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Ghrelin, a Novel Growth Hormone-Releasing Acylated Peptide, Is Synthesized in a Distinct Endocrine Cell Type in the Gastrointestinal Tracts of Rats and Humans*

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ABSTRACT

Ghrelin, a novel GH-releasing acylated peptide, was recently isolated from rat stomach. It stimulated the release of GH from the anterior pituitary through the GH secretagogue receptor (GHS-R). Ghrelin messenger RNA and the peptide are present in rat stomach, but its cellular source has yet to be determined. Using two different antibodies against the N- and C-terminal regions of rat ghrelin, we identified ghrelin-producing cells in the gastrointestinal tracts of rats and humans by light and electron microscopic immunohistochemistry and *in situ* hybridization combined with immunohistochemistry. Ghrelin-immunoreactive cells, which are not enterochromaffin-like cells, D cells, or enterochromaffin cells, accounted for about 20% of the endocrine cell population in rat and human oxyntic glands. Rat ghrelin was present in round, compact, electron-dense granules compatible with those of X/A-like cells whose hormonal product and physiological functions have not previously been clarified. The localization, population, and ultrastructural features of ghrelin-producing cells (Gr cells) indicate that they are X/A-like cells. Ghrelin also was found in enteric endocrine cells of rats and humans. Using two RIAs for the N- and C-terminal regions of ghrelin, we determined its content in the rat gastrointestinal tract. Rat ghrelin was present from the stomach to the colon, with the highest content being in the gastric fundus. Messenger RNAs of ghrelin and GHS-R also were found in these organs. Ghrelin probably functions not only in the control of GH secretion, but also in the regulation of diverse processes of the digestive system. Our findings provide clues to additional, as yet undefined, physiological functions of this novel gastrointestinal hormone. (*Endocrinology* **141:** 4255–4261, 2000)

SINCE THE DISCOVERY that met-enkephalin stimulates GH release from the anterior pituitary (1), small synthetic peptide and nonpeptide molecules, called GH secretagogues (GHSs), have been developed. GHS receptor (GHS-R), a G protein-coupled receptor that promotes calcium release from the endoplasmic reticulum, is present in the pituitary and hypothalamus of swine, rats, and humans (2, 3), indicating that an unidentified signaling molecule to the receptor does exist. Very recently, we discovered a new endogenous ligand for GHS-R, named ghrelin (from ghre the Proto-Indo-European root of grow), in rat stomach using an intracellular calcium influx assay of a stable cell line expressing rat GHS-R (4). This peptide consists of 28 amino acids, in which the Ser^3 residue is *n*-octanoylated. Rat ghrelin sequence directly follows the 23-residue signal peptide of a 117-residue prepro-ghrelin. Ghrelin stimulated GH release both *in vivo* and *in vitro*. Using rat ghrelin complementary DNA (cDNA), we also isolated human ghrelin cDNA from a stomach cDNA library (4). Human ghrelin differs from rat ghrelin by only two amino acids. Ghrelin circulates in healthy human blood at a plasma concentration of 117.2 ± 37.2 fmol/ml (mean ± SE) (4).

We have shown that ghrelin is likely to be present in gastric endocrine cells by *in situ* hybridization and immunohistochemistry (4). Gastrointestinal peptides, part of a complex biological signaling system, act as substrates for intracellular communication both in the digestive system and between the digestive system and body organs. More than 18 endocrine cell types have been identified in the gastrointestinal tract, and the number continues to grow. We used *in situ* hybridization histochemistry combined with immunohistochemistry, immunohistochemical double staining, and electron microscopy immunostaining to investigate the cellular origin of ghrelin in the digestive systems of rats and humans. We developed two RIAs specific for the N- and C-terminal regions of ghrelin. We also studied the distribution of ghrelin in rat gastrointestinal tract by the RIAs and RT-PCR, and its receptor by RT-PCR.

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Materials and Methods

Animals

Male Wistar rats, weighing 250–300 g (Charles River Japan, Inc., Shiga, Japan), were used in all experiments and were given standard laboratory chow and water *ad libitum*. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Peptide synthesis

C-Terminally Cys-extended rat ghrelin (position 1–11) with octanoylated Ser^3, C-terminally Tyr-extended rat ghrelin (position 1–28) with octanoylated Ser^3, N-terminally Cys- or Tyr-extended rat ghrelin (position 13–28), and des-acyl ghrelin (position 1–28) were synthesized using solid phase techniques. Cys-extended peptides were used for immunization and Tyr-extended peptides for radioiodination as described below. The validity of the synthesis was confirmed by amino acid analysis, sequencing, and spectrometric analysis.

Preparation and characterization of antisera

To generate antighrelin antisera, synthetic [Cys^{12}]ghrelin-(1–11) (4 mg) and [Cys^0]ghrelin-(13–28) (10 mg) were separately conjugated with maleimide-activated mariculture keyhole limpet hemocyanin (Pierce Chemical Co., Rockford, IL; 6 mg). Amino acid analysis of the conjugate showed that one hemocyanin molecule was coupled with an average 280 [Cys^{12}]ghrelin-(1–11) or 275 [Cys^0]ghrelin-(13–28) molecules. The antigenic conjugate solution (1.5–3 ml) was administered to three New Zealand White rabbits. The antirat ghrelin-(1–11) antiserum (G606) specifically recognized ghrelin with *n*-octanoylated Ser^3 and did not recognize des-acyl ghrelin. The antirat ghrelin-(13–28) antiserum (G107) equally recognized *n*-octanoyl-modified and des-acyl ghrelin. Both antisera had 100% cross-reactivity with human ghrelin-(1–28).

RIA procedure

Synthetic rat [Tyr^{29}]ghrelin-(1–28) and [Tyr^0]ghrelin-(13–28) were radioiodinated by the lactoperoxidase method. The ^{125}I-labeled peptides were purified on a TSK ODS SIL 120A column (Tosoh Co. Ltd., Tokyo, Japan) by reverse phase HPLC (RP-HPLC). The RIA incubation buffer was 50 mM sodium phosphate (pH 7.4) that contained 0.5% BSA treated with *N*-ethylmaleimide, 80 mM NaCl, 25 mM EDTA·2Na, 0.05% NaN_3, and 0.5% Triton X-100. A diluted sample or a standard peptide solution (100 μl) was incubated for 24 h with 100 μl of the antiserum diluent [final dilution of antighrelin-(1–11) antiserum, 1:620,000; that of antighrelin-(13–28) antiserum, 1:20,000]. The tracer solution (16,000 cpm in 100 μl) was added, and the mixture again incubated for 24 h. The bound and free ligands were separated by second antibody (200 μl). All procedures were performed at 4 C. Samples were assayed in duplicate. Half-maximum inhibition by rat ghrelin-(1–28) on the standard RIA curve with antighrelin-(1–11) antiserum was 3.8 fmol/tube, and that with antighrelin-(13–28) antiserum was 80 fmol. The dilution curves for the extracts of rat stomach and jejunum paralleled both standard curves. The respective intra- and interassay coefficients of variation in the RIA for ghrelin N-terminus were 3.5% and 3.2% at 50% binding, and those for ghrelin C-terminus were 3.7% and 3.3% at 50% binding. The recoveries of rat ghrelin-(1–28) (1 ng) and [^{125}I]rat ghrelin-(1–28) (5,000 cpm) added to the tissue homogenates in the extraction done with a Sep-Pak C_{18} cartridge (Waters Corp., Milford, MA), respectively, were 92.2 ± 0.4% (±SEM) and 88.9 ± 0.6%.

Quantification and chromatographic characterization of immunoreactive (ir-) ghrelin in stomach and intestine

The stomach and intestine were resected immediately after decapitation of three 12-week-old male Wistar rats fed *ad libitum*. The glandular stomach was divided into fundus and pylorus. The jejunum was resected 10–20 cm from the pyloric ring, the ileum 20–30 cm above the terminal ileum, and the colon 5–15 cm below the terminal ileum. The tissues were heated at 95–100 C for 10 min in a 10-fold volume of water to inactivate intrinsic proteases. After cooling to 4 C, CH_3COOH and HCl were added to the respective final concentrations of 1 M and 20 mM, after which the tissue was homogenized in a Polytron (Brinkmann Instruments, Inc., Westbury, NY) for 10 min. The homogenate was centrifuged at 11,500 × *g* for 30 min. The supernatants were applied to Sep-Pak C_{18} cartridges, then the peptides were eluted with 60% acetonitrile (CH_3CN) solution containing 0.1% trifluoroacetic acid (TFA). Some portions of the eluates were subjected to two RIAs for ghrelin, and other portions to RP-HPLC (Fig. 1, A and B). All HPLC fractions were quantified by the RIAs for ghrelin. Authentic rat ghrelin-(1–28) was chromatographed with the same HPLC system.

Quantification of ir-ghrelin in rat plasma

Truncal blood (4 ml) was obtained by decapitation from 10 free feeding male Wistar rats at 0900 h. It was drawn into chilled polypropylene tubes containing EDTA·2Na (1 mg/ml blood), aprotinin (500 U/ml blood), and Pefabloc SC (2.4 mg/ml blood; Roche, Nutley, NJ), then immediately centrifuged. After the plasma had been diluted to one half with 0.9% saline, it was applied to a Sep-Pak C_{18} cartridge preequilibrated with 0.9% saline. The cartridge was washed with saline and 10% CH_3CN solution containing 0.1% TFA. Adsorbed peptides were eluted with 60% CH_3CN solution containing 0.1% TFA, lyophilized, then subjected to RIAs.

In situ hybridization histochemistry

Three Wistar rats were anesthetized by an ip injection of sodium pentobarbital (75 mg/kg BW) and perfused transcardially for 10 min with 100 ml 0.1 M phosphate buffer (pH 7.4) containing heparin (100 U/100 ml), then for 15 min with 150 ml fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer. Their stomachs were removed, postfixed with the same fixative for 24 h at 4 C, then incubated for 24 h in 0.1 M PBS (pH 7.4) containing 30% sucrose. The glandular stomachs were quickly frozen on a dry ice and stored at −80 C until use for the *in situ* hybridization analysis. They were cut at −20 C with a cryostat in slices 12 μm thick, then thaw-mounted on silane-coated slides and kept at −80 C. The stored slides were allowed to dry for 10 min at room temperature, then fixed in 4% formaldehyde in PBS (pH 7.4) for 5 min and washed twice in PBS (pH 7.4). Next, they were incubated for 10 min in 0.9% saline containing 0.1 M triethanolamine and 0.25% acetic anhydride, dehydrated in a graded ethanol

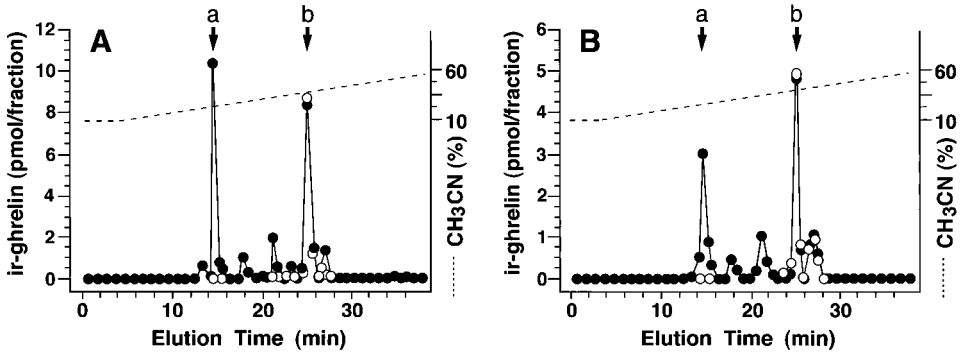

FIG. 1. Representative RP-HPLC profiles of ghrelin immunoreactivity in rat stomach and jejunum. ○, Data obtained using a RIA for ghrelin N-terminus; ●, data obtained using a RIA for ghrelin C-terminus. The fundus (A; 5 mg) and jejunum (B; 125 mg) were chromatographed on a TSK ODS SIL 120A column (4.6 × 150 mm). A linear gradient of 10–60% CH_3CN containing 0.1% TFA was run for 40 min at 1.0 ml/min. The fraction volume was 0.5 ml. *Arrows* indicate the elution positions of des-acyl rat ghrelin-(1–28) (a) and *n*-octanoylated rat ghrelin-(1–28) (b).

series, and delipidated in 100% chloroform for 5 min, after which they were immersed in 100% ethanol, then in 95% ethanol, and allowed to dry briefly in air. Hybridization was performed at 37 C overnight in 45 μl hybridization buffer containing 50% formamide and 4 × SSC (1 × SSC = 150 mM NaCl and 15 mM sodium citrate) with 500 μg/ml sheared salmon sperm DNA (Sigma, St. Louis, MO), 250 μl yeast total RNA (Sigma), 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrolidone, and 0.02% BSA), and 10% dextran sulfate (500,000 mol wt) under a Nescofilm (BDH/Merck, Dagenham, UK) coverslip. Three 45-mer antisense oligonucleotide probes against rat ghrelin cDNA (nucleotides 90–134, 213–257, and 421–465 in Ref. 4) were used. These probes are not closely homologous to other known cDNA sequences. A 100-fold molar excess of each unlabeled probe served as the controls. A total of 5×10^5 cpm/slide of 3′-end ^{35}S-labeled probes were used. After hybridization, sections were washed for 1 h in four changes of 1 × SSC at 55 C, then for 1 h in two changes of 1 × SSC at room temperature. After washing, they were dehydrated in a graded alcohol series and air-dried. The sections were coated with Kodak NTB3 emulsion (Eastman Kodak Co., Rochester, NY) for autoradiography and exposed for 24 h in light-tight boxes at 4 C. After development in Kodak D-19 and fixing in Fujifix (Fuji Photo Film Co., Ltd., Tokyo, Japan), they were rinsed with distilled water and coverslipped.

In the second protocol, after washing as described above some sections were subjected to immunohistochemical procedures. They were treated with 0.3% hydrogen peroxide for 30 min to inactive endogenous peroxidases, then incubated with normal goat serum for 1 h to block nonspecific binding. These sections were incubated overnight at 4 C with antisomatostatin (anti-SRIF) antiserum (DAKO Corp., Glostrup, Denmark; dilution, 1:200), antihistidine decarboxylase (anti-HDC) antiserum (EURO-DIAGNOSTICA, Malmö, Sweden; dilution, 1:2,000), antichromogranin A antiserum (DAKO Corp.; dilution, 1:500), or antiserotonin antiserum (DAKO Corp.; dilution, 1:5). After washing with PBS, the slides were incubated overnight at 4 C with goat biotinylated antirabbit IgG (Vectastain, Vector Laboratories, Inc., Burlingame, CA). They were stained for 10 min at room temperature using the avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit, Vector Laboratories, Inc.) with 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.006% hydrogen peroxide in 50 mM Tris-HCl buffer solution (pH 7.2). After dehydration in a graded alcohol series, they were covered with emulsion for autoradiography as described above and counterstained with hematoxylin. The number of cells that have ghrelin messenger RNA (mRNA) signal and the immunoreactivity of one of the above substances were quantified by counting two randomly selected visual fields in two sections from each of three rats under a light microscope (×20 objective lens; magnification, ×200). In this study we defined the selected visual fields as the areas that contain both ghrelin mRNA signal and the immunoreactivity of each of the above substances in the rat oxyntic gland. We also considered that cells with grain densities at least 5 times higher than the background densities are positively labeled for ghrelin mRNA, and a distinctive brown chromogen in the cell cytoplasm indicates the immunoreactivity of SRIF, HDC, chromogranin A, or serotonin.

Light microscopy immunostaining

Frozen 12-μm-thick sections of the glandular stomachs were prepared from the same three rats as those used in *in situ* hybridization histochemistry. Rat small intestine was fixed with 4% paraformaldehyde and 0.2% picric acid and embedded in paraffin. Human gastric fundi and small and large intestines obtained at autopsy from three patients who had died of cardiovascular disease were fixed and embedded in paraffin as described above, and the tissues were cut in 3-μm-thick slices. After pretreatment with 0.3% hydrogen peroxide and incubation with normal goat serum, all slices were incubated overnight at 4 C with antighrelin-(1–11) antiserum (final dilution, 1:10,000) or antighrelin-(13–28) antiserum (final dilution, 1:10,000). All of the sections were stained by the avidin-biotin complex method as described above. Control studies were performed with normal rabbit serum or antighrelin-(1–11) and antighrelin-(13–28) antisera that had been absorbed with 10 μg rat ghrelin.

Immunohistochemical double staining

The human gastric fundi sections were incubated with antighrelin-(1–11) antiserum, then with Alexa Fluor 488 goat antirabbit IgG (Molecular Probes, Inc., Eugene, OR). After being washed with PBS, they were incubated first with mouse antichromogranin A antiserum (DAKO Corp.; dilution, 1:500), then with Alexa Fluor 568 goat antimouse IgG (Molecular Probes, Inc.), after which they were observed under a BH2-RFC microscope (Olympus Corp., Tokyo, Japan). In the double staining for ghrelin *vs.* SRIF, HDC, and serotonin in human gastric fundi, ghrelin first was stained using the avidin-biotin complex method. The specimens then were washed with 0.1 M glycine-HCl buffer (pH 2.2) and incubated overnight at 4 C with anti-SRIF, -HDC, or -serotonin antiserum. They were stained by the streptavidin-alkaline phosphatase method using a labeled streptavidin biotin kit (DAKO Corp.) and AP Substrate Kit III (Vector Laboratories, Inc.). The number of cells in which two peptides were colocalized was quantified by counting two randomly selected visual fields in two sections from each of three human subjects under a fluorescence or light microscope (×20 objective lens; magnification, ×200).

Electron microscopy immunostaining

Three Wistar rats were perfused with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PBS. Their stomachs were excised and fixed at 4 C overnight with the above fixative, then postfixed at 4 C for 90 min with 1% osmium tetroxide in 0.1 M PBS. They were dehydrated in a graded ethanol series and embedded in Epon. Ultrathin sections of the specimens were cut and treated for 30 min with 5% sodium metaperiodate (5), after which they were immersed for 10 min in 5% normal goat serum and PBS containing 1% BSA, then incubated overnight at 4 C with antighrelin-(13–28) antiserum (dilution 1:1000). Next they were incubated with 8 nm colloidal gold conjugated antirabbit donkey IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; dilution, 1:50), after which the sections were counterstained with uranyl acetate and lead citrate. For the controls, antighrelin antiserum was omitted or replaced by normal rabbit serum. The sections were examined in a JEOL 1200EX electron microscope (JEOL USA, Inc., Tokyo, Japan). The sizes of 200 ghrelin-containing granules were measured.

RT-PCR for ghrelin and GHS-R

Total RNAs were extracted from the stomachs and small and large intestines of three Wistar rats by the acid guanidinium thiocyanate-phenol-chloroform method (6). First strand cDNA was synthesized from 2.5 μg of a RNA sample and 7 μM oligo(deoxythymidine)$_{18}$ primer with ReverTra Ace-α (Toyobo Co., Ltd., Osaka, Japan). The resulting cDNA was subjected to PCR amplification with 2 μM each of the sense and antisense primers and 2.5 U Pyrobest DNA polymerase (Takara Shuzo Co., Ltd., Shiga, Japan). The PCR primers specific for ghrelin were 5′-TTGAGCCCAGAGCACCAGAAA-3′ for sense and 5′-AGTTGCAGAGGAGGCAGAAGCT-3′ for antisense (nucleotides 112–132 and 437–458 in Ref. 4), and the primers specific for GHS-R were 5′-GAGATCGCTCAGATCAGCCAGTAC-3′ for sense and 5′-TAATCCCCAAACTGAGGTTCTGC-3′ for antisense (nucleotides 880–903 and 1170–1192 in accession no. AB001982, GenBank). The reaction volume was 25 μl, and the PCR conditions were 35 cycles of denaturation for 5 sec at 94 C, annealing for 10 sec at 65 C, and extension for 1 min at 72 C. The PCR products were electrophoresed on a 2% agarose gel (FMC BioProducts, Rockland, ME).

TABLE 1. Tissue content of rat immunoreactive ghrelin

Tissue	RIA for C-terminus (pmol/g wet wt)	RIA for N-terminus (pmol/g wet wt)
Stomach		
Fundus	4633.5 ± 440.1	1845.6 ± 290.0
Pylorus	120.7 ± 19.4	63.6 ± 21.1
Duodenum	262.5 ± 13.3	50.9 ± 7.2
Jejunum	102.3 ± 13.2	44.6 ± 8.4
Ileum	27.7 ± 1.1	1.5 ± 0.1
Colon	73.4 ± 19.2	11.7 ± 4.2

Values are the mean ± SEM (n = 6).

Results

Identification of ir-ghrelin molecule and its content

The homogenates of rat gastric fundus and jejunum were subjected to RP-HPLC. A large ghrelin-immunoreactive peak (peak b in Fig. 1) detected by two RIAs was eluted at the position identical to that of *n*-octanoylated ghrelin-(1–28). Another major peak (peak a in Fig. 1) in both stomach and jejunum was detected only by the RIA for ghrelin C-terminus. This molecule was eluted at a position identical to that of des-acyl ghrelin-(1–28), indicating that it was des-acyl ghrelin-(1–28). Ghrelin immunoreactivity was present from the stomach to the colon, with the highest content in the gastric fundus (Table 1). The plasma concentration of ir-ghrelin measured by RIA for C-terminus was 556.2 ± 43.8 fmol/ml (mean ± SE), and that measured by RIA for N-terminus was 94.6 ± 14.0 fmol/ml.

In situ hybridization histochemistry

Ghrelin mRNA hybridization signals were abundant from the neck to the base of the rat oxyntic gland (Fig. 2A) when the oligonucleotide probe 90–134 was used, but were infrequent in the pyloric gland (Fig. 2B). The signals disappeared

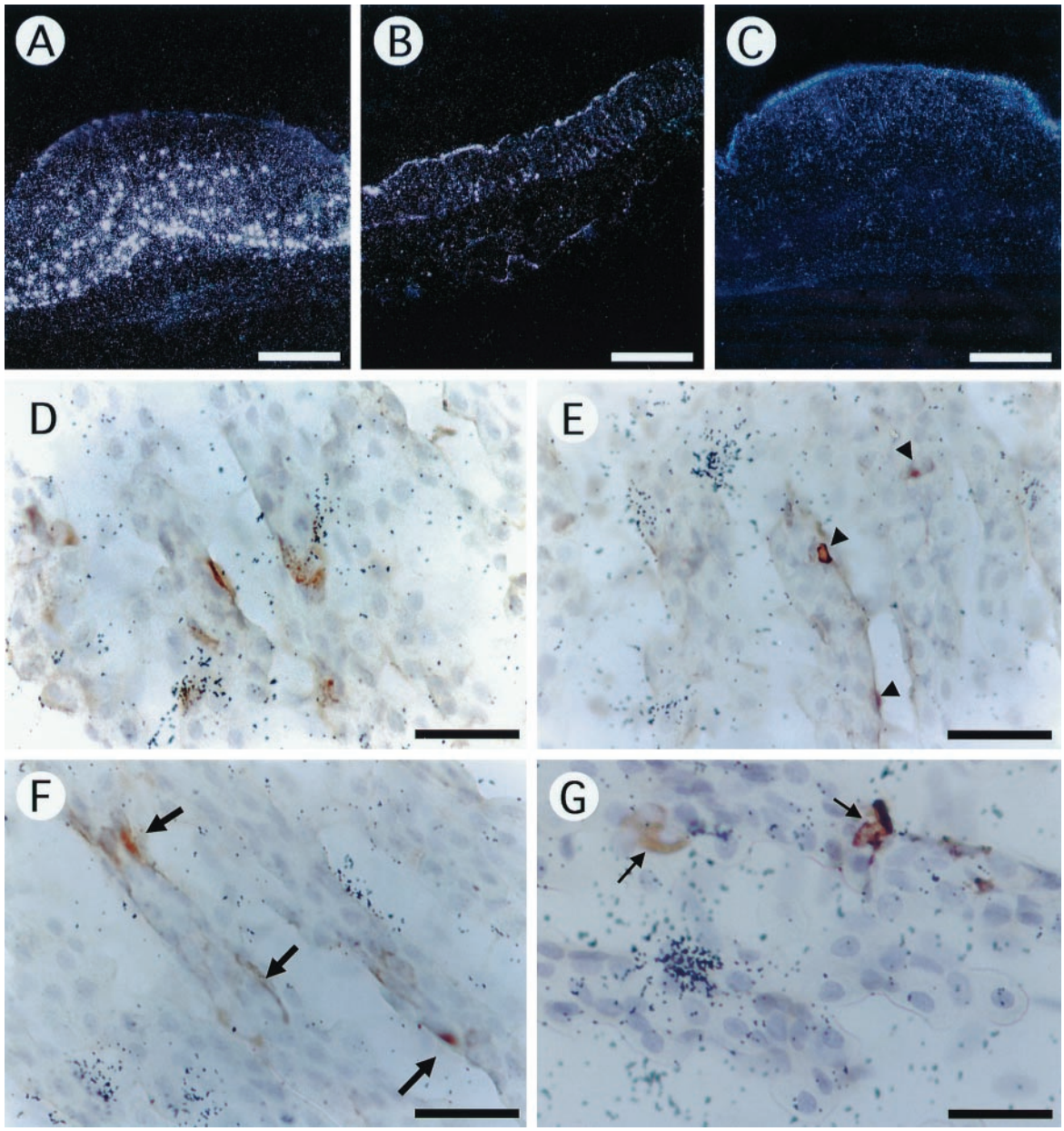

FIG. 2. A–C, Darkfield images of *in situ* hybridization for ghrelin mRNA in rat stomach. Hybridization signals are abundant from the neck to the base of the oxyntic gland (A), but are infrequent in the pyloric gland (B). C, No hybridization signals were seen with an excess of the unlabeled probe. D–G, *In situ* hybridization for ghrelin mRNA combined with immunohistochemistry. The sections were immunostained with antiserum for chromogranin A (D), SRIF (E), HDC (F), or serotonin (G) after *in situ* hybridization. Some chromogranin A-immunoreactive endocrine cells have hybridization signals, but SRIF- (*arrowheads*), HDC- (*large arrows*), and serotonin- (*small arrows*) immunoreactive cells do not. *Bar*, 500 μm in A–C; 30 μm in D–G.

when a 100-fold molar excess of the unlabeled probe was present (Fig. 2C). The same ghrelin mRNA localization patterns were detected when the other two probes were used (data not shown). Colocalization of ghrelin, chromogranin A, SRIF, HDC, and serotonin was investigated by *in situ* hybridization histochemistry combined with immunohistochemistry. Ghrelin mRNA hybridization signals were present in 21% (93 of 443) of chromogranin A-immunoreactive endocrine cells (Fig. 2D), but none was found in SRIF-, HDC-, or serotonin- immunoreactive cells (Fig. 2, E–G).

Immunohistochemistry

Ghrelin-immunoreactive cells were abundant from the neck to the base of the rat oxyntic gland, but were infrequent in the pyloric gland, as shown by *in situ* hybridization (Fig. 3, A and B). Small numbers of ghrelin cells were present in the upper small intestine (Fig. 3, C and D), and very small numbers were present in the lower small intestine and large intestine (not shown). Cells immunostained with antisera for ghrelin-(1–11) and -(13–28) were colocalized extensively in

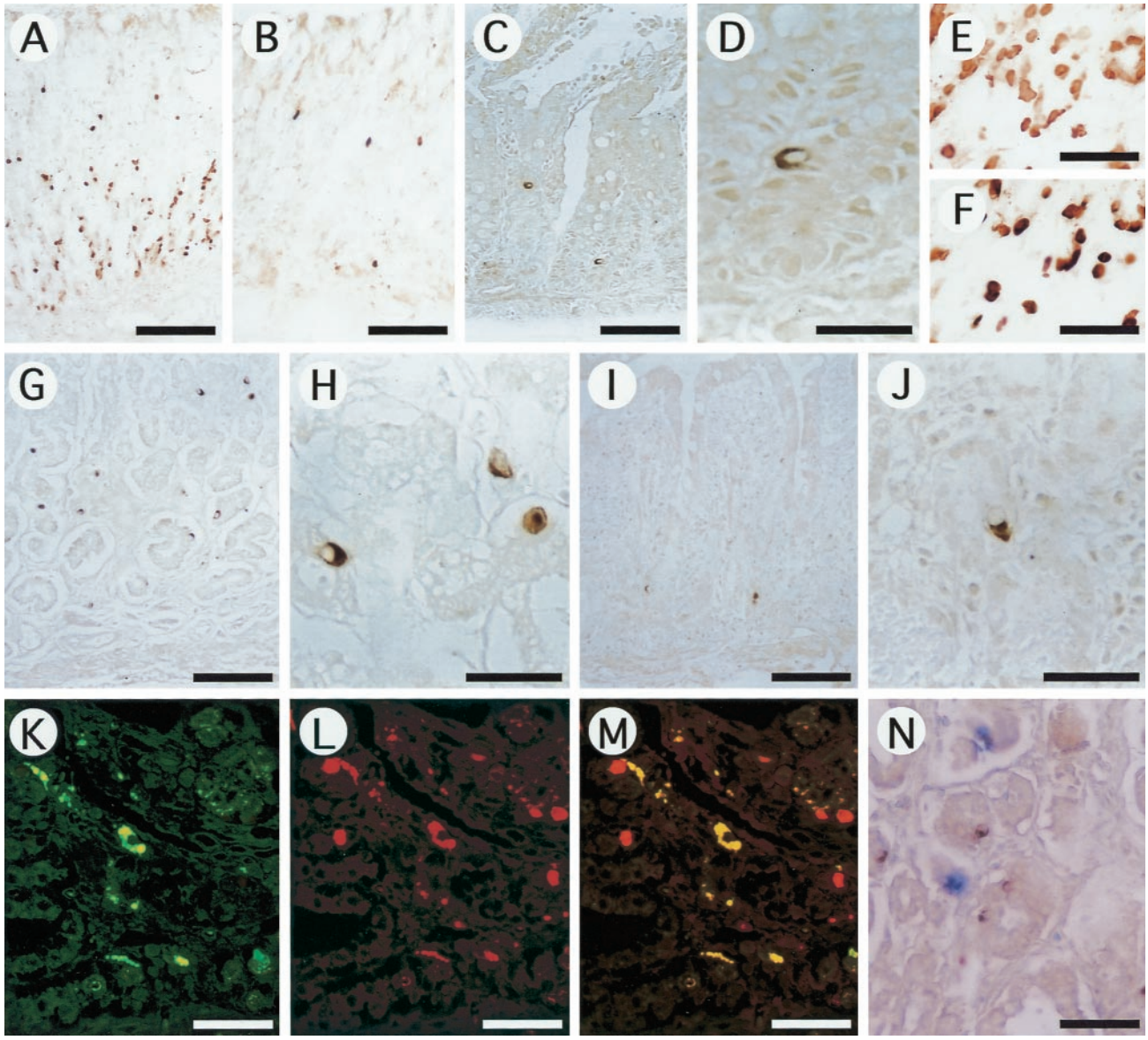

FIG. 3. Localization of ghrelin-immunoreactive cells in the gastrointestinal tracts of rats and humans. Antisera for ghrelin-(1–11) (A–E, G–K, M, and N), ghrelin-(13–28) (F), chromogranin A (L and M), and SRIF (N) were used. Ghrelin cells are distributed from the neck to the base of the rat oxyntic gland (A), are infrequent in the pyloric gland (B), and are very rare in the small intestine (C). D, High magnification of ghrelin cells in rat small intestine. Ghrelin cells in the rat oxyntic gland stained with antiserum for ghrelin-(1–11) (E) and with antiserum for ghrelin-(13–28) (F). G, Ghrelin cells are distributed from the neck to the base of the human oxyntic gland. H, High magnification of G. I, Ghrelin-immunoreactive cells in human small intestine. J, High magnification of ghrelin-immunoreactive cells in human small intestine. K–N, Immunofluorescence (K–M) and immunohistochemical double staining (N) of ghrelin cells in human oxyntic gland. K, Ghrelin cells are visible as *green* by Alexa Fluor 488 goat antirabbit IgG. L, Chromogranin A cells are visible as *red* by Alexa Fluor 568 goat antimouse IgG. M, Colocalization of ghrelin and chromogranin A shown in *yellow*. N, Ghrelin cells are stained *brown* by the avidin-biotin complex method, and SRIF is stained *blue* by the streptavidin-alkaline phosphatase method. *Bar*, 200 μm in A; 100 μm in B, C, E–G, and I; 50 μm in K–N; 30 μm in D, H, and J.

the gastrointestinal tract (Fig. 3, E and F). In humans, too, ghrelin cells were most abundant in the oxyntic gland and were infrequent in the pyloric gland and upper small intestine (Fig. 3, G–J). Ghrelin-immunoreactive materials were concentrated in the basal cytoplasm of ghrelin cells of both rats and humans (Fig. 3, D, H, and J). In the human gastric fundus, ghrelin cells accounted for 23% (30 of 128) of chromogranin A-immunoreactive endocrine cells in immunofluorescence double staining (Fig. 3, K–M). Human ghrelin cells did not have SRIF (Fig. 3N), HDC (not shown), or serotonin (not shown) immunoreactivity in double staining. No immunoreactivities for ghrelin-(1–11) and -(13–28) were detected in the tissues when normal rabbit serum or antiserum absorbed with excessive ghrelin was used (not shown).

Immunoelectron microscopy

In rat oxyntic gland, immunogold staining for ghrelin was localized on round, compact, electron-dense granules (Fig. 4). Ghrelin cells were round to ovoid and of a closed type that had no contact with the lumen. They were positioned close to the capillary. The ghrelin-containing granules were 120 ± 30 nm (mean ± SEM) in diameter, smaller than the granules of D cells and enterochromaffin-like (ECL) cells. Neither D nor ECL cells reacted with ghrelin antiserum (not shown).

RT-PCR amplification of ghrelin and GHS-R transcripts

A ghrelin transcript product corresponding to the predicted 347 bp size was present in rat stomach and small and large intestines (Fig. 5, *upper panel*). A GHS-R transcript product corresponding to the predicted 313 bp size also was present in all of these organs (Fig. 5, *lower panel*).

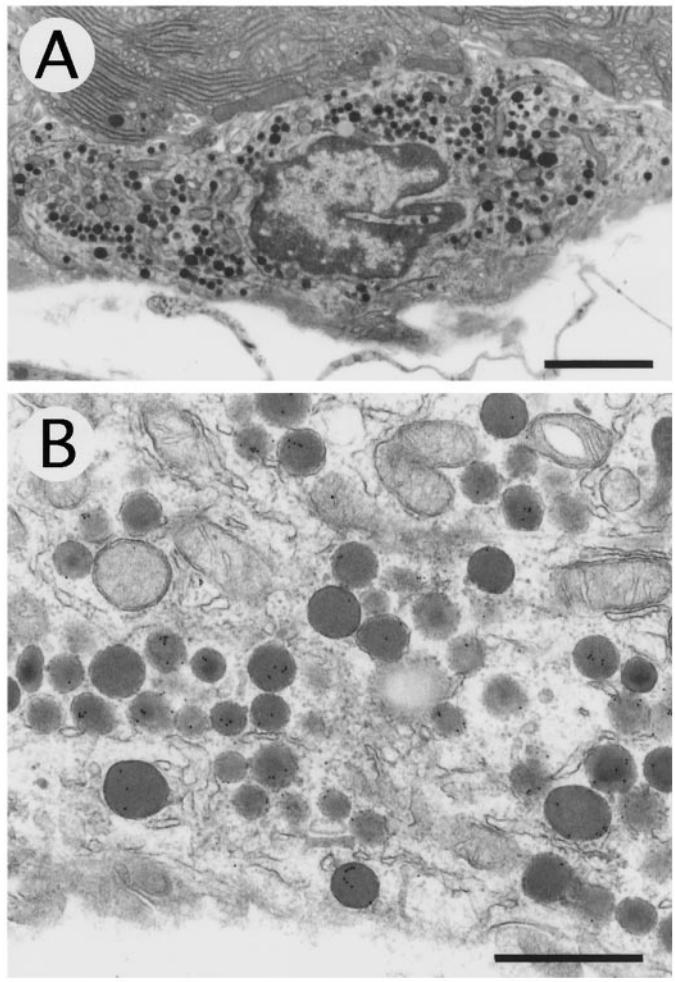

FIG. 4. Representative immunoelectron photographs of a ghrelin cell in rat oxyntic gland. A, The ovoid cell has many round, compact, electron-dense granules in its cytoplasm. *Bar*, 2 μm; magnification, ×7,000. B, Higher magnification of A. Granules in the cytoplasm are labeled with immunogold staining for ghrelin. *Bar*, 500 nm; magnification, ×36,000.

Discussion

Our survey of the endogenous ligand in rat systemic tissues for an orphan GH secretagogue receptor showed the highest receptor-stimulating activity in the stomach (4). Rat ghrelin was originally isolated from the stomach by this calcium influx assay. cDNA cloning and sequence determination also detected ghrelin in the human stomach. Ghrelin produced in the stomach may reach and act on the anterior pituitary via the blood circulation because its iv administration to rats rapidly increased the plasma GH concentration (4). Ghrelin also is synthesized in the hypothalamic arcuate nucleus (4), but the number of ghrelin neurons is small. Hypothalamic ghrelin might act on the anterior pituitary via the portal vein to release GH. Using two RIAs combined with HPLC, we found two major molecular forms of ghrelin, an *n*-octanoylated and des-acyl ghrelin, in rat stomach and intestine. Des-acyl ghrelin has no biological activity to increase the intracellular calcium concentration (4). Further studies are needed to determine whether des-acyl ghrelin is a premodified form or a *n*-octanoyl-deleted form.

We here identified ghrelin-producing endocrine cells in the digestive tracts of rats and humans. They were most abundant in the oxyntic mucosae of both species. To date, four types of endocrine cells, ECL, D, enterochromaffin (EC), and X/A-like cells, have been identified in the oxyntic mucosa by means of ultrastructural and immunohistochemical criteria (7, 8). The relative percentages of these four cells in rat oxyntic gland are 60–70% for ECL cells, 20% for X/A-like cells, 2–5% for D cells, and 0–2% EC cells; those in human oxyntic gland are 30% for ECL cells, 20% for X/A-like cells, 22% for D cells, and 7% for EC cells (9, 10). Major products in the granules of the first three cell types have been shown to be histamine and uroguanylin (11), SRIF, and serotonin, respectively, whereas no products have been reported in the granules of X/A-like cells. X/A-like cells represent a major endocrine cell population in the oxyntic mucosae of both rats and humans. X/A-like cells are round to ovoid, with round, compact, electron-dense granules (8, 12, 13). They are found mainly in the oxyntic gland and infrequently in the pyloric gland and small intestine. The localization, population, and ultrastructural features of ghrelin-immunoreactive cells in the gastrointestinal tract indicate that X/A-like cells, whose hormonal product has not previously been clarified, are ghrelin cells. Ghrelin cells can be abbreviated as Gr cells according to the precedented nomenclature of other enteroen-

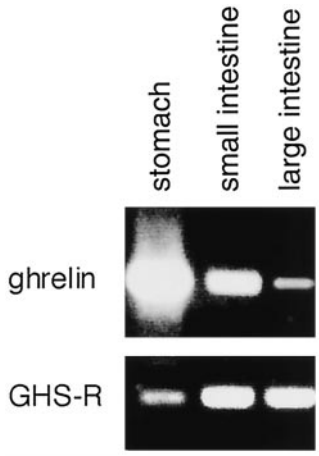

FIG. 5. Representative electrophoretic analysis patterns of the RT-PCR products of ghrelin and GHS-R mRNAs in rats. *Upper panel*, Ghrelin transcripts in the stomach and small and large intestines. *Lower panel*, GHS-R transcripts in those same organs.

docrine cells. Ghrelin cells in the oxyntic mucosa are closed-type cells that have no continuity with the lumen, suggesting that they respond to physical stimuli from the lumen, chemical stimuli from the basolateral site, or both. Ghrelin cells are closely associated with the capillary network running through the lamina propria in electron microscopy. Ghrelin circulates in the rat plasma. These findings suggest that ghrelin cells function in an endocrine fashion, a mechanism feasible for delivering ghrelin to remote tissues that express GHS-R.

Although Northern blot analysis of rat tissues showed that prepro-ghrelin mRNA occurs only in the stomach (4), RT-PCR analysis and RIAs detected it in the intestine as well. GHS-R mRNA also was present in the stomach and intestine. Ghrelin's possible function in the digestive tract, such as regulation of motility of the gut wall, gastric acid secretion, and renewal of gut epithelium, is a fascinating area that requires further investigation.

Future determination of ghrelin content and the amount of its mRNA in the gastrointestinal tract under various physiological and pathophysiological conditions should provide information on what mechanisms govern the biosynthesis and secretion of this peptide. The findings presented here will help establish new ways to clarify the additional, as yet undefined, physiological functions of this novel gastrointestinal hormone, ghrelin.

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