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Identification of sea lamprey GTHβ-like cDNA and its evolutionary implications

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Abstract

We have identified the first and perhaps only gonadotropin β-like protein by cDNA cloning in sea lamprey, a member of the oldest lineage of vertebrates, the agnathans. Two pituitary gonadotropins (GTHs: follicle-stimulating hormone (FSH) and luteinizing hormone (LH)) have been identified in representative species of all classes of vertebrates except the agnathans. The present study was undertaken to identify GTH in sea lamprey, *Petromyzon marinus*, to gain a further understanding of the origin and evolution of reproductive pituitary hormones and their respective genes in vertebrates. Sea lamprey preGTHβ-like cDNA was cloned from a plasmid cDNA library using an expressed sequence tag analysis. The preGTHβ-like cDNA encoded 150 amino acids, in which the GTHβ-like protein consisted of 134 amino acid residues. Sea lamprey GTHβ-like protein contained 12 Cys residues and two N-glycosylation sites at homologous positions to those of FSHβ and LHβ. The region of the molecule that has been proposed to control receptor binding specificity (i.e., the region between the 10th and 12th Cys residues) suggests that the proposed heterodimer would be more like a FSH than a LH. Sea lamprey GTHβ-like protein-producing cells were identified immunocytochemically in the ventral part of the proximal pars distalis of pituitary using antiserum prepared against a synthetic peptide of preGTHβ-like protein (52–68). Intraperitoneal administration of sea lamprey GnRH-I and -III at 100 μg/g body weight (twice at a 24 h interval) increased expression of GTHβ-like protein in the pituitary of adult female sea lamprey during the final maturational period. Thus, these results are the first to demonstrate the presence of a single GTH-like system in lampreys. Because the sea lamprey GTHβ-like protein is a clear out-group compared to those of the LH and FSH family based on phylogenic analysis, we propose that an ancestral glycoprotein hormone gave rise to only one GTH in lampreys and to the glycoprotein hormone family that gave rise to LH, FSH, and TSH during the early evolution of gnathostomes.
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1. Introduction

It has been well established that reproduction is regulated through the hypothalamus–pituitary–gonad axis in jawed vertebrates (gnathostomes). Gonadotropins (GTHs) are secreted from the pituitary in response to the hypothalamic gonadotropin-releasing hormone (GnRH) and stimulate synthesis of sex steroid hormones in the gonads, which in turn stimulate gonadal growth and maturation. Two gonadotropins (GTHs), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), together with thyroid-stimulating hormone (TSH) form a family of pituitary hormones. They are glycoproteins consisting of two noncovalently bound subunits, α and β. The α subunit is common within a single species (Kawauchi et al., 1989). The β subunits are homologous and convey hormone specificity. Therefore, these glycoprotein hormones are believed to

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have evolved from a common ancestral molecule through duplication of β-subunit genes and subsequent divergence (Dayhoff, 1976). Two GTHs have been identified in all taxonomic groups of gnathostomes, including actinopterygians (Kawauchi et al., 1989; Querat et al., 2000; Suzuki et al., 1988), sarcopterygians (Querat et al., 2004), and chondrichthyans (Querat et al., 2001), but not in jawless vertebrates (agnathans).

The agnathans probably arose as the first vertebrates about 530 million years ago immediately after the evolutionary explosion of multicellular organisms in the Cambrian period. Modern agnathans are represented by two groups, the lampreys and the hagfishes. Paleontological analysis of extinct agnathans had suggested that lampreys were more closely related to gnathostomes than either group is to the hagfishes (Forey and Janvier, 1993, 1994). However, Janvier and his collaborators reversed their position based on analysis of the complete mitochondrial DNA suggesting now that lamprey and hagfish form a clade (Delarbre et al., 2002), which is also supported by mitochondrial DNA analysis (Suga et al., 1999). Thus, these recent molecular analyses groups the hagfishes together with the lampreys in a single clade, making the lamprey an important model for evolutionary biology (Kuratani et al., 2002).

Despite this interest in agnathans, little is known about their reproductive pituitary hormones, even though two hypothalamic GnRHs have been identified in lampreys and shown by extensive functional, physiological, and immunocytochemical studies to control the pituitary–gonadal axis (Reviewed in Sower, 2003). In addition, the cDNAs of lamprey GnRH-I (Suzuki et al., 2000) and GnRH-III (Silver et al., 2004) have been cloned. Therefore, the identification of GTH in lampreys is essential for understanding the hypothalamic–pituitary axis and to assess the origin and evolution of reproduction pituitary hormones in vertebrates. We have identified the melanotropins (MSHs), corticotropin (ACTH) (Takahashi et al., 1995a,b), and growth hormone (GH) (Kawauchi et al., 2002) in the sea lamprey, *Petromyzon marinus*. The adenohypophysis of the lamprey pituitary gland is divided into three regions, the rostral pars distalis (RPD), the proximal pars distalis (PPD), and the pars intermedia (PI), as in gnathostome fish. It has been found that MSH cells are localized in the PI, ACTH cells in the RPD (Nozaki et al., 1995; Takahashi et al., 1995b), and GH cells in the dorsal half of the PPD (Kawauchi et al., 2002). Previous evidence from physiological and immunohistochemical studies strongly supported the presence of a GTH-like molecule in lampreys. Hypophysectomy and substitution therapy with pituitary extracts or mammalian GTHs indicated pituitary regulation of the gonads in river lampreys, *Lampetra fluviatilis*, (Larsen, 1965). Injection of salmon GTH preparation into adult spawning sea lamprey advanced ovulation by several weeks and elevated plasma estradiol levels (Sower et al., 1983). Two high affinity-binding sites for lamprey GnRH-I and -III were found in the PPD of sea lamprey pituitary (Knox et al., 1994) and the cDNA of one pituitary GnRH receptor was cloned (Silver et al., 2005). Moreover, GTH-like immunoreactivity was identified in cells distributed in the ventral half of the PPD (Nozaki et al., 1999, 2001). The present study on sea lamprey provides the first molecular identification of GTH in an agnathan and discusses the evolutionary implications of these findings.

2. Materials and methods

2.1. Pituitary

Adult landlocked female sea lampreys were caught in tributaries of Lake Huron during their upstream migration. The pituitaries were extirpated and immediately frozen on dry ice in June 2003 at the Hammond Bay Biological Station in Michigan. These pituitaries were used for cloning of preGTHβ-like cDNA. Adult, sea-run sea lampreys were collected in a trap at the Cocheco River in Dover, New Hampshire in May and June of 2004 and 2005 during their upstream spawning migration from the ocean. These fish were used to test the effects of GnRH-I and GnRH-III on the expression of pituitary hormones.

2.2. Preparation of pituitary cDNA library and sequencing analysis

Total RNA from 30 pituitaries (50 mg) of adult landlocked female sea lamprey was prepared by ISOGEN (Nihon Gene, Tokyo, Japan) as described by Moriyama et al. (2002). The concentration of total RNA was estimated by measuring the absorbance at 260 nm (conversion factor: 1 OD = 40 μg RNA/ml), and the purity was determined from the ratio of absorbance at 260/280 nm.

The landlocked female sea lamprey pituitary cDNA library was prepared from the total RNA according to the method of Kato et al. (2005). The ligated cDNA into the vector was transformed into DH12S competent cells (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. The cDNA clones were randomly selected and recombinant plasmid DNA was prepared by TempliPhi DNA Sequencing Template Amplification Kit (Amersham Biosciences, Piscataway, NJ). The 5′ sequence of clones was determined using a capillary DNA sequencer (ABI PRISM 3730 genetic analyzer, PE Applied Biosystems, Foster City, CA, USA) using a BigDye Terminator Cycle Sequencing Kit Ver. 3.1. (PE Applied Biosystems). DNASIS-Mac (Hitachi, Tokyo, Japan) was used for processing the sequence data and aligning the sequences. The BLASTX and BLASTP Internet programs from the Center for Information Biology and DNA Data Bank of Japan (Mishima, Japan) were used for homology searches for cDNA sequences.

2.3. Cloning lamprey GTHβ-like cDNA

The full-length putative lamprey preGTHβ-like cDNA was cloned using PCR. Oligonucleotide primers pGCAP1 (5′-TCCGGTGGTGGTG CAAATC-3′) and pGCAP-2 (5′-AAGCAGTGGTATCAACGCAGAGT $AC(T)_{30}$-3′) were designed for amplification of the full-length putative lamprey GTH cDNA. During PCR, 50 μl reaction mixes (25 μl *Taq* PCR Master Mix (QIAGEN, Hilden, Germany), 100 ng/4 μl plasmid, 2 μl each of primers (final concentration, 4 μM), and 17 μl RNase free water) were subjected to 35 cycles of amplification. After activation of *Taq* at 94 °C for 15 min, each cycle consisted of 1 min. denaturation at 94 °C, 1 min. Primer annealing at 50 °C, and 1 min. Primer extension at 72 °C. The final extension was done for 7 min at 72 °C.

The PCR-amplified cDNA product was electrophoresed on agarose gels (Nippon Gene) and visualized by ethidium bromide staining (Nippon Gene). The cDNA was extracted and purified from agarose gels using a QIAEX II Gel Extraction Kit (QIAGEN), ligated into pT7 Blue T-Vector (Novagen, Madison, WI, USA), and transformed into JM109 competent cells (Nihon Gene) according to the manufacturer's protocols. Recombinant plasmid DNA was prepared by the alkaline-SDS methods and

sequenced on both strands with a capillary DNA sequencer (ABI PRISM 3100 genetic analyzer) using a BigDye Terminator Cycle Sequencing Kit Ver. 1.1.

2.4. RNA probes

Two primers (laGTHβSP: CAAAAGG ATGGGTCCCCTTCAGCT GT and laGTHβASP: ATTTAAATTTGGCTCTATCGTAAACATT) were used to amplify the fragment of sea lamprey GTHβ-like cDNA (nt 70 to nt 649; 580 bp) by PCR using laGTHβ/pT7 Blue T-Vector plasmid as the template. The PCR-amplified fragment was purified using the QIAEX II gel extraction Kit, ligated into pT7 Blue T-Vector and transformed into JM109 competent according to the manufacturer's protocols. Three micrograms of the purified recombinant plasmid DNA was digested with *Bam*HI and purified by the phenol/chloroform method. Digoxigenin (DIG)-labeled sense and antisense RNA probes specific for GTHβ-like were generated by DIG RNA Labeling Kit (Roche, Penzberg, Germany) according to the manufacturer's instructions. The concentration was estimated by dot blot test, using DIG-labeled control RNA.

2.5. In situ hybridization

The pituitary glands of sea lamprey were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.4) for 24 h at 4 °C. Specimens were then immersed in 30% sucrose in PBS (pH 7.4) and embedded in OCT compound (Miles, Elkhart, IN). Frozen sections (12 μm) were cut and mounted on MAS-coated slides (Matunami Glass Ind., Osaka, Japan). For histological observations, the sections were treated with 1 μg/ml proteinase K for 5 min and post fixed with 4% PFA/PBS. Sections were acetylated in 0.1 M triethanolamine/1.0% HCl/0.2% acetic anhydride for 15 min and incubated in 1% Triton X-100/PBS for 30 min. Slides were incubated in prehybridization buffer (50% formamide, 5 × SSC, 1% SDS, 50 μg/ml yeast tRNA, 50 μg/ml Heparin, 0.1% Chaps, and 5 mM EDTA) for 2 h, and then in hybridization buffer containing 0.1 μg/ml DIG-labeled sense or antisense RNA probes for 16 h at 65 °C. Post hybridized sections were washed twice in 0.2 × SSC for 30 min at 65 °C. The slides were immersed in 1% Blocking Reagent (Roche) for 60 min, and incubated with anti-DIG-AP Fab fragments (Roche) diluted in 1% Blocking Reagent (1:1000) at room temperature for 2 h. Detection of alkaline phosphatase activity was performed in a color development solution containing 337 μg/ml NBT (Sigma, St Louis, MO), 175 μg/ml BCIP (Sigma) and 0.24 μg/ml levamisole hydrochloride (Wako, Tokyo, Japan). Stained sections were observed using a light microscope (Olympus BX50, Tokyo).

2.6. Immunohistochemistry

A rabbit antiserum was raised against a synthetic peptide corresponding to the preGTHβ-like sequence at positions 52–68 (YTWQLIGHNMRKI AQEV). Sections of lamprey pituitary were immunohistochemically stained as described previously (Nozaki et al., 1999) using a Vectastain ABC Elite Kit (Elite ABC, Vector Laboratories, Burlingame, CA, USA) and this specific antiserum (lot no. 0401), diluted 1:2500. To test the specificity of the immunostaining, the following control stains were done: replacement of primary antiserum with rabbit serum, and preabsorption of the primary antiserum with rabbit serum, and preabsorption of the primary antiserum with the synthetic peptide (100 μg/ml antiserum at working dilutions).

2.7. Injection of GnRHs

The female sea-run lamprey were injected intraperitoneally twice with either lamprey GnRH-I or GnRH-III at doses of 0, 50, and 100 μg/g body weight at 24 h. intervals as previously described (Sower et al., 1983, 1993). The fish were kept at the Anadromous Fish and Aquatic Invertebrate Research (AFAIR) laboratory in Durham, NH under normal photoperiod in a flow-through system supplied by ambient lake water at 18 °C. The pituitaries were dissected at 48 h. after the first injection, frozen in liquid nitrogen and stored at −80 °C until quantitative real-time PCR assay.

2.8. Quantitative real-time PCR assay

Total RNA from each pituitary was extracted with 0.25 ml Isogen, and single-strand cDNA were reverse transcribed using an Omniscript RT Kit (Qiagen) according to the manufacturer's protocols. Primers and TaqMan probes specific for lamprey GTHβ-like, GH, POC, and POM were designed with PrimerExpress software (PE Applied Biosystems) according to the manufacturer's protocols. The following primers were used:

GTH β-like Forward primer: 5′-CGCCGAGTGTCGTTACATCA-3′, Reverse primer: 5′-ACCTCCTGGGCAATCTTCCT-3′, TaqMan Probe: 5′-CTACACCTGGCAACTGATCGGGC AC-3′,

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1                          TGGAGTTTATCTGCACTCAGCCCATTCATTCATTCACAGAACAACAGCAACTCTGCACCCACTCCAAAACAAAAGG    76
77   ATGGGTCCCCTTCAGCTGTTCCAACTGGCTCTGTGGCTCGAGGTGGCTTACTCCAACTCCTTGTGCAAGCTGCACAACACCACCATAGCGGTGGAG    172
1     m  g  p  l  q  l  f  q  l  a  l  w  l  e  v  a  Y  S  N  S  L  C  K  L  H  N  T  T  I  A  V  E     32
                          Signal peptide
173  AAGAGTGGCTGCGCCGAGTGTCGTTACATCAACACCACCGTCTGCAGTGGCTACTGCTACACCTGGCAACTGATCGGGCACAATATGAGGAAGATT    268
33    K  S  G  C  A  E  C  R  Y  I  N  T  T  V  C  S  G  Y  C  Y  T  W  Q  L  I  G  H  N  M  R  K  I     64

269  GCCCAGGAGGTGTGCACGTACACGGACGTGGGCTACGAGACCGTGACCCTGCACGGCTGTGACCCCGGCGTTGACCCGACCCTCCACTATCCGGTG    364
65    A  Q  E  V  C  T  Y  T  D  V  G  Y  E  T  V  T  L  H  G  C  D  P  G  V  D  P  T  L  H  Y  P  V     96
                                Mature Protein
365  GCGCTCAGCTGCCAGTGCAGCCAATGCCAGACCGACACCACCGACTGCACCGTGCGCAGCCTGAGACCCGACTACTGCAGCCACCCGAGCCAGATC    460
97    A  L  S  C  Q  C  S  Q  C  Q  T  D  T  T  D  C  T  V  R  S  L  R  P  D  Y  C  S  H  P  S  Q  I    128

461  AAGGGGCCACCGCTTGGAGTCGATCTGACCAACGAAACCGTGCCAGCTGCGGGCAGCTACCGGGTTTAAGAAGAGGTTAACGGAGGGTTCATGGGG    556
129   K  G  P  P  L  G  V  D  L  T  N  E  T  V  P  A  A  G  S  Y  R  V  *                              150

557   TGGTGGGGGGGGGGCTGCTAAATTGTCTAAACTCGAGGGATGTGATCGACTGGCATTGCGTTTTTAATGTTTACGATAGAGCCAAATTTAAATCAT    652
653   AATCTCTTCATGTGAGCTTTATGTGGCTGTTCCGTGTTTTTGTATGTGCTTTCATTTAGCCATTGGTTTGAATACAGACCCCGCACTTTTTAACCA    748
749   GGTCAAATTATTTTGCAGGCTTGAAAAGGCTAACCTTAATGTATGTAACGGTAAGCAAATGATGTGCCGATAAATGTTTGTGTATTTAATAGAGGG    844
845   TATATAAAGTAATAGCAACGAGACAAACAACACCAATTGAGAAACGATGTTGTTGAATGAAGTAGCGACCAAGAATTGTAATAATTGTTCGCATTG    940
941   TGGTGGAGGGTGTGTGTGTGAATCGTTATGATCTGGAAAGAAACATCCAAAACCTATGCCCGGTGCCAATAAAGTTATAAAAATCTGGCGCACCAA   1036
1037  CCCGATTATAAGACGCACCACAAGTGATGCTCTGCGGGTGAATAATAGAAGCTTTCCAAATAACACATCTTACATTGAAGGAGCAATGTTGGCAGT   1132
1133  GGTATTATGGAATGAGTACACAAAGCACATTTCCACCACCACCAGTGTCTTTGGTAGTGTTTCAGCAGGTAGAAATATATATAGTTATGTGCTTGG   1228
1229  TCGTTTGAAGGAGTGTTTTAACGTTGTTGTAGCTATTATTTTCCGAAGCATTTTCTTTTAATGTTCCCTTACTGACAAATATTGATAAAACATTTG   1324
1325  CTGCACTCTTGCACAATGGCTGATTACTTTCGTGAAGGCCTCTTGACTGCTTTCAAAGTTGATGTAGTTGAGTGTTATTGTCATGGATTCTTGTAA   1420
1421  AAGTTCCACTTTCAAACAGTGAAAATAATTTCTAACTACTGTAATCCAAGTCCCATGAACTCAGCAGCACTTGAGTCAAATGTAATTTTTACTTTG   1516
1517  AAGCTTCCAAAAAGAGTACTATTGTTGTGCATCACCCCACAGGAAAACAATATTGGAATGTTGCAAAGAATAAACTTCTTAACGCCC            1603
```

Fig. 1. Nucleotide and deduced amino acid sequences of sea lamprey preGTH-like cDNA excluding the poly A tail. Nucleotide (upper line) and amino acids (lower line) are numbered from the initiation of methionine. Signal peptide (16 aa with small characters) is underlined with solid line. Stop codon is marked with an asterisk. A peptide corresponding to the underlined sequence (52–68) was synthesized and used for preparation of the antibody.

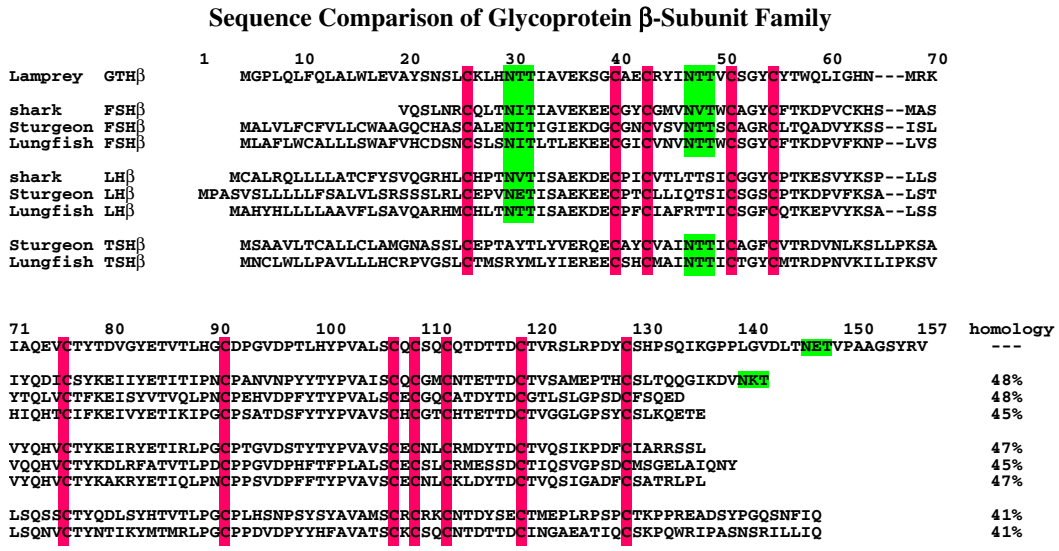

Fig. 2. Sequence comparison of sea lamprey GTHβ-like protein with the glycoprotein β-subunit family from sturgeon, lungfish, and catshark. The sequence identity compared to lamprey GTHβ-like protein was calculated with identical residues of 104 alignments between the first C and the last C.

GH Forward Primer: 5′-CAGACACTCTGTTGCCAAAAGC-3′, Reverse Primer: 5′-CGACCCCACGCGTCTCT-3′, TaqMan Probe: 5′-CTACAATGAAAGGAGGCTCTCTC GCGC-3′,

POC Forward Primer: 5′-TGCTGGAATGATGGGAAACTG-3′, Reverse Primer: 5′-GCCCGTGTCCCATTGCT-3′, TaqMan Probe: 5′-ACGGCTGGACCAGGGGTGCTTC-3′,

POM Forward Primer: 5′-GGCGTGCGAGAGCTGTCT-3′, Reverse Primer: 5′-CCCTCTGGCGCCTCATCT-3′, TaqMan Probe: 5′-CCCAGCCTGAGCCGCTCTGCT-3′,

β-actin Forward Primer: 5′-GACCTCACCGACTACCTGATGA A-3′, Reverse Primer: 5′-TGATGTCGCGCACGATCT-3′, TaqMan Probe: 5′-CGTTCACCACGACGGCCGAGC-3′.

Real time PCR was performed in 25 μl reaction mixtures consisting of 1× TaqMan Universal PCR Master Mix (PE Applied Biosystems), 900 nM of each primer, 250 nM TaqMan probes, and 1.5 ng first-strand cDNA (template) using an ABI PRISM 7000 (PE Applied Biosystems). The PCR thermal profile was 50 °C for 2 min and 95 °C for 10 min, followed by 40 reaction cycles of 95 °C for 15 s and 60 °C for 1 min each. For each reaction, the cycle threshold (C_t) at which fluorescence was detected above an arbitrary threshold (1.0) was determined. At the 1.0 threshold, C_t values are within the exponential phase of the amplification. To estimate the relative amounts of GTHβ-like, GH, POC, and POM mRNA in pituitaries from GnRHs treated and control fish, C_t values were normalized to those of the internal standard (β-actin) and compared.

2.9. Phylogenetic tree

Two phylogenetic trees of 86 and 154 vertebrate glycoprotein β subunits (deduced amino acids) were created following alignments (MegAlign Clustal W) using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0beta10 and trees were constructed using the neighbor joining method.

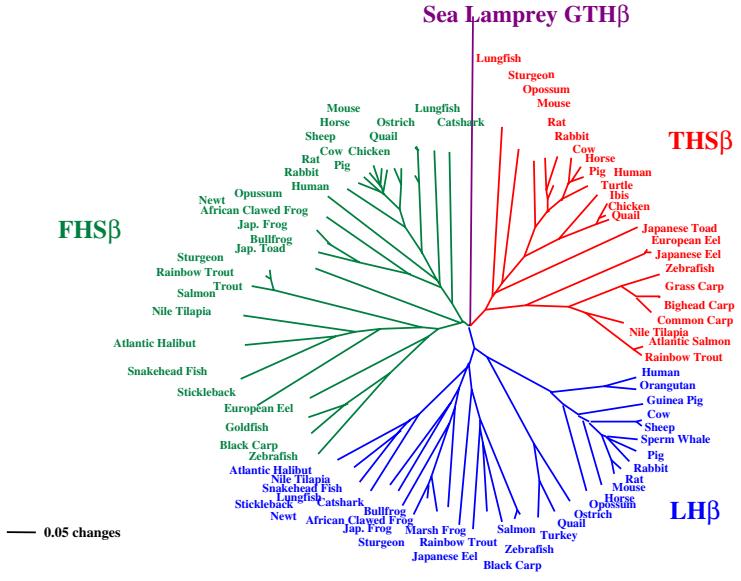

Fig. 3. Molecular phylogenetic tree of FSHβ, LHβ, and TSHβ. The neighbor joining method was used to construct this phylogenetic tree of 86 deduced amino acid glycoprotein β subunits. The lamprey GTHβ-like protein forms an outgroup.

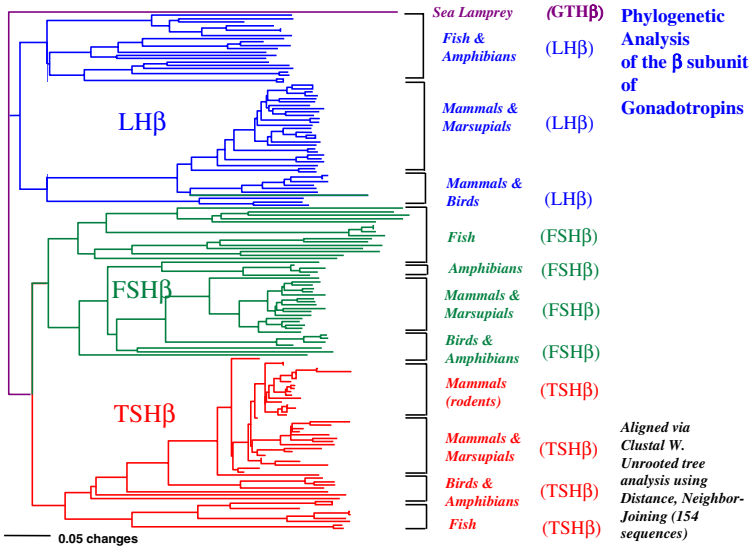

Fig. 4

2.10. Statistical analysis

All data are presented as the means ± standard error. Group comparisons were performed using two-way ANOVA, followed by Fisher's least significant difference test. Differences at $p < 0.05$ were considered significant.

3. Results

3.1. Expressed genes in landlocked female sea lamprey pituitary

From the cDNA library, 2208 clones were subjected to sequence analysis from the 5′ end, of which 281 clones corresponded to pituitary hormones; 155 clones for POM cDNA, 124 clones for POC cDNA, and 9 clones for GH cDNA. Three clones showed sequence similarity to glycoprotein hormone β subunits; for example, 51% sequence identity to carp LHβ.

3.2. Cloning sea lamprey preGTHβ-like cDNA

The putative lamprey GTHβ-like cDNA consists of 1603 nucleotides encoding a prehormone of 150 amino acids, with a putative signal peptide of 16 amino acid residues and a mature protein of 134 amino acid residues (Fig. 1) (GenBank Accession No. AY730276). The 5′ and

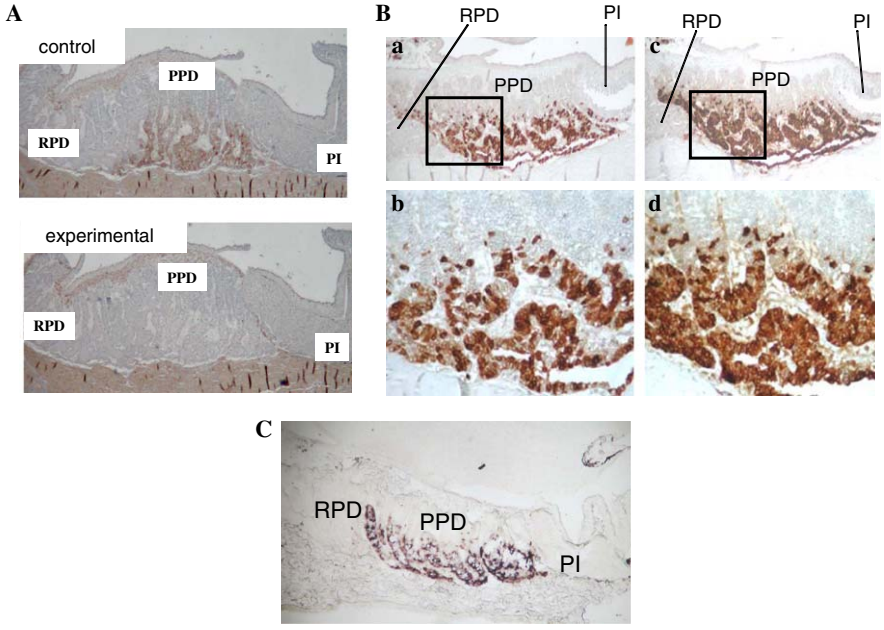

Fig. 5. Histochemical characteristics of sea lamprey pituitary: (A) Preabsorption of antiserum to sea lamprey GTH-β fragment with authentic peptide on sea lamprey pituitary gland; (B) an antiserum prepared against a synthetic peptide of sea lamprey preGTHβ-like protein (52–68); sea lamprey pituitary gland immunostained with (a, b) anti-lamprey GTHβ-fragment and (c, d) anti-ovine LHβ (Kawauchi et al., 2002); (C) labeled RNA probe specific for sea lamprey GTHβ-like mRNA (in situ hybridization).

Fig. 4. Molecular phylogenetic tree of FSHβ, LHβ and TSHβ. The neighbor joining method was used to construct this phylogenetic tree of 156 deduced amino acid glycoprotein beta subunits. The lamprey GTHβ- like protein forms an outgroup. The following list are the sequences with the branch number starting at the top of the tree and continues in descending order as follows: branch number, common name, genus species, accession # and name of glycoprotein hormone: *LH Beta Sequences* (1) Lamprey (*Petromyzon marinus*) AY730276, GTH beta; (2) three spined stickleback (*Gasterosteus aculeatus*) CAD59185, LHbeta; (3) Atlantic halibut (*Hippoglossus hippoglossus*) CAD10502, LHbeta; (4) Pejerrey Fish (*Odontesthes bonariensis*) AAP85607, LHbeta; (5) Convict rockcod (*Epinephelus septemfasciatus*) BAC78813, LHbeta; (6) Mozambique tilapia (*Oreochromis mossambicus*) AAS86813, LHbeta; (7) 1 Nile tilapia (*Oreochromis niloticus*) AAP49576, LHbeta; (8) Snakehead fish, (*Channa maculata*) AAS01609, LHbeta; (9) African clawed frog (*Xenopus laevis*) AAK49986, lutropin beta subunit; (10) bullfrog (*Rana catesbeiana*) S21196, lutropin beta chain; (11) Japanese frog (*Rana japonica*) BAD16756, LHbeta; (12) marsh frog (*Rana ridibunda*) CAC39252, LHbeta; (13) Japanese firebelly newt (*Cynops pyrrhogaster*) BAB92959, LHbeta; (14) Australian lungfish (*Neoceratodus forsteri*) CAE17335, LHbeta; (15) smaller spotted catshark (*Scyliorhinus canicula*) CAC43236, LHbeta; (16) black carp (*Mylopharyngodon piceus*) AAK07414, LHbeta; (17) zebrafish (*Danio rerio*) AAH75999, LHbeta 2; (18) Japanese eel (*Anguilla japonica*) BAD14302, LHbeta; (19) coho salmon (*Oncorhynchus kisutch*) AAO72300, LHbeta; (20) rainbow trout (*Oncorhynchus mykiss*) BAB17687, LHbeta; (21) Russian sturgeon (*Acipenser gueldenstaedtii*) AAP97490, LHbeta; (22) Siberian sturgeon (*Acipenser baerii*) CAB93502, LHbeta; (23) aye-aye (*Daubentonia madagascariensis*) AAL69734, LHbeta; (24) ruffed lemur (*Varecia variegata*) AAL69735, LHbeta; (25) Senegal galago (*Galago senegalensis*) AAL69736, LHbeta; (26) slender loris (*Loris tardigradus*), AAL69737, LHbeta; (27) rabbit (*Oryctolagus cuniculus*) AAT37165, LHbeta; (28) western tarsier (*Tarsius bancanus*) AAL69733, LHbeta; (29) cat (Felis catus) NP_001009277 , LHbeta; (30) Lyle's flying fox (fruit bat) (*Pteropus lylei*) AAL69739, LHbeta; (31) Pig (*Sus scrofa*) AAP92114, LHbeta; (32) white rhinoceros (*Ceratotherium simum*) AAB71983, LHbeta; (33) minke whale (*Balaenoptera acutorostrata*) PN0139, lutropin beta chain (LH); (34) sperm whale (*Physeter catodon*) PN0141, LHbeta; (35) Malayan flying lemur (*Cynocephalus variegatus*) AAL69738, LHbeta; (36) Djungarian hamster (Phodopus sungorus) AAF15966, LHbeta; (37) house mouse (*Mus musculus*) CAA71445, LHbeta; (38) Norway rat (*Rattus norvegicus*) NP_036990, LHbeta; (39) southern multimammate mouse (*Mastomys coucha*) AAQ55237, LHbeta; (40) golden hamster (*Mesocricetus auratus*) AAQ55238, LHbeta; (41) Mongolian gerbil (*Meriones unguiculatus*) AAQ74976, LHbeta; (42) cow (*Bos Taurus*) NP_776355, LHbeta; (43) sheep (*Ovis aries*) np_001009380, LHbeta; (44) donkey (*Equus asinus*) CAA56422, LHbeta; (45) zebra (*Equus burchellii*) CAA76146, LHbeta; (46) horses (*Equidae*) AAB22775, LHbeta; (47) Guinea pig (*Cavia porcellus*) AAQ75732, LHbeta; (48) red kangaroo (*Macropus rufus*) AAC96021, LHbeta; (49) silver-gray brushtail possum (*Trichosurus vulpecula*) AAC96019, LHbeta; (50) crab-eating macaque (*Macaca fascicularis*) CAH03730, LHbeta; (51) rhesus monkey (*Macaca mulatta*) AAL69721, LHbeta, (52) guereza (*Colobus guereza*) AAL69723, LHbeta; (53) spectacled langur (*Presbytis obscura*) AAL69722, LHbeta; (54) human (*Homo sapiens*) AAL69719, LHbeta; (55) orangutan (*Pongo pygmaeus*) AAL69720, LHbeta; (57) ostrich (*Struthio camelus*) S74085, LHbeta; (58) common quail (*Coturnix coturnix*) I51242, LHbeta; (59) turkey (*Meleagris gallopavo*) I51373, LHbeta. *FSH Beta Sequences* (56) douroucouli (*Aotus trivirgatus*) AAL69732, FSHb; (60) three spined stickleback (*Gasterosteus aculeatus*) CAD59058, FSHb; (61) Atlantic halibut (*Hippoglossus hippoglossus*) CAD10501, FSHb; (62) Snakehead fish (*Channa maculata*) AAS01610, FSHb; (63) Nile tilapia (*Oreochromis niloticus*) AAP49575, FSHb; (64) Pejerrey Fish (*Odontesthes bonariensis*) AAP85606, FSHb; (65) chinook salmon (*Oncorhynchus tshawytscha*) AAS75320, FSHb; (66) coho salmon (*Oncorhynchus kisutch*) AAO72299, FSHb, (67) rainbow trout (*Oncorhynchus mykiss*) BAB17686, FSHb; (68) Manchurian trout (*Brachymystax lenok*) AAR99810, FSHb; (69) black carp (*Mylopharyngodon piceus*) AAK07415, FSHb; (71) zebrafish (*Danio rerio*) AAV31152, FSHb; (72) North African catfish (*Clarias gariepinus*) AAO49013, FSHb; (73) conger (*Conger conger*) CAB93518, FSHb; (74) European eel (*Anguilla anguilla*) AAN64352, FSHb; (75) Russian sturgeon (*Acipenser gueldenstaedtii*) AAS92715, FSHb; (76) African clawed frog (*Xenopus laevis*) BAD14295, FSHb; (77) bullfrog (*Rana catesbeiana*) Q9PS36, FSHb; (78) Japanese frog (*Rana japonica*) BAD16757, FSHb; (79) marsh frog (*Rana ridibunda*) CAC39253, FSHb; (80) Japanese toad (*Bufo japonicus*) BAB93558, FSHb; (81) Australian brushtail possum (*Trichosurus vulpecula*) AAC71065, FSHb; (82) cow (*Bos taurus*) NP_776485, FSHb; (83) water buffalo (*Bubalus bubalis*) AAR13163, FSHb; (84) goat (*Capra hircus*) AAM81325, FSHb; (85) sheep (*Ovis aries*) NP_001009798, FSHb; (86) sika deer (*Cervus nippon*) AAN84782, FSHb; (87) crab-eating macaque (*Macaca fascicularis*) CAH03729, FSHb; (88) human (*Homo sapiens*) AAB02868, FSHb; (89) rabbit (*Oryctolagus cuniculus*) AAT37166, FSHb; (90) Guinea pig (*Cavia porcellus*) AAF68975, FSHb; (91) pig (*Sus scrofa*) NP_999040, FSHb; (92) horses (*Equus caballus*) P01226, FSHb; (93) Djungarian hamster (*Phodopus sungorus*) AAF15965, FSHb; (94) house mouse (*Mus musculus*) NP_032071, FSHb; (95) Norway rat (*Rattus norvegicus*) NP_001007598, FSHb; (96) southern multimammate mouse (*Mastomys coucha*) AAR21602, FSHb; (97) Mongolian gerbil (*Meriones unguiculatus*) AAQ83633, FSHb; (98) chicken (*Gallus gallus*) AAK31580, FSHb; (99) Japanese quail (*Coturnix japonica*) BAC01164, FSHb; (100) crested ibis (*Nipponia Nippon*) BAC07314, FSHb; (101) ostrich (*Struthio camelus*) S74084, FSHb; (102) Australian lungfish (*Neoceratodus forsteri*), CAE17337, FSHb; (103) Japanese firebelly newt (*Cynops pyrrhogaster*) BAB92958, FSHb; (104) smaller spotted catshark (*Scyliorhinus canicula*) CAC43235, FSHb. *TSH Beta Sequences* (70) goldfish (*Carassius auratus*) BAA13530, TSHb; (105) Norway rat (*Rattus norvegicus*) AAH58488, TSHb; (106) garden dormouse (Eliomys quercinus) CAD66066, TSHb; (107) Djungarian hamster (*Phodopus sungorus*), CAA62298, TSHb; (108) flying squirrel (*Glaucomys*) CAD66075, TSHb; (109) red squirrel (*Tamiasciurus*) CAD66074, TSHb; (110) Eastern chipmunk (*Tamias striatus*) AAP94768, TSHb; (111) rock dormouse (*Graphiurus platyops*) CAD66078, TSHb; (112) Woodland dormouse (*Graphiurus murinus*) CAD66079, TSHb; (113) spectacled dormouse (*Graphiurus ocularis*) CAD66080, TSHb; (114) small eared dormouse (*Graphiurus microtis*) CAD66076, TSHb; (115) Japanese dormouse (*Glirulus japonicus*) CAD66070, TSHb; (116) fat dormouse (*Myoxus glis*) CAD66073, TSHb; (117) forest dormouse (*Dryomys nitedula*) CAD66068, TSHb; (118) woolly dormouse (*Dryomys laniger*) CAD66067, TSHb; (119) hazel mouse (*Muscardinus avellanarius*) CAD66071, TSHb; (120) Roach's mouse-tailed dormouse (*Myomimus roachi*) CAD66069, TSHb; (121) mountain hare (*Lepus timidus*) AAP94781, TSHb; (122) cow (*Bos Taurus*) NP_776630, TSHb; (123) oribi (*Ourebia ourebi*) AF210240, TSHb; (124) Roan antelope (*Hippottragus equines*) AK67811, TSHb; (125) Horned oryx (*Oryx dammah*) AAK67810, TSHb; (126) dog (*Canis familiaris*) NP_001003290, TSHb; (127) horses (*Equus caballus*) AAA96826, TSHb; 128; pig (*Sus scrofa*) NP_999533, TSHb; (129) llama (*Lama glama*) AAB49315, TSHb; (130) human (*Homo sapiens*) AAB30828, TSHb; (131) mountain beaver (*Aplodontia rufa*) CAD66072, TSHb; (132) marsh rabbit (*Sylvilagus palustris*) AAP94780, TSHb; (133) brown hare (*Lepus capensis*) AAP94785, TSHb; (134) greater red rock rabbit (*Pronolagus crassicaudatus*) AAP947 93, TSHb; (135) house mouse (*Mus musculus*) NP_033458, TSHb; (136) Hispid hare (*Carprolagus hispidus*) AAP94772, TSHb; (137) rabbit (*Oryctolagus cuniculus*) AAP94770, TSHb; (138) gray short tailed opossum (*Monodelphis domestica*) AAL05938, TSHb; (139) chicken (*Gallus gallus*) AAQ16652, TSHb; (140) crested ibis (*Nipponia Nippon*) BAC07313, TSHb; (141) Common quail (*Corturnix coturnix*) AF541922, TSHb; (142) Chinese softshell turtle (*Pelodiscus sinensis*) AAT69236, TSHb; (143) Siberian sturgeon (*Acipenser baerii*) CAB93505, TSHb: (144) Japanese toad (*Bufo japonicus*) BAB93563, TSHb; (145) Australian lungfish (*Neoceratodus forsteri*) CAE17336, TSHb; (146) Atlantic salmon (*Salmo salar*) AAC77908, TSHb; (147) rainbow trout (*Oncorhynchus mykiss*) A48194, TSHb; (148) Nile tilapia (*Oreochromis niloticus*) BAC92374, TSHb; (149) bighead carp (*Aristichthys nobilis*) AAD51753, TSHb; (150) grass carp (*Ctenopharyngodon idella*) BAA20083, TSHb; (151) common carp (*Cyprinus carpio*) BAA20082, TSHb; (152) zebrafish (*Danio rerio*) NP_852471, TSHb; (153) European eel (*Anguilla anguilla*) CAA51908, TSHb; (154) Japanese eel (*Anguilla japonica*) AAO17791, TSHb.

3′ untranslated regions consist of 172 and 981 nucleotides, respectively. The polyadenylation signal (AATAAA) was at position 1585–1590, 13 bases upstream from the beginning of the poly (A) tail. Two N-glycosylation sites (Asn-X-Thr) were at position GTHβ-like (8–10) and (25–27), respectively. Two of them are homologous to those of FSHβ, one to LHβ and the other to TSHβ. The third one localized near the C-terminus.

The amino acid sequence of lamprey GTHβ-like protein is compared with those of three glycoprotein hormones from catshark (Querat et al., 2004), sturgeon (Querat et al., 2000), and lungfish (Querat et al., 2001) by aligning Cys residues and introducing deletions to obtain the maximal identity (Fig. 2). Lamprey GTHβ-like protein shows high sequence identity to LHβ and FSHβ but lower identity to TSHβ; 47, 45, and 47% identity with catfish, sturgeon, and lungfish LHβ, respectively; 48, 48, and 46% identity with catfish, sturgeon, and lungfish FSHβ, respectively; and 41 and 41% identity with sturgeon and lungfish TSHβ, respectively.

In the phylogenetic analysis of β subunits, sea lamprey GTHβ-like protein is far removed from the β-subunits of LH, FSH, and TSH, and takes a position as an out group, suggesting that the β-subunit of GTH separated after the agnathans and prior to the gnathostomes. Lamprey GTHβ-like protein groups a little closer to LHβs (Figs. 3 and 4).

3.3. GTHβ-like-producing cells

The replacement of primary antisera with normal rabbit serum yielded no stain in the pituitary gland (Fig. 5A). The cells in the ventral half of the PPD were found to express GTHβ-like protein and also specifically stained with the antiserum against the synthetic peptide (Fig. 5B). Expression of sea lamprey GTHβ-like mRNA by in situ hybridization was also shown to be in the ventral half of the PPD (Fig. 5C).

3.4. Effects of sea lamprey GnRHs on expression of sea lamprey GTHβ-like mRNA

Intraperitoneal injection of adult sea lamprey with synthetic GnRH-I and -III at doses of 50 and 100 μg/g body weight resulted in a significant elevation ($p < 0.05$) of GTHβ-like mRNA levels in the pituitary compared with the saline-injected control fish (Figs. 5 and 6). A significant increase ($p < 0.05$) in GH mRNA levels was also observed at 100 μg/g body weight of GnRH-III or GnRH-I (Figs. 6 and 7). However, no changes in POC and POM mRNA levels were observed at doses of 50 and 100 μg/g body weight (data not shown).

4. Discussion

In the present study, we have identified for the first time a single GTHβ-like protein by cDNA cloning from an agnathan, the sea lamprey. The GTHβ-like protein showed immunoreactivity in the ventral part of the proximal pars distalis of the pituitary. In addition, GnRH administration induced the expression of lamprey GTHβ-like mRNA levels in the pituitary. On the basis of phylogenetic analysis and our molecular studies, we propose that there is a single GTH in lampreys that has not diverged into FSH and LH as in gnathostomes. We propose that an ancestral glycoprotein hormone gave rise to lamprey GTH and then to the glycoprotein hormone family that gave rise to LH, FSH, and TSH that occurred during the early evolution of gnathostomes.

We have identified ACTH, MSHs, and GH and their cells in the RPD, PI, and the dorsal half of PPD in the lamprey pituitary, respectively, but had not yet identified GTH prior to the current study. Two high affinity-binding sites for lamprey GnRH-I and -III had been shown in the PPD of the adult sea lamprey pituitary (Knox et al., 1994), which coincided with GTH-like immunoreactivity identified in cells distributed in the ventral half of the PPD (Nozaki et al., 1999) and with GnRH receptor expression by in situ hybridization in the PPD (Silver et al., 2005). These pituitary cells in the PPD were stained intensely by anti-ovine LH including LHβ and moderately or weakly by several other antisera such as human LHβ. Therefore, it seemed that it would be easy to isolate GTH from pituitaries of adult sea lamprey that were close to spawning. One would expect a high content of GTH based on the reproductive stage and physiological data obtained from GnRH studies (Sower, 2003). For many years, we tried to isolate gonadotropin that we assumed to be a heterodimer glycoprotein, from the pituitary extracts from landlocked adult female sea lampreys. However, despite these exhaustive efforts, no molecule related to LH or FSH was found. During these early years, we did identify a glycoprotein homodimer called nasohypophysial factor (NHF) (Sower et al., 1995) that corresponded to the N-terminal peptide of proopiocortin (Takahashi et al., 1995b). This NHF molecule was always found as the most predominant glycoprotein (Sower et al., 1995). Moreover, there were numerous unsuccessful attempts using molecular techniques to clone α and β subunits with a number of primers corresponding to conserved regions for these subunits. Finally, the success of determining lamprey GTH-like protein as reported in this paper was accomplished by expressed sequence tag analysis of the pituitary cDNA library that allowed us to identify 3 out of 2208 clones showing sequence similarity to glycoprotein hormone β.

The mature protein contains 12 cysteine residues at homologous position to those of LH, FSH, and TSH and three N-glycosylation sites. Two of them are homologous to those of FSHβ, one to LHβ and the other to TSHβ. In addition, the region of the molecule that has been proposed to control receptor binding specificity (i.e., the region between the 10th and 12th Cys residues) suggests that the proposed heterodimer would be more like a FSH than a LH (Cosowsky et al., 1997). The mature protein showed similar sequence identity to LHβ and FSHβ of shark

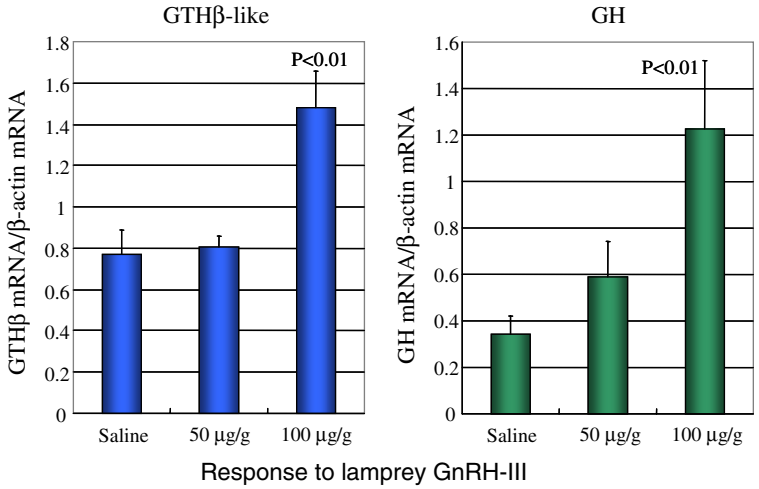

Fig. 6. The effects of sea lamprey GnRH-III (control, 50 μg GnRH-III/g body weight or 100 μg/g) on expression of GTHβ and GH mRNA in the pituitary (in vivo). An amplified internal fragment of β-actin was used as standard.

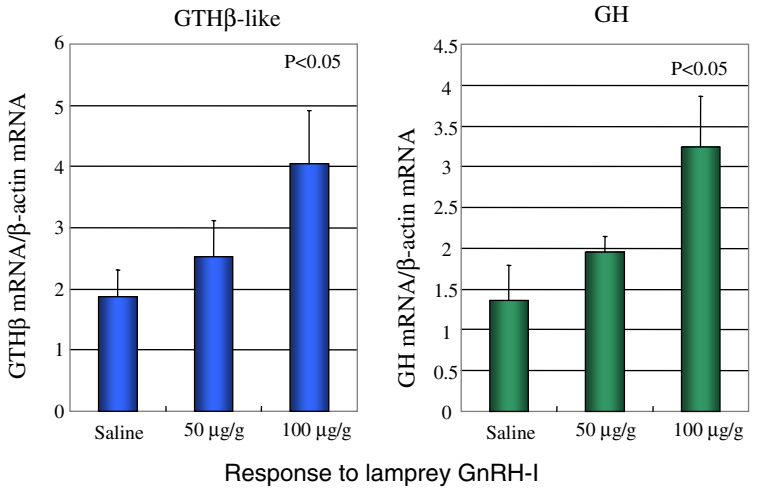

Fig. 7. The effects of sea lamprey GnRH-I (control, 50 μg GnRH-I/g body weight or 100 μg/g) on expression of GTHβ and GH mRNA 22–228.

(Querat et al., 2004), sturgeon (Querat et al., 2000), and lungfish (47%) (Querat et al., 2001) compared to TSHβ of sturgeon and lungfish (41%). We propose that the β subunit would likely combine with the α subunit since it has a hydrophobic residue (ile) that corresponds to hCG-β val44, a residue that fits into a hydrophobic pocket in the α subunit (Cosowsky et al., 1997; Moyle et al., 2004). This is a highly conserved subunit interaction in most, if not all gonadotropins, and slightly different than TSH. An unusual feature of the lamprey β-like protein is the tail that has a N-glycoyslation signal, a phenomenon not all that common in vertebrate β subunits. Perhaps this is to prolong its half-life. In accordance with the expression pattern of the isolated lamprey GTHβ-like protein, the antiserum against the synthetic peptide corresponding to the deduced amino acid sequence specifically stained most cells in the ventral half of the PPD, which had also been stained with anti-ovine LH (Nozaki et al., 1999). The results are in good agreement with the GnRH-binding study showing GnRH-binding sites in the proximal pars distalis of the pituitary (Knox et al., 1994). On the basis of sequence identity and histochemical characteristics, it is evident that this protein is a potential candidate for lamprey GTHβ. To obtain the definite proof, we examined whether GnRH could stimulate expression of the putative GTHβ gene in the pituitary of sea lamprey. After two intraperitoneal injections of 100 μg/g body weight at 24 h intervals in adult lamprey, both GnRH-I and GnRH-III stimulated the expression of mRNA of the putative GTHβ. In the same pituitary preparations, expression of other sea lamprey pituitary hormone genes such as GH, POC, and POM gene were investigated. The results demonstrated that lamprey GnRH also stimulated expression of GH, but not those of POC and POM in vivo. The stimulation of GH and GTH by GnRH is not novel in nonmammalian vertebrates. In previous studies, GnRH-induced GH and GTH secretion

from the goldfish pituitary (Marchant et al., 1989). These authors suggested that the secretion of GH and GTH in the goldfish are regulated, at least in part, through a common releasing factor, GnRH, whereas somatostatin and dopamine appear to act independently as GH and GTH release inhibitory factors, respectively (Marchant et al., 1989). Combining the biochemical characteristics, sequence identity, location of the GTH-like protein in the anterior pituitary and stimulation of GnRH, these data support that the identified glycoprotein hormone is gonadotropin-like in the lamprey pituitary.

The duality of gonadotropins or presence of LH and FSH has been established in all classes of gnathostomes. In this study, we found only a single GTHβ with intermediate sequence similarity to LHβ and FSHβ of phylogenetically adjacent species such as shark, sturgeon, and lungfish. In the molecular phylogenetic tree of β-subunits of glycoprotein hormones, sea lamprey is far removed from the β-subunits of LH, FSH, and TSH, and takes a position as an out group. In addition, immunohistochemical data suggest that there are no other cells that produce GTH; it has been shown that ACTH cells are in the RPD, GH, and GTH cells are in the PPD and MSH cells are in the PI (Nozaki et al., 1995). These results strongly suggest that duality of GTH was established after the divergence of gnathostomes and agnathans.

To date, there has been no clear evidence to support the presence of TSH in lampreys. In an earlier study from Sower et al. (1985), a partly purified salmon gonadotropin and an analog of GnRH stimulated the elevation of thyroxine in adult female sea lampreys. We suggested the possibility at that time that the thyroid may be activated by one glycoprotein hormone. More recently, two kinds of glycoprotein hormone receptors have been cloned in the sea lamprey: one is a GTH-like putative receptor located in the gonad and the other is a putative TSH receptor in the thyroid tissue (Freamat and Sower, 2002, 2005). Functional studies are ongoing with these putative receptors. Until further studies are completed, we could postulate that there is a TSH molecule in agnathans. Or another scenario as first proposed 1985 is that there is one glycoprotein hormone in lampreys that acts both as a GTH-like hormone and TSH-like hormone.

The key motifs and characteristics of the identified lamprey glycoprotein receptors reveals a mosaic of features common to all other classes of glycoprotein hormone receptor in vertebrates and indicating less specificity for glycoprotein hormones. It is proposed that the link between the glycoprotein and its receptor through time has facilitated the co-evolution of the ligand and its receptor and has led to increased specificities between certain receptor–ligand pairs, enabling the formation of preferred ligand (TSH, LH, and FSH) and receptor interactions in jawed vertebrates. Thus, there may have been much less specificity of the glycoprotein hormones for its receptors in agnathans. Whether there are two glycoprotein hormones in sea lamprey consisting of a single GTH and probably TSH remains to be determined. It is conceivable that an ancestral glycoprotein hormone gave rise to lamprey GTH and to the gonadotropin family that gave rise to LH, FSH, and TSH that occurred during the early evolution of gnathostomes.

Dayhoff (1976) originally proposed that the α- and β-subunits of the glycoprotein hormones evolved from a common ancestor by gene duplication and all three β subunits were derived from its ancestry by gene duplication and subsequent mutations. Li and Ford (1998) proposed that the first duplication produced an α subunit and a β subunit which was followed by a second duplication of the ancestral β subunit to yield the LHβ-subunit gene and the ancestral gene of the FSHβ and TSHβ-subunit genes. The third duplication produced FSH and TSH so that FSHβ is more related to TSHβ than to LHβ. In contrast, Querat et al. (2000, 2001, 2004) proposed that the ancestral GTH lineage and the TSH lineage derived from a primary duplication of an ancestral β gene and LH and FSH lineage arose from a duplication of an ancestral GTH lineage. Our results which include the structural information from agnathans implies that the initial duplication produced a GTH lineage and a TSH lineage and the second duplication occurred in GTH during the early evolution of gnathostomes to give rise to LH and FSH.

To confirm that we do indeed have the GTH hormone in lampreys, future, and ongoing studies include the identification of the alpha GTH, the production of recombinant lamprey GTH (α and β) to test in various physiological experiments; and GTH and GTH receptor structure–function studies. In addition, unannotated trace files (sequence data) have been released in 2005 from the lamprey genome project through the NCBI Genome Project. We have done some initial screening analyses and have not identified any other putative GTH or TSH to date. We plan to continue our screening efforts in the upcoming year.

In summary, the hypothalamic–pituitary–gonadal axis for reproductive endocrine system has now been completed in sea lamprey by the identification of GTHβ-like protein. We propose that an ancestral glycoprotein hormone gave rise to only one GTH in lampreys (agnathans) with the duality of the GTHs occurring only later in the gnathostomes.

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This paper is part of the special section dedicated to the Symposium in Honor of Hiroshi Kawauchi (author on this paper). As my role as one of the two senior authors, I dedicate this paper to Hiroshi Kawauchi (the other senior author)—it is been an honor and privilege to have collaborated with such an esteemed and distinguished scientist for the past 20 years. It has been actually the very best for our collaborations, friendship, and students and associates in our laboratories that it took 20 years to identify the gonadotropin in lamprey. This research has

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