serum albumin, 0.5% Triton X-100, 80 mM NaCl, 25 mM Na_2EDTA,A. O. and 0.05% NaN_3) containing 0.5% normal rabbit serum. The anti-rat ghrelin-(1–11) or anti-rat ghrelin-(13–28) antisera were used at final dilutions of 1:6,000,000 and 1:20,000, respectively. After incubation for 12 h, 100 μl of ^{125}I-labeled tracer (15,000 cpm) was added. Thirty-six hours later, 100 μl of anti-rabbit IgG goat serum was added. After 24 h of incubation, free and bound tracers were separated by centrifugation at 3,000 rpm for 30 min. Pellet radioactivity was counted with a gamma counter (ARC-600, Aloka, Tokyo). All assay procedures were performed in duplicate at 4 °C.

Both types of antisera exhibited 100% cross-reactivity with human and rat ghrelins. The anti-rat ghrelin-(1–11) antiserum specifically recognized the *n*-octanoylated portion at Ser^3 of ghrelin and did not recognize des-acyl ghrelin. The anti-rat ghrelin-(13–28) antiserum equally recognized *n*-octanoyl and des-acyl ghrelins. In the following sections, the RIA system using antiserum against the N-terminal fragment of rat ghrelin-(1–11) is termed N-RIA; the RIA system using antiserum against the C-terminal fragment (13–28) is termed C-RIA.

Detection of Ghrelin Activity by Calcium Mobilization Assay—CHO-GHSR62 cells, which stably express rat GHS-R (8, 12), were plated in flat-bottom, 96-well black-wall plates (Corning Inc., Corning, NY) at 4×10^4 cells/well for 12 h prior to the assay. The cells were loaded with 4 μM Fluo-4-AM fluorescent indicator dye (Molecular Probes, Inc., Eugene, OR) for 1 h in assay buffer (Hanks' balanced salts solution, 20 mM HEPES, 2.5 mM probenecid, 1% fetal calf serum) and washed four times in assay buffer without fetal calf serum. Intracellular calcium concentration ($[Ca^{2+}]_i$) changes were measured using a fluorometric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, CA). Maximum changes in fluorescence compared with the baseline were used to quantitate the agonist responses.

Purification of Human Ghrelins from Stomach—A human stomach mucosa (27 g) was minced and boiled for 5 min in 5× volumes of water to inactivate intrinsic proteases. The solution was adjusted to 1 M acetic acid (AcOH)-20 mM HCl. The stomach tissue was homogenized with a Polytron mixer. The supernatant of the extract, obtained after 30-min centrifugation at 11,000 rpm, was concentrated to ~25 ml by evaporation. The residual concentrate was subjected to acetone precipitation in 66% acetone. After removal of the precipitate, the supernatant was evaporated to remove the acetone and then loaded onto a 10-g cartridge of Sep-Pak C18 (Waters, Milford, MA), pre-equilibrated in 0.1% trifluoroacetic acid. The Sep-Pak cartridge was washed with 10% CH_3CN/0.1% trifluoroacetic acid, and the peptides were eluted in 60% CH_3CN/0.1% trifluoroacetic acid. The eluate was evaporated and lyophilized. The lyophilized materials were then redissolved in 1 M AcOH and applied to a Sephadex G-50 gel-filtration column (1.8 × 130 cm, Amersham Biosciences, Uppsala, Sweden). Five-milliliter fractions were collected. A portion of each fraction was subjected to ghrelin-specific RIA and the intracellular calcium influx assay using CHO-GHSR62 cells. The active fractions (#43–46) were separated by carboxymethyl (CM) ion-exchange high performance liquid chromatography (HPLC) on a TSK CM-2SW column (4.6 × 250 mm, Tosoh, Tokyo, Japan) using an ammonium acetate ($HCOONH_4$) (pH 4.8) gradient of 10 mM to 1 M in the presence of 10% CH_3CN and a flow rate of 1 ml/min for 100 min. One-milliliter fractions were collected and subjected to ghrelin-specific RIAs and intracellular calcium influx assays. The six active fractions (fractions A–F) separated by CM-HPLC were finally individually purified using C18 reverse-phase HPLC (RP-HPLC) columns (Symmetry 300, 3.9 × 150 mm, Waters). The amino acid sequences of the purified peptides were analyzed with a protein sequencer (494, Applied Biosystems, Foster City, CA).

Mass Spectrometric Analysis of Human Ghrelins—Electrospray ionization mass spectrometry (ESI-MS) was performed on a quadrupole mass spectrometer SSQ7000 (Finnigan, San Jose, CA) equipped with a Finnigan ESI source. A needle capillary was heated to 150 °C to evaporate the samples. Samples (~20 pmol) were dissolved in 50% (v/v) methanol, 1% AcOH and introduced into the +4.5 kV (positive ionization) ion source at a flow rate of 5 μl/min by direct infusion with a syringe pump. Molecular masses of the purified peptides were calculated using ICIS software Bioworks provided by Finnigan.

Human Ghrelin Peptide Synthesis—Peptide synthesis of acylated human ghrelin was performed as previously described for rat ghrelin (8, 12). Fully protected 27- and 28-amino acid peptides (with the exception of the exposed hydroxyl group of Ser^3) were synthesized by the Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) solid-phase method using a peptide synthesizer (433A, Applied Biosystems). The Ser^3 hydroxyl groups were acylated with *n*-octanoic acid or *n*-decanoic acid by the action of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in the presence of 4-(dimethylamino)pyridine.

Cloning of Human Prepro-ghrelin cDNA—Based on the amino acid sequence determined for purified rat ghrelin, we searched the GenBank™ expressed sequence tag data base. One rat expressed sequence tag sequence (accession number AI549172) contained the rat ghrelin sequence. Based on this expressed sequence tag sequence, we designed sense and antisense primers (5'-TTGAGCCCAGAGCACCAGAAA-3' and 5'-AGTTGCAGAGGAGGCAGAAGCT-3', respectively) and performed PCR on a rat stomach cDNA library. The conditions for the PCR involved 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min. A rat ghrelin cDNA of 501 bp was obtained. The amplified fragment was labeled with [^{32}P]dCTP and used as a screening probe for a human stomach cDNA library, which was constructed from 1 μg of human stomach poly(A)$^+$ RNA (Clontech, Palo Alto, CA) using a cDNA synthesis kit (Amersham Biosciences). By this method, a full-length human ghrelin cDNA was isolated. Several positive phages were isolated and subcloned into the plasmid pBS. Both strands of cloned cDNAs were sequenced.

In Vivo Assay of Growth Hormone-releasing Activity—Male Wister rats (270–300 g) were prepared with a single indwelling jugular catheter under sodium pentobarbital. Each rat received a 0.5- or 2-nmol injection of synthetic human ghrelins. Blood samples were collected at 0, 5, 10, 15, 20, 30, and 60 min after injection. All samples were centrifuged immediately, and the plasma samples were assayed for GH using the Biotrak rat GH enzyme immunoassay system (Amersham Biosciences, Buckinghamshire, UK).

Human Blood Sample Analysis—To study the changes in plasma levels of ir-ghrelin after gastrectomy, we followed three patients (one male and two females), 50–61 years of age, who underwent total gastrectomy due to gastric cancer. Blood samples were obtained before and within 30 min after gastrectomy, and then 3, 7, 14, 30, 150, and 240 days after gastrectomy for measurement of plasma ir-ghrelin levels. Four control males (60–70 years of age) who underwent standard partial colectomy due to cancer, were also examined. Their blood samples were obtained before and 1 day after surgical operation.

Preparation of Human Plasma Samples—Whole blood samples were collected with Na_2EDTA (2 mg/ml) and aprotinin (500 KIU/ml). Plasma, after centrifugation at 4 °C, was diluted with an equal volume of 0.9% saline. The samples were loaded onto a Sep-Pak C18 cartridge (Waters) pre-equilibrated with 0.9% NaCl, washed with 0.9% NaCl and 5% CH_3CN/0.1% trifluoroacetic acid, and then eluted with 60% CH_3CN/0.1% trifluoroacetic acid. After lyophilization of the eluates, they were subjected to RIAs for ghrelin as described above.

RESULTS

Purification of Human Ghrelins—We purified human ghrelin from stomach tissue extracts by gel-filtration chromatography and HPLC, using an intracellular calcium influx assay with a stable cell line expressing rat GHS-R (CHO-GHSR62) and two ghrelin-specific RIAs to screen fractions for the presence of ghrelin. Ir-ghrelin obtained by N-RIA specifically represents active acylated ghrelin, whereas ir-ghrelin acquired by C-RIA represents the total immunoreactivity of both acylated and des-acyl ghrelin. Fig. 1*a* depicts the gel-filtration chromatographic separation of stomach peptide extracts. Fractions possessing ghrelin immunoreactivity and promoting intracellular calcium influx were eluted at a molecular weight of roughly 3000. Active gel-filtration fractions were further separated by CM ion-exchange HPLC into six fractions, A–F (Fig. 1*b*). Fractions B–F induced intracellular calcium influxes and possessed ir-ghrelin as assessed by both C-RIA and N-RIA. Fraction A possessed only C-RIA ir-ghrelin and did not induce intracellular calcium influx. Each of the five factions, B–F, were separately purified to homogeneity by RP-HPLC and subjected to the calcium-mobilization assay (Fig. 1*c*). Each of the five fractions contained one active peak, except for fraction E, which had two (*peaks E-I* and *E-II*). Fraction A was also separated by RP-HPLC into two peaks (*peaks A-I* and *A-II*), which were found to possess ir-ghrelin by C-RIA (Fig. 1*d*). Each of the eight resulting purified active peaks contained a single peptide, and these peptides were then subjected to further analysis.

Structural Analyses of Human Ghrelins—The eight purified peptides were subjected to protein sequencer, showing that peaks C, E-I, E-II, and F shared the 28-amino acid sequence GSXFLSPEHQRVQQRKESKKPPAKLQPR, whereas peaks B

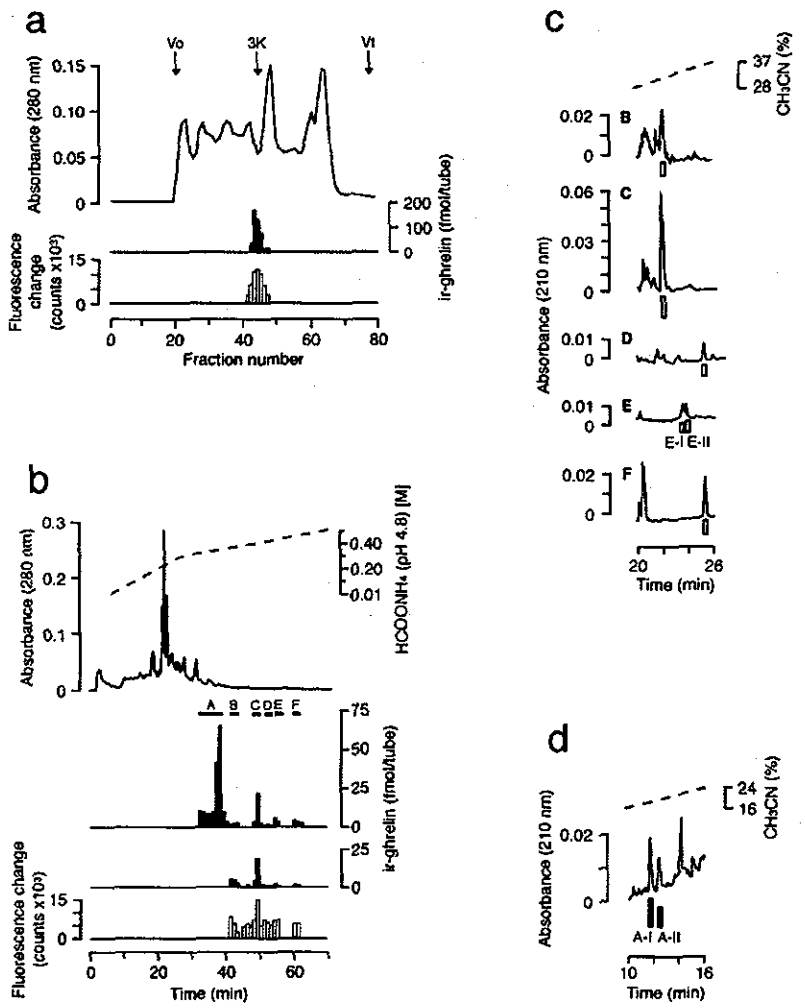

FIG. 1. **Purification of human ghrelin from stomach.** *Black bars* indicate ir-ghrelin content, and *open bars* indicate the fluorescence changes due to $[Ca^{2+}]_i$ increase in CHO-GHSR62 cells. The gradient profiles are indicated by the *dotted lines*. *a*, gel-filtration chromatography of human stomach extracts (27 g). Active fractions 43–46 were eluted at roughly M_r 3000 (*3 K*). *Vo*, void volume; *Vt*, total volume. An aliquot from each fraction (5 mg of wet tissue equivalent) was subjected to C-RIA. *b*, CM ion-exchange HPLC (pH 4.8) of gel-filtration-derived active fractions monitored by C-RIA (*upper*), N-RIA (*middle*), and calcium-mobilization assay (*bottom*) for ghrelin. Active fractions, indicated by *solid bars*, were separated into six fractions (*fractions A–F*). A portion of each fraction (5 mg of wet tissue equivalent) was subjected to RIAs for ghrelin. *c*, final purification of the each active fractions B–F derived from CM ion-exchange HPLC by RP-HPLC. *d*, final purification of fraction A derived from CM ion-exchange HPLC by RP-HPLC.

and D were of the sequence GSX′FLSPEHQRVQQRKESKKPPAKLQP, identical except for the lacked of the C-terminal arginine. Complementary DNA analysis of human ghrelin indicated that the third *X* and *X′* residues should be serine, and the serine residues were acyl-modified as described below. Moreover, the amino acid sequences of peak A-I and A-II were GSSFLSPEHQRVQQRKESKKPPAKLQPR and GSSFLSPEHQRVQQRKESKKPPAKLQP, respectively, the same as the acyl-modified ghrelin peptides. We did not detect des-Gln^{14}-ghrelin in this human stomach tissue.

To determine whether the purified peptides were also modified by *n*-octanoic acid at Ser^3 as is rat ghrelin, we subjected the peptides to ESI-MS and measured their molecular masses (Table I). The measured molecular mass of peak C, the major active peptide, was 3371.3 ± 0.1, and the calculated molecular mass of the 28-amino acid sequence is 3244.6. The discrepancy, 126.7 mass units, strongly suggests that the hydroxyl group of the Ser^3 in this peptide is indeed replaced by an *n*-octanoyl moiety (C8:0). The same was found for peak B, which had a measured mass (3214.6 ± 0.6) that was 126.2 mass units higher than the calculated molecular mass of the 27-amino acid peptide (3088.4), indicating modification by *n*-octanoic acid. The measured molecular masses of the peptides from peaks D and F were ~154 molecular mass units higher than the calculated molecular masses, indicating that these two peptides were modified by *n*-decanoic acid (C10:0). Peaks E-I and E-II were both 152.6 molecular mass units higher than the calculated molecular masses, 2 mass units smaller than what would be expected for decanoyl modification. Based on this result and the fact that peaks E-I and E-II were eluted at a time between the octanoyl (peaks C and E) and decanoyl-modified ghrelins (peaks D and F) by RP-HPLC, it is most likely that the peptides from peaks E-I and E-II are modified by decenoic acid (C10:1). The amounts of peptide purified from peaks E-I and E-II were very low, preventing a determination of the double-bond site of the decenoic acid. In conclusion, we were able to divide the collected ghrelins into four groups on the basis of acyl modification at Ser^3: non-acylated, octanoylated, decanoylated, and possibly decenoylated.

TABLE I
Structural analyses of human ghrelin and ghrelin derived molecules

Peak	Amino-acid sequence	Molecular mass		Linked fatty acid
		Measured	Measured-Calculated[a]	
B	Gly^1-Pro^{27}	3214.6 ± 0.6	126.2	C8:0
C	Gly^1-Arg^{28}	3371.3 ± 0.1	126.7	C8:0
D	Gly^1-Pro^{27}	3243.6 ± 0.4	155.2	C10:0
E-I	Gly^1-Arg^{28}	3397.2 ± 0.5	152.6	C10:1
E-II	Gly^1-Arg^{28}	3397.2 ± 0.8	152.6	C10:1
F	Gly^1-Arg^{28}	3398.9 ± 0.3	154.3	C10:0

[a] Calculated mass of amino acid sequence; Gly^1-Arg^{28}, 3244.6; Gly^1-Pro^{27}, 3088.4.

To verify the deduced structures, we synthesized four peptides, [*O*-*n*-octanoyl-Ser^3]-human ghrelin, [*O*-*n*-octanoyl-Ser^3]-

TABLE II
The molar ratio of purified human ghrelin and ghrelin derived molecules

Peak	Molecular form	Molar ratio
B	Ghrelin-(1–27) (C8:0)	2
C	Ghrelin (C8:0)	6
D	Ghrelin-(1–27) (C10:0)	1
E	Ghrelin (C10:1)	1
F	Ghrelin (C10:0)	2

human ghrelin-(1–27), [*O*-*n*-decanoyl-Ser^3]-human ghrelin, and [*O*-*n*-decanoyl-Ser^3]-human ghrelin-(1–27) and compared their characteristics with those of the purified peptides. The natural and synthetic peptides showed identical retention times by RP-HPLC and identical molecular masses. Moreover, the synthetic acyl-modified peptides had the same effects as purified ghrelin peptides on cells expressing GHS-R. These results confirmed our structural predictions for human ghrelin and the ghrelin-derived molecules. We designate the newly purified peptides as follows: [*O*-*n*-decanoyl-Ser^3]-human ghrelin as "human decanoyl ghrelin," [*O*-*n*-octanoyl-Ser^3]-human ghrelin-(1–27) as "human ghrelin-(1–27)," and [*O*-*n*-decanoyl-Ser^3]-human ghrelin-(1–27) as "human decanoyl ghrelin-(1–27)." The yield of purified human ghrelin was ~300 pmol from 27 g of stomach mucosa, and the molar ratio of the various subsets are shown in Table II.

Pharmacological Characterization of Ghrelins Using GHS-R-expressing Cells—Fig. 2 shows the dose-response relationships of the synthetic human ghrelin and the ghrelin-derived molecules on $[Ca^{2+}]_i$ changes in GHS-R-expressing cells. Four synthetic ghrelins potently induced increases in $[Ca^{2+}]_i$ in CHO-GHSR62 cells. Ghrelin, ghrelin-(1–27), decanoyl ghrelin, and decanoyl ghrelin-(1–27) had EC_{50} values of 2.7×10^{-9}, 2.8×10^{-9}, 2.5×10^{-9}, and 2.5×10^{-9} M, respectively, and displayed similar potency upon application to GHS-R-expressing cells.

In Vivo Effects of Human Ghrelins on GH Secretion—To confirm that human ghrelins possessed GH-releasing activity, we intravenously injected synthetic ghrelins into anesthetized rats and measured plasma GH concentrations. After injection of the each of the four synthetic ghrelins, plasma GH concentrations increased and reached a maximum within 10–15 min (Fig. 3). Each of the peptides displayed nearly identical dose-response relationships, confirming that the newly identified human ghrelin-derived molecules are endogenous GH-releasing peptides with similar potency to human ghrelin.

Structure of the Human Prepro-ghrelin cDNA—Using a rat ghrelin cDNA, we screened a human stomach cDNA library under low stringency conditions and obtained positive phages. Analysis of these clones yielded a deduced amino acid sequence for human prepro-ghrelin (a 117-amino acid precursor) (GenBank™ accession number AB029434), depicted in Fig. 4. The putative initiation codon ATG is located at nucleotides 34–36, preceded by the consensus initiation sequence, whereas a terminal codon TAG is found 117 codons downstream at position 385–387. A typical polyadenylation signal, AATAAA, is found at position 494–499.

Although nearly all of the cDNA clones isolated from human stomach encoded the prepro-ghrelin precursor, a few cDNA clones encoded the prepro-des-Gln^{14}-ghrelin precursor. Also, although we were not able to isolate des-Gln^{14}-ghrelin from the stomach extracts during this study, this result indicates that des-Gln^{14}-ghrelin is indeed present in very low amounts in the human stomach.

Characterization of Human Plasma Ghrelin Immunoreactivity—To confirm the presence of multiple molecular forms of ghrelin in human plasma in addition to the stomach, Sep-pak extracts of normal human plasma were fractionated by CM

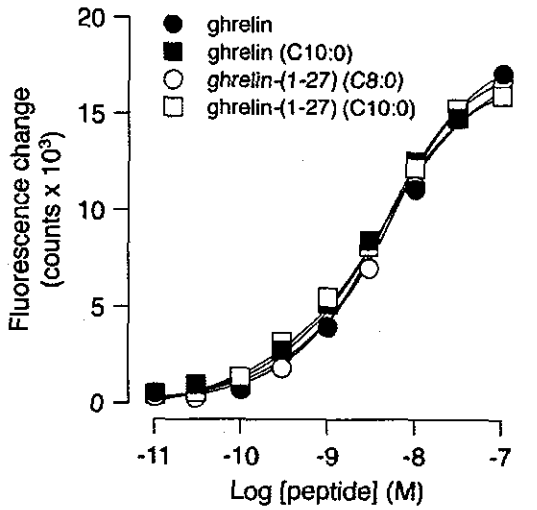

FIG. 2. **Pharmacological characterization of synthetic ghrelin and ghrelin-derived molecules using the GHS-R-expressing cells.** Dose-response relationships of $[Ca^{2+}]_i$ in CHO-GHSR62 cells, in response to treatment with human ghrelin, ghrelin-(1–27), decanoyl ghrelin, and decanoyl ghrelin-(1–27). Data points are means ± S.D. of three independent experiments.

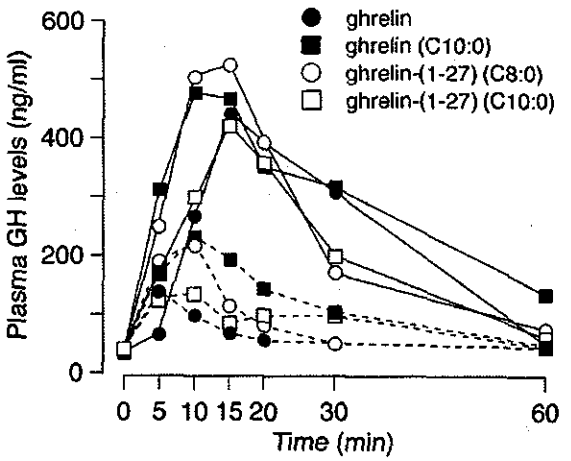

FIG. 3. **Growth hormone releasing activity of different synthetic forms of ghrelin *in vivo*.** Time courses of plasma growth hormone concentrations after intravenous injections of synthetic ghrelin and ghrelin-derived molecules into male rats. Each of the synthetic ghrelins was injected intravenously into male Wistar rats (270–300 g) anesthetized with pentobarbital, and blood samples were collected from the cervical artery. GH concentrations were measured by enzyme immunoassay. *Solid lines* and *broken lines* indicate doses of 2 and 0.5 nmol, respectively. Data represent the means of three experiments.

ion-exchange HPLC in exactly the same manner as those from the stomach. The HPLC pattern of plasma extracts in terms of the presence of ir-ghrelin was observed to be similar to that of the stomach extracts (Fig. 5), with peaks a–f emerging at positions identical to that of peaks A-F in Fig. 1*b*. Thus, it can be concluded that both ghrelin and the ghrelin-derived molecules circulate in human blood.

A minor unknown peak of ir-ghrelin (peak g) detected only by C-RIA was observed in human plasma. This peak accounted for ~15% of all ir-ghrelin by C-RIA. By RP-HPLC, this ir-ghrelin peak was eluted earlier than that of des-acyl ghrelin (data not shown), suggesting that the unknown ir-ghrelin in peak g results from digested ghrelin.

Plasma Ghrelin Levels in Gastrectomized Patients—To clar-

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GCAGGCCCACCTGTCTGCAACCCAGCTGAGGCCATGCCCTCCCCAGGGACCGTCTGCAGC   60
                                M  P  S  P  G  T  V  C  S       9

CTCCTGCTCCTCGGCATGCTCTGGCTGGACTTGGCCATGGCAGGCTCCAGCTTCCTGAGC  120
L  L  L  L  G  M  L  W  L  D  L  A  M  A  G¹ S  Ⓢ  F  L  S    29

CCTGAACACCAGAGAGTCCAGCAGAAAGGAGTCGAAGAAGCCCACCAGCCAAGCTGCAG  180
P  E  H  Q  R  V  Q  Q¹⁴ R  K  E  S  K  K  P  P  A  K  L  Q   49

CCCCGAGCTCTAGCAGGCTGGCTCCGCCCGGAAGATGGAGGTCAAGCAGAAGGGGCAGAG  240
P  R²⁸ A  L  A  G  W  L  R  P  E  D  G  G  Q  A  E  G  A  E    69

GATGAACTGGAAGTCCGGTTCAACGCCCCCTTTGATGTTGGAATCAAGCTGTCAGGGGTT  300
D  E  L  E  V  R  F  N  A  P  F  D  V  G  I  K  L  S  G  V    89

CAGTACCAGCAGCACAGCCAGGCCCTGGGGAAGTTTCTTCAGGACATCCTCTGGGAAGAG  360
Q  Y  Q  Q  H  S  Q  A  L  G  K  F  L  Q  D  I  L  W  E  E   109

GCCAAAGAGGCCCCAGCCGACAAGTGATCGCCCACAAGCCTTACTCACCTCTCTCTAAGT  420
A  K  E  A  P  A  D  K  *                                     117

TTAGAAGCGCTCATCTGGCTTTTCGCTTGCTTCTGCAGCAACTCCCACGACTGTTGTACA  480

AGCTCAGGAGGCGAATAAATGTTCAAACTGT                               511
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FIG. 4. **Nucleotide and deduced amino acid sequence of human prepro-ghrelin cDNA (GenBank™ accession number AB029434).** The predicted amino acid sequence of prepro-ghrelin is denoted *below* the nucleotide sequence. The *dotted line* indicates the signal peptide. The human ghrelin-(1–28) sequence is *double-underlined*. The *circled S* indicates an *n*-acyl-modified serine. The termination codon is marked with an *asterisk*. The AATAAA sequence, a polyadenylation signal, is *underlined*. The *boxed AG* of Gln^{14} may be used as a splicing acceptor site at the 3′-end of the intron to produce des-Gln^{14}-ghrelin.

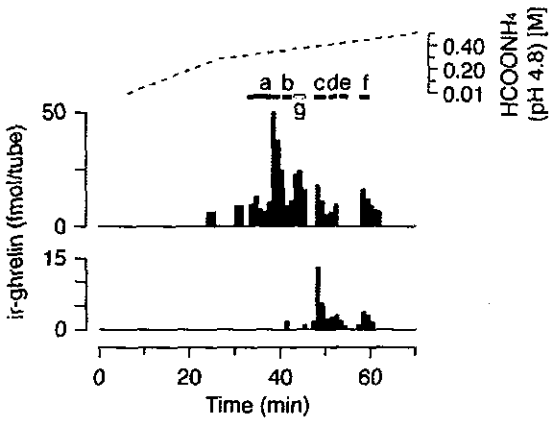

FIG. 5. **CM ion-exchange HPLC of human plasma extract monitored by radioimmunoassays for ghrelin.** Human plasma extracts from a Sep-Pak C18 cartridge were fractionated by CM ion-exchange HPLC (pH 4.8) in an identical manner as the stomach extracts. Each sample (2.5-ml plasma volume equivalent) was monitored by C-RIA (*upper*) and N-RIA (*lower*). The ir-ghrelin recovery of this CM ion-exchange HPLC step was ~90%. Active fractions were separated into seven fractions. Fractions *a–f* indicated by *solid bars* correspond to the active fractions *A–F* of the first CM ion-exchange HPLC step from the stomach extracts (Fig. 1*b*). Fraction *g* indicated by the *open bar* probably contained a C-terminal fragment of ghrelin and des-acyl ghrelin to be cleaved by proteases.

ify whether circulating ghrelin is indeed drastically reduced after gastrectomy as would be expected, plasma ir-ghrelin was measured before and after total gastrectomy in three patients (Fig. 6). Within 30 min after gastrectomy, plasma ir-ghrelin was found to decrease to approximately half of its pre-surgery levels. The levels remained depressed for roughly a week, but after that they began to increase. By the end of the day 240, two of the patients had ir-ghrelin levels that were two-thirds of their original levels, and one patient's ghrelin levels had completely normalized. In contrast, the subjects who underwent par-

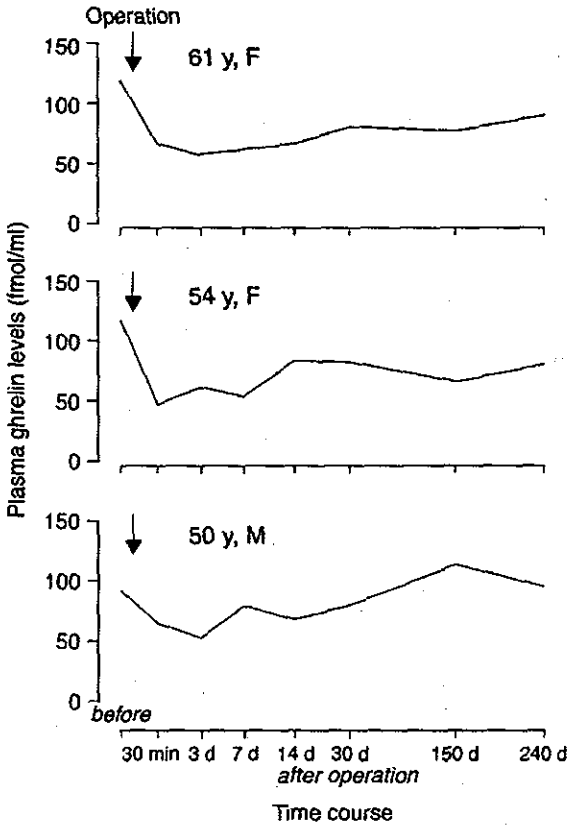

FIG. 6. **Time course of plasma ghrelin levels before and after total gastrectomy.** Individual changes in plasma ghrelin levels in three patients before and after total gastrectomy. Plasma levels of ir-ghrelin were measured by C-RIA.

tial colectomy showed no change in plasma ir-ghrelin before (75.4 ± 22.0 fmol/ml, mean ± S.D.) and 1 day after operation (69.6 ± 17.8 fmol/ml). These results suggest that the stomach is the major source of circulating ghrelin, and the other tissues compensate to maintain circulating ghrelin levels after gastrectomy.

DISCUSSION

Here, we reported the purification and characterization of human ghrelin and the other minor ghrelin-derived molecules from the stomach. The major active form of human ghrelin is a 28-amino acid peptide with an *n*-octanoyl modification at Ser^3. This peptide is identical to rat ghrelin with the exception of two residues (Arg^{11}-Val^{12}). The ghrelin-derived molecules we observed include octanoyl ghrelin-(1–27), decanoyl ghrelin, decanoyl ghrelin-(1–27), and decenoyl ghrelin. Moreover, the nonactive forms des-acyl ghrelin and des-acyl ghrelin-(1–27) were also present in the human stomach. As described, we were able to classify human ghrelin and the ghrelin-derived molecules into two groups on the basis of amino acid length and into four groups by type of acylation at Ser^3. Furthermore, all of these molecular forms of ghrelin were found in human plasma as well as in the stomach. In human stomach, the processing product ratio of 27-amino acid to 28-amino acid ghrelins was observed to be ~1:3.

It is likely that the 27- and 28-amino acid ghrelin molecules isolated in this study are produced through alternative C-terminal processing of the same ghrelin precursor. It is well known that peptide hormones are cleaved by processing proteases to product multiple forms, such as the enkephalins (16),

endorphins, dynorphins (17), corticotropins, and β-lipotropins (18). Many of the known proteolytic precursor cleavage events occur at pairs of basic amino acid residues (Lys or Arg), and both basic residues are usually absent from the resultant products (19). However, some proteolytic cleavages, as in the case of cholecystokinin, occur immediately after a C-terminal single basic residue (especially Arg) (20). Ghrelin-(1–28) may fit into this category, because cleavage to produce this peptide occurs following the C-terminal Pro^{27}-Arg^{28}. Interestingly, this basic arginine residue remains at the C terminus of ghrelin-(1–28) but is removed from ghrelin-(1–27) (which terminates in proline). A similar cleavage profile is seen in the case of α-neo-endorphin (YGGFLRKY-Pro-Lys) (21) and β-neo-endorphin (YGGFLRKY-Pro) (22), whose precursor possesses the Lys-Arg basic pair followed by a C-terminal proline. It is thought that production of these peptides occurs through cleavage at the C terminus of paired basic Lys-Arg residues, followed by removal of the C-terminal basic residue by a carboxypeptidase B-like enzyme (23). Peptide bonds involving proline are resistant to common proteases such as this. However, the removal of C-terminal lysine in α-neo-endorphin occurs partially to generate β-neo-endorphin. In a similar manner, ghrelin-(1–27) may be produced by removal of the C-terminal arginine of ghrelin-(1–28) by a carboxypeptidase B-like enzyme. Although ghrelin-(1–27) was present only at a very low level in rat stomach, it is likely that these processing mechanisms control of the maturation of human ghrelin.

The human prepro-ghrelin we isolated is predicted to encoded a 117-residue precursor peptide. We previously reported that there are two types of ghrelin precursors from rat stomach cDNA analysis, a 117-amino acid precursor (prepro-ghrelin) and a 116-amino acid precursor (prepro-des-Gln^{14}-ghrelin) (12). Des-Gln^{14}-ghrelin, a splice variant of ghrelin, is the second endogenous ligand for the GHS-R. Only a small percentage of the ghrelin clones isolated from the human stomach library encoded the des-Gln^{14}-ghrelin precursor, and the des-Gln^{14}-ghrelin peptide was not identified in human stomach. The ratios observed between the two precursor populations, ghrelin and des-Gln^{14}-ghrelin, was 5 to 1 in rat stomach, and 6 to 5 in mouse stomach (24). These differences are likely species-specific.

Ghrelin was the first example discovered of a bioactive peptide modified by an *n*-octanoic acid moiety. Although acyl modification of many proteins has been observed, including G-proteins and some G-protein-coupled receptors, the modifications are most often myristoylations (25) and palmitoylations (26). In this study, we further showed that ghrelin can be modified by *n*-decanoic acid. All of the acyl-modified ghrelins and ghrelin-derived molecules studied here have the same potency to induce an increase of $[Ca^{2+}]_i$ in the GHS-R-expressing cells and stimulate GH release in anesthetized rats, and *de novo* synthesis of molecules concretely demonstrated that octanoic acid is not the only Ser^3 modification that will confer full activity to ghrelin (27, 28). We recently reported the identification of bullfrog ghrelin acylated with *n*-decanoic acid and found that this ghrelin species comprises 33% of total isolated bullfrog ghrelin (29). In the human stomach, the ratio of octanoylated to decanoylated ghrelin was found to be roughly 3:1. Because acylation of ghrelin is essential for its activity, the enzyme that catalyzes this modification step should be an important regulator of ghrelin biosynthesis. However, the mechanism by which ghrelin is acylated during post-translational processing is still unclear.

We also analyzed the change in human plasma ir-ghrelin levels before and after total gastrectomy. We already reported that plasma levels of ir-ghrelin in totally gastrectomized patients were reduced to 35% of those in normal controls (14). In this study, C-RIA was used due to the instability of acylated ghrelin relative to its des-acylated counterpart, making its measurement from stored plasma samples unreliable. Plasma levels of ir-ghrelin were promptly reduced by approximately half within 30 min after total gastrectomy. Half-lives after intravenous administration of human ghrelin was about 10 min (30). Interestingly, the levels then began to increase in these cases, and in one patient even completely normalized. Significant amounts of ir-ghrelin were detected in the rat duodenum, jejunum, ileum, and colon (13), suggesting that these organs may be responsible for this compensation.

Further work will be required to determine the physiological significance of the various different forms of human ghrelin and ghrelin-related peptides discovered during the course of this study. Although all of these molecules displayed similar dose-response profiles from the tests performed in this study, it is possible that the various ghrelin forms have different signaling properties or stability.

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Peptide Purification, Complementary Deoxyribonucleic Acid (DNA) and Genomic DNA Cloning, and Functional Characterization of Ghrelin in Rainbow Trout

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We have identified ghrelin from the stomach of rainbow trout. Four isoforms of ghrelin peptide were isolated: the C-terminal amidated type of rainbow trout ghrelin (rt ghrelin) composed of 24 amino acids (GSSFLSPSQKPQVRQGKGKPPRV-amide) is a basic form; des-VRQ-rt ghrelin, which deleted three amino acids ($V^{13}R^{14}Q^{15}$) from rt ghrelin; and further two types of rt ghrelin that retained the glycine residue at the C terminus, rt ghrelin-Gly, and des-VRQ-rt ghrelin-Gly. The third serine residue was modified by octanoic acid, decanoic acid, or the unsaturated form of those fatty acids. In agreement with the isolated peptides, two cDNAs of different lengths were isolated. The rt ghrelin gene has five exons and four introns, and two different mRNA molecules are predicted to be produced by alternative splicing of the gene. A high level of ghrelin mRNA expression was detected in the stomach, and moderate levels were detected in the brain, hypothalamus, and intestinal tracts. Des-VRQ-rt ghrelin stimulated the release of GH in the rat *in vivo*. Furthermore, des-VRQ-rt ghrelin stimulated the release of GH, but not the release of prolactin and somatolactin in rainbow trout *in vivo* and *in vitro*. These results indicate that ghrelin is a novel GH secretagogue in rainbow trout that may affect somatic growth or osmoregulation through GH. Because ghrelin is expressed in various tissues other than stomach, it may play important role(s) in cellular function as a local regulator. (*Endocrinology* 144: 5215–5226, 2003)

GHRELIN IS A 28-amino acid peptide identified in rat and human stomach (1) as an endogenous ligand for the GH secretagogue (GHS) receptor (GHS-R) (2, 3). The third serine residue is uniquely modified by octanoylation, and the acylation of the peptide is essential for both receptor binding (4) and eliciting biological activity (1). When ghrelin is injected iv or ip in rats, plasma GH levels increase in anesthetized or free-moving rats (1, 5–7). Release of GH is also observed after intracerebroventricular injection of ghrelin (5, 8). Ghrelin has also been shown to increase plasma GH levels in humans (9, 10). The mechanism(s) by which ghrelin stimulates GH release has been investigated. Date *et al.* (11) demonstrated that the gastric vagal afferent is the major pathway conveying ghrelin signals for GH secretion. Tamura *et al.* (12) indicated that the arcuate nuclei (Arc) are involved in the regulation of GH secretion into the systemic circulation through the use of monosodium glutamate-treated Arc-ablated rats. Shuto *et al.* (13) further demonstrated that the GHS-R in the Arc is involved in GH secretion using transgenic rats expressing antisense GHS-R mRNA. In addition, ghrelin can stimulate the release of GH by directly acting on the pituitary (1). Therefore, it is likely that, in mammals, the regulation of GH secretion by ghrelin is controlled by both direct and indirect pathways that converge on the pituitary.

Abbreviations: Arc, Arcuate nuclei; BCM, basic culture medium; $[Ca^{2+}]_i$, intracellular calcium concentration; CHO, Chinese hamster ovary; CM, carboxymethyl; GHS, GH secretagogue; GHS-R, GH secretagogue receptor; GSP, gene-specific primer; PRL, prolactin; rt ghrelin, rainbow trout ghrelin; RACE, rapid amplification of the cDNA ends; RP, reverse-phase; RT, reverse transcriptase; SL, somatolactin.

In teleost fish, several factors have been implicated in regulating GH release. Species-related or age-related differences, however, have been observed (14). For example, GHRH, a primary regulator of GH release in mammals, stimulates the release of GH in the goldfish, rainbow trout, and tilapia but has no effect on GH secretion in the eel (15). Shepherd *et al.* (16) demonstrated that ip injection of a ghrelin mimic, peptidyl-GHS secretagogue, KP-102, increases plasma GH levels but not plasma prolactin (PRL) levels in the tilapia, suggesting the presence of a ghrelin-GHS system in fish. Unniappan *et al.* (17) have recently reported the cloning of a cDNA encoding preproghrelin from the brain and intestine of goldfish. Kaiya *et al.* (18, 19) isolated ghrelin peptide from the stomach of the Japanese eel and Mozambique tilapia (*Oreochromis mossambicus*). Interestingly, the carboxyl termini of these peptides possess an amide structure, which has not been observed in tetrapod ghrelins including mammals, chicken, and bullfrog (20, 21). Eel and tilapia ghrelin potently stimulated the release of GH and PRL from organ-cultured tilapia pituitary (18, 19). Rat ghrelin, however, led to similar levels of GH and PRL release (22); thus, these were not species-specific effects. Taken together, these data support a model wherein ghrelin stimulates the release of GH and PRL at least in the tilapia, but the effect of ghrelin seems to differ *in vivo* or at the pituitary. In the present study, we report the isolation of ghrelin from the stomach of rainbow trout, a common model of fish physiology, and examined its effects on the release of GH, PRL, and somatolactin (SL), another pituitary hormone, both *in vivo* and in explanted rainbow trout pituitary.

Materials and Methods

Purification of rainbow trout ghrelin (rt ghrelin) from stomach

Ghrelin activity during the purification process was followed by measuring changes in intracellular calcium concentration ($[Ca^{2+}]_i$) in a cell line stably expressing rat GHS-R [Chinese hamster ovary (CHO)-GHSR62] as described previously (1, 20). Rainbow trout stomach was collected in Takatsuki-city, Osaka, Japan, in January. Frozen stomachs, approximately 17.5 g from 10 specimens, were used as the starting materials. The basic peptide-enriched SP-III fraction was prepared as described previously (20, 21). The SP-III fraction was subjected to ion exchange HPLC (TSKgel CM-2SW, 4.6 × 250 mm, Tosoh, Tokyo, Japan). Active fractions were purified by an antirat ghrelin[1–11] IgG immunoaffinity column. Adsorbed substances were separated by reverse-phase (RP)-HPLC using a μBondasphare C18 column (3.9 × 150 mm, Waters, Milford, MA) at a flow rate of 1 ml/min under a linear gradient from 10 to 60% acetonitrile/0.1% trifluoroacetic acid for 40 min. The active fractions were further purified by a diphenyl column (2.1 × 150 mm, 219TP5125, Vydac, Hesperia, CA) at a flow rate of 0.2 ml/min under a linear gradient from 10 to 60% acetonitrile/0.1% trifluoroacetic acid for 40 min. Fractions corresponding to each absorbance peak were collected. To analyze the peptide sequence, 5 pmol of the purified peptide were subjected to protein sequencing (model 494, PE Applied Biosystems, Foster City, CA). The molecular weight of the purified peptide was determined using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Voyage-DE PRO, PE Applied Biosystems). Acylation patterns were determined by the difference between detected molecular mass and theoretical molecular mass that was calculated from amino acid sequence of peptide (Table 1).

cDNA cloning

Rapid amplification of cDNA ends (RACE) PCR method was used for cDNA cloning. Total RNA was extracted from a stomach using TRIzol reagent (Invitrogen, Carlsbad, CA). Poly(A)$^+$ RNA was isolated using a mRNA purification kit (Takara Bio Inc., Kyoto, Japan). For 3′-RACE PCR, first-strand cDNAs were synthesized from 100 ng of poly(A)$^+$ RNA using an adaptor primer supplied by the 3′-RACE system (Invitrogen) and the SuperScript II reverse transcriptase (RT) (Invitrogen). One tenth of the cDNA served as a template. Primary PCR was performed as described previously (20, 21) using four degenerated primers, based on the N-terminal seven amino acid sequence of mammalian ghrelin (GSSFLSP): GRL-s7, 5′-GGGTCGAGYTTCTTRTCNCC-3′; GRL-s8, 5′-GGGTCGAGYTTCTTRAGYCC-3′; GRL-s9, 5′-GGGTCGAGYTTCCTNTCNCC-3′; and GRL-s10, 5′-GGGTCGAGYTTCCTNAGYCC-3′. Amplification was performed as follows: 94 C for 1 min, 35 cycles at 94 C for 30 sec, 58 C for 30 sec and 72 C for 1 min, and a final extension for 3 min at 72 C. Amplified products were purified using Wizard PCR preps DNA purification system (Promega, Madison, WI). For second-round nested PCR, a nested sense primer for eel ghrelin[7–13] (5′-CCCTCACAGAGACCGCAGGG-3′) was used. The amplification reaction was 94 C for 1 min, 35 cycles at 94 C for 30 sec, 52 C for 30 sec and 72 C for 1 min, and a final extension for 3 min at 72 C. The candidate ghrelin cDNA fragment was subcloned using a TOPO TA cloning kit (pCR II-TOPO vector, Invitrogen), and sequence of inserted DNA was determined.

For 5′-RACE PCR, first-strand cDNAs were synthesized from 200 ng of poly(A)$^+$ RNA with oligo-dT_{12-18} primer or random primer and the SuperScript II RT or Thermoscript RT (Invitrogen). RT reaction was conducted at 42 C for 1 h by the SuperScript II RT and at 42 C for 10 min followed at 60 C for 50 min by the Thermoscript RT. One fifth of the purified cDNA was subjected to a TdT-tailing reaction of the 5′-ends of the first-strand cDNA with deoxy CTP according to the manufacturer's protocol (Invitrogen). The resultant dC-tailed cDNAs served as a template. A gene-specific primer (GSP) was designed based on the sequence of the rt ghrelin cDNA as determined by 3′-RACE PCR: GSP-1, 5′-TTGGAGAACAGGGAATGGAGG-3′. Primary PCR was performed using GSP-1, an abridged anchor primer supplied with the 5′-RACE kit, and Ex *Taq* DNA polymerase with the following reaction conditions: 94 C for 1 min, 35 cycles at 94 C for 30 sec, 60 C for 30 sec and 72 C for 1 min, and a final extension for 3 min at 72 C. The resultant product was purified by PCR preps, and the second-round nested PCR was performed with the same GSP-1 and abridged universal amplification primers. The amplification reaction was 94 C for 1 min, 30 cycles at 94 C for 30 sec, 55 C for 30 sec and 72 C for 1 min, and a final extension for 3 min at 72 C. The candidate PCR product was subcloned into the pCR-II TOPO vector, and sequence of inserted DNA was determined. The nucleotide sequence of the isolated cDNA fragment was determined by automated sequencing (DNA sequencer: model 373 or 3100, PE Applied Biosystems) according to protocol for the Thermosequence II dye terminator cycle sequencing kit (Amersham Bioscience KK, Tokyo, Japan) or the BigDye terminator cycle sequencing kit (PE Applied Biosystems). Approximately 430 and 480 bp of cDNA were determined by 3′- and 5′-RACE PCR, respectively.

Cloning of the rt ghrelin gene

Genomic DNA was extracted using the GenomicPrep Cells and Tissue DNA isolation Kit (Bioscience KK) from rainbow trout stomach. PCR for full-length genomic DNA was performed using 500 ng genomic DNA as a template and Ex*Taq* DNA polymerase. GSPs that were used are as follows: cdna-s, 5′-GATATCAATGTCCAAGGTATATCTG-3′; and cdna-as, 5′-AAGGAAGCAGTGTTATTTTATTCA-3′. The amplification conditions were 94 C for 2 min, and 30 cycles of 94 C for 1 min, 53 C for 30 sec, and 72 C for 6 min, and a final extension for 3 min at 72 C. A product of approximately 3.2 kbp was amplified and subcloned using the TOPO XL PCR Cloning Kit (pCR-XL-TOPO vector, Invitrogen). The nucleotide sequence was determined as described above.

Gene expression analysis by quantitative RT-PCR

Total RNA of brain, hypothalamus, heart, stomach, pyloric appendage, intestinal tracts, liver, spleen, kidney, head kidney, and gill was extracted separately from four individuals to examine the variation in mRNA levels. First-strand DNA was synthesized from 5 μg of DNase

TABLE 1. Expected molecular forms of isolated ghrelins from rainbow trout stomach

Groups	Peaks	Mass [M + H]	Expected molecular form	Yields (pmol)
A	1	2264.1	GSS(C8:0)FLSPSQKPQGKGKPPRVG-OH	5
	2	2288.4	GSS(C10:2)FLSPSQKPQGKGKPPRVG-OH	33
B	3	2291.1	GSS(C10:1)FLSPSQKPQGKGKPPRVG-OH	5
C	4	2204.8	GSS(C8:1)FLSPSQKPQGKGKPPRV-amide	7
	5	2648.2	GSS(C8:0)FLSPSQKPQVRQGKGKPPRVG-OH	2
	6	2672.3	GSS(C10:2)FLSPSQKPQVRQGKGKPPRVG-OH	7
D	7	2206.6	GSS(C8:0)FLSPSQKPQGKGKPPRV-amide	16
	8	2230.7	GSS(C10:2)FLSPSQKPQGKGKPPRV-amide	54
E	9	2590.0	GSS(C8:0)FLSPSQKPQVRQGKGKPPRV-amide	4
	10	2613.9	GSS(C10:1)FLSPSQKPQVRQGKGKPPRV-amide	8
	11	2613.7	GSS(C10:1)FLSPSQKPQVRQGKGKPPRV-amide	5
	12	2235.0	GSS(C10:0)FLSPSQKPQGKGKPPRV-amide	2
F	13	2232.5	GSS(C10:1)FLSPSQKPQGKGKPPRV-amide	9
G	14	2616.1	GSS(C10:1)FLSPSQKPQVRQGKGKPPRV-amide	1.5

I (Invitrogen)-treated total RNA using the Superscript II RT (Invitrogen) and random primer at 25 C for 10 min followed by 42 C for 1 h. Synthesized cDNA was cleaned up with phenol-chloroform treatment followed by ethanol precipitation. PCR was performed using the HotStarTaq Master Mix Kit (QIAGEN GmbH, Hilden, Germany) with a sense primer (5′-ACTGATGCTGTGTACTCTGG-3′) and an antisense primer (5′-CACCATACTCCTGGAACTCC-3′). The amplification reaction was performed with 95 C for 15 min, subsequent 35 cycles at 95 C for 30 sec, 57 C for 30 sec, and 72 C for 1 min, and a final extension for 3 min at 72 C. Amplicon of 248 bp was expected. As an internal control, a 369 bp of 18S ribosomal RNA fragment (DDBJ/EMBL/GenBank accession no. AF243428) was amplified with a sense primer (5′-TTAGTTGGTGGAGCGATTTGT-3′) and an antisense primer (5′-AGTGGCGACGGGCGGTGTGTA-3′) following reaction conditions: 95 C for 15 min, subsequent 16 cycles at 95 C for 30 sec, 57 C for 30 sec, and 72 C for 1 min, and a final extension for 3 min at 72 C. Amplified products were electrophoresed on a 2% (wt/vol) agarose, and incorporated ethidium bromide was visualized with a FLA2000 (Fuji Photo Film Co. Ltd., Kanagawa, Japan). In addition, quantitative, real-time RT-PCR analysis was performed by the LightCycler system (Roche Applied Science, Mannheim, Germany) using a QutantiTect SYBR Green PCR kit (QIAGEN GmbH) and above primer sets for each ghrelin or 18S ribosomal RNA.

GH-releasing activity of ghrelin in rats

Octanoylated rt ghrelin and des-VRQ-rt ghrelin were synthesized at the Daiichi Suntory Pharma Co., Ltd., Institute for Medicinal Research and Development, as described previously (23), and were used for some biological experiments described below.

Male Sprague Dawley rats, weighing 250–280 g, were cannulated in the femoral artery and vein under pentobarbital sodium anesthesia. After sampling untreated blood (time 0), 2 nmol/250 g body weight of either des-VRQ-rt ghrelin 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) 5, 10, 15, 20, 30, and 60 min after injection. GH concentration in plasma was measured using a rat GH enzyme-immunoassay kit (Amersham Bioscience KK). Data were analyzed by two-way ANOVA to evaluate effects of time or time *vs.* ghrelin species.

In vivo effect of trout ghrelin on the secretion of pituitary hormones in rainbow trout

Immature rainbow trout, weighing about 100 g, were obtained from a commercial supplier in Iwate Prefecture (Kamaishi-city, Japan) fisheries experimental station. Trout were maintained in an outdoor tank with running fresh water at 10–16 C under natural photoperiod for more than 2 wk until use without feeding. This experiment was conducted between 1000 and 1600 h. Intraperitoneal injection of rt ghrelin or des-VRQ-rt ghrelin was conducted according to the method of Moriyama *et al.* (24). Fish were lightly anesthetized with 0.01% 2-phenoxyethanol, and the ghrelins were ip injected with either 25 or 250 ng/g body weight. Control fish received 0.9% NaCl solution only (1 μl/g body weight). Blood samples were collected from the caudal vessels at 0.5, 1, and 3 h after injection or at 1, 3, and 6 h after injection as indicated (n = 5 at each time point). Body weights and sex of fish used were: short-term experiments, 74.4 ± 4.8 g (mean ± SD), 27 males and 23 females; moderate-term experiments, 89.1 ± 5.4 g, 25 males and 25 females.

Effects of trout ghrelin on the secretion of pituitary hormones in trout pituitary gland

Immature rainbow trout, weighing about 100 g, were used in this study. After anesthesia with 0.1% 2-phenoxyethanol, trout were decapitated, and the pituitary gland was dissected out. Pituitaries were rinsed once with basic culture medium (BCM; Eagle's MEM with Earle's salts (Life Technologies, Inc., Gaithersburg, MD) with kanamycin [60 mg/ml (pH 7.1–7.4)] with sodium bicarbonate (2.2 g /liter) of double concentration of kanamycin (120 mg/ml) for disinfection, then rinsed once with BCM, and placed in a 96-well plate with one pituitary per well containing 200 μl/well of BCM. The pituitary glands were precultured for 48 h at 11 C. After preincubation, media were removed, and 200 μl/well of rt ghrelin or des-VRQ-rt ghrelin diluted with fresh BCM were replaced at a concentration from 0.1 to 100 nM (n = 5 in each dose). After a 24-h incubation, cultured media were collected and stored at −30 C until RIA.

Hormone measurements

Concentrations of GH, PRL, and SL in plasma were measured by homologous RIAs according to the methods of Bolton *et al.* (25), Hirano

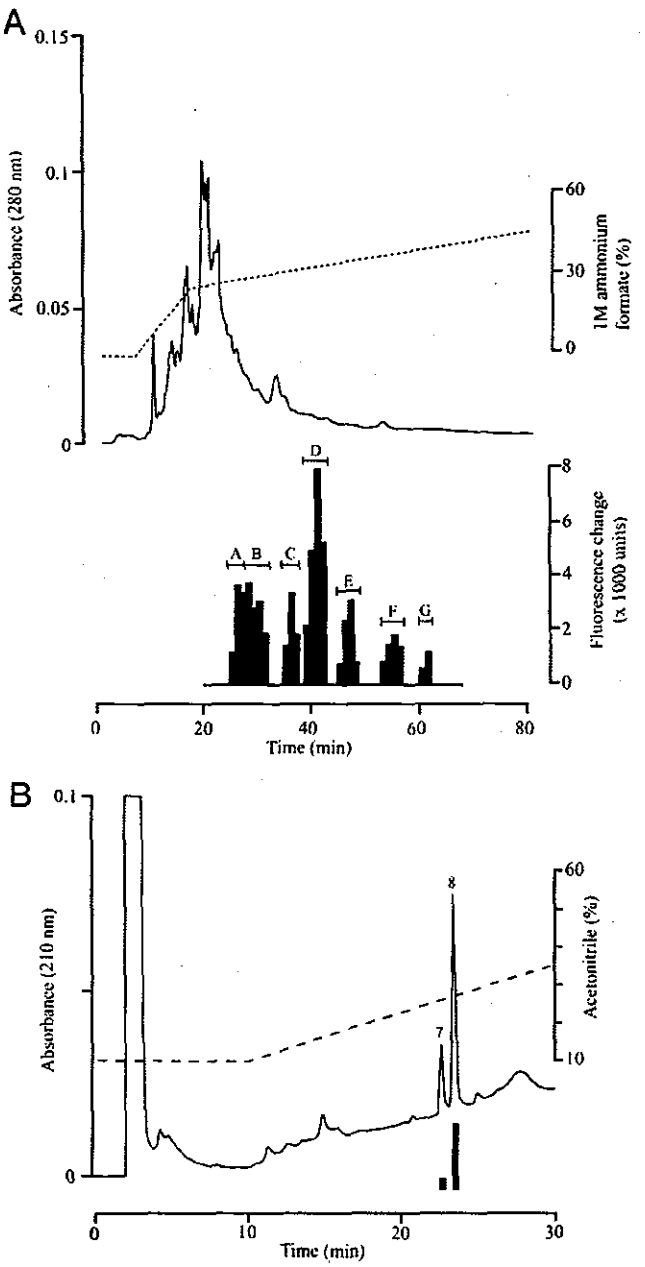

FIG. 1. Purification of rt ghrelin from stomach extract. *Black bars* indicate the fluorescence changes in $[Ca^{2+}]_i$ in CHO-GHSR62 cells. A, CM-cation exchange HPLC (pH 4.8) of SP-III fraction of stomach extract. Each active fraction (A–G) was separately purified by an antirat ghrelin[1–11] IgG immuno-affinity chromatography. B, Final purification of active fraction D in CM-HPLC by reverse-phase RP-HPLC. Two active ghrelins (Table 1, peaks 7 and 8) were isolated from this fraction.

et al. (26), and Kakizawa *et al.* (27) with some modifications. Antibody-bound hormone complexes were precipitated with 100 μl of 0.25% PANSORBIN Cells (Carbiochem, San Diego, CA) suspended in the RIA buffer, and the radioactivity of precipitate was counted by a γ counter (Packard Instrument Co., COBRA Quantum, Meriden, CT). The lowest detectable level of GH was 0.78 ng/ml, that of PRL was 0.37 ng/ml, and that of SL was 0.64 ng/ml. Intraassay coefficient of variation was less than 4%.

Statistics

All data are expressed as the mean ± SE. Group comparisons were performed using one-way ANOVA, followed by Fisher's least significant difference test. Differences of $P < 0.05$ were considered to be significant.

Results

Purification of rt ghrelin

Seven groups of ghrelin activity were identified by ion exchange HPLC (pH 4.8) for the SP-III fraction (Fig. 1A). Each active group was purified by passage over an antirat ghrelin[1–11] IgG immuno-affinity column, and followed by two different rounds of RP-HPLC. Figure 1B shows the final isolation of the major rt ghrelin from group D in Fig. 1A. Indeed, two active peaks were isolated in this group. We were able to isolate 14 active peptides from seven groups in carboxymethyl (CM)-HPLC (Table 1). Peptide sequence analysis revealed that peptides of two different lengths were isolated; one sequence was GSXFLSPSQKPQVRQGKGKPP,

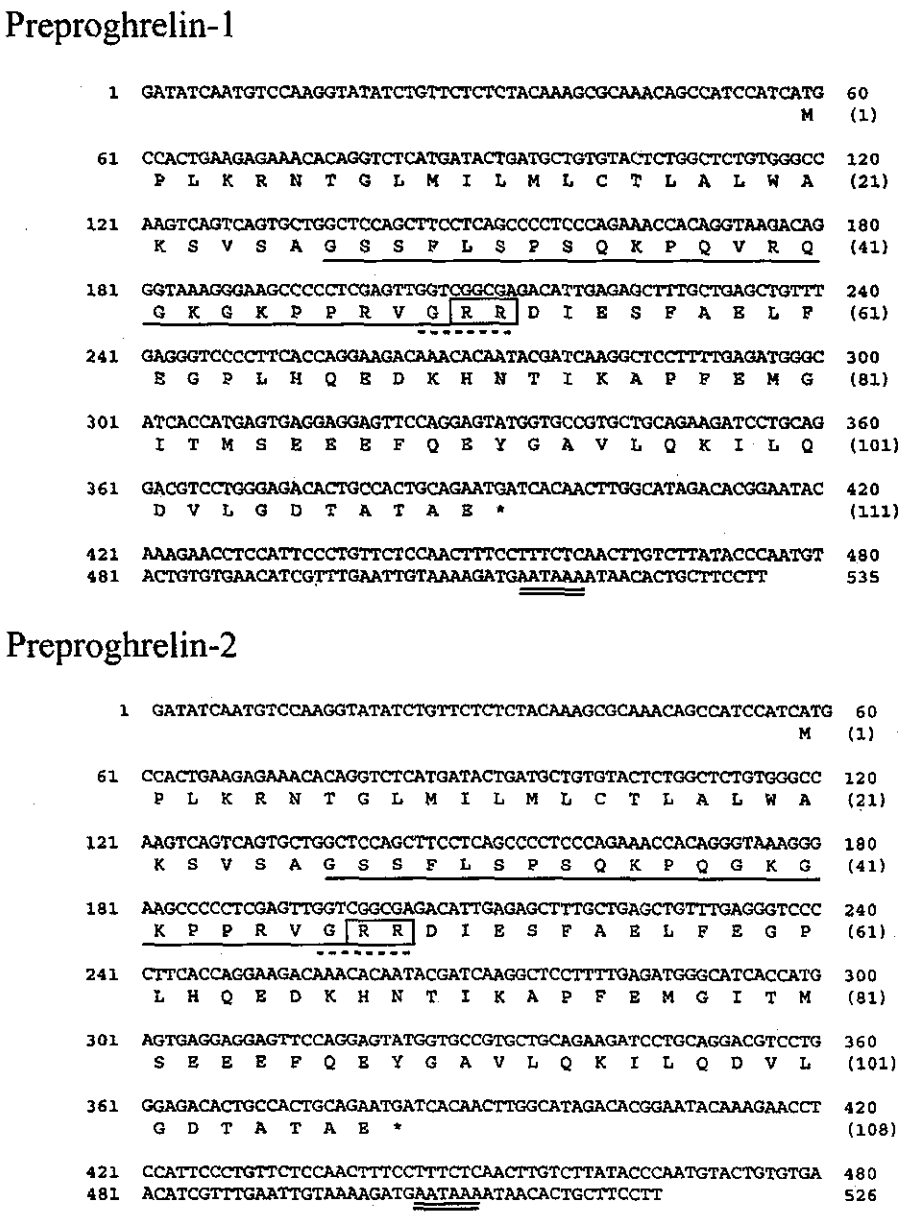

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the rt ghrelin cDNA. The rt ghrelin cDNA was 535 bp (preproghrelin-1) and 526 bp (preproghrelin-2) in length. The mature sequence of rt ghrelin is *underlined*. The C-terminal amidation signal is shown with a *dashed line*. A typical dibasic processing sequence is *boxed*. *Doubled underline* indicates the polyadenylation signal (AATAAA). The nucleotide sequence for the rt ghrelin precursor has been deposited in the DDBJ/EMBL/GenBank databases with the accession no. AB096919 (preproghrelin-1) and AB101443 (preproghrelin-2).

and the other one was GSXFLSPSQKPQGKGKPP (X was unidentified by the sequencer because of acyl modification). The latter form has a three amino acid deletion (VRQ) at positions 13 to 15 of the former. From the sequence homology compared with other ghrelins, we determined these isolated peptides to be rt ghrelins.

Cloning of rt ghrelin cDNA

cDNA encoding preproghrelin was isolated from stomach mRNA. cDNAs of two different lengths were isolated; both were identical in 5′-untranslated region and 3′-untranslated region length (57 bp and 142 bp, respectively), but differed in length of coding region between 336 bp (DDBJ/EMBL/GenBank accession no. AB096919) and 327 bp (DDBJ/EMBL/GenBank accession no. AB101443). Long-type cDNA was obtained only in the case that RT reaction was conducted using a random primer and Thermoscript RT enzyme. The deduced amino acid sequence of the coding regions of the two cDNAs indicated that the preproghrelin-1 and preproghrelin-2 are composed of 111 and 108 amino acids, respectively (Fig. 2). Figure 3 shows alignments of ghrelin precursors. The amino acid sequence of preproghrelin-1 in rainbow trout was 49, 57, and 47% identical to that of tilapia, eel, and goldfish, respectively (Fig. 3A). The identity to that of bullfrog, chicken, and human was 25, 40, and 33%, respectively (Fig. 3B). From this cDNA analysis, the unidentified third amino acid was determined to be serine, as seen in other species except bullfrog, in which this residue is threonine (20). An amidation signal Gly-Arg-Arg present in eel and goldfish ghrelin precursors followed at the C-terminal end of the mature ghrelin. It is predicted that posttranslational processing of the two isolated mRNAs will yield 23- or 20-amino acid peptides with an amidated C-terminal. Because a C-terminal amidated peptide is hard to read in a peptide sequencer, these results are in agreement with the results of peptide purification.

Structural determination

To determine the molecular weights of the rt ghrelins, isolated peptides were analyzed by MOLDI-TOF mass spectrometry. Table 1 shows the actual measured molecular mass of the isolated peptides, the expected molecular forms, and the isolated yields. From the molecular masses in addition to the analyses of peptide sequence, four types of amino acid sequence (20, 21, 23, and 24 amino acids) and two types of the C-terminal end (amidated and nonamidated forms) were

A

```
Rainbow trout-1      1 M-PLKRNTGLMILMLCTLALW-AKSVSAGSSFLSPSQKPQVRQGKGKPPRVGRRDIESFA   58
Rainbow trout-2      1 M-PLKRNTGLMILMLCTLALW-AKSVSAGSSFLSPSQKPQ---GKGKPPRVGRRDIESFA   58
Tilapia              1 MLLKRNTCLLAF-LLCSLTLWC-KSTSAGSSFLSPSQKPQNKV---KSSRIG-R-QAMEE   53
Eel                  1 MRQMKR-TAYIILLVCVLALW-MDSVQAGSSFLSPSQRPQGKD-K-KPPRVGRRDSDGIL   56
Goldfish             1 MPLRRRASHM-FVLLCALSL-CVESVKGGTSFLSPAQKPQGR----RPPRMGRRD--VAE   52
                         *              * * *    *  * ***** * **        * * *

Rainbow trout-1     59 ELF-EGPLHQED-KHNTIKAPFEMGITMSEEEFQEYGAVLQKILQDVLGDTATAE-----   111
Rainbow trout-2     59 ELF-EGPLHQED-KHNTIKAPFEMGITMSEEEFQEYGAVLQKILQDVLGDTATAE-----   108
Tilapia             54 P-N--QA--NED-KTITLSAPFEIGVTLRAEDLADYIVELQEIVQRLLGNTETAERPSPR   107
Eel                 57 DLFMRPPLQDEDIRHITFNTPFEIGITMTEELFQQYGEVMQKIMQDLLMDTPAKE-----   111
Goldfish            53 P-EI-PVI-KED-DQFMMSAPFELSVSLSEAEYEKYGPVLQKVLVNLLGDS-PLEF----   103
                          **       ***          *   *     *     *
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B

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Rainbow trout-1      1 MPLKRNTGLM-ILMLCTLALWAKSVSAGSSFLSP-SQKPQVRQGKG--KP-PRVGRRDIE   55
Rainbow trout-2      1 MPLKRNTGLM-ILMLCTLALWAKSVSAGSSFLSP-SQKPQ---GKG--KP-PRVGRRDIE   52
Bullfrog             1 MNFGKAAIFGVVL-FCLL--WTEGAQAGLTFLSPADMQKIAERQSQNKLRHGNMNRR---   54
Chicken              1 MFL-RVI-LLGILLLSILGT-ETAL-AGSSFLSP-TYK-NIQQQKDTRKPTARLHRRGTE   54
Human                1 MP-SPGTVCSLLL-LGML--WLDLAMAGSSFLSP-EHQRVQQRK-ESKKPPAKLQPRALA   54
                         *         *   *       **  ****                      *

Rainbow trout-1     56 SFAELFEGPLHQEDKHN-TIK---APFEMGITMSE-EEFQEYG-AVLQKILQDVLGDTA-   108
Rainbow trout-2     53 SFAELFEGPLHQEDKHN-TIK---APFEMGITMSE-EEFQEYG-AVLQKILQDVLGDTA-   105
Bullfrog            55 G-VEDDLAGEEIGVTPPLDMKMTQEQFQKQ-R-AAVQDFLYSSLLSLGS-VQDT--EDKN   108
Chicken             55 SFWDT-DETEGEDDNNSVDIKF-NVPFEIGVKITE-REYQEYG-QALEKMLQDILAENAE   110
Human               55 GWLRPEDGGQAEGAEDELEVRF-NAPFDVGIKLSGVQ-YQQHS-QALGKFLQDILWEEAK   111
                                             *                 *    **

Rainbow trout-1    109 TAE---                                                      111
Rainbow trout-2    109 TAE---                                                      108
Bullfrog           109 ENPQSQ                                                     114
Chicken            111 ETQTKS                                                     116
Human              112 EAPADK                                                     117
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FIG. 3. Comparison of amino acid sequence of preproghrelin. Mature ghrelin was *boxed*. *Asterisks* indicate identical amino acids across all species. Alignment in fish species (A) and in bullfrog, chicken, and human (B). Amino acid sequences are available from the DDBJ/EMBL/GenBank databases (accession no. AB062427, eel; AF454389, goldfish; AB077764, tilapia; AB058510, bullfrog; AB075215, chicken; and AB029434, human).

predicted. Both 21- and 24-amino acid ghrelins are produced by glycine retention before dibasic processing signal without C-terminal amidation. We concluded that all isolated peptides were rt ghrelin and its isoforms. The major rt ghrelin, with isolated yields of 54 pmol, was a peptide isolated from group D, peak 8. The expected peptide sequence was GSSFLSPSQKPQGKGKPPRV-amide with unsaturated decanoic acid (C10:2). Octanoylated and decanoylated ghrelins, which have primarily been identified in other animal species, were also identified, but those yields were relatively low (Table 1, peaks 5, 7, 9, and 12). We designated the 23-amino acid peptide (GSSFLSPSQKPQVRQGKGKPPRV-amide) as a basic form; rt ghrelin and rt ghrelin that deleted three amino acids of $V^{13}R^{14}Q^{15}$ were designated as des-VRQ-rt ghrelin (GSSFLSPSQKPQGKGKPPRV-amide). Furthermore, ghrelins composed of 24 amino acids (GSSFLSPSQKPQVRQGKGKPPRVG-OH) and 21 amino acids (GSSFLSPSQKPQGKGKPPRVG-OH) were designated as rt ghrelin-Gly and des-VRQ-rt ghrelin-Gly, respectively.

Cloning of genomic DNA of trout ghrelin

It was observed that the position of the deleted amino acids ($V^{13}R^{14}Q^{15}$) is similar to that seen in des-Gln^{14}-rat ghrelin, suggesting that alternative splicing of the ghrelin gene also occurred in the rt ghrelin gene. We cloned the rt ghrelin gene containing full-length ghrelin cDNA. The rt ghrelin gene is 3270 bp in length and is comprised of five exons and four introns (Fig. 4A; DDBJ/EMBL/GenBank accession no. AB100839). The overall genomic organization is similar to that of the mouse and rat ghrelin genes. Figure 4B shows a portion of the nucleotide sequence of the rt ghrelin gene in exon 2, intron 2, and bounded exon 3. This exon-intron boundary is consistent with the AG-GT rule of the splicing, but two consensus sequences for 5′ splice sites exist within 15 bases of the initial sequence of intron 2. These two AG nucleotides may be used as a splicing acceptor site at the 3′-end of intron 2. From this hypothesis, two lengths of ghrelin mRNA, 535 bp and 526 bp, would be produced.

Expression of ghrelin mRNA in rainbow trout tissues

Figure 5 shows expression pattern of mRNA for ghrelin and 18S ribosomal RNA from four different individuals in a representative amplification condition. High levels of ghrelin mRNA were observed in the stomach in all fishes. A detailed mRNA expression analysis based on quantitative PCR also demonstrated highest mRNA levels in the stomach, followed by moderate expression levels in the central nervous system and intestinal tracts (Table 2).

Effects of rt ghrelin on rat GHS-R

We examined the ability of synthesized rt ghrelin and des-VRQ-rt ghrelin to stimulate the rat GHS-R using GHSR62 cells. Both octanoylated rt ghrelin and des-VRQ-rt ghrelin increased $[Ca^{2+}]_i$ in a dose-dependent manner with similar potency to rat ghrelin, a full agonist for the GHS-R (Fig. 6A). No differences were seen among three ghrelins. Next, we examined the effect of ghrelin on the release of GH in the rat. Only des-VRQ-rt ghrelin was examined because the similar potency was shown between the two rt ghrelins in the rat GHS-R assay. Plasma GH levels were increased by a bolus iv injection of des-VRQ-rt ghrelin or rat ghrelin with similar kinetics and to the same extent (Fig. 6B).

Intraperitoneal injection of rt ghrelin into rainbow trout

Plasma GH levels increased 30 min after ip injection of des-VRQ-rt ghrelin (Fig. 7A). The increased levels of GH

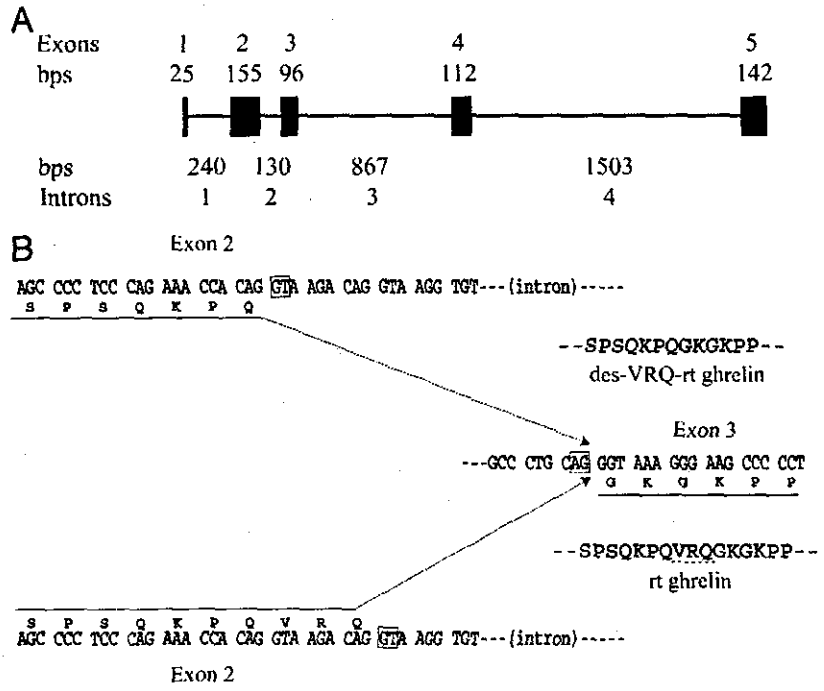

FIG. 4. Schematic representation of the rt ghrelin gene (A) and the model for splicing junction (B). A, Exons are shown by *boxes*. The sequence of the gene is available from the DDBJ/EMBL/GenBank databases (accession no. AB100839). B, The genomic sequence of the exon-intron boundaries of intron 2 of the ghrelin gene is shown. The splicing signals, GTs for the 5′-side and AG for the 3′-side of intron, are *boxed*.

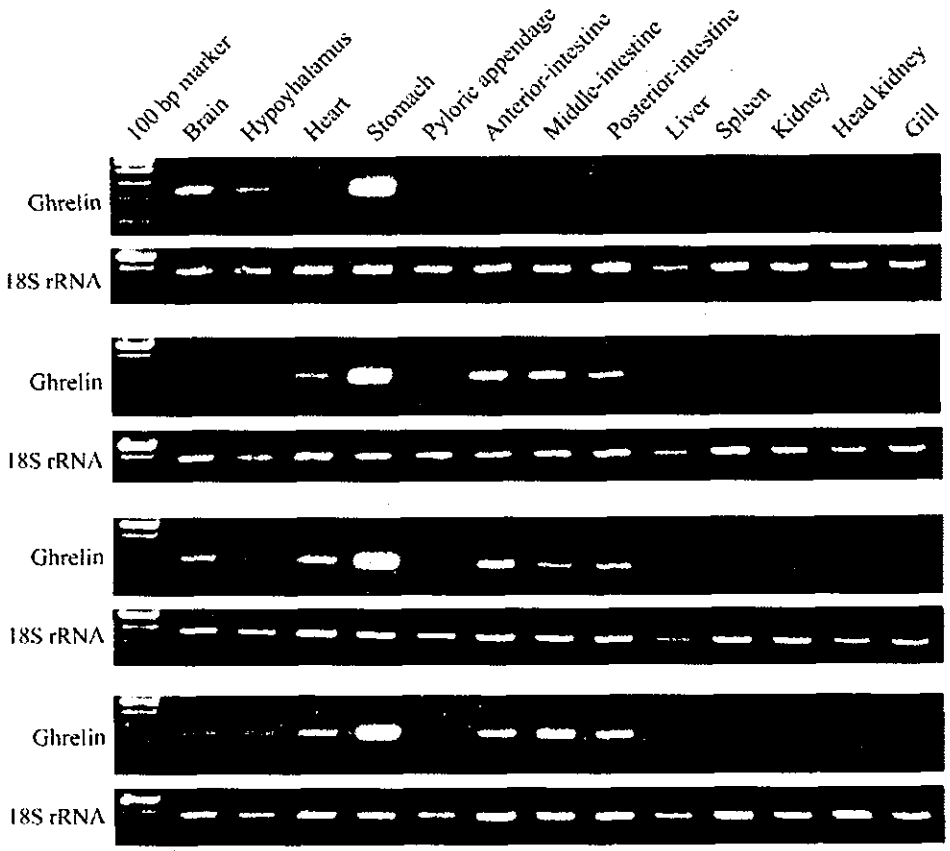

FIG. 5. Gene expression analysis of the rt ghrelin by RT-PCR. Gene expression pattern of ghrelin was examined in four different individuals. PCR product of each ghrelin and 18S ribosomal RNA was obtained with the amplification of 35 and 16 cycles, respectively. Detailed mRNA levels by quantitative PCR analysis are shown in Table 2.

were maximal 1 h after injection and gradually decreased to basal levels 6 h after injection (Fig. 7B). Conversely, no changes in plasma PRL (Fig. 8, A and B) and SL (Fig. 8, C and D) were observed at each dose during the 6-h experimental period. Octanoylated rt ghrelin demonstrated similar effects (data not shown).

In vitro study using trout pituitary

We examined whether ghrelin isolated in rainbow trout stimulates the release of adenohypophyseal hormones using organ-cultured rainbow trout pituitary. In this experiment, we examined only des-VRQ-rt ghrelin because similar effects were seen between rt ghrelin and des-VRQ-rt ghrelin in *in vivo* study (Figs. 7 and 8). A significant increase in the release of GH was observed during a 24-h incubation at a dose of 1 nM (Fig. 9). GH release was maximal after treatment with 10 nM; GH release was inversely related to ghrelin dose at 100 nM. In these samples, no significant changes in the release of PRL and SL were observed as demonstrated in *in vivo* study (Fig. 9).

Discussion

The present study demonstrated that ghrelin is present in rainbow trout and that it can alter GH levels *in vivo* and *in vitro*. This is in agreement with previous *in vivo* or *in vitro* studies that demonstrated that ip injected peptidyl GHS, KP-102, stimulates the release of GH in the tilapia (16), or rat, eel, and tilapia ghrelins stimulate the release of GH from the tilapia pituitary (18, 19, 22). Furthermore, we show for the first time in fish that peripheral administration of ghrelin stimulates the release of GH. The effects of ghrelin were specific for the release of GH but not that of PRL and SL both *in vivo* and *in vitro*. This differs from previous observations in the tilapia pituitary (22). It is possible that GHS-R is not present in PRL and SL cells in rainbow trout. It needs to be tested further for other aspects, for example PRL or SL synthesis.

Acyl modification

The major form of acyl modification in all the known mammalian and nonmammalian ghrelins is *n*-octanoic acid or *n*-decanoic acid (1, 18–21, 28). In the present study, saturated octanoylated and decanoylated ghrelins were isolated, but the amounts were relatively low. Most of the isolated ghrelin in rainbow trout was modified by unsaturated *n*-decanoic acid. Additional forms of ghrelin were also isolated with other types of acyl modifications including unsaturated octanoylated forms. In purified chicken ghrelin, several different types of acyl modifications were also observed (Kaiya, H., unpublished observations). In the bull-

TABLE 2. *Quantitative PCR analyses of ghrelin and 18S ribosomal RNA*

Sample	Specimens				Mean	SD
	1	2	3	4		
Ghrelin copies (raw data)						
Brain	1063.0	261.9	1374.0	868.0	891.7	468.7
Hypothalamus	412.7	551.9	856.5	972.8	698.5	260.4
Heart	146.6	929.8	1953.0	1552.0	1145.4	787.7
Stomach	194800.0	782300.0	483900.0	225200.0	421550.0	273250.2
Pyloric appendage	130.0	97.2	261.5	238.5	181.8	80.4
Anterior intestine	135.1	5751.0	2134.0	1856.0	2469.0	2359.8
Middle intestine	54.0	4952.0	949.7	5300.0	2813.9	2698.4
Posterior intestine	72.2	1697.0	1111.0	2209.0	1272.3	917.3
Liver	27.7	51.9	99.4	120.9	75.0	42.7
Spleen	157.1	213.4	234.3	239.4	211.1	37.7
Kidney	50.2	141.9	814.0	130.5	284.2	355.6
Heard kidney	90.7	107.1	435.6	286.5	230.0	163.3
Gill	124.9	65.8	357.0	180.7	182.1	125.7
18S rRNA (×1E + 8) copies (raw data)						
Brain	0.48	0.60	0.48	0.99	0.64	0.24
Hypothalamus	0.37	0.44	0.39	0.48	0.42	0.05
Heart	1.40	1.30	2.03	2.08	1.70	0.41
Stomach	2.04	0.95	1.70	1.08	1.44	0.52
Pyloric appendage	0.71	0.65	0.83	0.78	0.74	0.08
Anterior intestine	0.77	0.84	1.23	1.41	1.07	0.31
Middle intestine	0.95	1.91	1.08	1.48	1.35	0.43
Posterior intestine	2.67	2.39	0.88	2.11	2.01	0.79
Liver	0.25	0.43	0.29	0.29	0.32	0.08
Spleen	1.76	2.82	1.65	2.01	2.06	0.53
Kidney	1.35	1.51	1.25	0.80	1.23	0.30
Heard kidney	0.52	0.90	0.61	2.78	1.20	1.06
Gill	0.91	1.60	1.16	0.80	1.12	0.36
Ghrelin normalized with 18S rRNA (1E + 8)						
Brain	2201.7	433.0	2891.4	874.7	1600.2	1142.8
Hypothalamus	1118.4	1255.5	2173.3	2048.0	1648.8	538.7
Heart	104.8	716.9	961.6	747.6	632.7	368.4
Stomach	95443.4	824428.3	284312.6	209488.4	353418.2	323467.1
Pyloric appendage	182.9	149.4	315.3	305.5	238.3	84.5
Anterior intestine	175.7	6814.8	1729.3	1312.6	2508.1	2945.3
Middle intestine	56.9	2595.4	878.5	3583.5	1778.6	1602.0
Posterior intestine	27.0	710.3	1267.4	1049.4	763.5	541.9
Liver	108.9	120.4	346.5	416.0	248.0	156.6
Spleen	89.2	75.7	142.1	118.9	106.5	29.8
Kidney	37.2	93.7	653.8	162.2	236.7	282.7
Heard kidney	174.7	118.8	715.4	103.2	278.0	293.2
Gill	136.9	41.1	308.3	226.9	178.3	115.2

frog, eel, and chicken, octanoylated and decanoylated ghrelins were isolated in similar amounts (18, 20, 21), but decanoylated ghrelin was the major form in the tilapia (19). Several types of posttranscriptional modification have been reported in human ghrelin (28). The mechanisms governing the acylation of ghrelin are still unknown, but a condition of feeding may influence the type and amount of acyl modification in the ghrelin polypeptide.

Peptide structure

Peptide sequencing led to the identification of two ghrelin isoforms that differ in the deletion of three amino acids ($Q^{13}V^{14}R^{15}$). This was confirmed from the predicted peptide sequence resulting from translation of two isolated cDNAs; we have designated the shortened form as preproghrelin-2. Analysis of the full-length ghrelin gene suggests that mRNAs of two different lengths are generated by alternative splicing of intron 2. Similar alternative splicing of the ghrelin gene has been reported in the production of rat des-Gln^{14}-ghrelin (29), but not in other fish species (18, 19). In the analysis of partial sequence of the goldfish ghrelin gene (17) and the eel ghrelin gene (Kaiya, H., unpublished observation), there was no tandem AG-GT sequence in intron 2 as seen in the rainbow trout gene. In a recent deposited ghrelin gene of the tilapia, *Oreochromis niloticus*, such a sequence is also not found (DDBJ/EMBL/GenBank accession no. AB104860; Parhar, I. S., unpublished observation). This is the first case to show the presence of ghrelin splice variants in fish.

Amide and nonamide structure

Rainbow trout ghrelin has an amide structure at its C terminus; a similar modification is seen in other fish including eel, goldfish, and tilapia, but not in mammalian, chicken, or bullfrog ghrelin (1, 17–21, 28). It is most likely that the C-terminal amide structure is a conserved characteristic in fish ghrelin. Amidated eel ghrelin did not potentiate the release of GH in rats *in vivo*, and this effect was the same as nonamidated rat ghrelin (18). Rat and tilapia ghrelins seem to have no difference in their ability to stimulate GH release

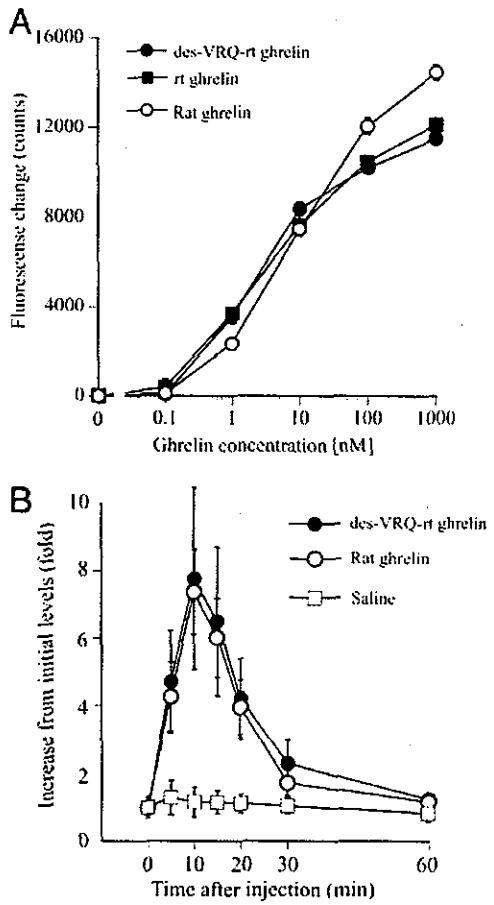

FIG. 6. Biological activity of rt ghrelin in rat models. A, Dose-response relationships of changes in $[Ca^{2+}]_i$ concentrations after treatment of CHO-GHSR62 cells with octanoylated rt ghrelin, octanoylated des-VRQ-rt ghrelin, and rat ghrelin. The maximum value of fluorescence changes was used for data calculation. Values represent means ± SEM (n = 3). B, Time-course of changes in plasma GH levels after iv injection of octanoylatd des-VRQ-rt ghrelin or rat ghrelin into male Sprague Dawley rats. Due to variation in the initial baseline levels, values (means ± SEM; n = 5) are expressed in terms of the ratio of each time point to the initial level (des-VRQ-rt ghrelin, 141.7 ± 8.8 ng/ml; rat ghrelin, 118.8 ± 19.1 ng/ml; saline, 201.9 ± 62.4 ng/ml).

in the tilapia (19, 22). To date, the importance of the amide structure in ghrelin remains unclear.

Nonamidated rt ghrelin and des-VRQ-rt ghrelin, designated as rt ghrelin-Gly and des-VRQ-rt ghrelin-Gly, were isolated in this study. Although a glycine residue within the amidation signal originally contributes to the formation of the amide structure, this residue was found at the C terminus of nonamidated ghrelins. This type of ghrelin molecule was not isolated in the previous purification of eel or tilapia ghrelin. There appears to be a species-specific amidation of ghrelin in the stomach of fish. We did not examine their biological activities in rainbow trout. These findings need to be further studied for their biological significance, including possible different activities compared with amidated rt ghrelin.

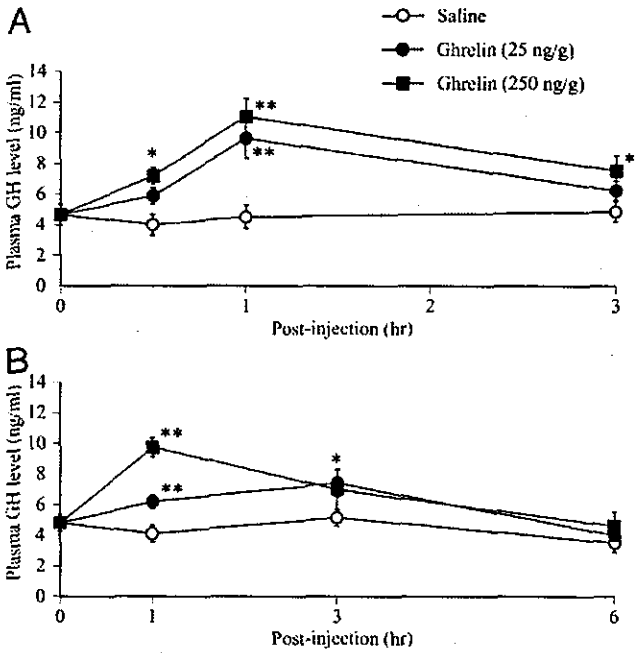

FIG. 7. Changes in plasma levels of GH after ip injection of rt ghrelin. Changes seen 0.5–3 h after injection (A) and 1–6 h after injection (B) are presented. The data result from synthetic octanoylated des-VRQ-rt ghrelin. Similar changes were seen in the administration of rt ghrelin (data not shown). Significant difference is expressed by *, $P < 0.05$; **, $P < 0.01$, compared with the values of saline injection at each time point.

Gene structure

We determined the partial structure of the rt ghrelin gene. The overall gene length is 3720 bp and is similar to that of the mouse ghrelin gene (3748 bp) (30), but it is longer than that of the goldfish ghrelin gene (980 bp) (17). The rt ghrelin gene is organized with five exons and four introns. This arrangement is the same as the rat and mouse genes, but not the goldfish gene (17) and the recent reported tilapia ghrelin gene (Parhar, I. S., unpublished observation), which each have four exons and three introns. The composition is also similar to those of the rat and mouse genes, in which a noncoding 25-bp short exon 1 was found (30).

mRNA expression

Ghrelin is primarily expressed in the stomach in all species previously examined. Although goldfish lack a stomach, the intestine is the major site of ghrelin production (17). These findings suggest that ghrelin is primarily synthesized in gastrointestinal tracts in vertebrates. In the present study, high levels of ghrelin mRNA expression were observed in the stomach, consistent with findings in other species. Expression of ghrelin mRNA has also been detected throughout the intestinal tract of different species of fish; the local role of ghrelin in the intestine is not known. Ghrelin mRNA expression was also observed in the brain and hypothalamus. Central ghrelin, produced in the hypothalamic Arc in the rat, seems to participate in the regulation of GH secretion from the pituitary (8). In addition to GH regulation, centrally ad-

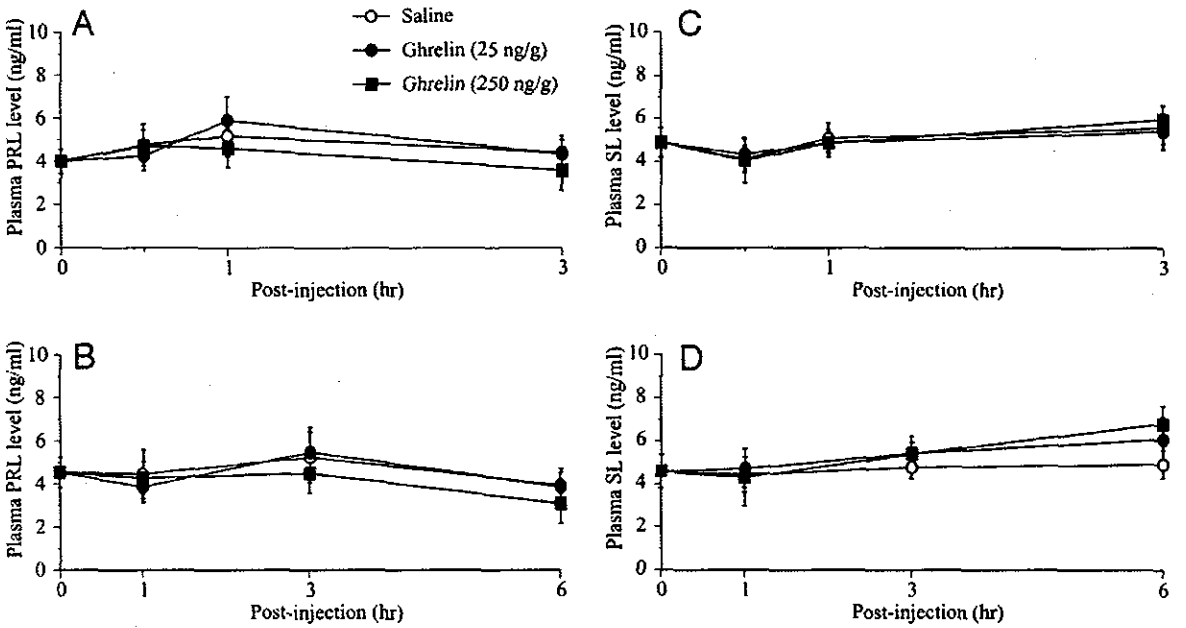

FIG. 8. Changes in plasma levels of PRL and SL after ip injection of rt ghrelin. Changes seen 0.5–3 h after injection (A and C) and 1–6 h after injection (B and D) are presented. The data result from synthetic octanoylated des-VRQ-rt ghrelin. Similar changes were seen in the administration of rt ghrelin (data not shown). No significant difference was seen compared with the values of saline injection at each time point.

ministered ghrelin modifies feeding behavior in rats (5, 31). In fish, intracerebroventricular injection of octanoylated goldfish ghrelin into the goldfish stimulated food intake (17). In addition to stomach-derived ghrelin, hypothalamic ghrelin may also regulate pituitary function and feeding in rainbow trout.

Quantitative PCR analysis clearly demonstrated that ghrelin mRNA, although the level was very low, was expressed in all tissues examined. Recent reports demonstrate the expression of ghrelin mRNA in the spleen of goldfish (17) or in the kidney and gills of the eel and tilapia (18, 19). Expression of ghrelin mRNA was demonstrated in the kidney, glomerulus, and renal cells of rodents (32) and in the kidney of humans (33). Autocrine or paracrine action of ghrelin is assumed, but a role(s) in these tissues and cells remains unclear. In fish, the kidney and gills are the major osmoregulatory organs, and the functions are governed by several endocrine or autocrine factors (34, 35). Ghrelin may play a role(s) in the functional regulation of these organs. A recent paper reported that ghrelin potently inhibited water drinking in eels acclimated in seawater (36), suggesting ghrelin may be related to osmoregulation in fish.

Bioactivity

Shepherd *et al.* (16) reported that, in the tilapia, ip injection of KP-102, a peptidyl-GHS, stimulated the release of GH after 6 h of injection. In contrast, plasma GH levels increased 30 min after injection of ghrelin with the same ip injection in the present study, and the increased levels returned to basal levels 6 h after injection. The release of GH in the rat occurs rapidly (within 5 min) after iv injection of ghrelin. The alteration of GH levels within 30 min after injection did not examine, but we have presented similar evidence here in the rainbow trout. Date *et al.* (11) have reported the involvement of the vagal nerve in conveying a peripheral ghrelin signal to the brain in the rat. A similar neural pathway may exist in rainbow trout.

The present study demonstrated that rt ghrelin stimulates the release of GH in rainbow trout when administered to explanted rainbow trout pituitaries. Similarly, a recent paper reported that tilapia ghrelin stimulated the release of GH from their pituitaries (19). These data indicate that ghrelin plays a role as a GHS in fish. In cultured whole pituitary of rainbow trout, a significant increase in the release of GH was observed at 1 nM after 24-h incubation. In other studies, a relatively short period of time for incubation was sufficient to induce the release of GH by ghrelin from the whole pituitary (18, 19, 22), but ghrelin could still stimulate the release of GH after 24-h incubation as shown by Riley *et al.* (22). Our results are consistent with these findings and suggest a direct effect of ghrelin on rainbow trout pituitary.

In addition to GH regulation, PRL secretion was potently stimulated by rat, eel, and tilapia ghrelins in the cultured tilapia pituitary (18, 19, 22). Similar effects on PRL secretion have been observed in human *in vivo* and bullfrog *in vitro* (9, 20, 37). In contrast, no effects on the release of PRL and another pituitary hormone, SL, were observed in the present *in vivo* and *in vitro* studies. It is possible that GHS-R is not present in PRL and SL cells in rainbow trout. For clarifying this issue, further aspects need to be examined, for example, PRL or SL synthesis or expression of ghrelin receptor in these cells.

In summary, ghrelin mRNA is found in high levels in the stomach of rainbow trout, and ghrelin can be isolated from the stomach. Four isoforms of ghrelin are produced through the amidation of alternatively spliced ghrelin gene products.

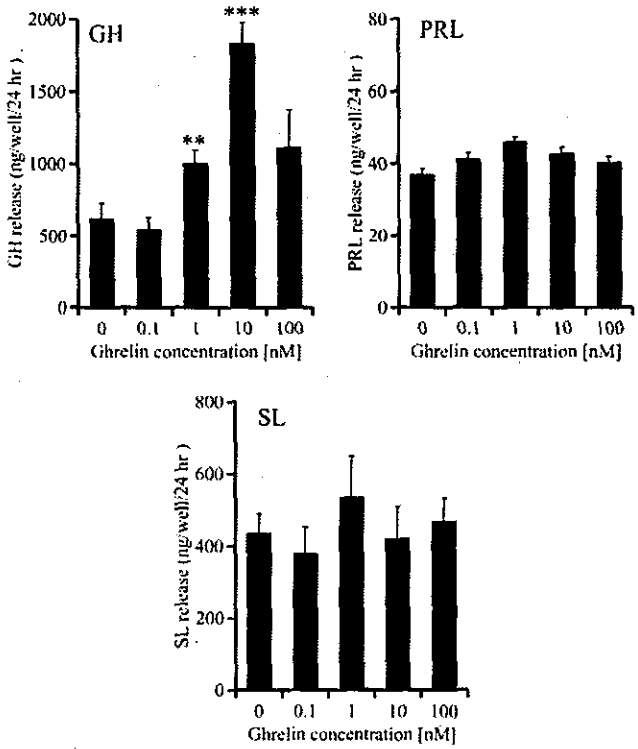

FIG. 9. Effects of rt ghrelin on the release of GH, PRL, and SL from the rainbow trout pituitary *in vitro*. Synthetic octanoylated des-VRQ-rt ghrelin was used for this assay. Values are expressed as mean ± SEM (n = 5). Significant difference is expressed by **, $P < 0.01$; ***, $P < 0.001$.

Furthermore, multiple types of acylation were observed, but all ghrelins were biologically active, as demonstrated by the assay using GHSR62 cells expressed rat GHS-R. The rt ghrelin gene is comprised of five exons and four introns, an arrangement identical to the rat and mouse ghrelin genes. Rainbow trout ghrelin stimulated the release of GH, but not PRL and SL both *in vivo* and *in vitro*. There are species-specific differences in the effects of ghrelin on the release of pituitary hormones in fish. Physiological functions of ghrelin in fish other than secretory regulation of pituitary hormone will be the next subject.

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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)FLSPSQRPQGKDKKPP RV-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.

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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 (Ser^3) 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,

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chicken ghrelin, which like mammals possesses a Ser^3, 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