Emergence of an Ancestral Glycoprotein Hormone in the Pituitary of the Sea Lamprey, a Basal Vertebrate

Stacia A. Sower, Wayne A. Decatur, Krist N. Hausken, Timothy J. Marquis, Shannon L. Barton, James Gargan, Mihael Freamat, Michael Wilmot, Lian Hollander, Jeffrey A. Hall, Masumi Nozaki, Michal Shpilman, and Berta Levavi-Sivan

Center for Molecular and Comparative Endocrinology (C.A.S., W.A.D., K.N.H., T.J.M., S.L.B., J.G., M.F., M.W., J.A.H.), Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, New Hampshire 03824; Robert H. Smith Faculty of Agriculture, Food, and Environment (L.H., M.S., B.L.-S.), The Hebrew University of Jerusalem, Rehovot, Israel 76100; and Sado Marine Biological Station (M.N.), Niigata University, Tassha, Sado, Niigata 952–2135, Japan

The gnathostome (jawed vertebrates) classical pituitary glycoprotein hormones, FSH, LH, and TSH, consist of a common α-subunit (GpA1) and unique β-subunits (GpB1, -2, and -3), whereas a recently identified pituitary glycoprotein hormone, thyrostimulin, consists of GpA2 and GpB5. This paper reports the identification, expression, and function of an ancestral, nonclassical, pituitary heterodimeric glycoprotein hormone (GpH) consisting of the thyrostimulin A2 subunit with the classical β-subunit in the sea lamprey, Petromyzon marinus, a jawless basal vertebrate. Lamprey (l) GpA2, and lGpH were shown to form a heterodimer by coimmunoprecipitation of lGpA2 with FLAG-tagged lGpH after the overexpression in transiently transfected COS7 cells using a bipromoter vector. Dual-label fluorescent in situ hybridization and immunohistochemistry showed the coexpression of individual subunits in the proximal pars distalis of the pituitary. GnRH-III (1) significantly increased the expression of lGpH and lGpA2 in in vitro pituitary culture. Recombinant lamprey GpH was constructed by tethering the N terminal of lGpA2 to the C terminal of lGpH with a linker region composed of six histidine residues followed by three glycine-serine repeats. This recombinant lamprey GpH activated the lamprey glycoprotein hormone receptor I as measured by increased cAMP/luciferase activity. These data are the first to demonstrate a functional, unique glycoprotein heterodimer that is not found in any other vertebrate. These data suggest an intermediate stage of the structure-function of the gonadotropin/thyroid-stimulating hormone in a basal vertebrate, leading to the emergence of the highly specialized gonadotropin hormones and thyroid stimulating hormones in gnathostomes. (Endocrinology 156: 3026–3037, 2015)
hormone (GpH)-α or GpA1) and unique β-subunits (FSHβ/LHβ/TSHβ). In general, FSH and LH regulate gonadal activity, and TSH regulates thyroidal activity. In recent years, a novel pituitary glycoprotein hormone was identified and named thyrostimulin because it activated the TSH receptor (2), but its function has not been established (3, 4). Compared with other GpHs, thyrostimulin has distinct subunits called GpA2 (α-subunit) and GpB5 (β-subunit), which are found in invertebrates and vertebrates.

The study of the pituitary GpHs in basal and later evolved vertebrates can provide insight into the molecular understanding of the structure-function of these hormones. Both jawed and jawless vertebrates are considered to have evolved from a common, jawless ancestor after a split that took place ca. 500 million years ago. The lamprey pituitary gland is well differentiated and similar with jawed vertebrates, consisting of the adenohypophysis and neurohypophysis (5). In lampreys, an extant Agnathan and jawless vertebrate, the organization of the HPG axis is similar with jawed vertebrates in its most fundamental features but with a simplified structure, overlapping with the pituitary-thyroid axis and involving a putative single GpH interacting with potentially two receptors (1). However, the full structure of the lamprey pituitary GpH has remained unknown. In contrast to jawed vertebrates, only one pituitary GpH heterodimer was identified in hagfish (6) and only one subunit, GpHβ (formerly GTHβ), was identified in the lamprey (7). The typical α-subunit of a functional lamprey gonadotropic GpH heterodimer, similar with those in the rest of the vertebrates, remained elusive despite extensive molecular and biochemical studies over many years (1, 7, 8). However, the presence of a heterodimeric gonadotropin in lampreys is strongly supported by substantial physiological and immunohistochemical studies (reviewed in references 1 and 8).

We considered that a canonical requirement for a functional pituitary gonadotropic hormone, regardless of its subunit composition, is to be released upon GnRH stimulation and to activate a GpH receptor localized in the gonadal tissue. Therefore, we further investigated the hypothesis that an atypical GpA2/GpHβ heterodimer might be the solution selected for the pituitary control of reproductive (and/or thyroid) physiology in this animal. Our overarching hypothesis is that there is a primitive, overlapping, yet functional hypothalamic-pituitary-gonadal and hypothalamic-pituitary-thyroid endocrine systems present in lampreys.

In this study, we report the identification and characterization of the full coding sequence of a GpA2 ortholog in sea lamprey and have performed expression, phylogenetic analysis and functional studies that provide evidence for an ancestral nonclassical, pituitary heterodimeric GpH consisting of the thyrostimulin A2 subunit with the classical β-subunit, providing insight into the molecular evolution of the pituitary glycoprotein hormone family.

Materials and Methods

Animals

For all studies, a total of approximately 95 adult sea run lampreys were collected at the Cocheco River fish ladder (Dover, New Hampshire). The lamprey were then transported to the Anadromous Fish and Invertebrate Research Laboratory (Durham, New Hampshire), where they were maintained in an artificial spawning channel with flow-through reservoir water at ambient temperatures (10–18°C) following University of New Hampshire Institutional Animal Care and Use guidelines. The animals were killed and dissected tissues were immediately snap frozen in liquid nitrogen and stored at −80 C.

Cloning of cDNA-encoding lamprey GpA2

cDNA for lamprey (l) GpA2 was prepared after the RNA extraction and using 5’-rapid amplification of cDNA ends (RACE) and 3’ RACE. Total RNA was extracted from lamprey tissues with 1.0 mL of QIAzol lysis reagent (QIAGEN) and digested with 5 U of RNase-free deoxyribonuclease (DNase; Promega) to remove genomic DNA. An RNasey lipid tissue minikit (QIAGEN) was used for total RNA extraction from ovaries according to the manufacturer’s protocol. After DNase treatment, total RNA was isolated with 0.3 mL of QIAzol lysis reagent. The concentration was determined by Nanodrop (Thermo Scientific).

5’ RACE for lGpA2 was performed with GeneRacer kit (Invitrogen) according to the manufacturer’s direction. Briefly, 4.5 μg of DNase-treated pituitary total RNA was dephosphorylated. After phenol chloroform extraction and ethanol precipitation, decapped RNA was capped and reisolated by phenol chloroform extraction and ethanol precipitation to obtain 5’ RACE-ready oligo-capped RNA. First-strand cDNA was reverse transcribed from the oligo-capped RNA. 3’ RACE for lGpA2 was performed with a SMART RACE cDNA amplification kit (CLONTECH) according to the manufacturer’s protocol. 3’RACE-ready cDNA was obtained from 1 μg of DNase-treated pituitary total RNA. First-round PCR for GpA2 3’ RACE was performed. In both reactions, and nested PCR was performed with one tenth of the first PCR product; PCR products were subcloned with pGEM-T easy vector system (Promega) for DNA sequencing.

Molecular, phylogenetic, and syntenic analyses of GpH subunits

One hundred thirty-nine invertebrate and vertebrate glycoprotein hormone sequences were used to make a phylogenetic tree by the maximum likelihood technique as previously described (6). The resulting tree was visualized and finished in FigTree version 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

To investigate the conservation of the gene organization in the neighborhood of the glycoprotein encoding genes, we examined the genes nearby to gpa1 in the lamprey genome and compared these with other vertebrates. Gene loci organization in the
genomic regions was obtained from comparing data from the lamprey genome assembly (9), Genomicus (10), Ensembl Genome Browser (http://wwwensembl.org), and the University of California, Santa Cruz, Genome Browser (http://genome.ucsc.edu/).

**Tissue distribution of lamprey GpH subunit transcripts**

Total RNA of each tissue from three males or three females were mixed, respectively, and first-strand cDNA was reverse transcribed from 3 µg of the pooled total RNA using the RNaseasy lipid tissue kit (QIAGEN). RT-PCR was performed on one tenth of first-strand cDNA using forward and reverse primers (GpH) primers: IGPHb forward and IGPHb reverse; e110 primers: PM E1A forward and PM E1A reverse; GPA2 primers: IGPA-qF1 and IGPA-qR1; GPB5 primers: GPB5_F5 and GPB5_R5. GpHβ was amplified using forward primer IGPHb forward (CGC-GGATGTCGGTCATGCA) and reverse primer IGPHb reverse (ACCTCCTGGGCAATCTTCCT), and lamprey GpA2 was amplified using forward primer IGPA-qF1 (ACTACCGCCACACATCACCA) and reverse primer IGPA-qR1 (TGAAAACCTC-CATCACTGGGT). The reference gene, ef1α, was amplified with forward primer PM E1A forward (CTGGCCA-CAGGGACCTCATC) and reverse primer PM E1A reverse (ACCGGCTCCTAAACCTACCA), and lamprey GpB5 was amplified using forward primer GPB5_F5 (GCACACTGGCC-CCTGACTGTCTGGTG) and reverse primer GPB5_R5 (CCACAGCGCCTGGTGACAGA).

**Fluorescent in situ hybridization**

Dual-label in situ hybridization was performed for IgPα2 and IgPβ in the pituitaries of adult lampreys following the procedures as described (11). For in situ hybridization, full-length, dual-labeled digoxigenin (DIG)/fluorescein riboprobes for IgPα2/IgPβ (GenBank accession numbers AC127885 and AV730276, respectively) were synthesized from respective CD-NAIs in pGEM-T Easy vectors (Promega) [IGPα2: 468 bp (antisense), 458 bp (sense); IGPHβ: 555 bp (antisense), 545 bp (sense)]. The plasmid DNA was purified using a GeneJet plasmid miniprep kit (Thermo Scientific) per the manufacturer’s protocol; the concentration and purity of the total DNA was determined by UV spectroscopy. Purified plasmids were then sent for sequencing at the University of New Hampshire’s Hubbard Genome Center to verify the sequence and establish insert orientation. Antisense and sense riboprobes for lamprey GpH subunits, GpA2 and GpHβ, were tested for their specificity by dot blot analysis, as previously described (12). Briefly, double-stranded full-length probe template cDNA (consisting of sense and antisense DNA strands) for GpA2 and GpHβ (amplified from purified plasmid DNA) were denatured and spotted onto a Nytran SuPerCharge TurboBlotter 0.45-µm nylon membrane (Whatman), and UV cross-linked. A “no” probe control with only cDNA was performed. All other dot blots were hybridized with GpA2 antisense and sense riboprobes and GpHβ antisense and sense riboprobes, respectively. After the hybridization, membranes underwent high-stringency washes, they were blocked, and antidigoxigenin-alkaline phosphatase antibody (Roche) was applied. Colorimetric detection was performed using filtered nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt (Roche). As expected, the fidelity of the riboprobes for GpA2 and GpHβ was confirmed by specifically binding to the complementary strand of double-stranded template cDNA (consisting of sense and antisense strands) for GpA2 and GpHβ, respectively, and exhibiting strong specific signal.

The purified plasmid DNAs were linearized with Ncol and Sal restriction enzymes to generate sense and antisense probes and subsequently purified with a GeneJet purification kit (Thermo Scientific), following the manufacturer’s protocol. In vitro transcription of the sense and antisense digests was performed separately, using the Riboprobe Combination System-SP6/T7 RNA polymerase kit (Promega), 10× DIG-RNA labeling mix (Roche) for IgPα2, and 10× fluorescein-RNA labeling mix (Roche) for IgPβ, as previously described (11). In situ hybridization was performed as previously described (11) with the following modifications: (20 µm) sagittal tissue sections were cut using a cryostat (Reichert-Jung, Leica Instruments) at −18°C. Tissue sections were hybridized with either antisense or sense dual-labeled riboprobes for IgPα2 and IgPβ, which were detected fluorescently, using anti-DIG-peroxidase (Roche) and TSA Cy3 (PerkinElmer), and antifluorescein-horseradish peroxidase (PerkinElmer) and TSA fluorescein (PerkinElmer), respectively. The slides were overslipped using Fluoro-Gel (with Tris buffer) (Electron Microscopy Services) and viewed on a LSM 510 Meta laser-scanning confocal microscope (Zeiss) at the University of New Hampshire with visualization of the TSA fluorescein/Cy3 fluorescence by excitation with 488-nm argon multiline and 543-nm helium/neon lasers, respectively, according to the following specifications: emitted fluorescence collected from 505–530 nm and 560–615 nm, respectively; ×8 and ×16 scan averaging for tiled and single images, respectively; pixel time 1.60 µsec; 1.0 and 1.4 zoom on a ×20 objective for tiled and single images, respectively.

**Immunohistochemistry**

For immunohistochemistry, two rabbit antisera were raised against the synthetic peptides corresponding to the lamprey pre-GPα2 (GpA2–3, lot 127, amino acids 27–41). Anti-IgPα2 (lot –127 GpA2 diluted 1:300) and anti-IgPβ (LH3-0404 diluted 1:750) (7) were used as primary antisera. Two types of immunohistochemical staining was performed: one was ordinary single immunostaining using a Vectastain avidin-biotin peroxidase complex (ABC) Elite kit (Vector Laboratories) on two successive sections, each exposed to one of the two antibodies, visualized separately by 3,3′-diaminobenzidine tetrahydrochloride (Sigma); the other was double immunostaining of a single section, using the ABC Elite kit followed by the Vectastain avidin-biotin alkaline phosphatase complex kit (Vector Laboratories), visualized by 3,3′-diaminobenzidine tetrahydrochloride and Vector Red (Vector Laboratories), as previously described (13, 14). To confirm the specificity of the immunoreactions, the following control experiments were done: replacement of primary antisera with normal rabbit serum and preabsorption of the primary antisera with corresponding antigens (synthetic lamprey GpA2). After preabsorption, there were no immunoreactive cells in the proximal pars distalis, confirming the specificity of the antisem. We did not perform preabsorption studies with the primary antisera for anti-IgPβ because this was previously done and specificity was confirmed for this antisem (7).
In vitro biological activity studies and real-time PCR

To examine the effect of lamprey GnRH-III on the expression of lGpA2 and lGpHβ at the pituitary, in vitro culture of lamprey pituitary incubated with lamprey GnRH-III was performed as a time course (0, 6, 12 h; n = 3 pituitaries per time point). Briefly, dissected pituitaries were pooled in preincubation media (Hanks’ buffer/25 mM HEPES; In Vitrogen) as previously described (15). After washing, the individual pituitary was preincubated for 24 hours in 0.5 mL in the preincubation media in each well of 24-well plates. After preincubation, the medium was replaced with 0.5 mL of the medium (Hanks’ buffer/25 mM HEPES) containing 1 μM lamprey GnRH-III, and the pituitary was incubated for 6 or 12 hours. Total RNA from individual pituitary was extracted with 1.0 mL of QIAzol lysis reagent (QIAGEN) and digested with 2 U of RQ1 ribonuclease-free DNase (Promega) for 1 hour at 37°C to remove genomic DNA. After the DNase treatment, the total RNA was isolated with 0.3 mL of QIAzol lysis reagent, and TaqMan real-time PCR was performed. The reaction mixture contained one tenth of first-strand cDNA, 1× TaqMan gene expression master mix (Applied Biosystems), 900 nM forward and reverse primers, and 250 nM lGpH and lGpHtem (Applied Biosystems). The relative expression values of the PCR products of the respective target cDNA sequences were spanned an intron/exon boundary and the probes were positioned to form a fusion gene that encodes a tethered poly-His tag ELISA detection kit (GenScript), generally according to the manufacturer’s instructions. Briefly, a His-tagged protein (molecular mass 12.7 kDa) is precoated on the microwell plate. Serial dilutions of His protein standards (11.3 kDa) and samples were added to each well test well, and then the anti-His tag monoclonal antibody was added to the plate. After washing steps, a horse-radish peroxidase-conjugated secondary antibody is added to each well to react with the anti-His tag monoclonal antibody. The final step involves the addition of 3,3′,5,5′-tetramethylbenzidine substrate, followed by the addition of stop solution for signal development. The OD reading indicated expression level of the His-tagged proteins. Overall, the purification procedure yielded 25 μg of pure recombinant lamprey GpH from 1 L of medium. The amount of the protein used in the receptor activation assay was according to the His tag ELISA.

The recombinant lGpH and deglycosylated hormone were electrophoresed on 15% polyacrylamide running gel, with a 5% stacking gel, blotted onto nitrocellulose membranes (Schleicher and Schuell), and blocked with 3% BSA, at 4°C overnight. The membranes were incubated in PBS plus 3% BSA with the His antibodies (1:7500, QIAexpress; QIAGEN) for 1 hour at room temperature. After washing, the membrane was treated with enhanced chemiluminescence reagent to reveal immunoreactive bands.

To measure the exact amount of the protein, we used a HIS tag ELISA detection kit (GenScript), generally according to the manufacturer’s instructions. Briefly, a His-tagged protein (molecular mass 12.7 kDa) is precoated on the microwell plate. Serial dilutions of His protein standards (11.3 kDa) and samples were added to each test well, and then the anti-His tag monoclonal antibody was added to the plate. After washing steps, a horse-radish peroxidase-conjugated secondary antibody is added to each well to react with the anti-His tag monoclonal antibody. The final step involves the addition of 3,3′,5,5′-tetramethylbenzidine substrate, followed by the addition of stop solution for signal development. The OD reading indicated expression level of the His-tagged proteins. Overall, the purification procedure yielded 25 μg of pure recombinant lamprey GpH from 1 L of medium. The amount of the protein used in the receptor activation assay was according to the His tag ELISA.

Construction, production, and receptor activation assay of recombinant lGpH

Using the methylochromic yeast Pichia pastoris, a recombinant lGpH was produced as a single-chain polypeptide, using the pPIC9K expression vector, according to published reports (17–19). The lGpA2 and lGpHβ mature protein-coding sequences were joined to form a fusion gene that encodes a tethered polypeptide in which the GpHβ subunit forms the N-terminal part and GpA2 forms the C-terminal part. A linker sequence composed of 12 amino acids (three Gly-Ser pairs and six His residues) was placed between GpHβ and GpA2 to enable purification of the recombinant protein. The supernatant of the induced culture obtained using pPIC9K-lGpHHis was purified using a one-step nickel batch purification according to the manufacturer’s instructions (QIExpressionist; QIAGEN). Reduced and denatured rGpH (100 ng) was incubated, according to the supplier’s recommendations (Roche Applied Science), overnight at 37°C in the presence or absence of N-glycosidase F, which hydrolyzes all types of N-glycan chains. Deglycosylated proteins were analyzed by Western blot analysis.

To determine the exact amount of the protein, we used a HIS tag ELISA detection kit (GenScript), generally according to the manufacturer’s instructions. Briefly, a His-tagged protein (molecular mass 12.7 kDa) is precoated on the microwell plate. Serial dilutions of His protein standards (11.3 kDa) and samples were added to each test well, and then the anti-His tag monoclonal antibody was added to the plate. After washing steps, a horse-radish peroxidase-conjugated secondary antibody is added to each well to react with the anti-His tag monoclonal antibody. The final step involves the addition of 3,3′,5,5′-tetramethylbenzidine substrate, followed by the addition of stop solution for signal development. The OD reading indicated expression level of the His-tagged proteins. Overall, the purification procedure yielded 25 μg of pure recombinant lamprey GpH from 1 L of medium. The amount of the protein used in the receptor activation assay was according to the His tag ELISA.

The IgpH receptors I and II (IgpH-R I and IgpH-R II) (GenBank accession numbers AY750688 and AA804061, respectively) were cloned in pcDNA3.1 expression vector (Zeo-; Invitrogen) under the control of the cytomegalovirus promoter. Transient transfection, cell procedures, and stimulation protocols were generally according to other published reports (19–21). Briefly, COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, 1% glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Biological Industries) under 5% CO2 until confluent. Cotransfection of either pc-GpHR1 or pc-GpHR2 (at 3 μg/plate) was carried out with FuGene 6.0 reagent (Roche). The cells were serum starved for 36 hours, stimulated with vehicle or various concentrations of IgpH for 6 hours and then harvested and analyzed. Lysates prepared from the harvested cells were assayed for both luciferase activity and β-galactosidase activity, which was used as an internal standard to normalize the luciferase activity. Transfection experiments were performed in triplicate with three independently hormone preparations. The concentrations of ligand used were from 5 pg/mL to 5 μg/mL.

Determination of heterodimeric GpH formation

To determine heterodimeric GpH formation of lGpA2 and lGpHβ, recombinant proteins produced from COS-7 cells were analyzed by Western blotting after FLAG immunoprecipitation. Full-length lGpA2 and lGpHβ cDNAs were amplified by RT-PCR from lamprey pituitary cDNAs and subcloned to pGEM-T
Tissue distribution of lGpA2 and lGpHβ

Tissue distribution of lamprey glycoprotein hormone subunit transcripts (Figure 4) was determined by RT-PCR. Both lGpA2 and lGpHβ mRNAs were detected in the brain, pituitary, and gonad, suggesting coexpression of lGpA2 and lGpHβ in lamprey tissues.

lGpA2 and lGpHβ were also detected in the testis. In contrast to the expression pattern of lGpA2, lGpHβ was more ubiquitously distributed in different tissues such as eye, intestine, thyroid, and heart.

Coexpression of GpA2 and GpHβ in the pituitary by in situ hybridization and immunohistochemistry

Dual-label fluorescent in situ hybridization showed that lGpA2 and lGpHβ transcripts were moderately to highly coexpressed in the proximal pars distalis of the pituitary of adult female sea lampreys [Figure 5, A–D, Fluor (Figure 5B), Fluor/Cy3 (Figure 5C), and Cy3 (Figure 5D)]. In addition, there was also coexpression in the pars intermedia and posterior neurohypophysis. The GpA2 and GpHβ antisense probes individually exhibited strong specific signal as confirmed by the notable absence of sense probe signal. Immunohistochemistry showed the protein to be slightly to moderately coexpressed in the proximal pars distal (Figure 5, E–I), with weaker expression in the neurohypophysis and pars intermedia. The preabsorption control for GpA2 is shown in Figure 5E, a small box outlined in gray.

Biological activity of lamprey GnRH on expression of glycoprotein hormone subunits

To characterize the expression of lGpA2 and lGpHβ in the hypothalamic-pituitary axis, the pituitaries were cultured in vitro with lamprey GnRH-III for a time-course study. Gene expression was measured by quantitative real-time-PCR relative to EF1α (Figure 6). The expression of lGpA2 and lGpHβ was higher at 6 hours of incubation with lGnRH-III compared with 0 hours.

Production of recombinant lamprey GpH and receptor functional assays

We produced lGpH as a single-chain polypeptide in the methylotrophic yeast P. pastoris. The immunoreactive glycosylated recombinant lGpH was revealed as an 18- to 35-kDa smear (Figure 7A, lane 3), probably due to a high glycosylation rate. After deglycosylation the hormone revealed as a 30-kDa band (Figure 7A, lane 2). Lamprey gonadotropin contains many glycosylation sites and hence it is highly glycosylated. Because the amount of the protein produced was low, the higher smear was not detectable. After the deglycosylation, all the variants of the protein

Results

Cloning, phylogenetic, and syntenic analysis of lGpA2

lGpA2 full-length cDNA encodes 121 amino acid residues including 20 amino acid residues of the putative signal peptide and one putative N-glycosylation site (Figure 1A). Amino acid sequence alignment of GpA2 between lamprey and vertebrates shows lGpA2 has 10 conserved cysteine residues at homologous positions to gnathostome GpA2 and one of two putative N-glycosylation sites (Figure 1, A and B).

Phylogenetic tree of glycoprotein hormone subunits (Figure 2) revealed that lamprey GpA2 belongs to the vertebrate GpA2 clade, whereas lGpHβ is an outgroup of gnathostome FSH/LH/TSH β-subunits, which is similar to hagfish GpHβ. Syntenic analysis showed orthologous genome regions of gnathostome GpA1 loci in the lamprey genome, suggesting the lamprey lost GpA1 (Figure 3).

Easy vectors (Promega). C-terminal FLAG and hemagglutinin (HA)-tagged lGpHβ and lGpA2, respectively, were generated by PCR with primers encoding FLAG tag sequence (DYKDDDDK) and HA tag sequence (YPYDVPDYA) and pGEM-T easy vectors containing lGpA2 and lGpHβ cDNAs as the templates. PCR products for C-terminal FLAG and HA tagged lGpHβ and lGpA2 were subcloned into the bipromoter expression vector pBluescript II (Invitrogen). The insert sequences were confirmed by DNA sequencing before transfection. COS-7 cells were maintained in 10% fetal bovine serum in DMEM (Invitrogen) at 37°C before transfection. pBluescript II bipromoter vectors containing FLAG-tagged and HA-tagged insert DNAs were transfected into COS-7 cells with Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the media were replaced by serum-free DMEM, and the cultures were continued for 6 days. After 3 and 6 days of serum-free culture, the media were harvested and concentrated with Amicon Ultra-15 (10K MWCO) (Millipore). FLAG-tagged proteins were isolated from the concentrated media by anti-FLAG M2 affinity gel (Sigma) according to the manufacturer’s protocol. The HA sequence exhibited no cross-reactivity with anti-FLAG antibodies used to prepare the affinity gel. The purified FLAG-tagged proteins were mixed with sample activity with anti-FLAG antibodies used to prepare the affinity gel. The purified FLAG-tagged proteins were mixed with sample.
were united into one band, which is stronger and visible in a Western blot (Figure 7).

Recombinant lGpH was used in a functional expression analysis to evaluate the response, binding selectivity, and signal transduction of lGpH-R I and lGpH-R II. Graded concentrations of the recombinant lGpH were applied to COS-7 cells that expressed lGpH-R I or lGpH-R II. Only GpH-R I induced a dose-response increase of CRE-luciferase activity with an EC50 of 3.74 ng/mL (Figure 7B).

**Heterodimeric formation studies**

The ability of lGpA2 and lGpH2 to form a heterodimer was demonstrated by the coimmunoprecipitation of both subunits when overexpressed together in transiently transfected COS-7 cells. LGpA2-HA as well as lGpH2-HA-FLAG could be detected both before and after FLAG immunoprecipitation (ip), showing the formation of a LGpA2/lGpH2 heterodimer (Figure 8). Under reducing conditions, Western analysis revealed the presence of lGpHβ-FLAG (red) and lGpA2-HA (green) prior to ip (lane 3). After FLAG-specific ip (lane 2), lGpHβ-FLAG (red) was detected as a large smear at 25–37 kDa, likely due to heavy glycosylation, and lGpA2-HA (green) was detected at approximately 17 kDa.

**Discussion**

In this study, we report the identification, expression, and function of an ancestral nonclassical, heterodimeric pituitary GpH consisting of the thyrostimulin A2 subunit with the classical GpH2 subunit in the sea lamprey, an Agnathan. We hypothesize this novel lGpH may regulate both reproductive and thyroid activities because lampreys possess...
only one GpH compared with the three glycoprotein hormones in gnathostomes, LH, FSH, and TSH. lGpHβ and lGpA2 were shown to coprecipitate in immunoprecipitation experiments, and a recombinant tethered lGpA2/lGpHβ hormone stimulated cAMP signaling in cells transfected with lGpH-R I. Dual-label fluorescent in situ hybridization and immunohistochemistry showed coexpression of individual subunits in the proximal pars distalis of the pituitary. Lamprey GnRH-III treatment induced an up-regulation of gene expression for both lGpA2 and lGpHβ in cultured lamprey pituitary explants. This is the first report of the identification and characterization of a thyrostimulin homolog in lampreys that forms a unique functional heterodimeric GpH with GpHβ that has not been reported in any other vertebrate. From our phylogenetic analysis of glycoprotein hormone subunits, lamprey GpA2 is shown to belong to the vertebrate thyrostimulin GpA2 clade, whereas lGpHβ is an outgroup of gnathostome FSH/LH/TSH-subunits and considered an ancestral vertebrate α-subunit. Each of the two glycoprotein hormone subunits of this newly identified gonadotropin is believed to have evolved from a common ancestral molecule that through duplication events within the gnathostomes (jawed vertebrates) led to three pituitary glycoprotein hormones, LH, FSH, and TSH, that are found in all species of gnathostomes.

Amino acid sequence alignment of lGpA2 compared with human, rat, mouse, and zebrafish GpA2 shows lGpA2 has 10 conserved cysteine residues at homologous positions to gnathostome GpA2 and one of two putative N-glycosylation sites. Whereas a homolog of the vertebrate GpHβ-subunit in lampreys had been identified and reported in 2006 (7), the existence of a GpH heterodimer and the identity of its binding partner remained elusive. Extensive molecular screens did not yield any evidence of GpA1, the typical vertebrate GpA1 ortholog of LH, FSH, and TSH. Syntenic analysis of the lamprey genomic regions orthologous to gene suites centered on gnathostome GpA1 loci also did not provide any support for the existence of this gene. In contrast, hagfish GpHα was identified as a vertebrate GpA1 ortholog that contains 8 of 10 Cys residues and two N-glycosylation sites conserved at positions homologous to gnathostome GpA1 sequence.

To date, no attempts have been reported in identifying the thyrostimulin GpA2/GpB5 subunits in hagfish or in any other fish. Genomic and syntenic analyses suggested that the chordate gap-gpb locus was duplicated prior to the first round of genomic duplication and the newly created gapbβ was transferred to vertebrate protochromosome D, whereas gap2, gpb5, and gapα localized on vertebrate protochromosome G (22). It was further suggested that paralogs of gap2-gpb5-gapα and gapbβ environments

Figure 2. Phylogenetic analyses of glycoprotein hormone subunits. The molecular phylogenetic analysis was constructed using the maximum-likelihood method (6) using 139 invertebrate and vertebrate glycoprotein hormone sequences retrieved from the National Center for Biotechnology Information database. lGpA2 is included in the vertebrate GpA2 clade and lGpHβ is an outgroup of gnathostome FSHβ/LHβ/TSHβ.
and the University of California, Santa Cruz, Genome Browser (10), Ensembl Genome Browser (http://www.ensembl.org), and the University of California, Santa Cruz, Genome Browser (http://genome.ucsc.edu/). Shared synteny supports the loss of gpa1 in lamprey. The orientation of each chromosome (chr.) and scaffold (sf.) is indicated with line arrows. A pointed box represents the orientation of each gene. Rectangles with X’s indicate lost gpa1 loci.

Figure 3. Synteny of glycoprotein hormone subunits. To investigate the conservation of the gene organization in the neighborhood of the glycoprotein-encoding genes, we examined genes nearby to gpa1 in the lamprey genome and compared these with other vertebrates. Gene loci organization in the genomic regions was obtained from comparing data from the lamprey genome assembly (9), Genomius (10), Ensembl Genome Browser (http://www.ensembl.org), and the University of California, Santa Cruz, Genome Browser (http://genome.ucsc.edu/). Shared synteny supports the loss of gpa1 in lamprey. The orientation of each chromosome (chr.) and scaffold (sf.) is indicated with line arrows. A pointed box represents the orientation of each gene. Rectangles with X’s indicate lost gpa1 loci.

were then created through two whole genome duplications. Therefore, we suggest that a gpa1 paralog created by one duplication event was subsequently lost in lamprey while preserved in hagfish, and that it is highly likely that hagfish also have a GpA2. The findings of gpa1 in hagfish clearly delineated the timing of the emergence of this subunit in the course of the vertebrate evolution. The proposed emergence of gpa1 and gphβ are supported by the absence of gpa1 and gphβ genes in the amphioxus genome (3, 23, 24). Phylogenetic analysis of glycoprotein hormone subunits shows that IGpA2 belongs to the vertebrate GpA2 clade, whereas lamprey and hagfish GpHβs are outgroups of gnathostome FSH/LH/TSH β-subunits and consided ancestral vertebrate β-subunits.

IGpA2 and IGpHβ mRNA transcripts were detected in the brain, pituitary, and gonad by RT-PCR, suggesting coexpression of IGpA2 and IGpHβ in these lamprey tissues. In contrast to the expression pattern of IGpA2, IGpHβ was more ubiquitously expressed in different tissues such as the eye, intestine, thyroid, and heart. Dual-label fluorescent in situ hybridization (FISH) and immunohistochemistry (IHC) showed coexpression of individual subunits in the proximal pars distalis of the pituitary similar to our previous IHC studies showing IGpHβ expressed in the ventral portion of the proximal pars distalis (7). However, in comparison with our previous ISH expression studies, FISH also showed coexpression and colocalization of the transcripts in the pars intermedia and neurohyposis. Additionally, current IHC showed major expression of IGpA2 and IGpHβ in the proximal pars distalis with weaker expression in the neurohyposis and pars intermedia, consistent with the FISH results. These data from the present study are much more extensive and comprehensive than the IHC and in situ hybridization studies performed for IGpHβ in our previous paper (7). The procedures used for ISH differed between the 2006 and present studies. As an example, in contrast to the current study, in the paper by Sower et al (7), the dehydration/delipidation steps were not performed and the high-stringency washes may have been overly stringent, thereby limiting expression. In situ hybridization of the pituitary showed coexpression and colocalization of the transcripts in the pars intermedia and neurohyposis, whereas IHC showed IGpA2 and IGpHβ in the proximal pars distalis with weaker expression in the neurohyposis and pars intermedia. These data may suggest that some of the transcripts are not processed to the mature protein in the pars intermedia and neurohyposis.

The distribution of the IGpH and its subunits needs to be examined much further in the lamprey pituitary. Once a recombinant lamprey GpH is made in sufficient quantities, antiserum can be generated and used in future IHC studies to fully determine the distribution in the pituitary and whether the mature GpH is expressed more broadly within the proximal pars distalis and in other areas of the pituitary as suggested by the current data. At this time, it
is unknown what cell types produce the glycoprotein heterodimeric subunits in lamprey pituitaries. Previous IHC studies for lGpH/Hβ indicated that this subunit was produced in the ventral portion of the proximal pars distalis in the presumed gonado-tropes (7). It is possible that more than one cell type within the lamprey pituitary could produce the glycoprotein hormone subunits. In the human pituitary, GpA2 and GpB5 have been shown to be produced in the corticotropes rather than the gonadotropes (25).

The ability of lGpA2 and GpHβ to form a heterodimer was demonstrated by the coimmunoprecipitation of both subunits when overexpressed together in transiently transfected COS-7 cells. With the exception of salmonids and some orders of Acanthopterygii, gnathostome FSH, TSH, and LH heterodimer assembly is achieved by two main disulfide bridges, known as the latch and tensor disulfides, and hydrogen bonding (26–28). Structurally, thyrostimulin GpA2 is quite similar to GpA1, differing only slightly in its distribution of cysteine residues. Both GpA1 and GpA2 have 10 conserved cysteine residues, six of which participate in three disulfide bridges bearing the cystine knot structure similar to other proteins in the eight-amino acid ring cystine knot protein family (29). GpA2 differs from GpA1 among other cystine-knot proteins in the nonhomologous location of two cysteine residues. Cysteines absent from loops 1 and 3 of GpA2 are offset by the presence of a cysteine at the N terminal and another located within the eight-amino acid ring structure. Due to the absence of cysteines in loops 1 and 3, GpA2 lacks a disulfide bridge formed between these residues. Instead, a disulfide bridge forms between the displaced cysteines of the N terminal and the...
eight-amino acid ring (29). In the gnathostome pituitary, glycoprotein hormone GpA1 is noncovalently bound to the \( \alpha \)-subunit of FSH, LH, TSH, and/or CG to form the heterodimer stabilized by the seatbelt region, through which loop 2 of GpA1 is threaded (30, 31). \( \alpha \)GpH has 12 cysteine residues at conserved locations and is thus expected to form a seatbelt region similar to gnathostome \( \alpha \)-subunits, suggesting it uses a similar mechanism for securing \( \alpha \)GpA2. The nuanced structural differences between GpA2 and GpA1 would not appear to hinder heterodimer formation between \( \alpha \)GpA2 and \( \alpha \)GpHβ.

In mammals, the GpH/GpH-receptor (R) system exhibits two characteristics tightly related to their proper function under normal physiological conditions: the specificity of their temporal and tissue expression profiles and selectivity in their interaction with the ligands (32). These characteristics have evolved during divergent evolution of the ancestral duplicated genes that were inherently neither specific in their expression nor selective in their ligand affinities. We produced \( \alpha \)GpH as a single-chain polypeptide in the methylotrophic yeast, \( \Phi \) pastoris, which was used in a functional expression analysis to evaluate the response, binding selectivity, and signal transduction of \( \alpha \)GpH receptors. Graded concentrations of the recombinant \( \alpha \)GpH were applied to COS-7 cells that expressed \( \alpha \)GpH-R I or \( \alpha \)GpH-R II. Only \( \alpha \)GpH-R I induced a dose-response increase of CRE-luciferase activity with an \( EC_{50} \) of 3.74 ng/mL. The assay was performed three times. Data are presented from one assay as mean ± SEM (n = 3).

Figure 6. In vitro studies: relative RNA expression of lamprey glycoprotein hormone subunits in response to lamprey GnRH-III. To examine the effect of \( \alpha \)GnRH-III on the RNA expression of \( \alpha \)GpA2 and \( \alpha \)GpHβ at the pituitary, in vitro culture of individual lamprey pituitaries incubated with lamprey GnRH-III (1 \( \mu \)M) was performed as a time course (0, 6, 12 h). Relative gene expression was measured by quantitative RT-PCR. Individual expression values are plotted as histograms at 0, 6, and 12 hours of incubation relative to EF1α. Data are represented as mean ± SEM of gene expression values of each time point (n = 3). The expression of \( \alpha \)GpA2 and \( \alpha \)GpHβ was higher at 6 hours of incubation with \( \alpha \)GnRH-III compared with 0 hours.

Figure 7. A, Western blot of recombinant lamprey glycoprotein hormone. Western blot staining with anti-His antibody of nickel batch purified \( \alpha \)GpA2/\( \alpha \)GpHβ heterodimer. Lane 1 is the protein marker. \( \alpha \)GpA2/\( \alpha \)GpHβ was incubated with (lane 2) or without (lane 3) N-glycosidase F. The glycosylated form of the heterodimer appears as a poorly concentrated smear, ranging from approximately 18 to 35 kDa (lane 3), and after deglycosylation all variants of the protein became concentrated as a major, more visible product of approximately 30 kDa (lane 2). B, Receptor activation assay of recombinant lamprey glycoprotein hormone. Recombinant \( \alpha \)GpH was used in a functional expression analysis to evaluate the response, binding selectivity, and signal transduction of \( \alpha \)GpH receptors. Graded concentrations of the recombinant \( \alpha \)GpH were applied to COS-7 cells that expressed \( \alpha \)GpH-R I or \( \alpha \)GpH-R II. Only \( \alpha \)GpH-R I induced a dose-response increase of CRE-luciferase activity with an \( EC_{50} \) of 3.74 ng/mL. The assay was performed three times. Data are presented from one assay as mean ± SEM (n = 3).
vous studies, a synthetic ligand containing the lGpHβ tethered with a mammalian GpHα activated both lGpH-R I and lGpH-R II (35).

Further extensive studies will be required to determine the cognate receptor(s) for lGpH as well as to examine the functional and signaling mechanisms of GpH with each of the two lamprey glycoprotein hormones to understand its role in regulating reproduction and/or metabolism.

Lampreys have three hypothalamic GnRHs (-I, -II, and -III) that have been shown by extensive functional, immunohistochemical, and anatomical studies to regulate the pituitary-gonadal axis in the control of reproduction (1, 36). Even though the gonadotropin/glycoprotein hormone has not been fully identified in the lampreys, there is substantial direct and indirect evidence of pituitary responsiveness to lamprey GnRHs (36). As an example, in our 2006 in vivo studies (7), administration of lGnRH-III at 100 µg/kg body weight (twice at a 24 h interval) increased expression of lGpHβ RNA (formerly called lGTHβ) in the pituitary of adult female sea lamprey. To further characterize the expression of lGpA2 and lGpHβ in the hypothalamic-pituitary axis, the pituitaries were cultured in vitro with the hypophysiotropic form, lamprey GnRH-III, for a time-course study. The RNA expression of lGpA2 and lGpHβ in all samples was higher at 6 hours of incubation with lGnRH-III than 0 hours. The results suggest that both lGpA2 and lGpHβ are up-regulated in the pituitary by lGnRH-III, confirming a functional hypothalamic-pituitary axis.

In summary, the results of this study provide evidence that lGpA2 and lGpHβ form an active heterodimeric glycoprotein hormone capable of transducing endocrine signals in vivo from the pituitary to peripheral glands and therefore may act as a gonadotropin and/or thyroid-stimulating hormone. Given that previous molecular studies failed to show a GpA1 homolog in the lamprey, this might be the missing endocrine factor that completes a hypothalamic-pituitary-peripheral gland pathway controlling reproduction and metabolism in this organism. Regardless of the molecular factors that were involved, discovery of a functional HPG axis in lamprey can provide important clues for understanding the forces that ensured a common organization of the hypothalamus and pituitary as essential regulatory systems in all vertebrates. We hypothesize the existence of a primitive, overlapping, yet functional hypothalamic-pituitary-gonadal and possibly hypothalamic-pituitary-thyroid axes in lampreys involving a pituitary level atypical GpA2-GpHβ heterodimer. These data suggest an intermediate stage of the structure-function of the gonadotropin/thyroid-stimulating hormone in a basal vertebrate, leading to the emergence of the highly specialized gonadotropin hormones and thyroid stimulating hormones in gnathostomes.

**Acknowledgments**

We thank Professor Zvi Yaron for the critical review of the manuscript. We also thank Takayoshi Kosugi for his technical assistance.

Address all correspondence and requests for reprints to: Stacia A. Sower, PhD, Center for Molecular and Comparative Endocrinology, Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, NH 03824. E-mail: sasower@unh.edu.

This work was supported by National Science Foundation Grants IOS-0849569 and IOS-1257476 (to S.A.S.) and a University of New Hampshire Undergraduate Research Award (to T.J.M.). Partial funding was provided by the New Hampshire Agricultural Experiment Station. This is Scientific Contribution Number 2561.

Disclosure Summary: The authors have nothing to disclose.

**References**

1. Sower SA, Freamat M, Kavanaugh SI. The origins of the vertebrate hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pitu-


Alvarez E, Cahoceau C, Combarroux Y. Comparative structure analyses of cystine knot-containing molecules with eight aminoacyl ring including glycoprotein hormones (GPH) α and β subunits and GPH-related A2 (GP2A) and B5 (GP2B) molecules. Reprod Biol Endocrinol. 2009;7:90.


