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Enterochromaffin cells of the digestive system: Cellular source of guanylin, a guanylate cyclase-activating peptide

(gut hormones/entero-endocrine cells/intestine/heat-stable enterotoxin receptor/diarrhea)

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ABSTRACT **Guanylin, a bioactive peptide, has recently been isolated from the intestine; this peptide activates intestinal guanylate cyclase (i.e., guanylate cyclase C) and thus is potentially involved in the regulation of water/electrolyte transport in the gastrointestinal mucosa. As yet, the cells involved in synthesis, storage, or secretion of guanylin have not been identified by immunocytochemistry. We raised antisera against guanylin and investigated the entire gastrointestinal tract of guinea pigs by light and electron microscopical immunocytochemistry. Extracts of various intestinal segments and plasma analyzed on a Western blot revealed a peptide band corresponding to the molecular mass of guanylin. Localization studies in the entire digestive tract showed that guanylin is exclusively confined to enterochromaffin (EC) cells. Remarkably, most EC cells contacted the gut lumen by cell processes that were highly immunoreactive for guanylin. In addition to the well known secretion in an endocrine fashion, EC cells by circumstantial evidence may release guanylin into the gut lumen to activate guanylate cyclase C that is immediately located on the brush border of adjacent enterocytes. The unique localization of guanylin in EC cells may indicate that these cells are involved in the regulation of fluid secretion in the gastrointestinal mucous membrane.**

Heat-stable enterotoxins (STa), small peptides of 18 or 19 amino acids, cause secretory diarrhea when secreted into the intestine by enterotoxigenic strains of *Escherichia coli* (1). A high-affinity receptor for this class of diarrheal enterotoxins has been demonstrated in the intestinal brush border membranes (2, 3). Recently, guanylate cyclase C (GC-C) has been identified as the cell surface receptor for STa (4, 5); it has been proposed that STa merely mimic the actions of an endogenous ligand for GC-C (6, 7). Guanylin, a 15-amino acid peptide, has been isolated from jejunal extracts and identified as endogenous activator of GC-C (8). As described for STa (6, 9), guanylin elicits an increase in cellular cGMP that mediates the increase in chloride secretion and the decrease in water absorption, finally causing secretory diarrhea (8, 10). Human, rat, and mouse cDNAs encoding the precursor of guanylin have been cloned and characterized (10–12). The prohormone consists of 115 amino acids containing at its C terminus the peptide isolated from the intestine (8).

Northern blot analysis of human and rat guanylin mRNA levels in various organs revealed that guanylin is mainly expressed in the intestine (10, 12) with the highest expression levels in the colon (11, 12); by using *in situ* hybridization techniques, guanylin mRNA was found in cells located at the base of small intestinal crypts, namely Paneth cells (10), a cell type that has a tendency to bind nonspecifically nucleic acid probes (13). Moreover, this cell type is restricted almost completely to the small intestine and is not present in the colon of most species (13, 14). On the other hand, high amounts of circulating high molecular mass guanylin were demonstrated in the blood (15), indicating that guanylin may originate primarily from endocrine sources in the gut. Therefore, we raised antisera against the midportion and against the C terminus of the guanylin molecule, characterized them by Western blot analysis, and report here the cellular and subcellular localization of guanylin in the guinea pig gastrointestinal tract.

MATERIALS AND METHODS

Peptide Synthesis. From the published human guanylin sequence (10, 11), the following guanylin peptides were synthesized: guanylin-(34–46), guanylin-(101–115), and [Lys^{100}]guanylin-(101–115). Guanylin-(34–46) was synthesized on a SMPS 350 automated multiple peptide synthesizer (Zinsser, Frankfurt, F.R.G.) using the standard Fmoc protocol (16). Peptide chains were assembled by TBTU/DIPEA/HOBT activation on a Wang resin. The sequence was synthesized as a linear peptide and as an octameric multiple antigenic peptide (for immunization; see ref. 17). Guanylin-(101–115) and [Lys^{100}]guanylin-(101–115) were synthesized manually on Fmoc-Cys(Trt)-Wang resin using double coupling cycles, monitoring by Kaiser test, and end-capping with acetic acid anhydride/pyridine, 2:1 (vol/vol). The cystine bridge between residues 107 and 115 was introduced by oxidation with potassium ferricyanide (III), and the cystine bridge between residues 104 and 112 was introduced by oxidation with iodine. The purity and sequence of the synthesized peptides were checked by reverse-phase HPLC [Vydac (Hesperia, CA) C_{18}], capillary zone electrophoresis (Bifocus 3000, Bio-Rad), mass spectrometry (Sciex API III, Perkin–Elmer), automated Edman degradation (model 473A protein sequencer, Applied Biosystems), and amino acid analysis (Aminoquant 1090L, Hewlett–Packard).

Immunization Procedure. [Lys^{100}]Guanylin-(101–115) (0.5 mg per rabbit) was conjugated to limpet hemocyanin (Sigma) by using carbodiimide as coupling agent. Guanylin-(34–46) (0.5 mg of multiple antigenic peptide per rabbit) was dissolved in saline (1 mg/ml). Rabbits (New Zealand White, five for each antigen) were immunized subcutaneously with guanylin-(34–46) and [Lys^{100}]guanylin-(101–115) conjugates emulsified in complete Freund's adjuvant at 1:1 (vol/vol). They were given booster injections every 4 weeks and bled 14 days after each booster injection. The titer of the antisera against the corresponding linear peptides was tested in enzyme-linked immunosorbent assay. Out of the antisera raised, the antisera K42 recognizing guanylin-(34–46) and

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Abbreviations: GC-C, guanylate cyclase C; EC, enterochromaffin.
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K605 recognizing guanylin-(101–115) had the highest titers and were used for detailed investigations.

Extraction of Guanylin from Plasma and Tissues. Guinea pigs (n = 2) were anesthetized by ether inhalation. Blood (2 ml) was collected into ice-chilled tubes containing K_2EDTA and centrifuged at 2000 × g for 20 min at 4°C. Plasma (1 ml) was diluted 1:1 (vol/vol) with 0.01 M HCl and adjusted to pH 3.0 by concentrated HCl. Tissue specimens from duodenum, jejunum, ileum, and colon were flushed of luminal contents with ice-cold saline. Skeletal muscle was taken as control tissue. All tissues were immediately boiled in 1 M acetic acid for 10 min and homogenized with an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, F.R.G.). The homogenates were centrifuged at 20,000 × g for 20 min at 4°C and the supernatants were filtered through a 0.45-μm (pore size) filter. Plasma and acid tissue extracts were applied to an octadecasilyl (C_{18}) Sep-Pak cartridge (Waters). The column was washed with 0.01 M HCl and material was eluted with 30% (vol/vol) 2-propanol/30% (vol/vol) methanol/0.01 M HCl. The eluted protein fractions were lyophilized and stored at −80°C until use.

Western Blots. For immunoblot analysis, plasma and tissue extracts were incubated for 7 min at 95°C in sample buffer with 4% (wt/vol) SDS (Merck), 50 mM Tris·HCl (pH 8.45), 1 mM EDTA, 3.24 mM dithiothreitol (Roth, Karlsruhe, F.R.G.), 12.5% (wt/vol) glycerol (Merck), and 0.002% bromphenol blue (Merck) (reducing conditions). The samples were separated by tricine–SDS/PAGE in 16.5% gels by the method of Schägger and von Jagow (18). Low molecular mass markers (Boehringer Mannheim) were used for the molecular mass calibration. After electrophoresis, proteins were electroblotted onto hydrophobic polyvinylidene fluoride-based membranes (Pall). To block unspecific binding of antibodies, blot strips were incubated in 5% (wt/vol) skin milk in Tris-buffered saline (TBST) containing 10 mM Tris·HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20. After washing in TBST, the membranes were incubated overnight at 4°C with antisera K42 (diluted 1:1500 in TBST) and K605 (diluted 1:250 in TBST). Immunoreactive proteins were visualized after incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:8000; Sigma) using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as chromogens (Sigma). Proteins remaining in the gels were fixed in 30% methanol/10% acetic acid for 45 min before they were stained with Coomassie brilliant blue in 10% acetic acid/30% methanol for 1–2 h.

Tissues and Tissue Preparation for Immunocytochemistry. Small specimens of the mucosa of all segments of the guinea pig gastrointestinal tract (n = 10) were snap-frozen in Freon 22 precooled with liquid nitrogen, freeze-dried, and fixed by vapor-phase paraformaldehyde (19) or diethyl pyrocarbonate (20). For immunoelectron microscopy, small specimens from the same tissues were fixed by immersion in periodate/lysine/paraformaldehyde (PLP) (21) for 18 h without postosmification, rinsed overnight in 0.05 M sodium phosphate-buffered (pH 7.3) 0.15 M NaCl (PBS) and dehydrated in a graded ethanol series. All specimens were embedded in epoxy resin (Araldite).

Antisera for Immunocytochemistry. The following antisera were used to identify cells: both guanylin antisera (K42 and K605), antisera against chromogranin A and lysozyme, and 13 rabbit antisera against the various gastrointestinal hormones—bovine adrenal medulla dodecapeptide (BAM-12P), cholecystokinin, gastrin, gastric inhibitory polypeptide, glucagon, histamine, motilin, neurotensin, bovine pancreatic polypeptide, peptide tyrosine tyrosine, secretin, serotonin, and somatostatin. Most of these antisera have been characterized and used in previous investigations (see refs. 22–27).

Immunocytochemical Protocol. *Light microscopy.* Serial semithin sections were cut at 0.5 μm and mounted on microscope slides. After removal of the epoxy resin by sodium methoxide (22, 23), the sections were immunostained by the avidin–biotin–peroxidase complex (ABC) technique (28): serial sections were alternatively incubated with guanylin antisera (diluted 1:2000–4000) and with the antisera for cell identification (diluted 1:1000–32,000) for 24 h at 4°C, followed by incubation with the second antibody, biotin-labeled goat anti-rabbit IgG (Jackson ImmunoResearch) for 30 min diluted 1:200. The sections were then incubated for 30 min with a preformed complex of biotin–peroxidase/streptavidin (Jackson ImmunoResearch), diluted in PBS (final concentrations: biotin-peroxidase, 0.7 μg/ml; streptavidin, 5 μg/ml). The antigen–antibody binding sites were visualized by incubation of the sections in 0.7 mM diaminobenzidine hydrochloride/0.002% H_2O_2 in 0.05 M Tris·HCl (pH 7.6). PBS was used as diluent for the antisera and as rinsing solution.

Electron microscopy. Ultrathin sections were immunostained by both guanylin antisera (diluted 1:1000) using the immunogold technique as described (23). Thereafter, the sections were air-dried, counterstained with uranyl acetate for 10 min, rinsed in H_2O for 2 min, and viewed in an electron microscope (Zeiss EM 9S-2).

Specificity Controls. Method-dependent nonspecificities were excluded by running controls as described (see refs. 22 and 23). Antibody specificities were tested by preadsorption of the antisera with homologous and heterologous antigens (see below) at 6.25–100 μg/ml of antiserum (working dilution). Preadsorption of the antiserum K42 with guanylin-(34–46) at concentrations as low as 6.25 μg/ml completely blocked immunostaining. The same was true for preadsorption of the antiserum K605 with guanylin-(101–115). Preadsorption of the antiserum K42 with guanylin-(101–115), as well as with various antigens (listed below), at concentrations up to 100 μg/ml had no effect on immunostaining. Likewise, immunostaining with antiserum K605 was not affected by preadsorption with guanylin-(34–46) and with various unrelated antigens at concentrations up to 100 μg/ml. The antisera against chromogranin A and serotonin could be blocked by preadsorption with the corresponding antigens (at 6.25 μg/ml) but not with guanylin-(34–46) and guanylin-(101–115) (at 100 μg/ml).

Antigens for Preadsorption Controls. Chromogranins (purified from adrenal medullary extracts) were provided by M. Gratzl (Ulm, F.R.G.). Chromostatin was obtained from D. Aunis (Strasbourg, France). Cholecystokinin was provided by W. Schlegel (Munster, F.R.G.). Bovine pancreatic polypeptide was a gift of R. E. Chance (Indianapolis). Guanylin-(34–46) and guanylin-(101–115) (see above); glucagon, serotonin creatinine sulfate, and histamine hydrochloride (all from Serva); BAM-12P, gastrin, gastric inhibitory polypeptide, limpet hemocyanin, motilin, and peptide tyrosine tyrosine (all from Sigma); insulin (Novo Industries, Bagsvaerd, Denmark); and α-neo-endorphin, dynorphin A, neurotensin, pancreastatin, secretin, somatostatin, and substance P (all from Peninsula Laboratories) were obtained as indicated.

RESULTS

Western Blot Analysis. Both guanylin antibodies (K42 and K605) recognized a major intestinal peptide of 10–12 kDa in all lanes loaded with intestinal tissue extracts (Fig. 1). In addition, intensive labeling of a plasma peptide in the same range (10–12 kDa) was obtained with the antiserum K605 but not with K42. With both antisera, a faintly stained band was also visualized at 17–19 kDa in lanes loaded with intestinal proteins. Any crossreactions with the second goat anti-rabbit antibody were excluded by appropriate controls. Western blot analysis of skeletal muscle extracts (control), processed

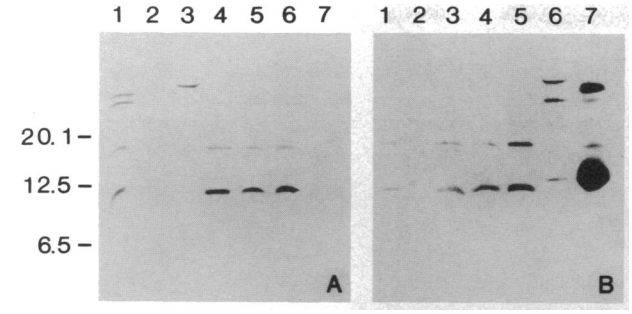

FIG. 1. Western blot of guinea pig tissues and plasma extracts (after tricine–SDS/PAGE electrophoresis) immunostained with antiserum K42 (*A*) and antiserum K605 (*B*). (*A*) Lanes: 1, colon; 2, plasma; 3, skeletal muscle; 4, ileum; 5, jejunum; 6, duodenum; 7, molecular mass markers. (*B*) Lanes: 1, colon; 2, molecular mass markers; 3, duodenum; 4, jejunum; 5, ileum; 6, skeletal muscle; 7, plasma. Note the predominant immunoreactive band of 10–12 kDa obtained with both antisera recognizing different epitopes in the guanylin molecule. Molecular mass markers are indicated (20.1 kDa, a trypsin inhibitor; 12.5 kDa, cytochrome *c*; 6.5 kDa, aprotinin).

on the same gel, showed neither the major immunoreactive band of 10–12 kDa nor the minor band at 17–19 kDa.

Immunocytochemistry. *Light microscopy.* Both guanylin antisera coincidingly immunostained distinct cells located in the epithelium throughout the gastrointestinal tract (Figs. 2 and 3). Within the gastric mucosa, such cells were rarely present; in the upper small intestine, they were numerous; in the lower small intestine and especially in the colon, guanylin-immunoreactive cells occurred rarely. In serial sections, guanylin-immunoreactive cells were also immunostained for chromogranin A, a glycoprotein used as marker for various endocrine cells (24, 29). Hence, guanylin was localized in endocrine cells. Of the various endocrine cell types in the entero-endocrine system (24–26, 30) immunostained by appropriate antisera, exclusively the enterochromaffin (EC) cells (identified by the serotonin antiserum) exhibited immunoreactivity toward both guanylin antisera (Figs. 2 and 3). These cells were located predominantly in the crypt regions but they were also present in the epithelium of the villi of the small intestine. Basically, EC cells of both "closed" and "open" types (26, 30) showed immunoreactivities for guanylin (Fig. 3). However, dependent on the segment investigated, the EC cells were heterogeneous with respect to their guanylin immunoreactivities. In the stomach, EC cells predominantly of the antral pyloric region exhibited immunoreactivity for both guanylin peptides (Fig. 2). In the duodenum, all EC cells detected showed guanylin immunoreactivity. Although many EC cells were present in the remaining intestinal segments, the frequency of guanylin-immunoreactive EC cells continuously decreased from jejunum toward colon, as estimated by comparison of serial sections alternatively immunostained by serotonin and guanylin antisera. Particularly in the colonic mucosa, most EC cells were unreactive toward both guanylin antisera. Beyond such variations in frequency, differences in staining characteristics of the guanylin antisera in the same EC cells were evident. Although in all gastrointestinal segments generally the same EC cells were immunostained by both guanylin antisera, a minority of EC cells displayed immunoreactivity for guanylin-(101–115) but lacked completely or showed faint immunoreactivity for guanylin-(34–46). In no case was immunoreactivity for guanylin found in other epithelial or nonepithelial cells including gastric gland cells and Brunner's gland cells. Paneth cells of the small intestine identified by their lysozyme content (14) were definitely unreactive toward both guanylin antisera.

Electron microscopy. Guanylin-(34–46) and guanylin-(101–115) immunoreactivities were exclusively localized in polymorphous secretory granules typical for EC cells (Fig. 3) that also contain serotonin and chromogranin A (27). The secretory granules were densely and homogeneously labeled by both guanylin antisera.

DISCUSSION

By cDNA analysis, the primary amino acid sequence of guanylin has been elucidated (10–12). Northern blot analyses in various organs showed high-level expression of guanylin mRNA restricted to the intestine (10). Recently, high amounts of guanylin were found in plasma (15), indicating that this peptide may originate mainly from endocrine sources in the intestine. Therefore, the present study was focused on the endocrine system of the gut to determine the cellular and subcellular localization of guanylin. By using two guanylin antisera directed against different epitopes, a predominant immunoreactive peptide in the range of 10–12 kDa was identified by Western blot analyses of extracts of all segments of guinea pig intestine but not of skeletal muscle extracts (negative control). This molecular mass is in accordance with the mass of circulating human guanylin (10.3 kDa; see ref. 15) as well as with the molecular mass deduced for the guanylin prohormone from the cDNA sequence (12.5 kDa; see ref. 10). As described for humans (15), guanylin seems to circulate as a high molecular mass peptide in guinea pig

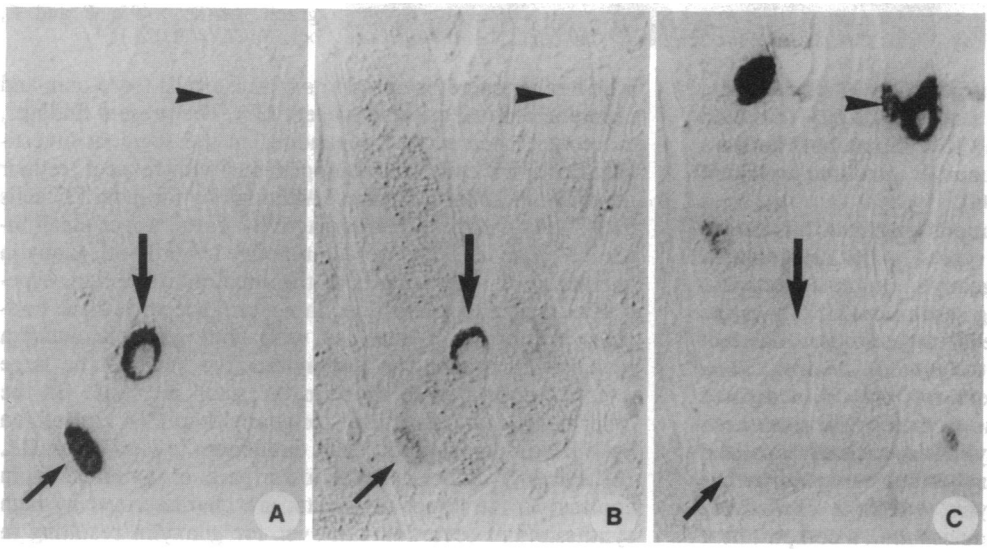

FIG. 2. Three semithin (0.5 μm) serial sections of guinea pig pyloric mucosa immunostained for serotonin (*A*), guanylin (antiserum K42) (*B*), and gastrin (*C*). Guanylin immunoreactivity is present only in EC cells identified by the serotonin antiserum. The EC cells shown contain strong (large arrows) or faint (small arrows) immunoreactivity for guanylin. Gastrin cells are devoid of guanylin immunoreactivity (arrowheads). Interference-contrast microscopy. (×520.)

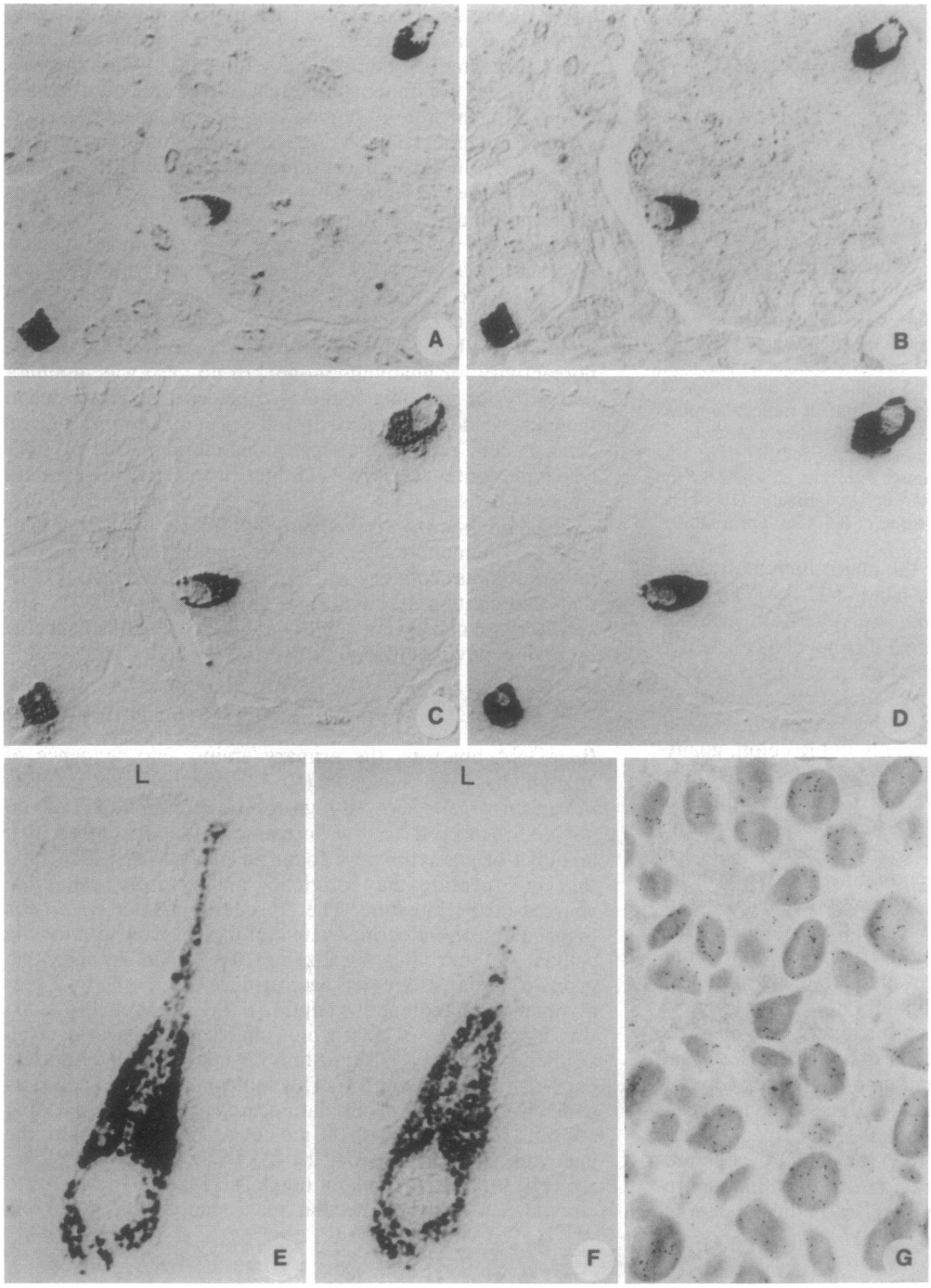

FIG. 3. (*A–D*) Four semithin (0.5 μm) serial sections of the duodenal mucosa immunostained by the guanylin antisera K42 (*A*) and K605 (*B*) and antisera against serotonin (*C*) and chromogranin A (*D*). Three EC cells, identified by the serotonin antiserum, simultaneously exhibit strong immunoreactivities for guanylin-(34–46), guanylin-(101–115), and chromogranin A. (*E* and *F*) Two semithin sections of the jejunal mucosa immunostained for serotonin (*E*) and guanylin-(101–115) (antiserum K605) (*F*). An EC cell (jejunal mucosa) contains strong serotonin and guanylin immunoreactivities predominantly localized at the supranuclear site. This EC cell contacts the lumen (L) through a slender cellular process. (*G*) Subcellular localization of guanylin (antiserum K605) in a duodenal EC cell by the immunogold technique. Guanylin immunoreactivity is confined to polymorphous secretory granules typical for EC cells. *A–F*, interference-contrast micrographs; *G*, electron micrograph. (*A–D*, ×495; *E* and *F*, ×1270; *G*, ×20,025.)

blood, since immunoreactivity of a 10- to 12-kDa plasma peptide was detected with the antibody K605 (directed against the C terminus of guanylin). In addition, both antisera did recognize with lower affinity another intestinal protein of higher molecular mass (17–19 kDa).

Immunocytochemical investigations revealed that among the various entero-endocrine cell types (24–26, 30) identified here, only the EC cells contain guanylin immunoreactivity. The EC cell, regularly containing serotonin (31, 32), is the most abundant endocrine cell type in the gastrointestinal tract and is common to all segments of the gut (30). The coinciding staining by both guanylin antisera not only in a defined endocrine cell population but also in mostly the same cells unequivocally indicates that guanylin is specifically localized in EC cells of the gut. This is further substantiated by the subcellular localization of guanylin, which is exclusively confined to EC-cell secretory granules—i.e., a compartment where other secretory products of these cells (serotonin and chromogranin A) are also present (27). The present findings, moreover, showed that depending on the segment investigated, the EC cells are heterogeneous with respect to their guanylin immunoreactivities. Although by far most EC cells displayed guanylin immunoreactivity in the upper small intestine, many EC cells were unreactive toward both guanylin antisera in the lower parts of the small intestine and, especially, in the large intestine. In accordance with these findings, Western blot analyses with both guanylin antisera revealed faint guanylin immunoreactive band in the large intestine compared to those in the small intestine. On the other hand, increasing levels of guanylin mRNA expression have been demonstrated from duodenum toward colon (11, 12). Possibly, EC cells in the lower parts of the intestine, in contrast to the upper segments, are characterized by high synthesis and secretion turnover for guanylin resulting in

minimal content or in lack of guanylin storage in these cells thus escaping detection by the respective antisera at dilutions used. Remarkably, in the gastric mucosa a small number of EC cells exhibited immunoreactivity for guanylin, although recent studies showed lack of guanylin mRNA expression in the stomach (11, 12), possibly due to minimal amounts of guanylin mRNA in the stomach falling below the sensitivity of the detection system.

From the fact that both guanylin antisera, recognizing different epitopes in the guanylin molecule, yield immunoreactivities concomitantly localized in most EC cells of all tissues investigated, we assume that these cells contain the entire guanylin molecule. The heterogeneous immunoreactivities for guanylin-(101–115) and guanylin-(34–46) found in a small subpopulation of EC cells may be related to intercellular differences in posttranslational modifications or processing of the guanylin molecule but also to a possible existence of unknown guanylin-related peptide(s) in EC cells, especially in view of the immunoreactivity pattern of the guanylin antisera in intestinal extracts as verified by Western blots.

EC cells as typical members of the "diffuse neuroendocrine system" (33) are scattered in the gastrointestinal epithelium. In this location, closed type and open type EC cells have been identified based on their relationship to the gut lumen (30). EC cells are generally accepted as typical endocrine cells of the gut; release of serotonin, the main EC-cell product, from the gastrointestinal tract into the circulation is well documented (34, 35). However, increasing evidence exists also for an endoluminal release of EC-cell secretory constituents (36–38); such a luminocrine secretory pathway is attributed to open type EC cells that reach the gut lumen by apical cell processes (30, 36). Indeed, release of serotonin into the gut lumen has clearly been demonstrated (38–40). Since guanylin is present in EC-cell secretory granules where serotonin is also localized, EC cells predictably release guanylin not only into the circulation but also into the gut lumen. Secretion of guanylin by EC cells through an endocrine pathway is certainly in line with the strong guanylin immunoreactivity in guinea pig plasma as demonstrated here by Western blot analysis and with the high amounts of guanylin found in human plasma (15). In addition, a luminocrine secretion of guanylin from EC cells is quite conceivable by circumstantial evidence and would allow interaction of this peptide with GC-C immediately localized on the brush border of enterocytes (2, 3) to regulate intestinal fluid secretion. Although the EC cell is the most numerous endocrine cell type in the gastrointestinal tract known for several decades, its true function(s) hitherto remained enigmatic. At least one function of EC cells is apparently related to local regulation of water and electrolyte secretion in the gastrointestinal epithelium through a luminocrine secretory pathway. The luminocrine secretory activity of EC cells is obviously under vagal control (39, 40), and interpreted retrospectively, diarrhea observed after stimulation of EC cells (41, 42) may be attributed to guanylin secreted by these cells into the gut lumen. In this respect, future studies should focus on the regulation of guanylin biosynthesis in EC cells and in tumors derived from them (e.g., carcinoids) of which one predominant and common effect is diarrhea.

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