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Identification of Growth Hormone in the Sea Lamprey, an Extant Representative of a Group of the Most Ancient Vertebrates

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GH was identified in the sea lamprey, an extant representative of a group of the most ancient vertebrates, the Agnatha. A putative GH-cDNA was cloned from the pituitary by RT-PCR. The entire coding region comprised an open-reading frame of 203 amino acids (aa). The mature protein was also isolated from pituitaries, and fractionated by gel filtration and reverse-phase HPLC. A putative GH was monitored by Western blotting with a rabbit antiserum against a synthetic peptide corresponding to pre-GH sequence (aa 29–45). Sequence analysis of the purified protein demonstrated that the prehormone consists of a signal peptide of 22 aa and the mature protein of 181 aa, which shows 25% sequence identity with sturgeon GH. The site of production was identified through immunohistochemistry to be cells of the dorsal half of the proximal pars distalis of the pituitary. Following cDNA cloning of lamprey IGF cDNA, it was shown using RT-PCR that lamprey GH stimulates IGF expression in lamprey liver. This is the first study in which a member of the GH/prolactin/somatolactin family has been identified in an agnathan. In addition, GH appears to be the only member of this hormone family in the sea lamprey. Evidence suggests that GH is the ancestral hormone in the molecular evolution of the GH family and that the endocrine mechanism for growth stimulation was established at an early stage of vertebrate evolution. (*Endocrinology* 143: 4916–4921, 2002)

GH, PROLACTIN (PRL), and somatolactin (SL) form a family of pituitary hormones that are similar in structure, function, and gene organization, and thus are believed to have evolved from a common ancestral gene through duplication and subsequent divergence (1). GHs have been identified in all taxonomic groups of the jawed vertebrates (gnathostomes) (2), but there has been no clear evidence for the presence of this family in jawless vertebrates (agnathans). Therefore, the evolutionary origin of this family still remains to be resolved 30 yr after the original proposal (3). Although functions of PRL and SL are diverse, GH almost exclusively stimulates somatic growth of the gnathostomes primarily through induction of IGF (4). However, a definitive link between GH and IGF has yet to be demonstrated in the agnathans.

The agnathans probably arose as the first vertebrates about 550 million years ago (5), 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 suggests that lampreys are more closely related to gnathostomes than either group is to the hagfishes (5), although recent molecular analysis groups the hagfishes together with the lampreys in a single clade (6).

The pituitary system in the agnathans had been an enigma until we identified the melanotropins (MSHs) and corticotropin (ACTH) in the sea lamprey, *Petromyzon marinus* (7). These hormones in the lamprey are significantly different in structure from those of gnathostomes. Moreover, MSH and ACTH are encoded in two distinct genes in sea lampreys, whereas they are encoded on a single gene in gnathostomes (8). On the other hand, there has been no conclusive evidence for the presence of other pituitary hormones in this species except for some immunohistochemical evidence for GH (9) and gonadotropin (GTH) (10).

Both IGF and insulin were demonstrated in tunicate (protochordate) (11) and hagfish (agnathan) (12). Thus, these hormones have maintained separate gene lineages in both vertebrate and protochordate evolution for at least 550 million years. Two homologous IGFs, IGF-I, and IGF-II, have been identified in gnathostomes, whereas a single molecular form of IGF has been cloned in hagfish (12) and in two protochordate species, amphioxus (Cephalochordata) (13) and tunicate (Urochordata) (11). Therefore, lampreys represent a critical nodal point to detect possible duplication of the IGF gene.

The present study on sea lamprey provides the first description of GH and a GH-IGF system in an agnathan and discusses the evolutionary implications of these findings.

Abbreviations: aa, Amino acids; GTH, gonadotropin; nt, nucleotides; ODS, octadecylsilane; PRL, prolactin; RACE, rapid amplification of cDNA ends; SL, somatolactin.

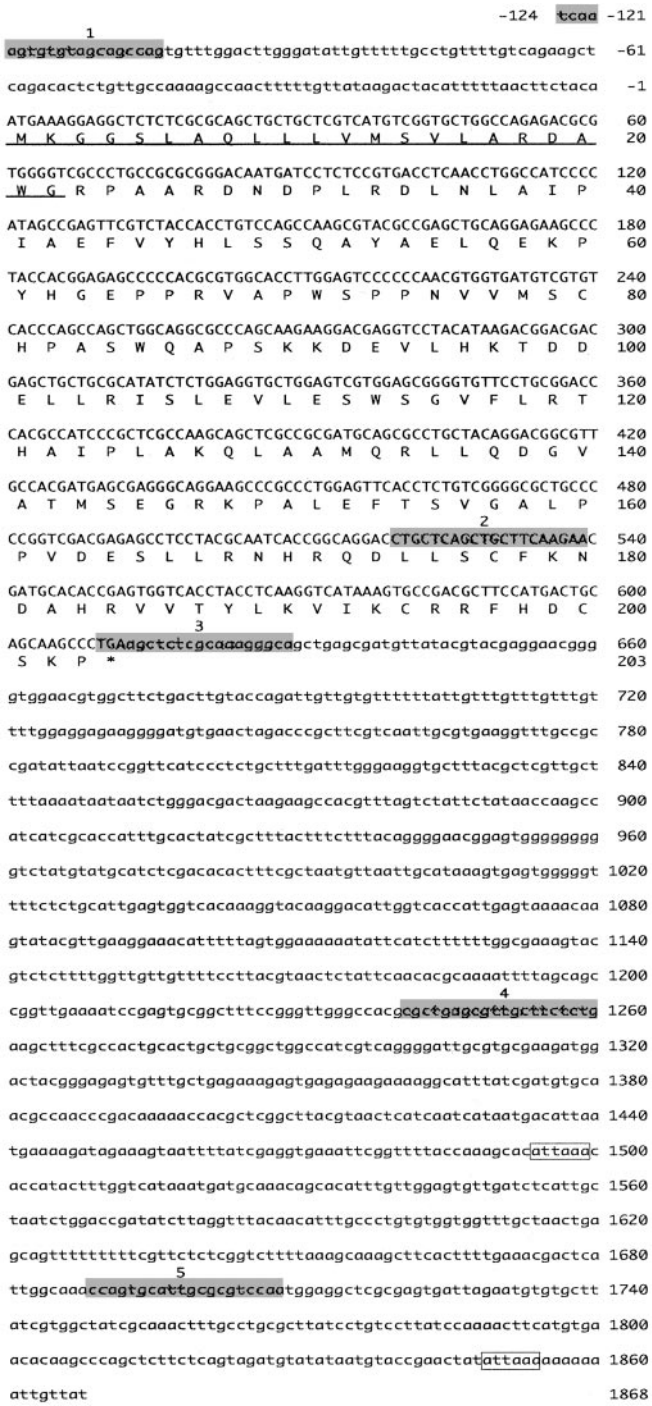

FIG. 1. Nucleotide and deduced aa sequences of lamprey GH cDNA excluding the poly A tail. Nucleotides (*upper line*) and amino acids (*lower line*) are numbered from the initiation methionine. Signal peptide (22 aa) is *underlined with solid line*. Stop codon is marked with an *asterisk*. Putative poly-adenylation sites are *boxed in open rectangles*. Primer positions are *shadowed*. The accession number in the DDBJ/EMBL/GenBank nucleotide sequence databases is AB081461.

Materials and Methods

Tissues

The pituitaries of 4600 adult landlocked sea lampreys (*Petromyzon marinus*), in their upstream migration, were extirpated and immediately frozen on dry ice in June 1999 at Hammond Bay Biological Station in Michigan. These tissues were used for cloning of GH cDNA and isolation of GH. Adult, sea-run, sea lampreys were collected in a trap at the Cocheco River in Dover, New Hampshire, in May and June 1999 during their upstream spawning migration from the ocean. The livers dissected from freshly killed lampreys were used for IGF cDNA cloning and incubation with lamprey GH to measure the expression of its mRNA.

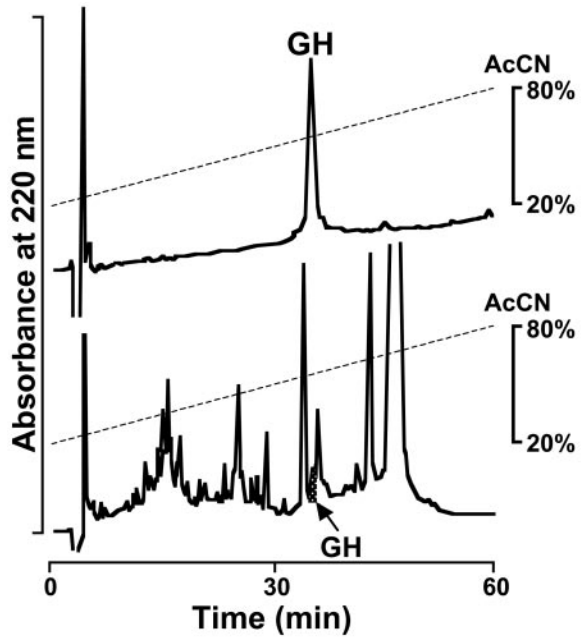

FIG. 2. Reverse-phase HPLC of putative lamprey GH. Putative GH was fractionated on an ODS column (the *lower chromatogram*) after gel filtration on a Sephadex G-100 column of a pituitary extract and detected with an antiserum raised against a synthetic peptide, pre-GH sequence (aa 39–45) and purified by rechromatography (the *upper chromatogram*).

Cloning of cDNAs encoding GH and IGF

Double-strand cDNA prepared from lamprey pituitary glands was inserted into pGM5zf plasmid. A cDNA encoding GH-like sequence was obtained from one of the 45 randomly selected *Escherichia coli* colonies transformed with the recombinant plasmid. Lamprey GH cDNA was isolated from a cDNA library using the cDNA as a probe. The nucleotide sequence of the lamprey GH cDNA was confirmed by sequencing of cDNAs amplified from lamprey pituitary total RNA by RT-PCR for the internal region and rapid amplification of cDNA ends (RACE) methods for 3′ ends using AmpliTaq Gold (PE Applied Biosystems, Foster City, CA) and First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Combinations of primers were:

1 (5′-TCAAAGTGTGTAGCAGCCAG-3′) and 3 (5′-TGCCCTTTGCGAGAGCTTCA-3′),

2 (5′-CTGCTCAGCTGCTTCAAGAA-3′) and 5 (5′-TTGGACGCGCAATGCACTGG-3′), and

4 (5′-CGCTGAGCGTTGCTTCTCTG-3′) and *Not*I provided by Amersham Pharmacia Biotech for 3′region. The amplified cDNAs were inserted into pT7-Blue T vector (Novagen, Madison, WI) for cloning and subsequent sequencing.

IGF cDNA was amplified from total RNA prepared from lamprey liver and sequenced by the methods described above and 5′RACE using 5′RACE System (Life Technologies, Inc., Gaithersburg, MD). Lamprey IGF cDNA was cloned from the liver by RT-PCR. The internal region of cDNA between nucleotides (nt) 214 and 318 was amplified by degenerated primers that encoded the amino acid (aa) sequences of VDLQFVC and LLEMYCA. Primer to 1 (5′-TGAAGATGGGTGCCGCACGAAGTAG-3′) was used to amplify the 5′ region (nt –145 and 227) by the 5′ RACE method. Primer 2 (5′-GAAGGGCATCGTGGAAGAAT-3′) in a

combination with the *Not*I was used to amplify the 3′ region. The full-length sequence of lamprey IGF cDNA was derived by overlapping all the sequence of the partial clones.

The cDNA nucleotide sequence was determined by sequencing according to the dideoxy chain termination method with a DNA sequencer (model 377, PRISM, PE Applied Biosystems). DNASIS-MAC (Hitachi, Tokyo, Japan) was used for processing the sequence data, aligning the sequences, and calculating sequence identity.

Immunohistochemistry

A rabbit antiserum was raised against a synthetic peptide corresponding to the pre-GH sequence (aa 29–45), *i.e.* DPLRDLNLAIPIAEFV. Sections of lamprey pituitary were immunohistochemically stained as described previously (9) using a Vectastain ABC Elite kit (Elite ABC, Vector Laboratories, Inc., Burlingame, CA) and this specific antiserum (lot no. 9901), diluted 1:5,000. An antilamprey ACTH (lot no. 9308) (14) and ovine LH β (NHPP) (10) sera were diluted 1:1500 and 1:8000, respectively. 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 the synthetic peptide.

Purification of GH from pituitaries

Frozen lamprey pituitaries (5 g) were extracted in 50 mM ammonium acetate (pH 9.0) and centrifuged at 20,000 $\times$ *g* for 30 min at 4 C. The resulting supernatant was subjected to gel filtration on Sephadex G-100 (3 $\times$ 80 cm) in 50 mM ammonium bicarbonate (pH 9.0). Immunoreactivity was monitored by Western blotting with the antiserum described above. The immunoreactive fractions were lyophilized (60 mg) and further purified by a C18 reverse-phase column [octadecylsilane (ODS)-120T, 0.46 $\times$ 25 cm; TOSOH, Tokyo, Japan]. Proteins were eluted with a linear gradient of 20–80% CH_3CN in 0.1% trifluoroacetic acid at a flow rate of 1 ml·min^{-1}. The immunoreactive fractions were pooled and rechromatographed on the same column. Amino acid sequences were determined by use of an automatic sequencer (Shimadz PSQ-1, Shimadz, Tokyo, Japan).

RT-PCR of IGF mRNA in the liver

Liver pieces (3 mm^3 each) were maintained in 0.5 ml MEM (Earle's salts) (Life Technologies, Inc.) in 50 μl/ml kanamycin for 24 h at 18 C and treated with medium alone (0), 5, 50, or 500 ng putative lamprey GH. Total RNA was extracted from 20 mg of the incubated liver pieces with

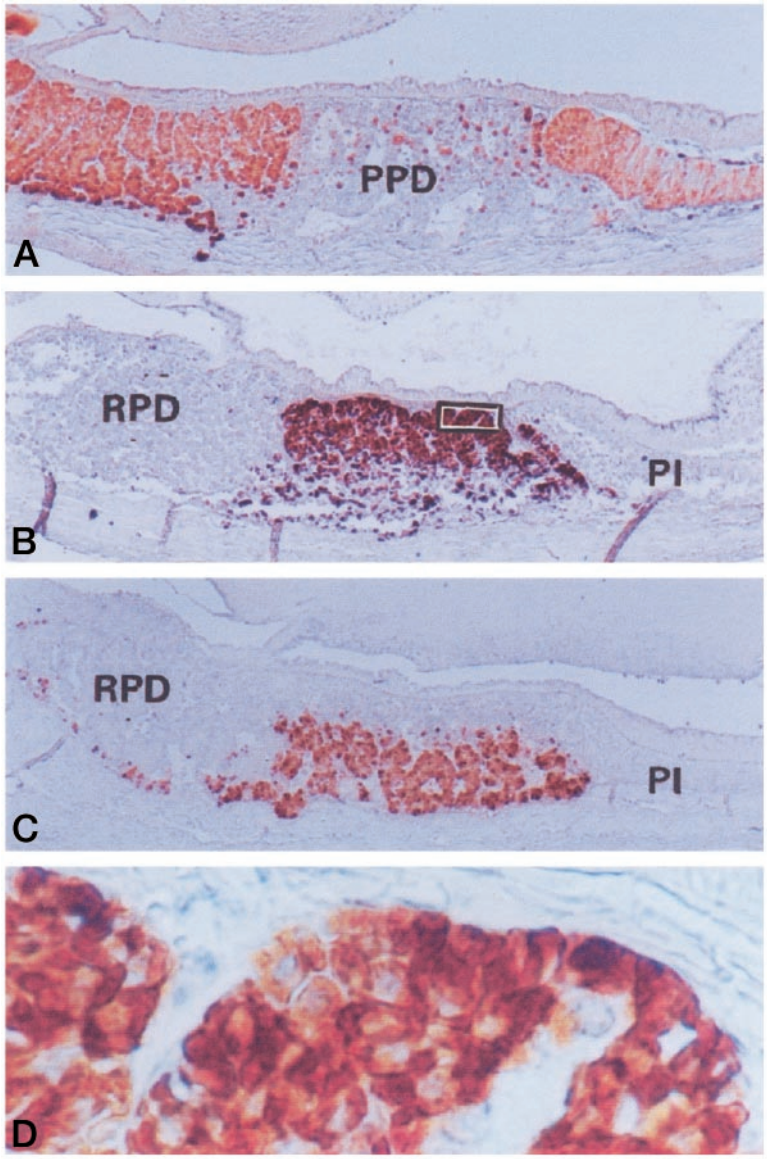

FIG. 3. Immunostaining of lamprey pituitary. Sections of adult lamprey pituitary were immunohistochemically stained using a Vectastain ABC Elite kit (Elite ABC) and specific antisera: A, antilamprey ACTH (lot no. 9308, diluted 1:1500) (10); B, antilamprey GH made to the pre-GH sequence (aa 29–45) (lot no. 9901), diluted 1:5000; and C, antiovine LH β (NHPP, diluted 1:8000) (14). The *area outlined by a rectangle* in B is enlarged and shown in D. In A, notice that ACTH-like and MSH-like cells are found in most parts of the rostral pars distalis (RPD) and the pars intermedia (PI), respectively. In B–D, notice that GH-like and GTH-like cells occupy the dorsal and ventral halves of the proximal pars distalis (PPD), respectively. A–C, Magnification, $\times$60; D, magnification $\times$880.

0.25 ml Isogen (Nippon Gene, Tokyo, Japan), and single-strand cDNAs were synthesized from total RNA by RT. A 594-bp cDNA was amplified by PCR using primers corresponding to lamprey IGF cDNA (nt –145 to –123) and (nt 428 to 449). The amplified internal fragment of β-actin was also used as a standard. PCR products were subjected to gel electrophoresis and semiquantified by densitometry.

Phylogenetic tree

The phylogenetic tree of GH was created by the neighbor-joining method using a computer program in Genetyx-Mac, version 11.2.1 (Software Development Co., Tokyo, Japan).

Results

Identification of sea lamprey GH

A sea lamprey cDNA cloned from the pituitary consisted of 1992 bp that encode a prehormone of 203 aa residues, including 5′ and 3′ noncoding sequence of 124 bp and 1259 bp, respectively (Fig. 1). To determine whether this product was a GH cDNA, we next isolated its mature protein. Proteins were extracted from pituitaries and fractionated by gel filtration and reverse-phase HPLC. A putative GH was monitored by Western blotting with a rabbit antiserum against a synthetic peptide corresponding to pre-GH sequence (aa 29–45). The immunoreactive protein was collected and purified by rechromatography on an ODS column (Fig. 2). The yield was 180 μg from 2000 pituitaries (5 g wet tissue). N-terminal sequence of this protein was determined to be RPAARDNDPLRDL-, which revealed that the prehormone consists of a signal peptide of 22 aa and the mature protein of 181 aa. This protein shows slightly higher sequence identity with GH (25%) (15) than with SL (21%) (16) and PRL (18%) (17) in an early-evolved osteichthyes, the sturgeon, *Acipenser gueldenstaedtii*.

FIG. 4. Nucleotide and deduced aa sequence of the lamprey IGF cDNA excluding poly A tail. Nucleotides (*upper line*) and aa (*lower line*) are numbered from the initiation methionine. The signal peptide (53 aa) and E-domain (13 aa) are *underlined with solid line* and *broken line*, respectively. Mature peptide between these two segments is composed of 66 aa. Primer positions are shadowed. The accession number in the DDBJ/EMBL/GenBank nucleotide sequence databases is AB081462.

GH-producing cells

The adenohypophysis of the sea 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. The MSH gene is expressed in the PI, and the ACTH gene in the RPD (Fig. 3A; Ref. 14), but hormones in the PPD have remained unidentified. Previous studies showed that antisera against salmon and sturgeon GHs and ovine GTH (Fig. 3C) stained the cells in the dorsal and the ventral half of the lamprey PPD, respectively (9, 10). Indeed, in the present study, the antiserum against the synthetic peptide specifically stained most cells in the dorsal half of the PPD (Fig. 3, B and D), which is the expected site of the distribution of GH cells in the pituitary of gnathostome fishes.

Cloning lamprey IGF

The sea lamprey prepro-IGF cDNA consists of 851 bp. Sequence comparison suggested that it encodes a signal peptide of 53 aa, a mature hormone of 66 aa, and an E domain of 13 aa (Fig. 4). The lamprey IGF shows 76% identity with hagfish IGF (12) and 58 and 62% with dogfish IGF-I and -II, respectively (18).

Expression of sea lamprey IGF mRNA by GH

Liver pieces from sea lamprey were incubated with the putative GH at three different doses. A 594-bp cDNA was amplified from total RNA from the tissues by RT-PCR using specific lamprey IGF primers. A maximal expression of IGF mRNA was seen at 100 ng/ml in both sexes (Fig. 5). The results confirmed that the cDNA cloned from the pituitaries of lamprey encodes GH and that the GH stimulates IGF mRNA expression in liver; the latter hormone could be involved in a central mechanism for growth regulation, as in gnathostomes (19).

Discussion

In this study, we used cDNA cloning and protein isolation to identify, for the first time, a homolog of the GH/PRL/SL family from the pituitary of sea lamprey. Our data show high sequence similarity to GH and also GH-immunoreactivity in the pituitary; collectively, these two results are good evidence for existence of GH in lampreys. Thus, we set out to demonstrate its biological activity. In other vertebrates, after GH is released from the pituitary, it binds to receptors in peripheral tissues such as the liver and stimulates synthesis and release of IGF-I, which in turn increases the rate of DNA and protein synthesis (19) in tissues such as cartilage and muscle. Thus, we cloned an IGF cDNA from the liver of sea lamprey by RT-PCR and demonstrated that the putative GH stimulated expression of the IGF gene.

A molecular phylogenetic tree of GH was constructed by choosing at least one representative species from each class of the subphylum Vertebrata (Fig. 6). The evolution of GH in

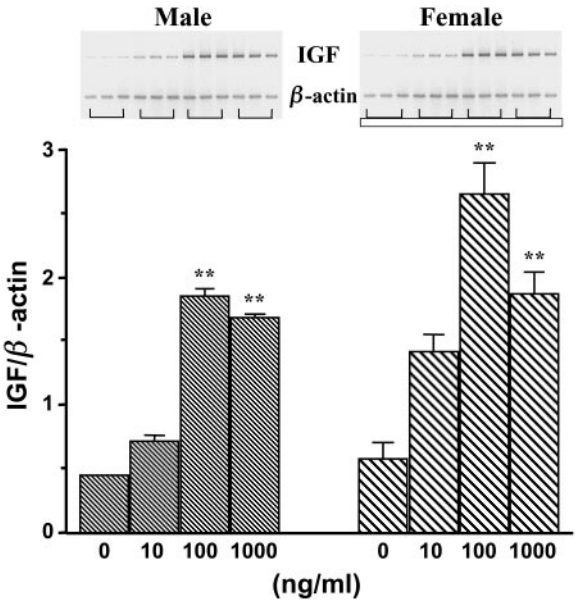

FIG. 5. The effects of lamprey GH on expression of IGF mRNA in the liver. Pieces of sea lamprey liver were incubated with putative GH isolated from sea lamprey pituitary at three concentrations. IGF cDNA was amplified from total RNA from the incubated tissues by RT-PCR using specific primers corresponding to lamprey IGF cDNA (nt –145 to –123) and (nt 428–449) (Fig. 4). An amplified internal fragment of β-actin was used as standard. PCR products were subjected to gel electrophoresis and semiquantified by densitometry. Expression of IGF mRNA peaked at 100 ng per ml in both sexes. Data are shown as means ± SEM (n = 3). Significant differences from the control are indicated by ** ($P < 0.01$).

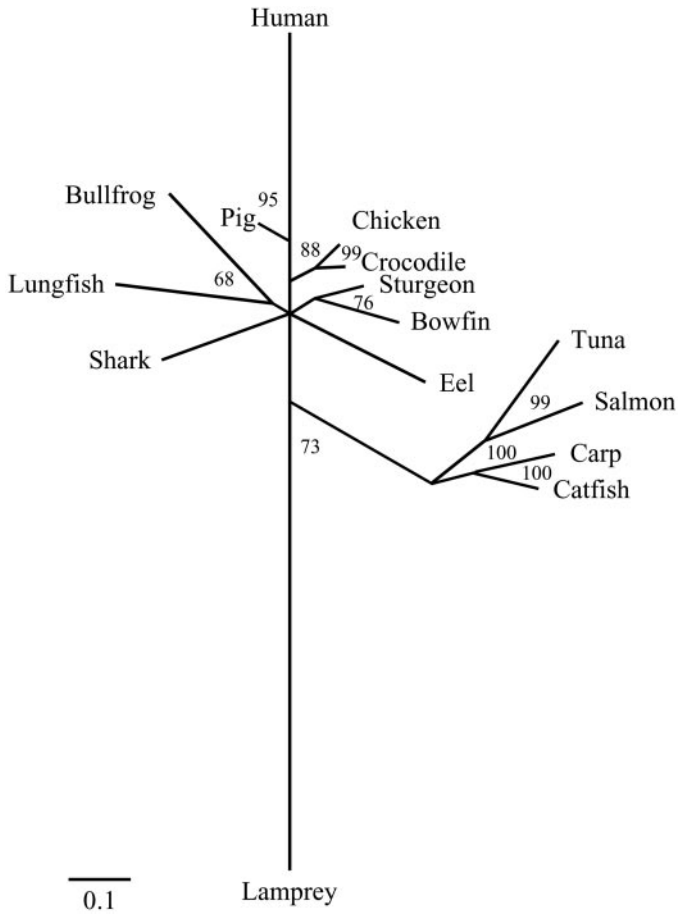

FIG. 6. The phylogenetic tree of GH. Bootstrap majority consensus values on 100 replicates are indicated at branch points. *Scale bar* refers to a phylogenetic distance of 0.1 amino acid substitutions per site.

primates and in teleost fishes appears to have been rapid (20, 21). Lamprey GH is far removed from gnathostome GHs, suggesting that GH diversified after separation of agnathans and gnathostomes.

In terms of the molecular evolution of the GH/PRL/SL family, it is not known which of the hormones in this family is closest to the ancestral hormone. The present study provides conclusive evidence that GH is present in agnathans and, therefore, in all classes of vertebrates. PRL has versatile functions including the eft-water drive response, which cannot be mimicked by any other pituitary hormone (22, 23). This activity was identified in Chondrichthyes, but not in the lampreys (24). Therefore, PRL is probably present in the Chondrichthyes, and thus all classes of gnathostomes, but not in agnathans. However, in an elasmobranch, the dogfish, no cells were stained with variety of heterologous PRL antisera and an elasmobranch PRL cDNA has not been cloned despite the use of primers designed from highly conserved regions of known PRLs. At the present time, we cannot rule out the possible existence of an elasmobranch PRL, for it may have a markedly different primary structure to the PRL of other species. SL, on the other hand, is a newly discovered, multifunctional hormone limited to the Osteichthyes (16), regulating acid-base balance (25), reproduction (26), background adaptation (27), and phosphate metabolism (28) in teleosts. In the dogfish pituitary, SL and GH cells were identified in the PI and the PPD, respectively, and their cDNAs were cloned (SL: our unpublished data). Figure 3 shows that GH, ACTH, MSH, and possibly GTH, are major pituitary hormones in the sea lamprey. Based on the current studies and other related studies, there has been no evidence that PRL and SL are present or functional in extant agnathans including hagfish (our unpublished data).

Our current findings on a vertebrate of ancient lineage indicate that GH is the ancestral hormone and a forerunner of the GH family and that its gene duplicated during the early evolution of gnathostomes to form PRL and/or SL. If gene duplication occurred during early evolution of agnathans, the only gene to endure was the GH gene, which likely was important for the survival of the descendants of the extinct ostracoderms. While GH has maintained its original function of growth stimulation throughout vertebrate evolution, the later derived hormones, PRL and SL, may have contributed to the expansion of vertebrates into new environments.

Sea lamprey IGF is more closely related to hagfish (the only other living member of Agnatha) IGF than to dogfish IGFs. This supports the view of the monophyly of agnathans (6), and that the prototypical IGF molecule duplicated and diverged in an ancestor of the extant gnathostomes.

In conclusion, GH was identified for the first time in the sea lamprey, a representative of one of the two most ancient extant vertebrate groups. Lamprey GH stimulates IGF expression as in mammals and other gnathostome vertebrates.

In addition, GH appears to be the only member of the GH family in the sea lamprey, which suggests that GH is the ancestral hormone that originated first in the molecular evolution of the GH family in vertebrates.

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