Amidated fish ghrelin: purification, cDNA cloning in the Japanese eel and its biological activity

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Abstract

We purified ghrelin from stomach extracts of a teleost fish, the Japanese eel (Anguilla japonica) and found that it contained an amide structure at the C-terminal end. Two molecular forms of ghrelin with 21 amino acids were identified by cDNA and mass spectrometric analyses: eel ghrelin-21, GSS(O-n-octanoyl)FLSPSQRPQGKDKKPPRV-amide and eel ghrelin-21-C10, GSS(O-n-decanoyl)FLSPSQRPQGKDKKPPPRV-amide. Northern blot and RT-PCR analyses revealed high gene expression in the stomach. Low levels of expression were found only in the brain, intestines, kidney and head kidney by RT-PCR analysis. Eel ghrelin-21 increased plasma growth hormone (GH) concentrations in rats after intravenous injection; the potency was similar to that of rat ghrelin. We also examined the effect of eel ghrelin on the secretion of GH and prolactin (PRL) from organ-cultured tilapia pituitary. Eel ghrelin-21 at a dose of 0·1 nM stimulated the release of GH and PRL, indicating that ghrelin acts directly on the pituitary. The present study revealed that ghrelin is present in fish stomach and has the ability to stimulate the secretion of GH from fish pituitary. A novel regulatory pathway of GH secretion by gastric ghrelin seems to be conserved from fish to human.


Introduction

In mammals, the secretion of growth hormone (GH) is regulated primarily by growth hormone-releasing hormone (GHRH) and somatostatin released from the hypothalamus (Bluet-Pajot et al. 1998). In fish, various hypothalamic peptides such as GHRH, gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH), neuropeptide Y (NPY), cholecystokinin (CCK), pituitary adenylate cyclase activating polypeptide and corticotropin-releasing hormone have been shown to be involved in GH release in different teleost species (Peng & Peter 1997, Montero et al. 2000), but species differences have been found in their effects. GHRH stimulates the release of GH in goldfish, rainbow trout and tilapia, but has only a weak effect in salmon (Parker et al. 1997). GHRH does not show any effect on GH secretion in the eel (Montero et al. 1998, Rousseau et al. 1999). GnRH stimulates the release of GH in goldfish, common carp and tilapia, but no effect is seen in the trout (Blaise et al. 1995), catfish (Bosma et al. 1997) or eel (Rousseau et al. 1999). TRH induces the release of GH in the goldfish and common carp, but not in the tilapia (Melamed et al. 1995) or eel (Rousseau et al. 1999). NPY and CCK stimulate the release of GH in the goldfish, but not in the eel (Rousseau et al. 1999). There is no consensus regarding the identity of a common regulator of GH release in fish.

We recently identified an endogenous ligand for the orphan G-protein-coupled growth hormone secretagogue receptor (GHS-R) (Kojima et al. 1999). This novel molecule, a 28-amino acid peptide named ‘ghrelin’, possesses a unique serine residue at the N-terminal position 3 (Ser³) with an acyl modification by n-octanoic acid. This acylation is essential for biological activity (Kojima et al. 1999) and receptor binding (Muccioli et al. 2001). Ghrelin exhibits potent GH-releasing activity in the rat in vivo and in vitro. However, little is known about its structure or function in non-mammalian vertebrates. Recently, we purified ghrelin and cloned its cDNA in the bullfrog and chicken (Kaiya et al. 2001, 2002). Both species of ghrelin possess an n-octanoylated third residue, as seen in mammalian ghrelin. In the bullfrog, however, the third amino acid is a threonine, not a serine. Bullfrog ghrelin stimulates secretion of GH and prolactin (PRL) in cultured adenohypophyseal cells, but only a weak effect on release of GH was observed in the rat in vivo. In contrast,
chicken ghrelin, which like mammals possesses a Ser\(^3\), is capable of stimulating the release of GH in both chicken and rat \textit{in vivo}.

In fish, three species of receptor gene belonging to the GHS-R family have been identified in the pufferfish (Palyha \textit{et al.} 2000). A recent \textit{in vivo} study in tilapia demonstrated that plasma GH concentrations were increased by a growth hormone secretagogue (GHS), KP-102 (Shepherd \textit{et al.} 2000). Furthermore, release of GH from the tilapia pituitary \textit{in vitro} is stimulated by rat ghrelin (Riley \textit{et al.} 2002). These results strongly suggest that a ghrelin–GHS-R system may exist and have a role in the release of GH in fish. Recently, Unniappan \textit{et al.} (2002) have identified fish ghrelin cDNA and gene structure from goldfish brain and intestine. Goldfish ghrelin had some unique structures that have not been seen in other ghrelin: the C-terminal amide structure and a second amino acid substitution to threonine from serine.

In the present study, we purified ghrelin peptide from the stomach of the Japanese eel, \textit{Anguilla japonica}, and determined cDNA encoding the precursor protein. Because this species belongs to the group of Elopomorphs and determined cDNA encoding the precursor protein. We report here that eel ghrelin possesses the C-terminal amide structure and a second amino acid substitution to threonine from serine.

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In addition, eel ghrelin stimulates the release of GH in the rat \textit{in vivo} and the release of GH and PRL in the tilapia \textit{in vitro}.

### Materials and Methods

#### Purification of eel ghrelin from stomach tissue

During the purification process, ghrelin activity was followed by measuring changes in intracellular calcium concentration ([Ca\(^{2+}\)]\textit{i}) using a fluorimetric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA, USA) in a cell line expressing rat GHS-R (Chinese hamster ovary (CHO)–GHSR62) as described previously (Kojima \textit{et al.} 1999, Kaiya \textit{et al.} 2001, 2002). Crude acetic acid (AcOH) extracts prepared from fresh eel stomach (231±7 g) were treated with cold acetone at a final concentration of 66%. After centrifugation (13 600 \(g\) for 30 min), the supernatant was treated with two Sep–Pak Vac 35 cc (10 g) C18 cartridges (Waters Corp., Milford, MA, USA), to enrich the peptide component. The resulting peptide-rich fraction was subjected to cation-exchange chromatography (SP-Sephadex C-25, H\(^+\)-form, Amersham Pharmacia Biotech). Successive elution of 1 M AcOH, 2 M pyridine and 2 M pyridine-AcOH (pH 5–0) yielded three fractions: SP-I, SP-II, and SP-III respectively. The basic peptide-enriched SP-III fraction was subjected to a Sephadex G-50 column (fine grade, 2.9 × 144.5 cm, Amersham Pharmacia Biotech) at flow rate of 15 ml/h. The eluate was collected in 15-ml fractions. A 15-mg tissue equivalent of each fraction was assayed by FLIPR. The active fractions were subjected to carboxymethyl cation-exchange HPLC (TSKgel CM-2SW, 4.6 × 250 mm, Tosoh, Tokyo, Japan) at a flow rate of 1 ml/min. A two-step gradient profile was made from solvent A (10 mM ammonium formate (pH 4.8)/10% acetonitrile (ACN)) to 25% solvent B (1 M ammonium formate (pH 4.8)/10% ACN)) for 10 min and then to 55% solvent B for 90 min. The eluate was collected in 1-ml fractions. A 20-mg tissue equivalent of each fraction was assayed by FLIPR. One-tenth of the active fractions (approximately 23 g tissue equivalent) were desalted by a Sep–Pak Light C18 cartridge (Waters Corp.). The lyophilized sample was dissolved in 100 mM phosphate buffer (pH 7.4) and applied to an anti-rat ghrelin(1–11) immunoglobulin G (IgG) immuno-affinity column (50 µl gel volume) to purify ghrelin-immunoreactive substances. Adsorbed substances were eluted with 0.5 ml of a 60% ACN–0.1% trifluoroacetic acid (TFA) solution and then separated by reverse-phase (RP) HPLC using a µBondasphare C18 column (3.9 × 150 mm, Waters Corp.) at a flow rate of 1 ml/min under a linear gradient from 10% to 60% ACN–0.1% TFA for 40 min. The eluate was collected in 0.5 ml-fractions. Active fractions were further purified on a µBondasphare C18 column (2.1 × 150 mm, Waters Corp.) at a flow rate of 0.2 ml/min under a linear gradient from 10% to 60% ACN–0.1% TFA for 40 min. The eluate corresponding to each absorbance peak was collected. To analyze peptide sequences, 10 pmol of the purified peptide was applied to a protein sequencer (model 494, PE Applied Biosystems, Foster City, CA, USA). To analyze amino acid composition, 50 pmol of the purified peptide was applied to an amino acid analyzer (model L-8500A, Hitachi, Tokyo, Japan). The molecular weight of the purified peptide was determined using MALDI-TOF mass spectrometry (Voyager systems, PE Applied Biosystems).

#### 3’-Rapid amplification of cDNA ends PCR

Eel ghrelin cDNA was cloned from an eel stomach cDNA library constructed with 3 µg poly(A\(^+\)) RNA using an eel ghrelin cDNA fragment prepared by 3’-rapid amplification of cDNA ends (RACE) PCR for a probe. Poly(A\(^+\)) RNA was isolated from total RNA using a mRNA purification kit (TaKaRa, Kyoto, Japan). First-strand cDNA was synthesized from 500 ng poly(A\(^+\)) RNA using an oligo(dT)-containing adapter primer supplied by the 3’-RACE system (Invitrogen Life Technologies). The reaction mixture was purified with a Wizard PCR prep DNA purification system (Promega Corp.) and eluted in 50 µl sterilized water. One-tenth of this purified cDNA served as a template for PCR using four degenerate sense-primers, based on the N-terminal seven amino acid sequence conserved in mammalian ghrelin (GSSFLSP): S1, 5’-GGTGGTGGYTTTTTTCNCC-3’; S2, 5’-GGTGGTGGYTTTTTTCNCC-3’; S3, 5’-GGTGGTGC
GYTTCCCTNTNC-C-3'; S4, 5'-GGGTCGAGYTTCCT TNAGYCC-3'. Primary PCR was performed with these degenerate sense-primer, a 3'-universal amplification primer (UAP) supplied with a 3'-RACE kit, and Ex Taq DNA polymerase (TaKaRa). Sequences were amplified at 94 °C for 1 min, with 35 subsequent cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. Next, nested PCR was performed with one-tenth of the purified primary PCR product, a gene-specific sense-primer (5'-CCC TCACAGAGACCAGGGG-3', nucleotides 153–172), based on the amino acid sequence of the purified eel ghrelin (7–13), and UAP under the following conditions: 94 °C for 1 min, and 30 subsequent cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min. The candidate product was subcloned using a TOPO TA cloning kit (pCR II-TOPO vector, Invitrogen). The nucleotide sequence was determined with a DNA sequencer (model 373, PE Applied Biosystems), according to the Thermo-sequence II dye terminator cycle sequencing kit procedure (Amersham Pharmacia Biotech). An approximately 400-bp EcoRI-digested fragment of eel ghrelin cDNA was used as a screening probe for the cDNA library described below.

cDNA cloning

Double-stranded cDNA was synthesized from 3 µg stomach poly(A)+ RNA using a cDNA synthesis kit (Amersham Pharmacia Biotech) with SuperScript II reverse transcriptase (Invitrogen). Complementary DNA was ligated to adapters. The cDNA was size-selected on a Sephacryl S-500 HR column (Invitrogen) and ligated into the EcoRI site of the ZAP II vector. The titer of the cDNA library was 8.9 × 10^5 pfu/ml. Phage DNA (40,000 pfu/plate × 8 plates) was transferred onto nylon membranes (Biodyne B, Pall East Hills, NY, USA), and hybridized with an EcoRI-digested eel ghrelin cDNA fragment labeled with [α-32P]dCTP for 24 h at 37 °C in a hybridization buffer [5 × SSPE (750 mM NaCl, 50 mM NaH2PO4), and 5 mM EDTA, pH 7.4], 5 × Denhardt's solution, 50% formamide, 0.5% SDS and 100 ng/ml calf thymus DNA]. Hybridized membranes were washed twice with 2 × SSC–0.1% SDS at 55 °C for 30 min and then subjected to autoradiography on X-ray films (Kodak, Tokyo, Japan) at −80 °C for 24 h. Eight phages that gave positive plaques were isolated in the secondary screening, and these phages were infected to XL 1-Blue MRF strain (Stratagene, LaJolla, CA, USA), a resultant plasmid containing full-length eel ghrelin cDNA was analyzed.

Gene expression analyses

Northern blot analysis was conducted using 2 µg poly(A)+ RNA prepared from nine eel tissues: whole brain, heart, stomach, anterior, middle and posterior intestine, body kidney, head kidney and gill. RNA was electrophoresed on a 1% agarose-formamide gel for 2 h at 50 V and then transferred onto a nylon membrane (Zeta-probe, Bio-Rad, Hercules, CA, USA). 32P-Labeled full-length eel ghrelin cDNA was hybridized to the membrane. The hybridization and wash conditions followed the procedure as described above. Membrane was exposed to an Image plate (Fuji photo film Co., Ltd, Kanagawa, Japan) for 3 h. The intensity of the plate was scanned using a BAS-5000 bioimaging analyzer (Fuji photo film Co., Ltd).

Reverse transcription-PCR analysis was conducted on cDNA from the same nine tissues. Template cDNA was made from 50 ng poly(A)+ RNA using SuperScript II reverse transcriptase. One-tenth of the cDNA was used as a template. PCR was performed using HotStarTaq Master Mix (Qiagen GmbH). The 20-µl reaction mixture contained 4 µl cDNA solution (10 ng mRNA equivalent), 1 µl 10 µM sense-primer (eel ghrelin-full-s2; 5'-TAC ATCATCCTGCTGGTCTGC-3', nucleotides 81–101) and 1 µl 10 µM antisense-primer (eel ghrelin-full-as1; 5'-TTGGCAGGTGTGGTCTGCG-3', nucleotides 365–385). The amplification reaction was performed at 95 °C for 15 min, with subsequent 30 or 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and final extension for 3 min at 72 °C. Resultant PCR products (305–bp) were electrophoresed on a 2% agarose gel containing ethidium bromide.

In vivo effect of eel ghrelin on release of GH in the rat

Eel ghrelin was synthesized at the Suntory Institute for Medicinal Research & Development as described previously (Matsumoto et al. 2001). Male Sprague–Dawley rats weighing 250–280 g were cannulated in the femoral artery and vein under pentobarbital sodium anesthesia. After untreated blood had been sampled (time 0), 2 nmol/250 g body weight of either synthetic eel ghrelin-21 or rat ghrelin was injected into the femoral vein. Blood (150 µl) was collected from the femoral artery in a syringe containing EDTA (1 mg/ml blood) at 5, 10, 15, 20, 30 and 60 min after injection. GH concentration in plasma was measured using a rat GH enzyme immunoassay kit (Biotrak, Amersham Pharmacia Biotech). Data were analyzed by two-way analysis of variance (ANOVA) to evaluate the effects of time of or time compared with ghrelin species.

In vitro effects of eel ghrelin on the secretion of GH and PRL in the tilapia

Mozambique tilapia, Oreochromis mossambicus, weighing 50–200 g, were used. They were maintained in fresh water (25 ± 2 °C) and fed twice daily. Whole pituitaries were removed and pre-incubated in a 96-well plate for 18–20 h at 37 °C in 100 µl isosmotic bicarbonate-Ringer solution (330 mOsmol) with essential additives as described below.

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described previously (Wigham et al. 1977), supplemented with 0.025 µg/ml gentamicin. The pre-incubation medium was removed and replaced with fresh medium (100 µl) containing increasing concentrations of eel ghrelin-21 (0, 0.1, 1 and 10 nM). Medium samples were removed at 2, 4 and 8 h, and replaced with fresh medium containing appropriate concentrations of eel ghrelin. Medium samples were stored at −20 °C until required for analysis for GH and PRL (PRL188) by homologous radioimmunoassay (Ayson et al. 1993) as modified by Yada et al. (1994). Release of GH and PRL was expressed as ng/100 g body weight, because significant correlation was observed between the amounts of hormone released from the pituitary and body weight (Eckert et al. 2002). Group comparisons were performed using one-way ANOVA, followed by the least significant test or by Mann–Whitney U-test for each time point. Correlation was determined using the Pearson correlation test. Calculations were performed using a computer program, Statistica (StatSoft, Tulsa, OK, USA).

Results

**Purification and structural determination of eel ghrelin**

The SP-III fraction (51.3-mg protein) in which ghrelin activity was detected by the FLIPR assay was separated by Sephadex G-50 gel-permeation chromatography (Fig. 1a). Ghrelin activity was eluted in fractions 43–48, which correspond to a molecular weight of approximately 3000 Da. Active fractions 43–47 were subjected to carboxymethyl cation-exchange HPLC (Fig. 1b). Ghrelin activity was observed in 25 consecutive fractions from 29 to 53. Fractions 42–47, showing high activity (P1), were purified by an anti-rat ghrelin(1–11) IgG immuno-affinity column. Two peaks (I and II) containing high ghrelin activity were isolated by secondary RP-HPLC after the immuno-affinity chromatography (Fig. 1c). The yields of peak I and II peptides were estimated to be approximately 230 and 180 pmol respectively. Amino acid sequences of both peptides were identical: GSXFLPSQRPQGKD KKPPR (X, unidentified). The unidentified X residue at position 3 was predicted to contain an acyl modification, as seen in other ghrelins. Amino acid composition analysis revealed that one more serine (Ser) and one more valine (Val) were present in the purified peptides (data not shown).

Figure 1 Purification of eel ghrelin from eel stomach extract.
(a) Sephadex G-50 gel-permeation chromatography of the SP-III fraction. (b) Carboxymethyl cation-exchange HPLC (pH 4.8) of the active fraction from gel-permeation chromatography. The active fraction (P1) was then purified by anti-rat ghrelin(1–11) IgG immuno-affinity chromatography. (c) Secondary RP-HPLC of immuno-affinity chromatography binding peptides. Peaks I and II were isolated. Black bars indicate the changes in fluorescence in [Ca^{2+}] in CHO-GHSR62 cells.
shown). From peptide sequence and amino acid composition analyses, we predicted that the unidentified X residue is a Ser from the homology of mammalian ghrelins, and that the C-terminal end of these two peptides may contain an additional Val residue. Thus the expected eel ghrelin comprised 21 amino acids with the following sequence: GSSFLSPQRPGKKPRPV-amide.

To determine the complete sequence, we isolated its cDNA encoding precursor protein from an eel stomach cDNA library. The isolated full-length eel cDNA was 503 bp long, containing a 56 bp 5' untranslated region (UTR), a 336-bp coding region and a 110 bp 3' UTR (Fig. 2). Although two ATG codons are present in the 5' UTR, it was predicted that the first ATG codons (nucleotides 57–59) encode the initial methionine by Kozak's rule (A or GnnATGA or G) and by homology of other fish ghrelin precursor sequences (data not shown). A typical polyadenylation signal (AATAAA) was identified in the 3' UTR. The deduced amino acid sequence of the coding region indicated that the eel ghrelin precursor is 111-amino acids long (Fig. 2). The unidentified third amino acid and the C-terminal end of amino acid were determined to be Ser and Val, respectively, as predicted by the amino acid composition analysis. A typical amidation signal, Gly-Arg-Arg, was followed at the C-terminal Val of the mature peptide. Thus eel ghrelin is likely to be terminated with Val-amide.

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We examined the molecular weight of the purified eel ghrelin isofoms (peak I and II) by MALDI-TOF mass spectrometry. Molecular weights of peak I and II were 2421·7 Da and 2449·2 Da respectively; the values are approximately 126 and 154 mass units greater than the theoretical mass calculated from the 21-residue eel ghrelin sequence (2295·5 Da). These findings indicate that the Ser3 hydroxyl groups of peak I and II peptide are modified by n-octanoic acid or n-decanoic acid respectively. The structures determined for the eel ghrelin isoforms are shown in Fig. 3. We designated these two peptides as eel ghrelin-21 and eel ghrelin-21-C10, respectively. Amino acid sequences of various ghrelins determined so far are shown in Fig. 4. The first seven amino acids of eel ghrelin exhibit 100% sequence homology to those of mammalian ghrelins.

### Tissue expression of eel ghrelin mRNA

A strong signal derived from ghrelin mRNA (approximately 0·5 kb) was detected only in the stomach by northern blot analysis (Fig. 5a). RT-PCR with 30 cycles of amplification detected expression of ghrelin gene in the anterior intestine, outside the stomach (Fig. 5b, top).
When the amplification cycle was increased to 35 cycles, gene expression was detected in the brain, heart, intestines, body kidney and head kidney, but not in the gill (Fig. 5b, bottom).

**Biological activity of eel ghrelin**

We examined the potency of eel ghrelin at the rat GHS-R using GHSR62 cells. Administration of 0·1–1000 nM eel ghrelin-21 dose-dependently increased [Ca\(^{2+}\)]\(_i\), with a potency similar to that of rat ghrelin, a full agonist for the GHS-R (Fig. 6a). Furthermore, eel and rat ghrelin elicited a similar level and pattern of release of GH in the rat, despite the considerable difference in the sequence between the two peptides (Fig. 6b). Plasma GH concentrations increased 5 min after intravenous injection of ghrelin, reaching a maximum at 10 min and returning to initial values at 60 min.

To examine whether ghrelin stimulates the release of GH in fish, we used a bioassay system of organ-cultured whole pituitaries from tilapia. Release of PRL was also measured to determine the specificity of the effect of ghrelin on the release of GH. Eel ghrelin-21 stimulated the release of GH from the cultured tilapia pituitary (Fig. 7a). Significant stimulation was observed at concentrations of 1 and 10 nM up to 8 h after treatment. Ghrelin at 0·1 nM was without effect at 4 h, but did elicit some activity after 8 h. The effect of GH release was significantly \((P<0.05)\) dose-dependent during the experimental period. Eel ghrelin was also effective in stimulating the release of PRL at all concentrations examined and at all time points (Fig. 7b). No dose–response relationship was observed, however, which may suggest that the lowest concentration (0·1 nM) was sufficient to saturate the response.

**Discussion**

This paper describes the purification of ghrelin peptides from the stomach of a teleost fish, the Japanese eel. Ghrelin has also been cloned from the brain and intestine of
goldfish (Unniappan et al. 2002). Eel ghrelin was capable of stimulating the release of GH, a typical biological activity of ghrelin, in the rat both in vivo and in vitro bioassay using cultured pituitaries of the tilapia. These findings support recent observations showing that the release of GH is regulated by a ghrelin–GHS-R system in fish (Palyha et al. 2000, Shepherd et al. 2000, Riley et al. 2002).

The Ser\(^3\) hydroxyl group of all of known mammalian ghrelins is acylated with \(n\)-octanoic acid (Kojima et al. 1999). Des-acyl ghrelin, which lacks the acyl modification, has no effect on \([\text{Ca}^{2+}]\), in CHO-GHSR62 cells (Kojima et al. 1999) and does not bind to the GHS receptor (Muccioli et al. 2001). This acylation, therefore, is essential for ghrelin activity. Similarly, the Ser\(^3\) residue of eel ghrelin is also octanoylated, indicating that this modification is likely to be a conserved characteristic for receptor binding of ghrelin in many other vertebrates. A recently identified chicken ghrelin also has the same modification of the Ser\(^3\) residue (Kaiya et al. 2002). Although the acylated amino acid in bullfrog ghrelin is a Thr, not a Ser, it contains a hydroxyl group in the side chain, allowing octanoylation to occur (Kaiya et al. 2001).

In addition to the \(n\)-octanoylated form, we also isolated an eel ghrelin acylated with \(n\)-decanoic acid. Decanoylated ghrelin has been identified in the bullfrog (Kaiya et al. 2001) and human (Hosoda et al. 2002), but not in the rat (H. Hosoda et al., unpublished observation). In human, 25% of ghrelin isolated from the stomach is decanoylated. In the bullfrog, the decanoylated form represented 33% of total isolated ghrelin. In the case of eel, 44% of the total ghrelin isolated was the decanoylated form. It is noteworthy that the percentage of the decanoylated form tends to increase in lower classes of vertebrates, although the mechanisms governing the acylation of ghrelin remain unknown. We did not test whether eel ghrelin–21-C10 is biologically active; the decanoylated form of ghrelin is biologically active in the rat and bullfrog, and its efficacy was similar to that of the octanoylated form (Matsumoto et al. 2001, Kaiya et al. 2001).

The complete amino acid sequence of eel ghrelin has only 46–48% identity to those of mammalian ghrelins. The first seven amino acids of eel ghrelin (GSSFLSP), however, exhibit 100% sequence homology to both mammalian and chicken ghrelins. Nevertheless, eel ghrelin–21 showed the same potency as rat ghrelin with respect to GH-releasing activity and increase in intracellular calcium in rat models. A similar level of inter-species cross-activity was also seen with chicken ghrelin (Kaiya et al. 2002). In contrast, bullfrog ghrelin did not alter plasma GH concentrations in rats and showed only low affinity to rat GHS–R, but potently stimulated the release of GH and PRL from bullfrog pituitary cells (Kaiya et al. 2001). These differences in biological activity of ghrelin could be explained, in part, by species-specific binding of ghrelin to the GHS receptor for the animal. The N-terminal tetrapeptide (GSSF), including acyl modifications, is the ‘active core’ of ghrelin (Bednarek et al. 2000, Matsumoto et al. 2001). The active core of human, rat, chicken and eel ghrelins is 100% identical, but bullfrog ghrelin contains two amino acid substitutions, to Leu\(^2\)-Thr\(^3\) from Ser\(^2\)-Ser\(^3\), in the active core. Thus the structure of the ligand-binding site of the GHS-R is likely to be similar in mammals, birds and fish (Palyha et al. 2000). It is noteworthy that recently identified goldfish ghrelin has one substitution in the region, from serine to threonine in the second amino acid (Unniappan et al. 2002).

Eel ghrelin possesses a unique amide structure at the C-terminal end. This structure was first identified in goldfish ghrelin (Unniappan et al. 2002). Mature eel ghrelin is produced by a typical amidation signal (Gly-Arg-Arg) at the C-terminal end. The dibasic processing

**Figure 7** Effect of eel ghrelin on release of (a) growth hormone (GH) and (b) prolactin (PRL) from tilapia pituitary in vitro. Values are expressed as means ± S.E.M. (n=8). Significant difference at each time point were evaluated by least significant test or Mann–Whitney U-test. *P<0.05, **P<0.01 compared with control.
signal (Arg-Arg) is conserved in non-mammalian vertebrates, including chicken (Kaiya et al. 2002), bullfrog (Kaiya et al. 2001) and goldfish (Unniappan et al. 2002). Amidation for the C-terminal carboxyl group of ghrelin fragments enhances ghrelin activity (Matsumoto et al. 2001). In the present study, however, the potency of eel ghrelin for release of GH in the rat was the same as that of rat ghrelin. Furthermore, non-amidated rat ghrelin stimulates the release of GH from fish pituitary (Riley et al. 2002). It is likely that the C-terminal amide structure does not affect receptor binding and activity of ghrelin. The physiological significance of the C-terminal amidation of ghrelin in fish, in addition to the diversity of the C-terminal amino acid sequence, remains to be elucidated.

To evaluate the effect of eel ghrelin on the release of GH in fish, we used organ-cultured tilapia pituitaries, because a homologous radioimmunoassay for eel GH is not currently available. We have reported earlier that intraperitoneal injection of a GHS, KP-102, significantly increased plasma GH concentrations in the tilapia, suggesting that a specific GHS receptor is present in the tilapia (Shepherd et al. 2000). Recently, we have shown that rat ghrelin stimulates the release of GH from cultured tilapia pituitaries (Riley et al. 2002). In the present study, eel ghrelin stimulated the release of GH from tilapia pituitaries, indicating that eel ghrelin is capable of stimulating GH release in fish by acting directly on the pituitary. The release of GH from the pituitary increased twofold above control levels. A similar response was observed when rat pituitary cells were exposed to ghrelin (Kojima et al. 1999). In contrast, the in vivo response of GH release in the rat was sevenfold, compared with a threefold increase in vitro. In the rat, Date et al. (2002) have recently reported that the gastric vagal afferent is the major pathway conveying the ghrelin signal for GH release to the brain. This finding suggests that intermediate factor(s) may modify the signal of peripheral ghrelin.

In addition to GH release, PRL secretion was also stimulated by eel ghrelin in the tilapia pituitary. We have also shown that rat ghrelin stimulated the release of PRL from the tilapia pituitary (Riley et al. 2002). PRL-releasing activity of ghrelin has been observed in bullfrog pituitary cells (Kaiya et al. 2001). In contrast, no increase in PRL was observed in dispersed rat pituitary cells (Kojima et al. 1999). These results suggest a species-specific effect of ghrelin on PRL cells in lower vertebrates.

Ghrelin in the eel is synthesized predominantly in the stomach. This is in agreement with findings in other animals (Kojima et al. 1999, Kaiya et al. 2001, 2002). In the eel, relatively high expression of ghrelin gene was also observed in the anterior intestine by RT-PCR analysis. This was also observed in the goldfish (Unniappan et al. 2002). It is to be noted that goldfish lack a stomach, and therefore the intestine may be the primary site of ghrelin production. Furthermore, ghrelin mRNA was detected in the brain, heart, intestines, kidney and head kidney of the eel. These results are slightly different from that in the goldfish (Unniappan et al. 2002). The physiological significance of gene expression in the various tissues remains unknown, but brain-derived ghrelin seems to be involved in feeding, as seen in the goldfish.

In conclusion, we have shown that ghrelin is present in the eel stomach, stimulating the release of GH and PRL from the pituitary. Thus the regulatory function of pituitary activity by ghrelin through a novel gastropituitary pathway seems to be conserved in vertebrates, including fish, amphibians, avians and mammals. In fish, stimulation of GH secretion is controlled by various hypothalamic neuropeptides (Peng & Peter 1997, Montero et al. 2000), and gastric or brain-derived ghrelin could also be a primary regulator of GH release.

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