.-C., Edery, M., 1991. The prolactin/growth hormone receptor family. Endocr. Rev. 12, 235–251. https://doi.org/10.1210/edrv-12-3-235.

Kikuyama, S., 1994. Amphibian prolactin and control of its secretion. In: Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), Perspectives in Comparative Endocrinology. National Research Council, Ottawa, pp. 237–242.

Kikuyama, S., Kawamura, K., Tanaka, S., Yamamoto, K., 1993. Aspects of amphibian metamorphosis: hormonal control. In: Jeon, W.J., Jarvik, J. (Eds.), International Review of Cytology. Academic Press, New York, pp. 105–148.

Kikuyama, S., Yazawa, T., Abe, S., Yamamoto, K., Iwata, T., Hoshi, K., Hasunuma, I., Mosconi, G., Polzonetti-Magni, A.M., 2000. Newt prolactin and its involvement in reproduction. Can. J. Physiol. Pharmacol. 78, 984–993. https://doi.org/10.1139/y00-099.

Kobayashi, T., Kikuyama, S., 1991. Homologous radioimmunoassay for bullfrog growth hormone. Gen. Comp. Endocrinol. 82, 14–22. https://doi.org/10.1016/0016-6480(91)90291-D.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw054.

Marano, R.J., Ben-Jonathan, N., 2014. Minireview: extrapituitary prolactin: an update on the distribution, regulation, and functions. Mol. Endocrinol. 28, 622–633. https://doi.org/10.1210/me.2013-1349.

Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C.J., Lu, S., Chitsaz, F., Derbyshire, M.K., Geer, R.C., Gonzales, N.R., Gwadz, M., Hurwitz, D.I., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Geer, L.Y., Bryant, S.H., 2017. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucl. Acids Res. 45, D200–D203. https://doi.org/10.1093/nar/gkw1129.

Matsuda, K., Yamamoto, K., Kikuyama, S., 1990. Purification and properties of newt prolactin. Gen. Comp. Endocrinol. 77, 63–69. https://doi.org/10.1016/0016-6480(90)90206-2.

Mejía, S., Morales, M.A., Zetina, M.E., de la Escalera, G.M., Clapp, C., 1997. Immunoreactive prolactin forms colocalize with vasopressin in neurons of the hypothalamic paraventricular and supraoptic nuclei. Neuroendocrinology 66, 151–159. https://doi.org/10.1159/000127233.

Nakajima, K., Uchida, D., Sakai, M., Takahashi, N., Yanagisawa, T., Yamamoto, K., Kikuyama, S., 1993. Thyrotropin-releasing hormone (TRH) is the major prolactin-releasing factor in the bullfrog hypothalamus. Gen. Comp. Endocrinol. 89, 11–16. https://doi.org/10.1006/gcen.1993.1004.

Nakano, M., Hasunuma, I., Minagawa, A., Iwamuro, S., Yamamoto, K., Kikuyama, S., Machida, T., Kobayashi, T., 2018. Possible involvement of thyrotropin-releasing hormone receptor 3 in the release of prolactin in the metamorphosing bullfrog larvae. Gen. Comp. Endocrinol. 267, 36–44. https://doi.org/10.1016/j.ygcen.2018.05.029.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, Oxford.

Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786. https://doi.org/10.1038/nmeth.1701.

Roselli, C.E., Bocklandt, S., Stadelman, H.L., Wadsworth, T., Vilain, E., Stormshak, F., 2008. Prolactin expression in the sheep brain. Neuroendocrinology 87, 206–215. https://doi.org/10.1159/000114643.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454.

Seki, T., Kikuyama, S., 1986. Effect of thyrotropin-releasing hormone and dopamine on the in vitro secretion of prolactin by the bullfrog pituitary gland. Gen. Comp. Endocrinol. 61, 197–202. https://doi.org/10.1016/0016-6480(86)90197-8.

Stevens, F.M., Shaw, C., 1982. Prolactin-like immunoreactivity in human small-intestinal mucosa. Br. Med. J. Clin. Res. Ed. 284, 1014–1015.

Stricker, P., Grueter, R., 1928. Action du lobe antérieur de l'hypophyse sur la montée laiteuse. Compt. Rend. Soc. Biol. 99, 1978–1980.

Takada, M., Hokari, S., 2007. Prolactin increases Na^+ transport across adult bullfrog skin via stimulation of both ENaC and Na^+/K^+-pump. Gen. Comp. Endocrinol. 151, 325–331. https://doi.org/10.1016/j.ygcen.2007.01.040.

Takahashi, N., Yoshihama, K., Kikuyama, S., Yamamoto, K., Wakabayashi, K., Kato, Y., 1990. Molecular cloning and nucleotide sequence analysis of complementary DNA for bullfrog prolactin. J. Mol. Endocrinol. 5, 281–287. https://doi.org/10.1677/jme.0.0050281.

Takahashi, N., Yamamoto, K., Kikuyama, S., 1993. Cloning of a toad prolactin cDNA: expression of prolactin mRNA in larval and adult pituitaries. J. Mol. Endocrinol. 11, 343–349. https://doi.org/10.1677/jme.0.0110343.

Tang, Z.-J., Lue, S.-I., Tsai, M.-J., Yu, T.-L., Thiyagarajan, V., Lee, C.-H., Huang, W.-T., Weng, C.-F., 2014. The hormonal regulation of color changes in the sexually dichromatic frog *Buergeria robusta*. Physiol. Biochem. Zool. 87, 397–410. https://doi.org/10.1086/675678.

Taylor, A.C., Kollros, J.J., 1946. Stages in the normal development of *Rana pipiens* larvae. Anat. Rec. 94, 7–23. https://doi.org/10.1002/ar.1090940103.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.

Toyoda, F., Hasunuma, I., Yamamoto, K., Yamashita, M., Kikuyama, S., 2005. Prolactin acts centrally to enhance newt courtship behavior. Gen. Comp. Endocrinol. 141, 172–177. https://doi.org/10.1016/j.ygcen.2004.12.015.

Urtishak, S.L., Mckenna, E.A., Mastro, A.M., 2001. Prolactin and prolactin receptor expression in rat, small intestine, intraepithelial lymphocytes during neonatal development. Dev. Immunol. 8, 319–330. https://doi.org/10.1155/2001/10175.

Walker, A.M., 2007. S179D prolactin: antagonistic agony!. Mol. Cell. Endocrinol. 276, 1–9. https://doi.org/10.1016/j.mce.2007.06.001.

Yamaguchi, Y., Takagi, W., Kuraku, S., Moriyama, S., Bell, J.D., Seale, A.P., Lerner, D.T., Grau, E.G., Hyodo, S., 2015. Discovery of conventional prolactin from the holocephalan elephant fish, *Callorhinchus milii*. Gen. Comp. Endocrinol. 224, 216–227. https://doi.org/10.1016/j.ygcen.2015.08.020.

Yamamoto, K., Kikuyama, S., 1981. Purification and properties of bullfrog prolactin. Endocrinol. Jpn. 28, 59–64. https://doi.org/10.1507/endocrj1954.28.59.

Yamamoto, K., Kikuyama, S., 1982. Radioimmunoassay of prolactin in plasma of bullfrog tadpoles. Endocrinol. Jpn. 29, 159–167. https://doi.org/10.1507/endocrj1954.29.159.

Yamamoto, K., Kobayashi, T., Kikuyama, S., 1986. Purification and characterization of toad prolactin. Gen. Comp. Endocrinol. 63, 104–109. https://doi.org/10.1016/0016-6480(86)90187-5.

Yamamoto, T., Nakayama, Y., Matsuda, Y., Abé, S., 1998. Cloning and expression of a cDNA encoding a prolactin receptor of the Japanese red-bellied newt, *Cynops pyrrhogaster*. Zool. Sci. 15, 741–747. https://doi.org/10.2108/zsj.15.741.

Yamamoto, T., Nakayama, Y., Tajima, T., Abe, S., Kawahara, A., 2000. Cloning of a cDNA for *Xenopus* prolactin receptor and its metamorphic expression profile. Dev. Growth Differ. 42, 167–174.

Yamashita, K., Matsuda, K., Hayashi, H., Hanaoka, Y., Tanaka, S., Yamamoto, K., Kikuyama, S., 1993. Isolation and characterization of two forms of *Xenopus* prolactin. Gen. Comp. Endocrinol. 91, 307–317. https://doi.org/10.1006/gcen.1993.1131.

Yang, L., Meng, Z., Liu, Y., Zhang, Y., Liu, X., Lu, D., Huang, J., Lin, H., 2010. Growth hormone and prolactin in *Andrias davidianus*: cDNA cloning, tissue distribution and phylogenetic analysis. Gen. Comp. Endocrinol. 165, 177–180. https://doi.org/10.1016/j.ygcen.2009.07.010.

Yasuda, A., Yamaguchi, K., Kobayashi, T., Yamamoto, K., Kikuyama, S., Kawauchi, H., 1991. The complete amino acid sequence of prolactin from the bullfrog, *Rana catesbeiana*. Gen. Comp. Endocrinol. 83, 218–226. https://doi.org/10.1016/0016-6480(91)90024-Z.