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Glycoprotein hormone in the pituitary of hagfish and its evolutionary implications

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Abstract The pituitary gland is present in all vertebrates, from agnathans (jawless vertebrates) to mammals, but not in invertebrates. Reproduction in gnathostomes (jawed vertebrates) is controlled by two pituitary gonadotropins (GTHs), luteinizing hormone and follicle-stimulating hormone, which are part of the pituitary glycoprotein hormone (GPH) family. Hagfishes, which lack both jaws and vertebrae, are considered the most primitive vertebrate known, living or extinct. Accordingly, they are of particular importance in understanding the evolution of the pituitary GPHs and their functions related to vertebrate reproduction. Nevertheless, key elements of the reproductive endocrine system in hagfish have yet to be elucidated. Our current report has revealed the first identification of a functional GPH composed of two subunits that possess gonadotropic action at the pituitary of brown hagfish. It seems most likely that an ancestral GPH gave rise to only one GTH in hagfish pituitary and that multiplicity of GPHs arose later during the early evolution of gnathostomes. This paper briefly summarizes the latest findings on the hagfish GPH from an evolutionary point of view.

Keywords Hagfish · Pituitary gland · Glycoprotein hormone · Gonadotropin · Reproduction · Evolution

Abbreviations

FSH	Follicle-stimulating hormone
GPA	Glycoprotein alpha subunit
GPB	Glycoprotein beta subunit
GPH	Glycoprotein hormone
GTH	Gonadotropin
HPG axis	Hypothalamic–pituitary–gonadal axis
LH	Luteinizing hormone
TSH	Thyroid-stimulating hormone

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Introduction

Reproduction in jawed vertebrates (gnathostomes) is controlled by a hierarchically organized endocrine system called the hypothalamic–pituitary–gonadal (HPG) axis (Sower et al. 2009). The HPG axis, which

is specific to vertebrates, is considered to be an evolutionary innovation and seminal event that emerged prior to or during the differentiation of the ancestral jawless vertebrates (agnathans). In gnathostomes, two functional gonadotropins (GTHs), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), together with thyroid-stimulating hormone (TSH) form the pituitary glycoprotein hormone (GPH) family consisting of two non-covalently bound subunits, α and β (Kawauchi et al. 1989). The α subunit is homologous within a single species, while the β subunits are different and convey hormone specificity (Kawauchi and Sower 2006). Two gonadotropins are secreted from the pituitary and stimulate the gonads inducing the synthesis and release of sex steroid hormones, which in turn elicit growth and maturation of the gonads (Nagahama 1994). These GPHs are believed to have evolved from a common ancestral GPH through duplication of β subunit genes and subsequent divergence (Kawauchi and Sower 2006). Although two GTHs and one TSH have been identified in all taxonomic groups of gnathostomes (Kawauchi and Sower 2006), there had been no clear evidence for pituitary GPHs in agnathans, until current reports in hagfish (Uchida et al. 2010) and sea lamprey (Sower et al. 2006). By analyses of pituitary gland in most primitive vertebrate, hagfish, we have provided evidence for a unique functional GPH, with the isolation of one GPHα and single GPHβ subunits (Uchida et al. 2010). This review provides an overview of the latest information on hagfish GPH and its molecular and functional evolution in the earliest divergent vertebrate.

Hagfish pituitary gland

The extant representative of the agnathans, hagfish, which lack both jaws and vertebrae, is considered the most primitive vertebrate (Janvier 1996; Hall 1998). They mostly inhabit a deep marine environment that is relatively free of circadian, or even seasonal changes (Martini 1998). Since hagfish represent the most basal vertebrate that diverged over 550 million years ago (Hall 1998), they serve as key animals in understanding the evolution of the HPG axis related to vertebrate reproduction. The adenohypophysis of the hagfish is considered primitive compared to jawed vertebrates. As shown in Fig. 1, the adenohypophysis of hagfish consists of only a series of clusters of cells embedded in connective tissue below the neurohypophysis (Holmes and Ball 1974; Gorbman 1983; Nozaki 2008). The adenohypophysis and neurohypophysis are completely separated by a layer of the connective tissue, reflecting no anatomical relationship between them (Gorbman 1983; Nozaki 2008). In addition, there is no clear cytological differentiation between the pars distalis and the pars intermedia (Nozaki 2008) (Fig. 1a, b). These histological observations clearly reflect a primitive state of hagfish pituitary gland. Until our study (Uchida et al. 2010), none of the adenohypophysial hormones had been identified and sequenced, even though there was evidence for some of the typical pituitary hormones as suggested via immunohistochemical studies (Nozaki et al. 2007).

During the past five years, we showed immunoreactive (ir)-GTH in the adenohypophysis of brown hagfish, *Paramyxine atami* using antisera to mammalian LHβ (Miki et al. 2006). We also demonstrated that ir-GTH cells predominated in adults with developing gonads, providing evidence for the presence of GTH-like molecule in the adenohypophysis of hagfish (Miki et al. 2006). Moreover, injection with estradiol benzoate in juvenile brown hagfish resulted in a significant increase in the amount of GTH-like material in the adenohypophysis (Miki et al. 2006). These reports suggested that GTH-like material acts as a functional gonadotropin, and it is likely to be a crucial endocrine molecule related to hagfish HPG axis similar to that in more advanced gnathostomes. In support of these reports, the identity of a functional GPH related to reproduction has been elucidated from the pituitary of brown hagfish (Uchida et al. 2010). The molecular and functional aspects of hagfish GPH and its evolutionary implications are summarized in the following paragraphs.

Molecular and cellular aspects of hagfish GPH

Based on the expressed sequence tag analysis of the pituitary cDNA library, two GPH subunits, GPHα and GPHβ subunits, have been identified from brown hagfish (Uchida et al. 2010). The GPHα consists of a prohormone of 118 amino acids. The mature protein contains 8 out of 10 cysteine residues and two possible N-glycosylation sites at the homologous positions to GPHα subunits of jawed vertebrates (see Fig. 1 in

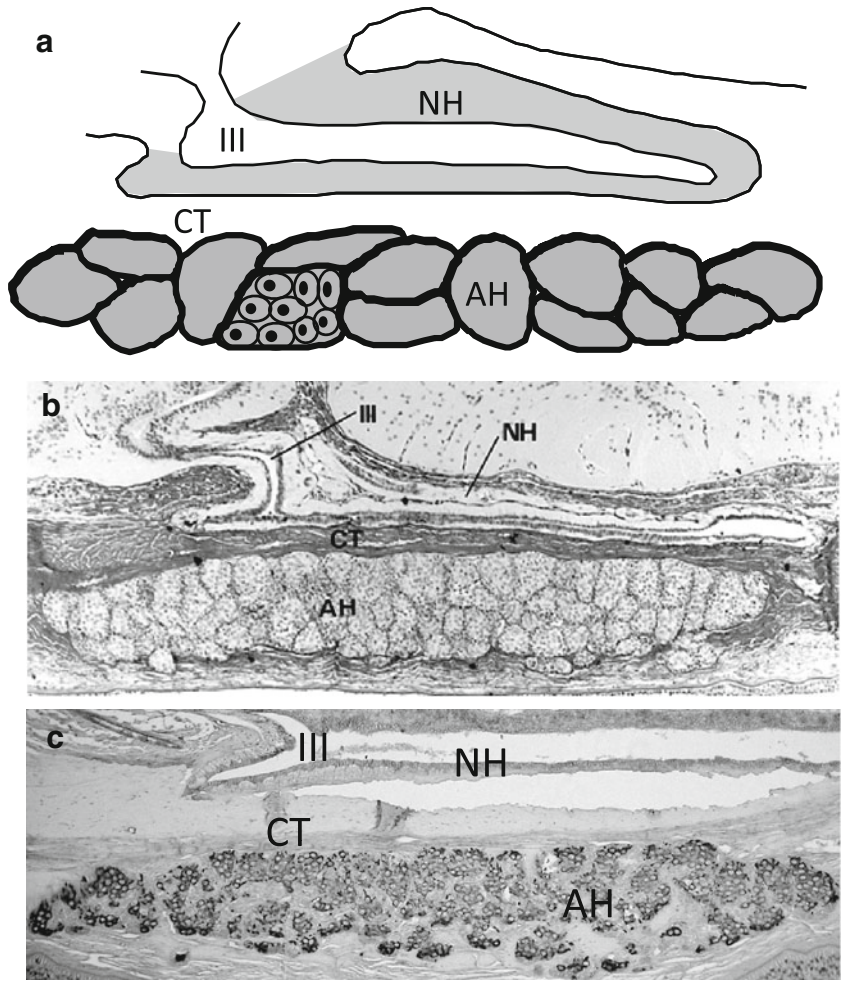

Fig. 1 The pituitary gland of the hagfish (*Paramyxine atami*). **a** Schematic diagram of the hagfish pituitary gland. **b**, **c** Pituitary sections stained with hematoxylin–eosin (**b**) and specific antiserum raised against hagfish GPHβ (**c**). Note that the adenohypophysis consists of only a series of clusters of cells and is embedded in connective tissue. *AH* adenohypophysis, *NH* neurohypophysis, *CT* connective tissue, *III* third ventricle

Uchida et al. 2010). The hagfish GPHα showed 41–43 % sequence identity with α subunits of GPHs of shark, sturgeon and lungfish. In comparison, the hagfish GPHβ has two possible N-glycosylation sites consisting of a prohormone of 126 amino acids and 12 cysteine residues that are exactly conserved at homologous positions to three kinds of gnathostome GPHβ subunits, LHβ/FSHβ/TSHβ (see also Fig. 1 in Uchida et al. 2010). The hagfish GPHβ shows similar sequence identities (32–43 %) to three kinds of GPHβ subunits of gnathostome species such as shark, sturgeon and lungfish, and to lamprey GTHβ (39 %). It has been reported that well-conserved cysteine residues found in GPH molecules seem to be important for its predicted cysteine-knot structure comprising of disulfide bridges and for specific binding to its putative receptors (Fan and Hendrickson 2005). From these molecular characteristics, hagfish GPH clearly belongs to pituitary GPH family in gnathostomes.

As demonstrated in Fig. 2a, hagfish GPHα has been far removed from GPHα of gnathostome species. In the phylogenetic analysis of vertebrate GPHβ subunits, the subunits of gnathostomes indicate distinct three clades, LHβ/FSHβ/TSHβ (Fig. 2b). Hagfish GPHβ is far removed from those three GPHβ clades, while it forms a clade with the TSHβ. These molecular and phylogenic results suggest that the two subunits from hagfish pituitary are related to gnathostome GPHα and β subunits and are evolutionary ancestors of vertebrate GPHs expressed specifically in the pituitary gland.

Our recent studies have demonstrated that an intense immunoreaction to anti-hagfish GPHα (data not shown) and GPHβ (Fig. 1c) was observed in the adenohypophysis, where approximately 30–60% of

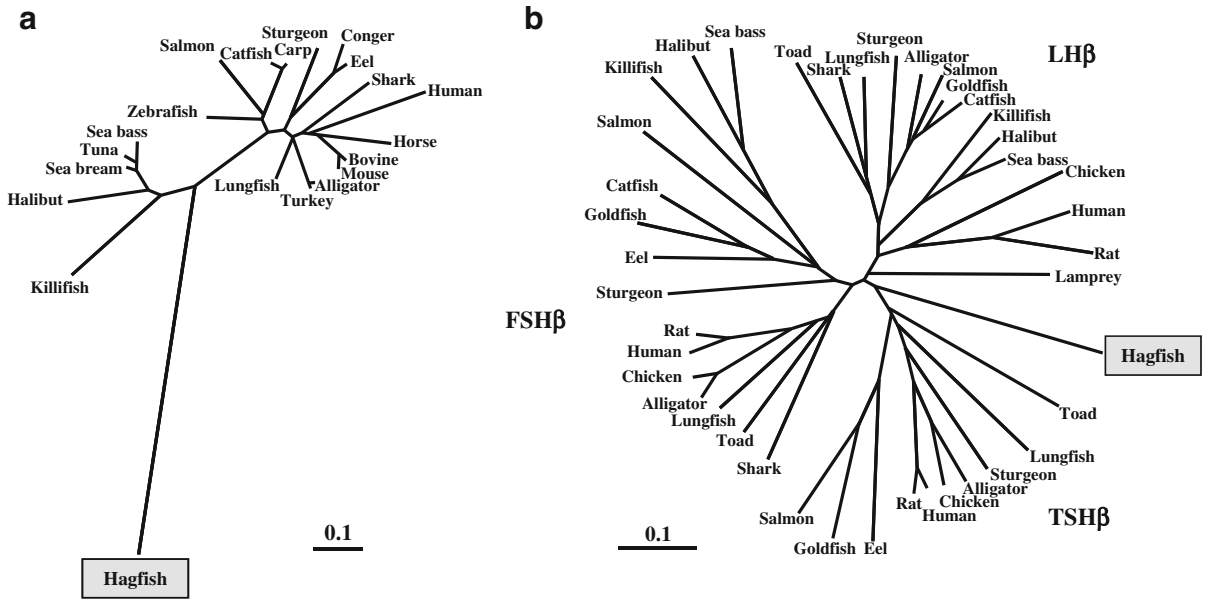

Fig. 2 Unrooted phylogenetic trees of vertebrate GPHα (**a**) and GPHβ (**b**), constructed with neighbor-joining method. Scale bars refer to a phylogenetic distance of 0.1 amino acid substitutions per site. The positions of the hagfish GPHα and GPHβ are highlighted with *gray boxes*. Note that hagfish GPHα forms an outgroup and shows evolutionary origin of vertebrate GPHαs. The GPHβs classify into three groups, FSHβ, LHβ and TSHβ, and hagfish GPHβ takes a position as an outgroup and forms a clade with the TSHβs. The DDBJ/EMBL/GenBank accession numbers of sequences used for analysis are listed in the supporting information (Table S1) of our previous report (Uchida et al. 2010)

cells in most adenohypophysial cell clusters. Cellular characteristics of both two subunits demonstrated that they were synthesized and colocalized in the same cells of the adenohypophysis as shown in gnathostomes (Uchida et al. 2010). Based on these cellular properties, the identified two subunits appear to be the typical GPHα and GPHβ subunits as found in gnathostome's pituitary gland.

Functional aspects of hagfish GPH

In jawed vertebrates, GTHs, in response to hypothalamic gonadotropin-releasing hormone (GnRH), are released from the pituitary and act on the gonads to regulate steroidgenesis and gametogenesis (Nagahama 1994). Lampreys have a functional HPG axis, which is supported by a vast number of biochemical, molecular, histochemical and functional studies (Sower et al. 2009). In contrast, our knowledge of the HPG axis of the hagfish has been almost completely lacking. The lack of information is mostly due to the fact that hagfish pituitary function is difficult to assess from cytological and physiological studies. For example, as demonstrated in Fig. 1, the pituitary gland represents the simplicity and primitiveness of its morphology (Nozaki 2008). Complete hypophysectomy in *Eptatretus stouti* has not provided any clear evidence for pituitary gonadotropic activity (Matty et al. 1976). On the other hand, in *Eptatretus burgeri*, there have been some indications that gonadal development and spermatogenesis may be retarded after hypophysectomy (Patzner and Ichikawa 1977). Thus, the pituitary–gonadal axis of hagfish was considered to be minimally, if at all, functional for the past several decades (Gorbman 1983).

However, recent reports have revealed that the activities of ir-GPH cells of brown hagfish were strongly correlated with the degrees of the developmental conditions of gonads (Uchida et al. 2010). For instance, a heavy accumulation of ir-GPHβ was found in many adenohypophysial cells in adults with well-developing gonads, while no or a faint accumulation in the cells in juveniles (Fig. 3a, b). In support of the cytological observations in brown hagfish, gene expression of two GPHs was also significantly higher

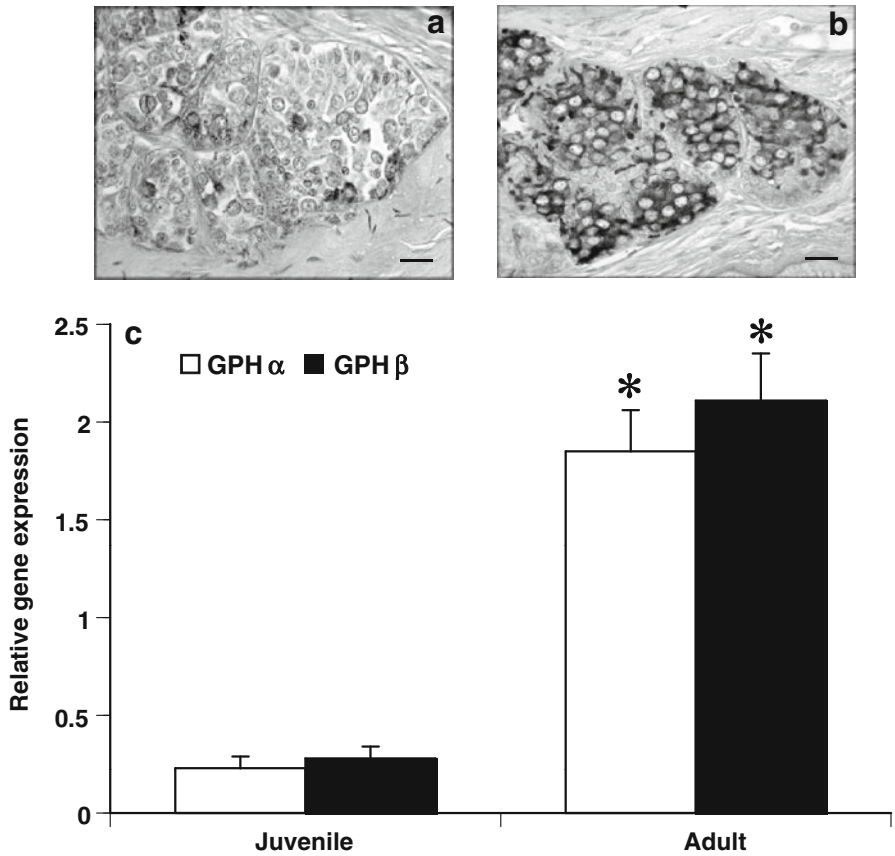

Fig. 3 Correlation between pituitary GPH activities and gonadal development in brown hagfish. Juvenile and mature brown hagfish were used for sampling. Cellular activities of GPHβ cells in the pituitary (**a**, **b**). Note that intense immunoreactions are observed in mature female (**b**), while faint reactions presented in juvenile (**a**). (*Scale bars*: 20 μm). Relative GPHα and GPHβ gene expressions in the pituitary of female hagfish (**c**). *Open bars* (*white*) represent GPHα gene expressions and *filled bars* (*black*) represent GPHβ gene expressions. The two mRNA levels were normalized by β-actin mRNA levels. Relative values are expressed as mean ± SE. ($n = 6$). Significant differences from the juvenile are indicated by $^*P < 0.001$. Note that the two GPH transcripts increase in well accordance with the developmental conditions of the gonad

both in maturing female (Fig. 3c) and male (data not shown) than in juveniles. Currently, we have identified two GPH subunits from the pituitary of Pacific hagfish, *E. burgeri*, and the gene expressions have been well-related to their developmental conditions of gonads (unpublished data). Thus, in general, hagfish do have two functional GPH subunits in their pituitary, and these molecules are strongly related to their reproduction. Finally, native GPH purified from the pituitary gland of brown hagfish led to dose-dependent increases in the releases of sex steroids from the gonad in vitro (Fig. 4). These data provide strong evidence that hagfish has a functional GTH-like hormone retaining a functional pituitary–gonadal axis that is highly conserved across all vertebrates.

Although three GnRHs (GnRH I, II and III) have been identified in the brain of sea lamprey and shown to have a hypothalamic role in reproduction (Kavanaugh et al. 2008; Sower et al. 2011), the gene or protein structure of hypothalamic GnRH in hagfish has yet to be characterized. However, the occurrence and distribution of GnRH-like molecule have been reported (Braun et al. 1995; Sower et al. 1995). Seasonal changes in brain GnRH measured by lamprey GnRH III antiserum, gonadal steroids, such as estradiol and progesterone, were demonstrated corresponding to gonadal reproductive stages in the Atlantic hagfish, *Myxine glutinosa* (Powell et al. 2004; Kavanaugh et al. 2005). These reports suggest an association of brain GnRH with gonadal maturity

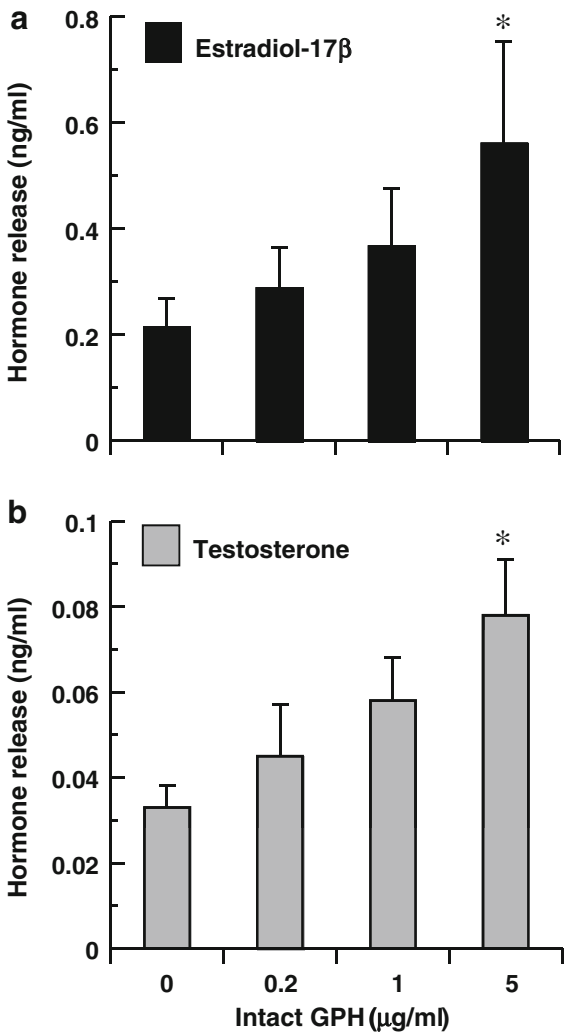

Fig. 4 In vitro effects of native GPH from hagfish pituitary on the releases of estradiol-17β (**a**, *black columns*) and testosterone (**b**, *gray columns*) from organ cultured testis. Values are expressed as mean ± SE. ($n = 6$–8). Significant differences from the control groups (0 μg/ml in **a**, **b**) are indicated by $^*P < 0.05$. Redrawn and modified from Fig. 5 in Uchida et al. (2010)

and sex steroid productions. Furthermore, four novel RFamide peptides, which had the C-terminal Pro–Gln–Arg–Phe–NH_2 structure, were recently characterized from the brains of brown hagfish (Osugi et al. 2011). The RFamide peptides belong to the PQRFamide peptide group that includes mammalian neuropeptide FF (NPFF) and NPAF, and localized in the infundibular nucleus of the hypothalamus. Interestingly, one of these peptides stimulates the expression of hagfish GPHβ mRNA in the cultured pituitaries, clearly indicating the presence of regulatory mechanisms of GPH by hypothalamic neuropeptides. It is highly probable that hypothalamic–pituitary axis is present in hagfish. The HPG axis appears to have been established during the early phase of vertebrate evolution and conserved across all vertebrates.

Interestingly, hagfish GPHβ forms a clade with the gnathostome TSHβ (Fig. 2a). However, to date, there has been no evidence to support the presence of TSH in agnathans (Kawauchi and Sower 2006). In lamprey, two functional GPH receptors, GTH-like and TSH-like receptors, have been identified, and it is proposed that single GTH-like molecule stimulates their gonad and thyroid gland through a GTH-like receptor and a TSH-like receptor, respectively (Freamat and Sower 2010). In hagfish, only one type of GPH-producing cells, containing both GPHα and β subunits, has been identified in their adenohypophysis (Nozaki 2008; Uchida et al. 2010), indicating the entirely colocalization of two subunits in the same cells. Thus, cellular differences in the occurrence and/or intensity of immunoreactivities between the two subunits would be evident, if the second form of GPH, possible TSH, is present. Moreover, the two genes encoding hagfish GPH subunits show equivalent expression during reproductive development in both sexes (Fig. 3c, Uchida et al. 2010). It is highly probable that the single GPH is a direct descendent of a common ancestor of the gnathostome GPH family and that hagfish GPH may act as both GTH- and TSH-like hormones as proposed in the lamprey (Sower et al. 2009). Further studies are needed to clarify the presence of GPH receptor(s) and the thyrotropic actions of the single GPH though its functional receptor(s) in hagfish.

Molecular and functional evolution of vertebrate GPHs

Recently, a fourth heterodimeric GPH has been discovered in the human genome and termed "thyrostimulin" due to its thyroid-stimulating activity (Nakabayashi et al. 2002). The thyrostimulin α subunit, called glycoprotein α subunit 2 (GPA2), is homologous but not identical to the common or pituitary-specific α subunit (GPHα or GPA1). With the discovery of GPA2 and glycoprotein β subunit 5 (GPB5, thyrostimulin β) homologs in other vertebrates and some invertebrates

including fly, nematode and sea urchin, it was proposed that these two subunits represent the ancient forms of GPH α and β subunits of vertebrate pituitary hormones, respectively (Park et al. 2005; Sudo et al. 2005). The basal lineage of chordates, tunicates and amphioxus, do not have a pituitary, and they possess GPA2 and GPB5 in their genomes but not GPHα and GPHβ, LHβ/FSHβ/TSHβ (Dos Santos et al. 2009; Tando and Kubokawa 2009a, b). Our molecular phylogenetic analysis has also revealed that the hagfish GPHα forms a clade with the gnathostome GPHαs, while GPHβ forms a clade with the pituitary-specific GPHβs (see Fig. 2 in Uchida et al. 2010), indicating that hagfish GPHα and GPHβ do not form a clade with GPA2 and GPB5. Since the hagfish GPHα and GPHβ subunits appear to be the typical GPH subunits found in gnathostome pituitaries, we propose the following evolutionary scenario of the GPH family (Fig. 5). Ancestral GPA- and GPB-like molecules likely existed in the ancestor of bilateria, and diverged or underwent gene duplication into two ancestral subunits (GPA2 and GPB5) after the split of protostomes and deuterostomes (Park et al. 2005). These two subunits evolved and were retained in the genome as functional GPA2

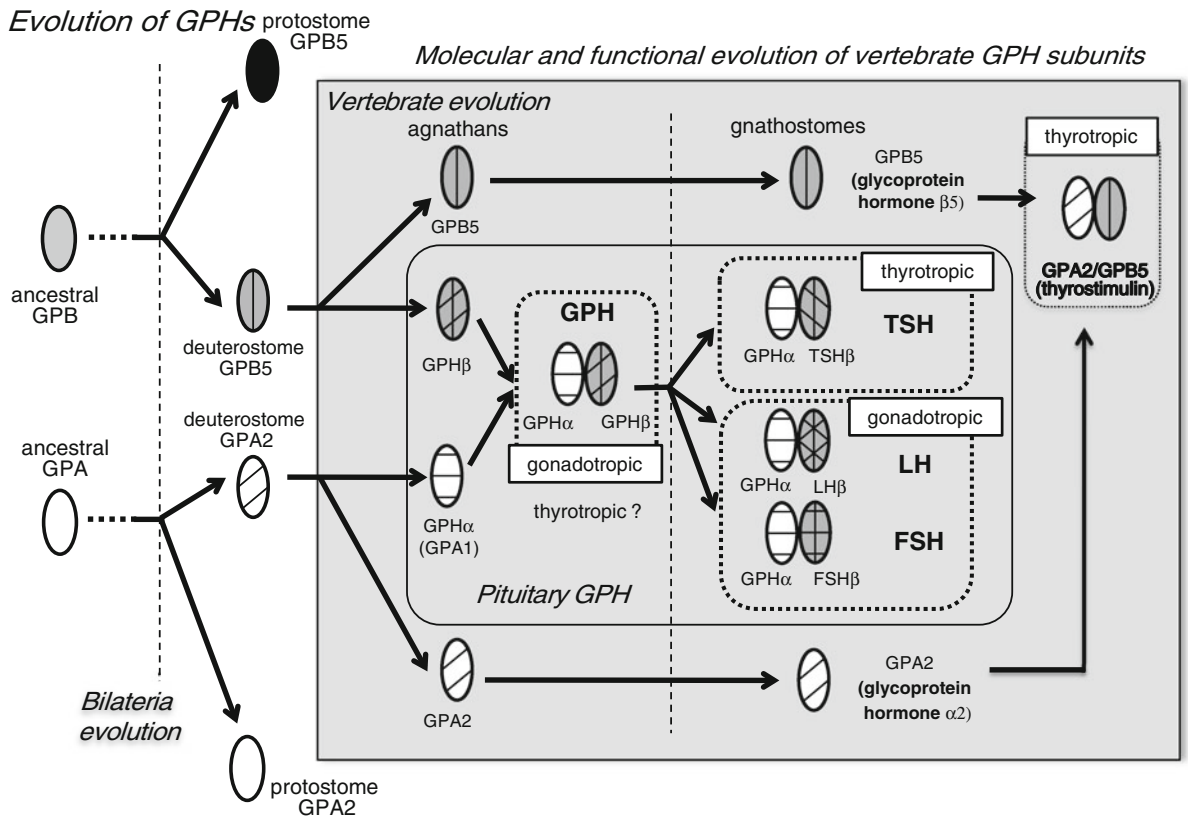

Fig. 5 Schematic diagram of the possible evolution of glycoprotein hormones (GPHs). The molecular and functional evolution of vertebrate GPH subunits is emphasized in the *gray box*. *Thick doted* enclosures are showing functional units of pituitary GPH α and β in agnathan (hagfish) and GPHα and LHβ/FSHβ/TSHβ in gnathostomes. *Thin doted* enclosures are showing GPA2/GPB5 (thyrostimulin) in jawed vertebrates. Ancestral GPA- and GPB-like molecules likely existed in the common ancestor of protostomes and deuterostomes, and diverged into two functional glycoprotein hormones (GPA2 and GPB5) in both lineages. Unlike the presence of single GPA2 and GPB5 in invertebrate species, an ancestral GPA2 and GPB5 in the lineage of deuterostomes diverged into GPA2 and GPHα, GPB5 and GPHβ, respectively, during the early phase of agnathan divergence. The GPA2 and GPB5 may have formed a heterodimer acting as a thyrotropic factor, thyrostimulin, after the split of gnathostome species. The GPHα and GPHβ formed a heterodimeric hormone in the pituitary and acted as the first adenohypophysial gonadotropic factor during the evolution of agnathan species, while the thyrotropic actions of this heterodimeric GPH remain to be determined. In the lineage to gnathostomes, this GPH heterodimer further diverged into three functional units of adenohypophysis, GPHα and LHβ/FSHβ as two gonadotropins, GPHα/TSHβ as a thyrotropin

and GPB5 in most groups of both lineages (Dos Santos et al. 2009). Actually, an *in slico* survey indicated that both *GPA2* and *GPB5* are present in metazoan genomes, and they are located close to each other in the genomic environment of non-vertebrate species (Dos Santos et al. 2011). As reported by Kuraku et al. (2009), two rounds of whole-genome duplications have been proposed to occur before the agnathan–gnathostome split in part based on the establishment of gene repertories. Current reports by performing the genomic clustering and synteny analysis suggest that vertebrates *GPHα* and *GPHβ* were generated concomitantly by a specific local duplication of the ancestral forms of *GPA2* and *GPB5* prior to the first round of genomic duplication, followed by a relocation of *GPHβ* into a new chromosomal environment, while *GPHα* was retained in the *GPA2/GPB5* locus (Dos Santos et al. 2011). Therefore, we propose that ancestral GPA2/GPB5 units of primitive chordates, such as tunicates and amphioxus, may have provided two functional units, GPA2/GPB5 (thyrostimulin) and GPHα/GPHβ during the early phase of agnathan divergence. The latter, which is specific to the vertebrate pituitary, may have further diverged into three functional units, GPHα and LHβ/FSHβ/TSHβ after the agnathan–gnathostome split, while the former unit may also have retained and independently evolved in the vertebrate lineages. It is therefore possible that hagfish have retained a GPA2/GPB5, but this has not yet been determined.

Concluding remarks

We have summarized the functional GPH in the pituitary of hagfish, the earliest divergent extant lineage and most primitive vertebrate. It seems most likely that an ancestral GPH gave rise to at least one GTH in hagfish and that the multiplicity of GPHs arose later during early evolution of gnathostomes, probably after the two rounds of whole-genome duplication that occurred early in the evolution of vertebrates. We hypothesize that the identity of a single functional GPH of the hagfish, hagfish GTH, provides critical evidence for the existence of a pituitary–gonadal system in the earliest divergent vertebrate that likely evolved from an ancestral, pre-vertebrate exclusively neuroendocrine mechanism by gradual emergence of a new control level, the pituitary, that is not found in the protochordates (Kubokawa et al. 2010).

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