Ghrelin, a novel 28-amino acid peptide with an n-octanoyl modification at Ser3, was isolated from rat stomach and found to be an endogenous ligand for the growth-hormone secretagogue receptor (GHS-R). This octanoyl modification is essential for ghrelin-induced GH release.

We report here the purification and identification of human ghrelin from the stomach, as well as structural analysis of the human ghrelin gene and quantitation of changes in plasma ghrelin concentration before and after gastrectomy. Human ghrelin was purified from the stomach by gel filtration and high performance liquid chromatography, using a ghrelin-specific radioimmunoassay and an intracellular calcium influx assay on a stable cell line expressing GHS-R to test the fractions. In the course of purification, we isolated human ghrelin of the expected size, as well as several other ghrelin-derived molecules. Classified into four groups by the type of acylation observed at Ser3, these peptides were found to be non-acylated, octanoylated (C8:0), decanoylated (C10:0), and possibly deoctanoylated (C10:1). All peptides found were either 27 or 28 amino acids in length, the former lacking the C-terminal Arg28, and are derived from the same ghrelin precursor through two alternative pathways. The major active form of human ghrelin is a 28-amino acid peptide octanoylated at Ser3, as was found for rat ghrelin. Synthetic octanoylated and decanoylated ghrelin produce intracellular calcium increases in GHS-R-expressing cells and stimulate GH release in rats to a similar degree. Both ghrelin and the ghrelin-derived molecules were found to be present in plasma as well as stomach tissue. Plasma levels of immunoreactive ghrelin after total gastrectomy in three patients were reduced to approximately half of their pre-gastrectomy values, after which they gradually increased. This suggests that the stomach is the major source of circulating ghrelin and that other tissues compensate for the loss of ghrelin production after gastrectomy.

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Structural Divergence of Human Ghrelin

IDENTIFICATION OF MULTIPLE GHRELIN-DERIVED MOLECULES PRODUCED BY POST-TRANSLATIONAL PROCESSING*

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Growth hormone (GH) secretion from the pituitary gland is regulated by two hypothalamic hormones, growth hormone-releasing hormone and somatostatin (1, 2). A third independent pathway responsible for regulation of GH release has recently emerged from studies of artificial GH secretagogues (GHSs) (3, 4). GHSs are synthetic compounds that are potent stimulators of pituitary GH release, acting through the GHS receptor (GHS-R) (5–7). Previously, we identified ghrelin, an endogenous ligand for GHS-R, from rat stomach (8). Ghrelin, a 28-amino acid peptide capable of stimulating GH release in vitro and in vivo, has a unique n-octanoyl modification at its third serine residue (Ser3), which is essential for this function (9–11). Subsequently, des-Gln14-ghrelin, also isolated from rat stomach, was identified as a second endogenous ligand for GHS-R (12). Des-Gln14-ghrelin is produced from the ghrelin gene by an alternative splicing mechanism and is also octanoylated at Ser3.

In the present study, we purified human ghrelin from the stomach, using a ghrelin-specific radioimmunoassay (RIA) (13) and an intracellular calcium influx assay on a stable cell line expressing GHS-R (8, 12). During the course of purification, we noticed several minor peptides with characteristics different from standard ghrelin that displayed ghrelin-like activity. We identified these stomach peptides as ghrelin-derived molecules and examined the levels of ghrelin as well as these ghrelin-derived molecules in human plasma.

The ghrelin gene is abundantly expressed in rat (8) and human (14) stomach, and in the rat, no other major sources of ghrelin production have been observed (13, 15). These results prompted us to question whether ghrelin should be drastically reduced following gastrectomy. To address this question, we examined the change in plasma levels of immunoreactive ghrelin (ir-ghrelin) in humans before and after total gastrectomy.

EXPERIMENTAL PROCEDURES

Radioimmunoassays for Ghrelin—RIAs specific for ghrelin were performed as previously described (13). In rabbits two types of polyclonal antibodies were raised against the N-terminal fragment (Gly1–Lys11) with O-n-octanoylation at Ser7) and the C-terminal fragment (Gln13–Arg28) of rat ghrelin. The RIA incubation mixture consisted of 100 µl of standard ghrelin or unknown sample, and 200 µl of antisemur diluted with RIA buffer (50 mM sodium phosphate buffer (pH 7.4), 0.5% bovine

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**§§§ The abbreviations used are: GH, growth hormone; GHS, growth hormone secretagogue; GHS-R, growth hormone secretagogue receptor; RIA, radioimmunoassay; ir, immunoreactive; CHO, Chinese hamster ovary; [Ca2+]i, intracellular calcium concentration; AcOH, acetic acid; CM, carboxymethyl; RP, reverse-phase; HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; N-RIA, N-terminal fragment of rat ghrelin (1–11); C-RIA, C-terminal fragment of rat ghrelin (13–28).

† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AB029434 and AB035700.
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**serum albumin, 0.5% Triton X-100, 80 mM NaCl, 25 mM Na2EDTA, A. O. and 0.05% NaN3 containing 0.5% normal rabbit serum. The anti-rat ghrelin-(1-11) or anti-rat ghrelin-(12-28) antisera were used at final dilutions of 1:6,000,000 and 1:20,000, respectively. After incubation for 12 h, 100 µl of [3H]-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 ghrelin. The anti-rat ghrelin-(1-11) antisera specifically recognized the n-octanoylated portion at Ser2 of ghrelin and did not recognize des-acyl ghrelin. The anti-rat ghrelin-(12-28) antisera equally recognized n-octanoyl and des-acyl ghrelin. In the following sections, the RIA system using antisera against the C-terminal fragment of rat ghrelin-(1-11) is termed N-RIA; the RIA system using antisera against the C-terminal fragment (12-28) is termed C-RIA.

Detection of Ghrelin Activity by Calcium Mobilization Assay—CHO-GHSR62 cells, which stably express rat GHS-R (9, 12), were plated at 4 × 105 cells/well for 12 h prior to the assay. The cells were loaded with 4 µM Fluor-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 glycine, 1% fetal calf serum) and washed four times in assay buffer without fetal calf serum. Intracellular calcium concentration ([Ca2+]i) changes were measured using a fluorometric imaging plate reader (FLIIPR, Molecular Devices, Sunnyvale, CA). Maximum changes in fluorescence compared with the baseline were used to quantitate the agonist responses.

**Purification of Human Ghrelin 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 subject to acetone precipitation in 66% acetone. After removal of the precipitate, the supernatant was subjected to the calcium-mobilization assay (Fig. 1A). Each of the five factions, B-F, induced intracellular calcium influxes and promoted intracellular calcium influx were eluted at a molecular weight of 4,500 Da. The ghrelin immunoreactivities in B-F were separated by CM ion-exchange HPLC into six fractions, A-F. Fractions A 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. 2 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 3,000. Active gel-filtration fractions were further separated by CM ion-exchange HPLC into six fractions, A-F (Fig. 1b). 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. 1c). Each of the five factions 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. 1d). 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 GXFSLSPHQRVQKRESKPPAKLQPR, whereas peaks B...
and D were of the sequence GSX/H11032FLSPEHQRVQQRKESKKP-PAKLQP, 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-Gln14-ghrelin in this human stomach tissue.

To determine whether the purified peptides were also modified by n-octanoic acid at Ser3 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 Ser3 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 Ser3: non-acylated, octanoylated, decanoylated, and possibly decenoylated.

To verify the deduced structures, we synthesized four peptides, [O-n-octanoyl-Ser3]-human ghrelin, [O-n-octanoyl-Ser3]-

| Peak | Amino-acid sequence | Molecular mass | Linked fatty acid |
|------|---------------------|----------------|------------------|
| B    | Gly1-Pro27          | 3214.6 ± 0.6   | C8:0             |
| C    | Gly1-Arg28          | 3371.3 ± 0.1   | 126.7 C8:0       |
| D    | Gly1-Pro27          | 3243.6 ± 0.4   | 155.2 C10:0      |
| E-I  | Gly1-Arg28          | 3397.2 ± 0.5   | 152.6 C10:1      |
| E-II | Gly1-Arg28          | 3397.2 ± 0.8   | 152.6 C10:1      |
| F    | Gly1-Arg28          | 3398.9 ± 0.3   | 154.3 C10:0      |

a Calculated mass of amino acid sequence; Gly1-Arg28, 3244.6; Gly1-Pro27, 3088.4.
human ghrelin-(1–27), \([O-n\text{-decanoyl-Ser}^3]\)-human ghrelin, and \([O-n\text{-decanoyl-Ser}^3]\)-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: \([O-n\text{-decanoyl-Ser}^3]\)-human ghrelin as “human decaoyl ghrelin,” \([O-n\text{-octanoyl-Ser}^3]\)-human ghrelin-(1–27) as “human ghrelin-(1–27),” and \([O-n\text{-decanoyl-Ser}^3]\)-human ghrelin-(1–27) as “human decaoyl 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 Ghrelin Using GHS-R-expressing Cells—Fig. 2 shows the dose-response relationships of the synthetic human ghrelin and the ghrelin-derived molecules on \([\text{Ca}^{2+}]_i\), changes in GHS-R-expressing cells. Four synthetic ghrelins potently induced increases in \([\text{Ca}^{2+}]_i\) in CHO-GHSR62 cells. Ghrelin, ghrelin-(1–27), decaoyl ghrelin, and decaoyl ghrelin-(1–27) had EC\(_{50}\) values of 2.7 × 10\(^{-7}\), 2.8 × 10\(^{-7}\), 2.5 × 10\(^{-7}\), and 2.5 × 10\(^{-7}\) M, respectively, and displayed similar potency upon application to GHS-R-expressing cells.

In Vivo Effects of Human Ghrelin on GH Secretion—To confirm that human ghrelin possessed GH-releasing activity, we intravenously injected synthetic ghrelin 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\textsuperscript{TM} 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 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. 1b. 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-

| 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           |
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 partial 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 Ser3. This peptide is identical to rat ghrelin with the exception of two residues (Arg11-Val12). The ghrelin-derived molecules observed include octanoyl ghrelin-(1–27), decanoyl ghrelin, decanoyl ghrelin-(1–27), and decenoyl ghrelin. Moreover, the non-active 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 Ser3. 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 ghrelin 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 produce multiple forms, such as the enkephalins (16),

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![Fig. 4. Nucleotide and deduced amino acid sequence of human prepro-ghrelin cDNA (GenBank™ accession number AB029843). 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 Gln14 may be used as a splicing acceptor site at the 3'-end of the intron to produce des-Gln14-ghrelin.](http://www.jbc.org/)

![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. 1b). Fraction g indicated by the open bar probably contained a C-terminal fragment of ghrelin and des-acyl ghrelin to be cleaved by proteases.](http://www.jbc.org/)

![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.](http://www.jbc.org/)
endorphins, dynorphins (17), corticotropins, and beta-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 Pro27-Arg28. 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 alpha-endorphin (YGGFLRKY-Pro-Lys) (21) and beta-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 alpha-endorphin occurs partially to generate beta-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 contribute to the maturation of human ghrelin.

The human prepro-ghrelin we isolated is predicted to encode 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-Gln14-ghrelin) (12). Des-Gln14-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-Gln14-ghrelin precursor, and the des-Gln14-ghrelin peptide was not identified in human stomach. The ratios observed between the two precursor populations, ghrelin and des-Gln14-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 acylation of many proteins has been observed, including G-proteins and some G-protein-coupled receptors, the modifications are most often myristoylations (25) and palmitoylations. C-terminal lysine in ghrelin is acetylated by acetyl-CoA acetyltransferase (23). Peptide bonds involving proline are resistant to common proteases such as this. However, the removal of C-terminal lysine in alpha-endorphin occurs partially to generate beta-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 contribute to the maturation of human ghrelin.

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