

Review

# The dawn and evolution of hormones in the adenohypophysis

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## Abstract

The adenohypophysial hormones have been believed to have evolved from several ancestral genes by duplication followed by evolutionary divergence. To understand the origin and evolution of the endocrine systems in vertebrates, we have characterized adenohypophysial hormones in an agnathan, the sea lamprey *Petromyzon marinus*. In gnathostomes, adrenocorticotropin (ACTH) and melanotropin (MSH) together with  $\beta$ -endorphins ( $\beta$ -END) are encoded in a single gene, designated as proopiomelanocortin (POMC), however in sea lamprey, ACTH and MSH are encoded in two distinct genes, proopiocortin (POC) gene and proopiomelanotropin (POM) gene, respectively. The POC and POM genes are expressed specifically in the rostral pars distalis (RPD) and the pars intermedia (PI), respectively. Consequently, the final products from both tissues are the same in all vertebrates, i.e., ACTH from the PD and MSH from the PI. The POMC gene might have been established in the early stages of invertebrate evolution by internal gene duplication of the MSH domains. The ancestral gene might be then inherited in lobe-finned fish and tetrapods, while internal duplication and deletion of MSH domains as well as duplication of whole POMC gene took place in lamprey and gnathostome fish. Sea lamprey growth hormone (GH) is expressed in the cells of the dorsal half of the proximal pars distalis (PPD) and stimulates the expression of an insulin-like growth factor (IGF) gene in the liver as in other vertebrates. Its gene consists of 5 exons and 4 introns spanning 13.6 kb, which is the largest gene among known GH genes. GH appears to be the only member of the GH family in the sea lamprey, which suggests that GH is the ancestral hormone of the GH family that originated first in the molecular evolution of the GH family in vertebrates and later, probably during the early evolution of gnathostomes. The other member of the gene family, PRL and SL, appeared by gene duplication. A  $\beta$ -chain cDNA belonging to the gonadotropin (GTH) and thyrotropin (TSH) family was cloned. It is expressed in cells of the ventral half of PPD. Since the expression of this gene is stimulated by lamprey gonadotropin-releasing hormone, it was assigned to be a GTH $\beta$ . This GTH $\beta$  is far removed from  $\beta$ -subunits of LH, FSH, and TSH in an unrooted tree derived from phylogenetic analysis, and takes a position as an out group, suggesting that lampreys have a single GTH gene, which duplicated after the agnathans and prior to the evolution of gnathostomes to give rise to LH and FSH.

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## 1. Introduction

The adenohypophysis of the pituitary gland secretes a number of peptide hormones that regulates a variety of the physiological processes of vertebrates. The adenohypophysial hormones can be classified, on the basis of structural and functional similarity, into three groups, the proopiomelanocortin (POMC) family, the growth hormone

(GH) family, and glycoprotein hormone family. Each family is believed to have evolved from an ancestral gene by duplication and subsequent mutations. Therefore, identification of these hormones throughout the vertebrate lineage will allow us to understand the processes of molecular evolution and functional divergence, which might have made the diversification and prosperity of vertebrates successful. To date, these hormones and genes of gnathostomes (jawed vertebrates) have been well characterized. On the other hand, the pituitary system in the agnathans (jawless vertebrates) had been an enigma until very recently.

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Within the superclass of gnathostomes there are two major classes of fish, the Chondrichthyes and Osteichthyes. The Osteichthyes include actinopterygians (ray-finned fish) and sarcopterygians (lobe-finned fish). 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). The lampreys are divided into three families: the *Petromyzonidae*, which are found in the northern hemisphere, and the two southern hemisphere families, *Geotriidae* and *Mordaciidae*. The *Petromyzonidae* consists of six genera: *Ichthyomyzon*, *Petromyzon*, *Caspiomyzon*, *Eudontomyzon*, *Tetrapleurodon*, and *Lampetra*. The *Geotriidae* and *Mordaciidae* each consist of only one genus, *Geotria* and *Mordacia*, respectively (Hubbs and Potter, 1971).

In 1985, we started the collaboration for the identification of the pituitary hormones in the sea lamprey, *Petromyzon marinus*. This species, found in the North Atlantic Ocean and many of its tributaries, invaded the upper Great Lakes after the construction of a canal to bypass a natural barrier, Niagara Falls, in 1829. Since adult sea lampreys are parasitic, sucking the blood of other fishes, they are serious pests to the fishing industry. Thus, the control program known as “integrated sea lamprey management” uses several techniques to attack the landlocked sea lampreys around the Great Lakes. One of the efforts is the sterile-male-release-technique, which includes trapping adult sea lamprey, sterilizing the males with bisazir, and releasing them back to the Great Lakes. The pituitary hormones were characterized from the tissues collected from the remaining captured female adult sea lampreys at Hammond Bay Biological Station in Michigan.

The first outcome of our studies was the identification of a neurohypophysial hormone, arginine vasotocin (Lane et al., 1988). Since then, the collaboration was expanded with the addition of Dr. Masumi Nozaki of Niigata University, Dr. Akiyoshi Takahashi and Dr. Shunsuke Moriyama of Kitasato University, Dr. Jean M.P. Joss of University of Macquarie, and Dr. John H. Youson of the University of Toronto at Scarborough and led to the great successes in identification of nasohypophysial factor (Sower et al., 1995), two MSHs, ACTH (Heinig et al., 1995; Takahashi et al., 1995a,b), GH (Kawauchi et al., 2002) and GTH (Sower et al., 2005). These adeno-hypophysial hormones in the lamprey are significantly different in structure from those of gnathostomes. On the basis of these data from the sea lamprey together with gnathostomes, the origin and evolution of hormones from adeno-hypophysis are discussed in this review.

## 2. Background: Lamprey pituitary glands and its putative hormones

The pituitary gland of lamprey consists of the adeno-hypophysis and neurohypophysis as in all other vertebrates (Fig. 1). The lamprey adeno-hypophysis is divided into the rostral pars distalis (RPD), the proximal pars distalis (PPD), and the pars intermedia (PI) as in teleosts. Both ACTH and MSH activities were demonstrated in the pituitary of lamprey, *Lampetra fluviatilis*, (Baker and Buckingham, 1983) and ACTH- and MSH-like substances were also demonstrated immunohistochemically in the RPD and PI of the brook lamprey, *Lampetra lamotruii*, respectively (Dores et al., 1984) (Fig. 1). There had been no clear evidence for the presence of GH family in lampreys except for an earlier study that cells in the dorsal half of the PPD of sea lamprey were stained by anti-rat PRL antisera (Wright, 1983). Recently, GH-like immunoreactivity was demonstrated by a hydrated autoclaved pretreatment of pituitary sections of sea lamprey (Ominato and Nozaki, 2002) (Fig. 1). In contrast, evidence from physiological and immunohistochemical studies strongly supported the presence of a GTH-like molecule and the presence of the hypothalamo-pituitary-gonadal axis in lampreys. In river lampreys, *Lampetra fluviatilis*, hypophysectomy and substitution therapy with pituitary extracts or mammalian GTHs indicated pituitary regulation of the gonads (Larsen, 1965). Moreover, 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). Moreover, GTH-like immunoreactivity was identified in cells distributed in the ventral half of the PPD (Nozaki et al., 1999) (Fig. 1). These cells were stained intensely by anti-ovine LH including LH $\beta$  and moderately or weakly by several other antibodies such as human LH $\beta$ , amphibian LH, and sturgeon FSH $\beta$ . No positive reaction was observed in the sea lamprey pituitary using antibodies to FSH-related GTHs, TSH or pituitary glycoprotein

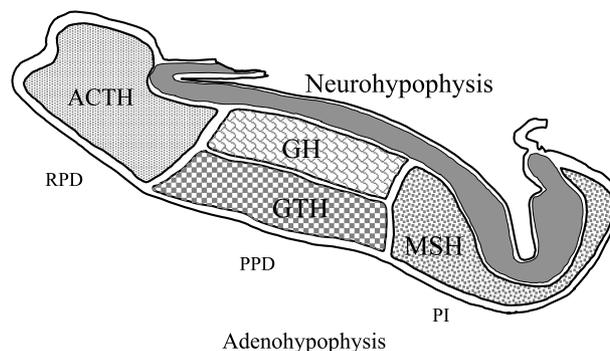


Fig. 1. Schematic representation of sea lamprey pituitary gland and localization of cells for adeno-hypophysial hormones. RPD, the rostral pars distalis; PPD, the proximal pars distalis; PI, the pars intermedia; ACTH, adrenocorticotropic; GH, growth hormone; GTH, gonadotropin; MSH, melanotropin.

hormones of teleostean origins. Thus, GTH-like substance in the sea lamprey pituitary seemed to be more closely related to LH, rather than to FSH or TSH (Nozaki et al., 1999, 2001).

### 3. Discovery of the lamprey pituitary hormones

#### 3.1. POMC family

##### 3.1.1. Gnathostomes

Proopiomelanocortin (POMC) is a common precursor protein for several hormonal peptides, such as adrenocorticotropin (ACTH), melanotropins (MSHs), and  $\beta$ -endorphin ( $\beta$ -END). These POMC-derived hormones exhibit a variety of physiological functions, which are associated with stress response and environmental adaptation. ACTH stimulates the adrenal cortex to produce and secrete adrenocortical hormones (e.g., corticosteroids, glucocorticoids). MSH causes dispersion of pigment granules of melanocytes, producing a rapid change in skin coloration.  $\beta$ -END binds to the opioid receptors in the brain and exhibits analgesic effects in mammals.

POMCs have been characterized from the pituitary glands of representatives of taxonomic groups in all classes of gnathostomes. Tetrapod POMCs are composed of  $\gamma$ -MSH, ACTH (or  $\alpha$ -MSH),  $\beta$ -MSH and  $\beta$ -END from the N-terminus (Nakanishi et al., 1979). Sarcopterygian POMCs contain three MSHs and one  $\beta$ -END as in tetrapod POMC, although  $\gamma$ -MSH of Australian lungfish (Dores et al., 1999; Lee et al., 1999b) but not African lungfish (Amemiya et al., 1999b) and coelacanth (Takahashi et al., 2003) has one substitution from Arg to His in the core sequence of His-Phe-Arg-Trp. This mutation appears to have occurred independently in the Australian lungfish lineage. Even though sturgeon (Alrubaian et al., 1999; Amemiya et al., 1997), paddlefish (Danielson et al., 1999), and gar (Dores et al., 1997) POMCs have three MSH domains as in the sarcopterygians, the  $\gamma$ -MSH domain of these early-evolved actinopterygians is probably nonfunctional due to one mutation of an essential residue in the core-sequence. All teleostean POMCs lack the  $\gamma$ -MSH domain entirely (Arends et al., 1998; Kitahara et al., 1988; Lee et al., 1999a; Okuta et al., 1996; Salbert et al., 1992; Takahashi et al., 2005a). These structural features suggest that the  $\gamma$ -MSH domain has progressively accumulated mutations in the lineage of early-evolved ray-finned fish and eventually deleted in the teleost lineage.

In Chondrichthyes, including elasmobranchs (Amemiya et al., 1999c, 2000; Dores et al., 2003) and holocephalans (Takahashi et al., 2004a), POMC genes encode another MSH, which is located between  $\alpha$ - and  $\beta$ -MSH, and was named  $\delta$ -MSH after  $\alpha$ -,  $\beta$ - and  $\gamma$ -MSH. On the basis of the sequence similarity and the length of the domains, these four MSHs could be classified into two groups; one is  $\alpha$ -MSH and  $\gamma$ -MSH and the other is  $\beta$ -MSH and  $\delta$ -MSH (Amemiya et al., 1999c, 2000). This grouping implies an internal gene duplication of  $\beta$ -MSH-

$\beta$ -END segment and subsequent mutation of the  $\beta$ -END sequence from the duplicated domain leaving  $\delta$ -MSH during evolution of Chondrichthyes (Amemiya et al., 1999c, 2000).

All POMC genes consist of three exons and two introns (Chang et al., 1980; Deen et al., 1991; Nakanishi et al., 1981; Notake et al., 1983; Uhler et al., 1983) and is expressed in both the PD and the PI of the pituitary, and the resulting pro-hormone is processed in tissue-specific manner to produce different final products, i.e., ACTH and  $\beta$ -END in the PD and  $\alpha$ -MSH and  $\beta$ -MSH in the PI.

##### 3.1.2. Agnathans

In Agnatha, three MSH-like peptides were isolated from the sea lamprey (Takahashi et al., 1995a). MSH-A and -B, consisting of 19 and 20 amino acids, respectively, are active in the frog skin assay, but differ significantly from gnathostome MSHs in terms of structure. The third MSH-like peptide consists of 60 amino acids and exhibits an ACTH activity in lamprey kidney. This is the largest ACTH molecule that has been characterized to date, since most gnathostome ACTH consist of 39 amino acid residues. As seen for gnathostomes, the first 22 amino acids of lamprey ACTH include an  $\alpha$ -MSH-like sequence that is followed by four basic amino acids and additional 34 amino acids.

cDNA cloning of the POMC gene in lamprey revealed the presence of two POMC-related cDNAs that are encoded as separate genes (Heinig et al., 1995; Takahashi et al., 1995b): one encoding ACTH and  $\beta$ -END, named proopiocortin (POC) and the other encoding two MSHs and a different  $\beta$ -END, named proopiomelanotropin (POM). Therefore, sea lamprey POC and POM are different from gnathostome POMC in which ACTH, MSHs, and  $\beta$ -EP are encoded on a single POMC gene. Although the amino acid sequence identity between POC and POM is only 30%, the locations of  $\beta$ -END sequences in both POC and POM are well conserved and MSH-B can be aligned with  $\alpha$ -MSH segment of ACTH in POC. POC gene is expressed in the PD to form ACTH and  $\beta$ -END and POM gene is expressed in the PI to form MSHs and another  $\beta$ -END (Ficele et al., 1998; Nozaki et al., 1995; Takahashi et al., 1995b) (Fig. 1). It should be noted that the final products derived from the processing of POC and POM are the same as in gnathostomes; i.e., ACTH from the RPD and MSHs from the PI (Takahashi et al., 2001). As in gnathostome POMC genes, both POC and POM genes consist of three exons and two introns and all hormonal peptides are encoded in the third exon (Takahashi et al., 2005b). *Mordacia mordax* and *Geotria australis* collected in Tasmania, Australia, were subjected to cloning of POC and POM genes. The data demonstrated that two lamprey species in the southern hemisphere also have two homologues but distinct POMC genes, suggesting that all modern lampreys have two subtypes of POMC genes (Takahashi et al. submitted to Gen. Comp. Endocrinol.).



The molecular evolution of GH and PRL in gnathostomes has previously been discussed (e.g., Rand-Weaver and Kawauchi, 1993; Rand-Weaver et al., 1993; Forsyth and Wallis, 2002) so that in this paper a few points are only mentioned briefly. In earlier studies using rat GH radioimmunoassay to non-mammalian vertebrate GHs, decreasing immunoreactivity was observed with increasing phylogenetic distance. To date, GHs have been identified from over 100 species. The molecular phylogenetic tree of GH including representative species from all classes of gnathostomes showed that the evolutionary rate of ray-finned fish GH appeared significantly higher than that of tetrapods (Kawauchi and Yasuda, 1988; Noso et al., 1995). Primate and ruminant GHs have also diverged rapidly during the evolution of mammals (Wallis et al., 2005b). PRLs have been identified in most classes of gnathostomes except for Chondrichthyes. In sharp contrast to GHs, an evident discontinuity in sequence identities to primate PRL separates tetrapod PRLs from teleost PRLs. Among tetrapods with the exception of rodents, PRLs are similar to each other, while exhibiting versatile functions as mentioned above. Conservation of structure and versatility in functions of PRL may reflect a significant diversity of expression sites of the PRL receptor. The diversification of PRL in primates has been analyzed by Wallis et al. (2005a).

SL was originally found in the pituitary of cod (Rand-Weaver et al., 1991b) and flounder (Ono et al., 1990). SL cells are localized in the periphery of the PI, while GH and PRL cells are in the PPD and RPD, respectively (Rand-Weaver et al., 1991a). SL has been implicated in many physiological processes as mentioned above. However, most of these functions were estimated by changes of plasma SL levels under given conditions. Recently, Sugimoto et al. (2004) have provided genetic evidence for SL function. They found medaka mutants which show abnormal proliferation and morphogenesis of leucophores and xanthophores but not any obvious morphological and physiological defects, and demonstrated that a truncated SL gene responsible for medaka “color interfere” mutants by positional cloning. This finding provided strong support of pigmentation as a function of SL. Recently, a SL receptor, a cytokine receptor homologous to GH and PRL receptors, has been cloned in salmon (Fukuda et al., 2005). The transcripts for the receptor were at the highest level in liver and fat, supporting earlier studies (Mingarro et al., 2002) that a function of SL is regulation of lipid metabolism (Fukuda et al., 2005). A possible explanation for versatility of SL functions is that all SLs reported so far are not true orthologs. Fugu and medaka seem to have only one copy of somatolactin, according to their genome databases. Eel (May et al., 1997), goldfish (Cheng et al., 1997), and rainbow trout (Yang and Chen, 2003) have a unique somatolactin with 35–48% sequence similarity with other SLs (Ono et al., 1990; Rand-Weaver et al., 1991b; Yang et al., 1997). Indeed, Zhu et al. (2004) demonstrated two paralogous

copies of zebrafish SL genes that are expressed in different cells within the PI. They proposed two distinct subgroups of the SL family, SL $\alpha$  and SL $\beta$ ; SL $\beta$  includes the single SL genes reported from goldfish, catfish, and eel, along with one of zebrafish SL and rainbow trout SLP. All other SLs, including two similar SL of sea bream (Cavari et al., 2000; Astola et al., 1996), belong to SL $\alpha$ .

SLs have also been cloned from sturgeon and lungfish (Amemiya et al., 1999a). Although SLs have been limited to the Osteichthyes, the PI of dogfish pituitary is immunohistochemically stained with anti-salmon SL serum and its cDNA has been cloned (SL: unpublished data). These non-teleostean SLs are more similar to SL $\alpha$ , suggesting that SL gene duplicated depending on the species during the diversification of teleosts. On the other hand, no SL has been found in tetrapod lineage after numerous attempts in the isolation and cloning and screening mouse or human genomes for SL suggesting that the SL gene had been lost during the evolution to tetrapods.

### 3.2.2. *Agnathans*

Recently, we cloned GH-like cDNA from the pituitary of sea lamprey using RT-PCR with degenerated primers based on conserved regions of GHs (Kawauchi et al., 2002). The precursor protein consists of 203 amino acid residues. The mature protein was subsequently isolated from a pituitary extract by Western blotting using a rabbit antiserum raised against a synthetic peptide fragment corresponding to the deduced amino acid sequence. Amino acid sequence analysis of the isolated protein revealed that the mature protein consists of 181 amino acid residues. The protein shows a slightly higher sequence identity with GH of sturgeon (25% identity; Yasuda et al., 1992), the most primitive ray finned fish, than with sturgeon SL (20% identity; Amemiya et al., 1999a) and PRL (17% identity; Noso et al., 1993), although this alignment includes five consecutive deletions in the lamprey protein. The antiserum stained most of cells in the dorsal half of the proximal pars distalis of the pituitary (Fig. 1), which is the expected site of distribution of GH in the pituitary of teleost fishes. The protein was finally identified to be GH by demonstrating an increase of expression of IGF gene in the liver. The sea lamprey genomic GH gene consists of five exons and four introns (intron A, 1600 bp; intron B, 3080 bp; intron C, 4313 bp; intron D, 2619 bp), spanning approximately 13.6 kb. There are several consensus sequences such as TATA box, Pit-1/GHF-1, TRE, and CRE within 697 bp of the 5' flanking region (Moriyama et al., 2005).

### 3.2.3. *Evolutionary implications*

In terms of the molecular evolution of the GH family, it is not known which of the hormones in this family is closest to the ancestral hormone. In earlier research, an attractive model for evolution of this family was proposed on the basis of the internal homology for human GH (Niall et al., 1971). These authors proposed that a small primordial gene was

repeatedly duplicated to form the precursor gene of the family. However, subsequent accumulation of sequence data of cDNAs and genes of GH from other species did not support the internal homology model. Thus, the evolutionary origin of this family remained an enigma 30 years after the original proposal (Bewley and Li, 1970). Sea lamprey GH 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 (Bern, 1983; Grant, 1959). This activity was identified in Chondrichthyes, but not in the lampreys (Bern and Nicoll, 1968). 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 (Nozaki personal communication) 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 PRL in Chondrichthyes, it may have a markedly different primary structure to the PRL of other species. In contrast, there has been no evidence that PRL and SL are present or functional in extant agnathans including hagfish. Therefore, it is highly conceivable 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 (Fig. 3).

The lamprey GH gene is found to be the largest GH in all known GH genes. It appears that these introns of GH gene are progressively shortened throughout evolution of vertebrates. The structure of the genes encoding GH is made up of 5 exons and 4 introns in mammals, bird, and teleosts such as carp (Chiou et al., 1990; Ho et al., 1991; Hong and Schartl, 1993), catfish (Tang et al., 1993) and loach (Noh

et al., 1999), elasmobranch (Moriyama et al. to be published in 2005), and lamprey (Moriyama et al. to be submitted to *Gen. Comp. Endocrinol.*), except for the cases of advanced teleosts such as salmon (Agellon et al., 1988; Du et al., 1993; Johansen et al., 1989; Male et al., 1992), tilapia (Ber and Daniel, 1992), barramundi (Yowe and Epping, 1995), yellowtail (Ohkubo et al., 1996), sea bream (Almuly et al., 2000), fugu (Venkatesh and Brenner, 1997), and flounder (Tanaka et al., 1995). These fish have 6 exons where an additional intron inserted in the 5th exon of the 5-exon type. The insertion of the 5th intron took place only within the ray-finned fish, after the evolutionary separation of cypriniformes, but before salmoniformes, periviformes, and tetradoniformes. All PRL and SL genes also have 5 exons (Chen et al., 1991; Poncelet et al., 1996; Swennen et al., 1992; Takayama et al., 1991; Xiong et al., 1992). Therefore, the 5-exon-type gene organization might reflect the structure of the ancestral gene for this family. 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 (Fig. 3).

All GHs contain two conserved disulfide bonds. The teleost PRLs share these characteristics with the GHs so that some of the overall molecular features of teleost PRLs resemble those of all vertebrate GHs. In contrast, tetrapod, lungfish, and sturgeon PRLs and all SLs so far identified have an additional disulfide bond in the N-terminal portion. Thus, questions can be raised whether the disulfide loop near the N-terminal region was deleted from a tetrapod PRL-like gene into the evolution of the teleost or inserted into a teleostean PRL-like or GH-like ancestral gene. There is no clear answer on this issue to date.

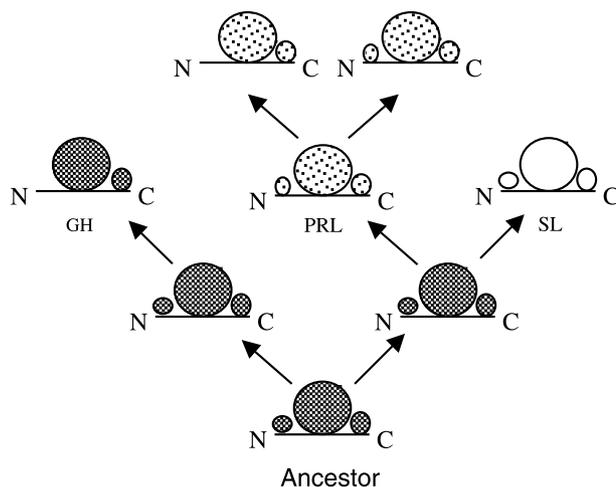


Fig. 3. Molecular evolution of growth hormone family. Molecules are shown schematically by emphasizing disulfide loops. N, the N-terminus; C, the C-terminus. The GH family probably originated from a molecule with a primordial function of growth promotion. The ancestral GH underwent subsequent duplications to form SL and PRL in an early stage of gnathostome evolution. The disulfide loop near the N-terminal region might be deleted into the evolution of the teleosts.

### 3.3. Glycoprotein hormone family

#### 3.3.1. Gnathostomes

Two GTHs, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) are heterodimeric glycoproteins consisting of two non-covalently bound, chemically distinct subunits, designated  $\alpha$ - and  $\beta$ -subunit. The  $\alpha$ -subunit is common among the hormones within a single species, whereas the  $\beta$ -subunit is specific for each hormone and thus confers hormone specificity.

In both sexes of mammals, LH stimulates secretion of sex steroids from the gonads. In Leydig cells of the testis, LH stimulates synthesis and secretion of testosterone. In theca cells of the ovary, LH stimulates secretion of testosterone, which is converted into estrogen by adjacent granulosa cells. In females, a large burst of LH secretion induces ovulation of mature follicles on the ovary. Residual cells within ovulated follicles proliferate to form corpora lutea, which secrete the steroid hormones progesterone and estradiol. FSH stimulates

the maturation of ovarian follicles and supports the function of Sertoli cells, which in turn support many aspects of sperm cell maturation. TSH stimulates the thyroid gland to produce and release the thyroid hormones, which in turn stimulate diverse metabolic activities most tissues, leading to an increase in basal metabolic rate.

Comparative studies revealed that two gonadotropins, which are homologous to LH and FSH, exist in most species of tetrapods studied except for snake in reptiles, in which a FSH-like gonadotropin had been suggested (Licht et al., 1979). These observations have been substantiated by the finding that snake FSH- $\beta$  is more conserved than LH- $\beta$  among reptilian species. However, since both LH and FSH are in turtles and crocodylians, it is unlikely that snakes are exceptional. Indeed, we have identified LH in addition to FSH and TSH from the brown tree snake by cDNA cloning (to be submitted to *Gen. Comp. Endocrinol.*). It had also been thought prior to the late 1980s, that teleosts had only a single gonadotropin homologous to mammalian LH, regulating all aspects of teleost reproduction. However, in the course of the characterization of salmon pituitary hormones, two chemically distinct GTHs were isolated and sequenced, originally designated as GTH I and GTH II, which are chemically and functionally homologous to FSH and LH, respectively (Kawauchi et al., 1989; Suzuki et al., 1988; Swanson et al., 1991). These GTHs are produced in two different cell-types; FSH $\beta$  cells are in the periphery of the glandular cord of the PPD and LH $\beta$  cells are in the central parts of the glandular cords of the PPD in salmon pituitary (Nozaki et al., 1990).

The plasma levels of FSH increased during the period of vitellogenesis and spermatogenesis, and significantly correlated with estradiol-17 $\beta$  in female and 11 keto-testosterone in male coho salmon (Swanson et al., 1991). FSH stimulated the process of vitellogenin uptake, whereas LH had no effect in rainbow trout (Tyler et al., 1991). In contrast, during the time of final maturation and spawning, LH levels increased, and were significantly correlated with 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one in both sexes of coho salmon (Swanson et al., 1991). All these results suggested that synthesis and secretion of FSH and LH are regulated differentially in a cell-specific manner during reproductive development. FSH regulates gametogenesis and growth, whereas LH regulates final maturation, ovulation and spermiation. The duality of GTHs in salmon was further substantiated by identification of two distinct receptors for FSH and LH (Oba et al., 1999a,b).

Since then, two GTHs were isolated from bonito (Koide et al., 1993), tuna (Okada et al., 1994), and yellowtail (Garcia-Hernandez et al., 1997), and their cDNAs have been cloned in several teleost species, early-evolved ray-finned fish (Quérat et al., 2000), lobe finned fish (Quérat et al., 2004), and elasmobranchs (Quérat et al., 2001). Thus, the duality of GTHs was established across all classes of gnathostomes.

On the other hand, TSH has been difficult to isolate due to its relative low content to GTHs in the pituitary, so that

fish TSH has only been characterized by cDNA cloning from rainbow trout (Itoh et al., 1993), eel (Salmon et al., 1993), sturgeon (Quérat et al., 2000), and lungfish (Quérat et al., 2004). Although TSH in the chondrichthyes has not been isolated, thyrotropin activity was found in extract of the ventral lobe (Dent and Dodd, 1961). Thus, the presence of two functionally different GTHs and one functional TSH in the pituitaries of gnathostomes had been established.

### 3.3.2. *Agnathans*

A number of glycoproteins and proteins have been purified from pituitary extracts of adult female sea lamprey by conventional and Con-A affinity chromatography in the past 20 years. The most predominant glycoprotein was nasohypophysial factor (Sower et al., 1995), which corresponded to the N-terminal peptide of POC, consisting of 121 amino acids with one glycosylation site (Sower et al., 1995). However, none of them were related to LH, FSH or TSH. This was an unexpected result, because the content of GTH should be high in the pituitaries of up-migrating adult female lampreys close to spawning. Thus, we set out to determine lamprey GTH by molecular cloning. However, numerous attempts to clone  $\alpha$ - and  $\beta$ -subunits with a number of primers corresponding to conserved regions for these subunits were unsuccessful. These negative results suggested that the structure of lamprey GTH is significantly different from those of gnathostomes GTHs.

Finally, the success of determining lamprey GTH was done by expressed sequence tag analysis of the pituitary cDNA library (Sower et al., 2005). Extracted mRNAs from the pituitary of adult female sea lampreys were reverse transcribed. The resulting cDNAs were cloned into cDNA library vectors. From this cDNA library, 2208 clones were subjected to sequence analysis from 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. The remaining 3e clones showed sequence similarity to glycoprotein hormone- $\beta$ .

The complete sequence analysis of the LH-like cDNA was determined with specific primers by 3'RACE. The entire coding region comprised an open-reading frame of 150 amino acids (a signal peptide of 16 amino acids and mature protein of 134 amino acids). 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 FSH $\beta$ , one to LH $\beta$  and the other to TSH $\beta$ . The third one localized near at the C-terminus. The mature protein showed similar sequence identity to LH $\beta$  and FSH $\beta$  of shark, sturgeon, and lungfish (47%) compared to TSH $\beta$  of sturgeon and lungfish (41%). The antiserum against the synthetic peptide corresponding to the deduced amino acid sequence specifically stained most cells in the ventral half of the PPD (Fig. 1), which was also stained with anti-ovine LH (Nozaki et al., 1999). It was evident that this protein was a potential candidate of lamprey GTH $\beta$ .

To obtain the definite proof, we examined whether GnRH could stimulate expression of the putative GTH $\beta$  gene in the pituitary of sea lamprey. Lamprey GnRH III was injected intraperitoneally to adult female sea lampreys, which were caught during the up-migration in Cochecho River, Dover, New Hampshire, USA, from the Atlantic Ocean. After two injections at 24 h interval, the pituitaries were dissected, removed, and frozen on dry ice. The expression levels of the putative GTH $\beta$  gene as well as GH, POC, and POM were measured by the real-time PCR. The results demonstrated that lamprey GnRH stimulated expression of the putative GTH $\beta$  and also GH, but not those of POC and POM *in vivo*. The results were in good agreement with the GnRH-binding study showing GnRH-binding sites in the proximal pars distalis of the pituitary (Knox et al., 1994).

### 3.3.3. Evolutionary implications

On the basis of amino acid sequences of mammalian pituitary glycoprotein hormones, Dayhoff (1976) originally proposed that the  $\alpha$ - and  $\beta$ -subunits of the glycoprotein hormones evolved from a common ancestor by gene duplication and all three  $\beta$ -subunits were derived from its ancestry by gene duplication and subsequent mutations. Two kinds of scenarios for molecular evolution of glycoprotein hormones, i.e., the order for duplication of  $\beta$ -subunits, have been proposed on the basis of sequence comparison of glycoprotein hormone  $\beta$ -subunits in gnathostomes. Li and Ford (1998) proposed that the first duplication produced an  $\alpha$ -subunit and a  $\beta$ -subunit and was followed by a second duplication of the ancestral  $\beta$ -subunit to yield the LH $\beta$ -subunit gene and the ancestral gene of the FSH $\beta$  and TSH $\beta$  subunit genes. The third duplication produced FSH and TSH so that FSH $\beta$  is more related to TSH $\beta$  than to LH $\beta$ . In contrast, Quérat et al. (2000, 2001, 2004) proposed that the ancestral GTH lineage and the TSH lineage derived from a primary duplication of an ancestral  $\beta$ -gene and LH and FSH lineage arose from a duplication of an ancestral GTH lineage.

The identification of sea lamprey GTH $\beta$  allows for the first time to perform phylogenetic analysis of the glycoprotein family including representative species from all major classes of vertebrates. A molecular phylogenetic tree of 120  $\beta$ -subunits of glycoprotein hormones including sea lamprey GTH $\beta$  was done by the neighbor-joining method using a computer program in Genetyx-Mac. In this unrooted tree, sea lamprey GTH $\beta$  is far removed from the  $\beta$ -subunits of LH, FSH, and TSH, and takes a position as an out group, suggesting that the  $\beta$ -subunit of GTH occurred prior to the separation of agnathans and gnathostomes, although lamprey GTH $\beta$  is a little closer to LH $\beta$ s. There is no clear evidence to support the presence of TSH. However, Freamat and Sower (2005) have cloned two kinds of glycoprotein hormone receptors in the sea lamprey: one is a GTH-like receptor located in the gonad and the other is a TSH-like receptor in the thyroid tissue. Therefore, it could be considered that TSH exists in agnathans. Or another scenario 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 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 (Fig. 4).

## 4. Conclusion

In summary, the initial aim of our collaboration was the identification of GTH in sea lamprey to elucidate the hypothalamo–pituitary–gonadal system in a representative species of the most ancient lineage of vertebrates. Unfortunately, the sea lamprey GTH eluded our extensive efforts for many years and was the last to come. Ironically, it was to our fortune that we had not initially identified the GTH because it let to our collaborative efforts in identifying most of the adeno-hypophysial hormones such as ACTH, MSHs, and GH and their genes during the long and tantalizing process and period of 20 years. Pituitary hormones that we have yet to identify and call them “escapees” include the  $\alpha$ -subunit of gonadotropin and perhaps TSH $\beta$ . The POMC gene has evolved by gene duplication, which increased the number of copies, and by internal and/or whole gene duplication and deletion of the MSH

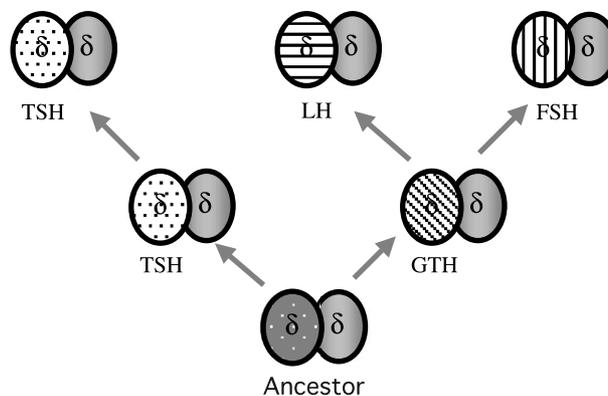


Fig. 4. Molecular evolution of glycoprotein hormone family. LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone.  $\alpha$  and  $\beta$  are subunits. The initial duplication produced GTH lineage and TSH lineage and the second duplication occurred in GTH gene during the early evolution of gnathostomes to give rise to LH and FSH.

domains that prompted the diversity of POMC as to the number of MSHs. GH appears to be the ancestral hormone in the GH family of sea lamprey and the GH-IGF endocrine mechanism for growth stimulation was established at an early stage of vertebrate evolution. The hypothalamo–pituitary–gonadal axis for reproductive endocrine system had been established early in evolution in the agnathans and has shown a conservation of this system among the hormones, receptors and their functions, with the major exception of having only one GTH molecule. The duality of GTHs appears to have arisen with the gnathostomes. We want to believe and propose that these are all of the adeno-hypophysial hormones in the sea lamprey. This proposal will be verified in the next few years, since on 4 August, 2004, the US National Human Genome Research Institute unveiled that 18 species including sea lamprey had been selected for genome sequencing which in time will reveal the presence of all pituitary hormones in this ancient lineage of vertebrates.

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