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

H Kaiya¹, M Kojima^{1,3}, H Hosoda¹, L G Riley², T Hirano²,
E G Grau² and K Kangawa¹

¹Department of Biochemistry, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan

²Hawaii Institute of Marine Biology, University of Hawaii, Kaneohe, Hawaii 96744, USA

³Division of Molecular Genetics, Institute of Life Science, Kurume University, 2432-3 Aikawa-machi, Kurume, Fukuoka 839-0861, Japan

(Requests for offprints should be addressed to H Kaiya; Email: kaiya@ri.ncvc.go.jp)

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)FLSPSQRPQGKDKKPPRV-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.

Journal of Endocrinology (2003) **176**, 415–423

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 ghrelins. 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 Ser³, is capable of stimulating the release of GH in both chicken and rat *in vivo*.

In fish, three species of receptor gene belonging to the GHS-R family have been identified in the pufferfish (Palyha *et al.* 2000). A recent *in vivo* study in tilapia demonstrated that plasma GH concentrations were increased by a growth hormone secretagogue (GHS), KP-102 (Shepherd *et al.* 2000). Furthermore, release of GH from the tilapia pituitary *in vitro* is stimulated by rat ghrelin (Riley *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 *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 ghrelins: 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, *Anguilla japonica*, and determined cDNA encoding the precursor protein. Because this species belongs to the group of Elopomorphs considered to be close to the origin of teleosts, we aimed to obtain information about the ancestral structure of ghrelin. We report here that eel ghrelin possesses the C-terminal amide structure, as seen in goldfish ghrelin. In addition, eel ghrelin stimulates the release of GH in the rat *in vivo* and the release of GH and PRL in the tilapia *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 ($[Ca^{2+}]_i$) 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 *et al.* 1999, Kaiya *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 preps 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 ghrelins (GSSFLSP): S1, 5'-GGGTCGAGYTTCTTRTCNCC-3'; S2, 5'-GGGTCGAGYTTCTTRAGYCC-3'; S3, 5'-GGGTCGA

GYTTCCTNTCNCC-3'; S4, 5'-GGGTCGAGYTTCC TNAGYCC-3'. Primary PCR was performed with these degenerate sense-primers, 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 TCACAGAGACCGCAGGG-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 (pCRII-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 *Eco*RI-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 *Eco*RI 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 $\times$ 8 plates) was transferred onto nylon membranes (Biodyne B, Pall East Hills, NY, USA), and hybridized with an *Eco*RI-digested eel ghrelin cDNA fragment labeled with [α -³²P]dCTP for 24 h at 37 °C in a hybridization buffer [5 $\times$ SSPE (750 mM NaCl, 50 mM NaH₂PO₄, and 5 mM EDTA, pH 7.4), 5 $\times$ Denhardt's solution, 50% formamide, 0.5% SDS and 100 ng/ml calf thymus DNA]. Hybridized membranes were washed twice with 2 $\times$ 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' with a helper phage. After *in vivo* excision with SOLR as a host 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'-TTGGCAGGTGTGTCCATCAGC-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 or of 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 $\pm$ 2 °C) and fed twice daily. Whole pituitaries were removed and pre-incubated in a 96-well plate for 18–20 h at 27 °C in 100 µl isosmotic bicarbonate–Ringer solution (330 mOsmol) with essential additives as

described previously (Wigham *et al.* 1977), supplemented with 0.025 μ g/ml gentamicin. The pre-incubation medium was removed and replaced with fresh medium (100 μ l) 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 radio-immunoassay (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: GSXFLSPSQRPGKD 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

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 $[\text{Ca}^{2+}]_i$ in CHO-GHSR62 cells.


```

10      20      30      40      50      60      70
TCCAAGAGGACTGGGTTCTCTTAAGTGCACAAATCCACTGTGAGCTTCAGACATGAGGCAGATGAA
M R Q M K (5)

80      90      100     110     120     130     140
ACGCACCGCATACATCTCTGCTGGTCTGCGCTGGCGCTGTGGATGAGCTCTGTCAGGCTGGCTCC
R T A Y I I L L V C V L A L W M D S V Q A G S (28)

150     160     170     180     190     200     210
AGCTTCCTCAGCCCCCTACAGAGACCGCAGGGGAAGGATAAGAAGCTCCAGGGTGGCAGACGAGACT
S F L S P S Q R P Q G K D K K P P R V G R R D (51)

220     230     240     250     260     270     280
CAGATGGGATCCTGGACCTGTTTATGAGGCCCTTCAGGATGAAGACATCAGACACATTACGTTTAA
S D G I L D L F M R P P L Q D E D I R H I T F N (75)

290     300     310     320     330     340     350
CACTCTCTTTGAGATCGGGATCACCATGACCTGAGGAGCTGTTCCAGCAATATGGAGAAGTGATGCAGAAG
T P F E I G I T M T E E L F Q Q Y G E V M Q K (98)

360     370     380     390     400     410     420
ATCATGAGGATTGCTGATGGACACCTGCCAAGAGTGAAGAAGTGGATATGATCTGGACTTCATA
I M Q D L L M D T P A K E * (111)

430     440     450     460     470     480     490
AAACCTCGCTCCCATATCTCTGCATATTGCATGACATAATCAACCAATTGTTAAACATTTAATAAA
AATTTGCAACGC

```

Figure 2 Nucleotide sequences and deduced amino acid sequence of the eel ghrelin. The eel ghrelin cDNA contains 503 nucleotides. Prepro-ghrelin is composed of 111 amino acids (parentheses at right). The mature sequence of eel ghrelin is underlined and a typical dibasic processing sequence is boxed. Dotted line, C-terminal amidation signal; double underline, polyadenylation signal (AATAAA).

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: GSSFLSPSQRPQGKDKKPPRV.

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.

We examined the molecular weight of the purified eel ghrelin isoforms (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

• Peak I (eel ghrelin-21):

• Peak II (eel ghrelin-21-C10):

Figure 3 Structure of eel ghrelin. The identity of the third serine residue (Ser³) was determined by cDNA analysis. The modifications of Ser³ with *n*-octanoic acid in peak I and with *n*-decanoic acid in peak II were determined by mass spectrometric analysis.

Eel	GSSFLSPS-QRPQG-KD-KKP-----PRV (21)
Goldfish-12	GTSFLSPA-QKPQ----- (12)
Goldfish-19	GTSFLSPA-QKPQGR----RP-----PRM (19)
Bullfrog	GLTFLSPADMOKIAERQSQNKLRH-GNMN (28)
Chicken	GSSFLSPTYKNIQQQKDRKPTARL--H- (26)
Human	GSSFLSPEHQRVQQRKESKKPPAKLQPR- (28)
Rat, Mouse	GSSFLSPEHQKAQQRKESKKPPAKLQPR- (28)
Bovine	GSSFLSPEHQKL-QRKEAKKPSGRLKPR- (27)
Pig	GSSFLSPEHQKVQQRKESKKPAALKPR- (28)
Dog	GSSFLSPEHQKLQQRKESKKPPAKLQPR- (28)
	* * * * *

Figure 4 Comparison of amino acid sequence of ghrelin. *Amino acids that are identical in all species. Numbers in parentheses at right are number of amino acids. Amino acid sequences are available from the DDBJ/EMBL/GenBank databases (accession numbers AB062427 (eel), AF454389 (goldfish), AB058510 (bullfrog), AB075215 (chicken), AB029434 (human), AB092433 (rat), AB03571 (mouse), AF350329 (bovine), AF308930 (pig) and AJ298295 (dog)).

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).

Figure 5 Gene expression analyses of eel ghrelin in various tissues. (a) Northern blot analysis. A hybridized signal (approximately 0.5 kb) was detected only in the stomach. (b) RT-PCR analysis: top panel, result of 30 cycles of amplification; bottom panel, result of 35 cycles of amplification. Poly(A)⁺ RNA (50 ng) was subjected to reverse transcription, and one-fifth of the resulting cDNA was used as a template for specific amplification (305-bp product). Each lane contains three-quarters of the reaction mixture.

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

Figure 6 Biological activity of eel ghrelin in rat models. (a) Dose-response relationships of change in intracellular calcium concentrations in response to eel and rat ghrelin in CHO-GHSR62 cells. The maximum value of the response was used for calculation of the data. Values represent means $\pm$ S.E.M. ($n = 3$). (b) Time-course of changes in plasma GH concentration after intravenous injection of eel ghrelin-21 or rat ghrelin into male Sprague-Dawley rats. Because of variation in the initial baseline concentrations, values (means $\pm$ S.E.M., $n = 5$) are expressed in terms of the ratio of each time point to the initial concentration (eel ghrelin, 145.1 ± 34.3 ng/ml; rat ghrelin, 118.8 ± 19.1 ng/ml; saline, 201.9 ± 62.4 ng/ml).

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

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 $\pm$ 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.

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 *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 Ser³ 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 $[Ca^{2+}]_i$ 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 Ser³ 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 Ser³ 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 Leu²-Thr³ from Ser²-Ser³, 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

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.

Acknowledgements

The nucleotide sequence for the eel ghrelin precursor has been deposited in the DDBJ/EMBL/GenBank databases with the accession number AB062427.

We thank Yasuo Kitajima, Masaru Matsumoto and Yoshiharu Minamitake at the Suntory Institute for Medicinal Research and Development for synthesizing eel ghrelin peptides.

This work was supported in part by a Grant-in-Aid for Scientific Research from the MEXT of Japan, the Promotion of Fundamental Studies in Health Science from the Organization for Pharmaceutical Safety and Research (OPSR) of Japan, a grant from Takeda Science Foundation, and also by grants from the National Science Foundation (IBN 01-33714), US Department of Agriculture (No. 9835206644) and University of Hawaii Sea Grant College Program (NOAA project R/AQ-62, Institutional Grant No. NA86RG0041, UNIH-SEAGRANT-JC-01-12).

References

- Ayson FG, Kaneko T, Tagawa M, Hasegawa S, Grau EG, Nishioka RS, King DS, Bern HA & Hirano T 1993 Effects of acclimation to hypertonic environment on plasma and pituitary levels of two prolactins and growth hormone in two species of tilapia *Oreochromis mossambicus* and *Oreochromis niloticus*. *General and Comparative Endocrinology* **89** 138–148.
- Bednarek MA, Feighner SD, Pong SS, McKee KK, Hreniuk DL, Silva MV, Warren VA, Howard AD, Van Der Ploeg LH & Heck JV 2000 Structure–function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. *Journal of Medicinal Chemistry* **43** 4370–4376.
- Blaise O, Le Bail P-Y & Weil C 1995 Lack of gonadotropin releasing-hormone action on *in vitro* and *in vivo* growth hormone release in rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology C* **110** 133–141.

- Bluet-Pajot MT, Epelbaum J, Gourdji D, Hammond C & Kordon C 1998 Hypothalamic and hypophyseal regulation of growth hormone secretion. *Cellular and Molecular Neurobiology* **18** 101–123.
- Bosma PT, Kolk SM, Rebers FE, Lescroart O, Roelants I, Willems PH & Schulz RW 1997 Gonadotrophs but not somatotrophs carry gonadotrophin-releasing hormone receptors: receptor localisation, intracellular calcium, and gonadotrophin and GH release. *Journal of Endocrinology* **152** 437–446.
- Date Y, Murakami N, Toshinai K, Matsukura S, Nijima A, Matsuo H, Kangawa K & Nakazato M 2002 The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* **123** 1120–1128.
- Eckert SM, Hirano T, Leedom TA, Takei Y & Grau EG 2002 Effects of angiotensin II and natriuretic peptides on prolactin and growth hormone release in the tilapia, *Oreochromis mossambicus*. *General and Comparative Endocrinology* (In Press).
- Hosoda H, Kojima M, Mizushima T, Shimizu S & Kangawa K 2002 Structural divergence of human ghrelin; identification of multiple ghrelin-derived molecules produced by post-transcriptional processing. *Journal of Biological Chemistry* (In Press).
- Kaiya H, Kojima M, Hosoda H, Koda A, Yamamoto K, Kitajima Y, Matsumoto M, Minamitake Y, Kikuyama S & Kangawa K 2001 Bullfrog ghrelin is modified by *n*-octanoic acid at its third threonine residue. *Journal of Biological Chemistry* **276** 40441–40448.
- Kaiya H, Van Der Geyten S, Kojima M, Hosoda H, Kitajima Y, Matsumoto M, Geelissen S, Darras VM & Kangawa K 2002 Chicken ghrelin: purification, cDNA cloning and biological activity. *Endocrinology* **143** 3454–3463.
- Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402** 656–660.
- Matsumoto M, Hosoda H, Kitajima Y, Morozumi N, Minamitake Y, Tanaka S, Matsuo H, Kojima M, Hayashi Y & Kangawa K 2001 Structure–activity relationship of ghrelin: pharmacological study of ghrelin peptides. *Biochemical and Biophysical Research Communication* **287** 142–146.
- Melamed P, Eliahu N, Levavi-Sivan B, Ofir M, Farchi-Pisanty O, Pentier-Delerue F, Smal J, Yaron Z & Naor Z 1995 Hypothalamic and thyroidal regulation of growth hormone in tilapia. *General and Comparative Endocrinology* **97** 13–30.
- Montero M, Yon L, Rousseau K, Arimura A, Fournier A, Dufour S & Vaudry H 1998 Distribution, characterization, and growth hormone-releasing activity of pituitary adenylate cyclase-activating polypeptide in the European eel, *Anguilla anguilla*. *Endocrinology* **139** 4300–4310.
- Montero M, Yon L, Kikuyama S, Dufour S & Vaudry H 2000 Molecular evolution of the growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide gene family. Functional implication in the regulation of growth hormone. *Journal of Molecular Endocrinology* **25** 157–168.
- Muccioli G, Papotti M, Locatelli V, Ghigo E & Deghenghi R 2001 Binding of ¹²⁵I-labeled ghrelin to membranes from human hypothalamus and pituitary gland. *Journal of Endocrinological Investigation* **24** RC7–RC9.
- Palyha OC, Feighner SD, Tan CP, McKee KK, Hreniuk DL, Gao YD, Schleim KD, Yang L, Morriello GJ, Nargund R, Patchett AA, Howard AD & Smith RG 2000 Ligand activation domain of human orphan growth hormone (GH) secretagogue receptor (GHS-R) conserved from pufferfish to human. *Molecular Endocrinology* **14** 160–169.
- Parker DB, Power ME, Swanson P, Rivier J & Sherwood NM 1997 Exon skipping in the gene encoding pituitary adenylate cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. *Endocrinology* **138** 414–423.
- Peng C & Peter RE 1997 Neuroendocrine regulation of growth hormone secretion and growth in fish. *Zoological Studies* **36** 79–89.
- Riley LG, Hirano T & Grau EG 2002 Rat ghrelin stimulates growth hormone and prolactin release in the tilapia, *Oreochromis mossambicus*. *Zoological Science* **19** 797–800.
- Rousseau K, Le Belle N, Marchelidon J & Dufour S 1999 Evidence that corticotropin-releasing hormone acts as a growth hormone-releasing factor in a primitive teleost, the European eel (*Anguilla anguilla*). *Journal of Neuroendocrinology* **11** 385–392.
- Shepherd BS, Eckert SM, Parhar IS, Vijayan MM, Wakabayashi I, Hirano T, Grau EG & Chen TT 2000 The hexapeptide KP-102 (D-Ala-D-β-Nal-Ala-Trp-D-Phe-Lys-NH₂) stimulates growth hormone release in a cichlid fish (*Oreochromis mossambicus*). *Journal of Endocrinology* **167** R7–R10.
- Unniappan S, Lin X, Cervini L, Rivier J, Kaiya H, Kangawa K & Peter RE 2002 Goldfish ghrelin: molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. *Endocrinology* **143** 4143–4146.
- Wigham T, Nishioka RS & Bern HA 1977 Factors affecting in vitro activity of prolactin cells in the euryhaline teleost Sarotherodon mossambicus (*Tilapia mossambicus*). *General and Comparative Endocrinology* **32** 120–131.
- Yada T, Hirano T & Grau EG 1994 Changes in plasma levels of the two prolactins and growth hormone during adaptation to different salinities in the euryhaline tilapia, *Oreochromis mossambicus*. *General and Comparative Endocrinology* **93** 2214–2223.

Received 10 September 2002

Accepted 11 November 2002