Molecular evolution of the growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide gene family. Functional implication in the regulation of growth hormone secretion

M Montero, L Yon, S Kikuyama, S Dufour and H Vaudry
European Institute for Peptide Research (IFRMP 23), Laboratory of Cellular and Molecular Neuroendocrinology, INSERM U413, UA CNRS, University of Rouen, 76821 Mont-Saint-Aignan, France
1Laboratory of Endocrinology, Department of Biology, School of Education, Waseda University, Nishi-Waseda 1-6-1, Tokyo 169–8050, Japan
2Laboratory of General and Comparative Physiology, CNRS UMR 8572, National Museum of Natural History, 75005 Paris, France

ABSTRACT
Growth hormone-releasing hormone (GHRH) and pituitary adenylate cyclase-activating polypeptide (PACAP) belong to the same superfamily of regulatory neuropeptides and have both been characterized on the basis of their hypophysiotropic activities. This review describes the molecular evolution of the GHRH/PACAP gene family from urochordates to mammals and presents the hypothesis that the respective roles of GHRH and PACAP in the control of GH secretion are totally inverted in phylogenetically distant groups of vertebrates. In mammals, GHRH and PACAP originate from distinct precursors whereas, in all submammalian taxa investigated so far, including birds, amphibians and fish, a single precursor encompasses a GHRH-like peptide and PACAP. In mammals, GHRH-containing neurons are confined to the infundibular and dorsomedial nuclei of the hypothalamus while PACAP-producing neurons are widely distributed in hypothalamic and extrahypothalamic areas. In fish, both GHRH- and PACAP-immunoreactive neurons are restricted to the diencephalon and directly innervate the adenohypophysis. In mammals and birds, GHRH plays a predominant role in the control of GH secretion. In amphibians, both GHRH and PACAP are potent stimulators of GH release. In fish, PACAP strongly activates GH release whereas GHRH has little or no effect on GH secretion. The GHRH/PACAP family of peptides thus provides a unique model in which to investigate the structural and functional facets of evolution.

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INTRODUCTION
Growth hormone-releasing hormone (GHRH) was initially characterized from a human pancreatic tumor on the basis of its ability to stimulate growth hormone (GH) secretion from adenohypophysial cells (Guillemin et al. 1982, Rivier et al. 1982).

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first identified from the ovine hypothalamus as a result of its capability of stimulating cAMP formation in rat anterior pituitary cells (Miyata et al. 1989). Soon after the discovery of PACAP, it became apparent that these two hypophysiotropic neuropeptides exhibit a
number of structural and functional similarities. (i) They stand among the largest hypothalamic neurohormones discovered so far. (ii) They possess considerable sequence similarity so that it is generally accepted that they arose by genomic duplication of the exon encoding a common ancestor. (iii) They both appeared very early during evolution prior to the emergence of vertebrates. (iv) They are widely distributed in peripheral organs, notably in the gut, pancreas, ovary and testis. (v) Their biological effects are mediated through 7-transmembrane domain receptors positively coupled to adenylyl cyclase. Besides these similarities, GHRH and PACAP display one major

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**Mammals**

PACAP gene:

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5'  1  2  3  4  5  3'
SP  PRP  PACAP
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GHRH gene:

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5'  1'  2'  3'  4'  5'  3'
SP  GHRH  C peptide
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**Infra-mammalian Vertebrates**

GHRH-PACAP gene:

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5'  1  2  3  4  5  3'
SP  GHRH  PACAP
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**Urochordate Invertebrates**

pacap1: GHRH-PACAP 1 gene:

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5'  1  2'  3'  4'  3'
SP  GHRH  PACAP
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pacap2: GHRH-PACAP 2 gene:

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5'  1  2  3  4  5  3'
SP  GHRH  PACAP
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**Figure 3.** Organization of the GHRH, PACAP and GHRH/PACAP genes of vertebrates and invertebrates. Exon numbering follows that used for the mammalian genes.
difference: the sequence of GHRH has been poorly conserved, even in closely related species, while the sequence of PACAP has been extremely well preserved during evolution.

Examination of the amino acid sequences of GHRH and PACAP clearly indicates that these two neuropeptides belong to a superfamily of regulatory peptides which encompasses glucagon, secretin, vasoactive intestinal polypeptide (VIP), peptide histidine methionine (PHM) or peptide histidine isoleucine (PHI), gastric inhibitory peptide (GIP), and glucagon-like peptides (GLPs) (Fig. 1) (Campbell & Scanes 1992, Kieffer & Habener 1999). Molecular characterization of the GHRH and PACAP precursors in several representative species of urochordates and vertebrates, together with studies on their physiological implication in the control of pituitary hormone secretion, have provided important information pertaining to the evolutionary aspects of their structures and functions. The aim of the present review is to summarize the current knowledge regarding the phylogenetic origin and the evolution of the respective roles of GHRH and PACAP in the regulation of GH secretion.

**MOLECULAR DIVERSITY OF GHRH AND PACAP**

**Evolution of the primary structures of GHRH and PACAP**

The sequence of GHRH was initially determined from a tumor which had caused acromegaly (Guillemin et al. 1982, Rivier et al. 1982). Subsequently, the primary structure of the peptide has been elucidated in a number of species, either directly determined after peptide purification or deduced from a cloned cDNA. In contrast to most hypophysiotropic neurohormones whose sequences have been generally well conserved during evolution, the primary structure of GHRH is highly variable (Fig. 2). In particular, rat and mouse GHRHs only exhibit 67% sequence identity, which is by far the lowest degree of similarity for regulatory neuropeptides observed between these two rodent species.

Two molecular forms of PACAP with 38 (PACAP38) and 27 (PACAP27) amino acids were originally isolated from the sheep hypothalamus (Miyata et al. 1989, 1990). The sequence of PACAP has been remarkably well preserved from the sea squirt (an urochordate invertebrate) to the human (Fig. 2). In particular, the sequence of PACAP38 is identical in all mammalian species studied so far. In other vertebrates, strong evolutionary pressure has acted to preserve the sequence of the N-terminal domain which corresponds to the biologically active region of the molecule (Gonzalez et al. 1998). It is noteworthy that the sequences of the two tunicate PACAP27 variants exhibit only one and four amino acid substitutions as compared with their mammalian ortholog (Miyata et al. 1990, McRory & Sherwood 1997). Such a high degree of peptide sequence conservation between two taxa which have diverged from a common ancestor some 600 million years ago (McRory & Sherwood 1997) indicates that PACAP fulfills important biological functions.

**Evolution of the structure of the GHRH and PACAP precursors**

In mammals, the GHRH and PACAP precursors are encoded by two distinct genes (Mayo et al. 1985a, Hosoya et al. 1992). Each of these precursors encompasses two related peptides: the GHRH precursor comprises GHRH and a C-terminal flanking peptide called C-peptide, while the PACAP precursor comprises PACAP and an N-terminal flanking peptide called PACAP-related peptide or PRP (Fig. 3). Comparison of the sequences of these peptides reveals a higher degree of similarity between PRP and GHRH than between PRP and PACAP (45% and 21% respectively in human).

In contrast, in all submammalian species investigated so far, a GHRH-like peptide and PACAP are located on the same precursor (Fig. 3). To date, the cDNA encoding the GHRH/PACAP precursor has been characterized in the chicken Gallus domesticus (McRory et al. 1997), the frog Rana ridibunda (Alexandre et al. 2000), the catfish Clarius macrocephalus (McRory et al. 1995) and the salmon Oncorhynchus nerka (Parker et al. 1993). In birds, amphibians and fish, alternative splicing of the primary transcript can generate either a long mRNA encompassing both the GHRH and PACAP sequences, or a short mRNA lacking the GHRH sequence (McRory et al. 1997, Parker et al. 1997, Alexandre et al. 2000). Two cDNAs encoding GHRH/PACAP precursors have also been cloned in an urochordate, the sea squirt Chelyosoma productum (McRory & Sherwood 1997). The organization of the GHRH/PACAP genes in urochordates exhibits strong similarities with those of vertebrates (Fig. 3). Sequence analysis of the two GHRH/PACAP precursors of the sea squirt reveals a higher degree of similarity between the two GHRH sequences (89%) and the two PACAP sequences (89%) than between the GHRH and PACAP sequences within each precursor (41% for the GHRH/PACAP1 precursor and 48% for the
GHRH/PACAP2 precursor), suggesting that exon duplication generating GHRH and PACAP occurred before duplication of the ancestral gene (Fig. 4).

Since only one GHRH/PACAP cDNA has been characterized to date in teleosts (Parker et al. 1993, McRory et al. 1995), amphibians (Alexandre et al. 2000) and birds (McRory et al. 1997) while two distinct cDNAs encoding either GHRH or PACAP have been cloned in rat (Mayo et al. 1985b, Ogi et al. 1990), mouse (Frohman et al. 1989, Suhr et al. 1989, Okazaki et al. 1995) and human (Gübler et al. 1983, Mayo et al. 1983, 1985a, Kimura et al. 1990, Hosoya et al. 1992, Ohkubo et al. 1992), it has been proposed that a gene duplication event occurred just before the emergence of mammals (Arimura 1998, Hoyle 1998, Vaudry et al. 2000). However, several observations suggest that a second gene encoding a GHRH-like peptide may well exist in non-mammalian species (Fig. 4). In particular, two GHRH/PACAP genes are present in urochordates, suggesting that duplication of the ancestral gene may have occurred very early during evolution (McRory & Sherwood 1997). In fact, the organization of the GHRH/PACAP1 gene of the sea squirt exhibits much similarity with that of the mammalian GHRH precursor gene, while the structure of the GHRH/PACAP2 gene of the sea squirt resembles that of the mammalian PACAP precursor gene (Fig. 4), suggesting that the two urochordate genes may be orthologous to the two mammalian genes. In that case, the existence of two GHRH/PACAP precursors should also occur in submammalian vertebrate species. Indeed, Southern blot analysis of salmon genomic DNA has revealed the existence of at least two bands hybridizing with a probe including the GHRH/PACAP coding region (Parker et al. 1993) and two variants of GHRH/PACAP cDNAs have recently been cloned in the goldfish (Leung et al. 1999), confirming the existence of more than one GHRH/PACAP gene in teleosts.

**EVOLUTION OF THE DISTRIBUTION OF GHRH AND PACAP NEURONS IN THE BRAIN**

In mammals, GHRH-containing neurons are strictly located in the arcuate and the dorsomedial nuclei of the hypothalamus (Mayo et al. 1995), whereas PACAP-containing perikarya are widely distributed, not only in the hypothalamus, but also in the thalamus, septum, cortex, amygdala, hippocampal area, cerebellum and pons (Arimura et al. 1991, Koves et al. 1991, 1994, Ghati et al. 1993, Legradi et al. 1994, Nielsen et al. 1998) (Fig. 5a).
In birds, the distribution of GHRH neurons has never been reported while the location of PACAP-containing neurons has been studied in the chicken forebrain (Peeters et al. 1998a). PACAP-immunoreactive cell bodies are particularly abundant in the ventral aspect of the supraoptic nucleus, as well as in the magnocellular subdivisions of the preoptic and paraventricular nuclei (Peeters et al. 1998a).

Figure 5. Schematic parasagittal sections through (a) rat, (b) frog and (c) eel brain depicting the distribution of GHRH and PACAP perikarya. Red triangles, GHRH-immunoreactive cell bodies; green triangles, PACAP-immunoreactive cell bodies. A, amygdala; ARC, arcuate nucleus; C, cerebellum; Cx, cortex; Hi, hippocampus; NPM, magnocellular preoptic nucleus; NPP, posterior preoptic nucleus; OB, olfactory bulb; PVN, paraventricular nucleus; S, septum; SON, supraoptic nucleus; TN, thalamic nucleus; TS, torus semicircularis. Adapted from Gonzalez et al. 1998, Montero et al. 1998 and Yon et al. 1992.
In amphibians, unfortunately, the distribution of GHRH neurons has been exclusively investigated in the hypothalamo-hypophyseal system, so that the presence of GHRH-immunoreactive cell bodies has only been described in the magnocellular part of the preoptic nucleus (Marivoet et al. 1988). The existence of PACAP-positive perikarya has been reported in the frog hypothalamus as well as in extrahypothalamic areas including the thalamus, amygdala and torus semicircularis (Yon et al. 1992) (Fig. 5b). Since, in frog, a GHRH-like peptide and PACAP are encoded by the same gene (Alexandre et al. 2000), it is conceivable that GHRH-like immunoreactivity could have a more widespread distribution than initially described (Marivoet et al. 1988). Thus, the location of GHRH-containing neurons in the amphibian brain outside the hypothalamic region deserves to be investigated.

In fish, GHRH-immunoreactive neurons are primarily located in the magnocellular and parvocellular portions of the preoptic nucleus; a few GHRH-positive neurons are also found in the rostral part of the nucleus lateralis tuberis (Marivoet et al. 1988, Oliveireau et al. 1990). Similarly, PACAP-positive cell bodies are found in the preoptic nucleus and in the nucleus lateralis tuberis (Matsuda et al. 1997b, Montero et al. 1998, Yoshida et al. 1999) (Fig. 5c). The expression of PACAP mRNA in the preoptic area has recently been confirmed by in situ hybridization (Satoh et al. 1999). The co-localization of GHRH and PACAP in the same regions of the fish diencephalon is consistent with the occurrence of a GHRH-like peptide and PACAP within the same precursor (Parker et al. 1993, McRory et al. 1995). Interestingly, in teleost fish, fibers immunoreactive for GHRH and PACAP directly innervate the pars distalis (Marivoet et al. 1988, Oliveireau et al. 1990, Matsuda et al. 1997b, Montero et al. 1998), suggesting that both GHRH and PACAP may be involved in the control of pituitary hormone secretion.

**EVOLUTION OF THE REGULATION OF GH SECRETION BY GHRH AND PACAP**

In mammals, GH secretion is primarily under the stimulatory control of GHRH and the inhibitory influence of somatostatin (Bertherat et al. 1995). Studies aimed at investigating the effect of PACAP on GH secretion have led to controversial results. Specifically, some data suggest that PACAP stimulates GH release in rat (Goth et al. 1992, Hart et al. 1992, Jarry et al. 1992, Leonhardt et al. 1992, Nagy et al. 1993, Wei et al. 1993, Velkeniers et al. 1994), sheep (Sawangjaroen et al. 1997) and cattle (Hashizume et al. 1994), whereas other reports indicate that PACAP has no effect on GH secretion (Miyata et al. 1989, Culler & Paschall 1991, Jarry et al. 1992, Sawangjaroen & Curlewis 1994). In humans, intravenous administration of PACAP does not modify plasma GH levels (Chioidera et al. 1996) and PACAP is less potent than GHRH in stimulating GH release from somatotrophic adenoma cells in primary culture (Adams et al. 1994). In birds, PACAP has been found to induce a dose-dependent stimulation of cAMP formation by chicken pituitary cells. However, PACAP causes only a modest increase of GH release as compared with the robust stimulatory effect of human GHRH (Peeters et al. 1998b). Taken together, these data indicate that PACAP probably plays a minor role in the control of GH secretion in mammals and birds (Table 1).

In amphibians, frog (f) PACAP stimulates GH secretion in the European green frog Rana ridibunda (Martinez-Fuentes et al. 1994) and the bullfrog Rana catesbeiana (S Kikuyama, unpublished data). Frog PACAP stimulates adenyl cyclase activity in frog pituitary slices (Yon et al. 1993) and increases cytosolic calcium concentration in cultured frog somatotrope cells (Gracia-Navarro et al. 1992). At least two types of PACAP receptors, i.e. type I receptors (PACAP-specific receptors) and type II receptors (PACAP-VIP receptors) are expressed in the distal lobe of frog and toad (Alexandre et al. 1999, Hu et al. 2000). Concurrently, it has been shown that human (h) GHRH stimulates GH secretion from bullfrog pituitary cells, and that the hGHRH-induced GH release is inhibited by somatostatin in a dose-dependent manner (Jeandel et al. 1998). In addition, we have recently found that fGHRH-like peptide and fPACAP are equipotent in stimulating GH secretion from cultured frog adenohypophyseal cells (S Kikuyama, unpublished data). These observations suggest that, in amphibians, GHRH and PACAP (in addition to

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Table 1. Relative implication of GHRH and PACAP in the control of GH secretion in vertebrates
somatostatin) are involved in the neuroendocrine control of somatotrope cell activity (Table 1).

In fish, GHRH has been reported to exert a modest stimulatory effect in the trout Oncorhynchus mykiss (Luo et al. 1990, Blaise et al. 1995), the carp Cyprinus carpio (Vaughan et al. 1992) and the tilapia hybrid Oreochromis niloticus × Oreochromis aureus (Melamed et al. 1995) both in vitro and in vivo. In the salmon Oncorhynchus nerka, native GHRH produces only a weak stimulation of GH release from pituitary cells (Parket al. 1997). In a primitive teleost, the European eel Anguilla anguilla, hGHRH is totally devoid of an effect on GH secretion (Montero et al. 1998). In contrast, both PACAP27 and PACAP38 provoke a robust stimulation of GH release from goldfish Carassius auratus (Wong et al. 1998) and eel pituitary cells (Montero et al. 1998). These observations strongly suggest that, in fish, PACAP rather than GHRH plays a physiological role in the control of GH secretion (Table 1). It should be noticed, however, that in the goldfish both type I PACAP receptor and a GHRH receptor are expressed in the pituitary (Chan et al. 1998, Wong et al. 1998), indicating that not only PACAP but also GHRH may exert some kind of hypophysiotropic activity. In addition, it has previously been shown that, in birds, amphibians and fish, several regulatory neuropeptides such as gonadotropin-releasing hormone (Marchant et al. 1989, Lin et al. 1993, Melamed et al. 1995), neuropeptide Y (Peng et al. 1990), thyrotropin-releasing hormone (Harvey et al. 1978, Gracia-Navarro et al. 1991, Trudeau et al. 1992), cholecystokinin (Himick et al. 1993), bombesin (Himick & Peter 1995) and corticotropin-releasing hormone (Rousseau et al. 1999) are involved in the control of somatotrope cell activity.

**CONCLUSIONS**

Since the identification of the sequence of secretin (Mutt et al. 1970), the characterization of multiple related peptides has provided insight into the molecular evolution of this large peptide superfamily. In the case of GHRH and PACAP, a series of exon and gene duplication events has led to the formation of a subfamily of neuropeptide precursors which exhibit the same overall organization. During vertebrate evolution, the primary structure of GHRH has markedly diverged while the sequence of PACAP has been strongly preserved. In submammalian vertebrates, GHRH and PACAP are arranged in tandem within the same precursor whereas, in mammals, the two peptides are generated from distinct precursors, suggesting that gene duplication may have occurred in a common ancestor during the transition period between birds and mammals (Fig. 4). Alternatively, submammalian vertebrates may possess two related GHRH/PACAP-encoding genes, one of which would remain to be characterized.

In all vertebrate species studied so far, the secretion of GH is under the dual control of inhibitory and stimulatory neuropeptides. While somatostatin acts as an inhibitor of GH release throughout the vertebrate phylum, the respective roles of GHRH and PACAP as stimulatory factors of somatotrope cells have been radically inverted during evolution (Table 1). Recently, a novel hypothalamic neurohormone, termed ghrelin, has been characterized for its ability to stimulate GH secretion in mammals (Kojima et al. 1999). Identification of this novel neuropeptide in representative submammalian species and investigation of its possible GH-releasing activity will be necessary to understand the phylogenetic history of the control of GH secretion during vertebrate evolution.

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Evolution of the GHRH/PACAP precursors

M MONTERO and others


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