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Biochemistry

Precursor structure, expression, and tissue distribution of human guanylin

(enterotoxin/heat-stable enterotoxin receptor/Paneth cells/intestine)

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ABSTRACT **Heat-stable enterotoxins (STa) are small, cysteine-rich peptides secreted by *Escherichia coli* that are able to induce diarrhea through the stimulation of an intestine-specific receptor-guanylyl cyclase known as STaR. A 15-amino acid peptide, guanylin, was recently purified from rat jejunum and proposed to be a potential endogenous activator of this receptor. We describe here the cloning and characterization of human and mouse cDNAs encoding precursor proteins of 115 and 116 amino acids, respectively, having guanylin present at their C termini. Expression of the human cDNA in mammalian cells leads to the secretion of proguanylin, an inactive 94-amino acid protein. Guanylin generated by either trypsin or acid treatment of proguanylin was purified and found to bind to, and activate, STaR. Northern blot and *in situ* hybridization show high-level expression of guanylin mRNA restricted to the intestine, with localization to Paneth cells at the base of the small intestinal crypts. These results demonstrate that guanylin is an endogenous activator of STaR.**

The recent cloning and characterization of membrane-bound forms of guanylyl cyclases have demonstrated that they function as receptors for small peptides. These receptor-guanylyl cyclases share a common topological organization with a single transmembrane domain separating the extracellular binding domain from the intracellular region. The latter contains two distinct domains, one having homology to protein kinases followed by a C-terminal guanylyl cyclase catalytic domain. The first receptor-guanylyl cyclases to be identified were the natriuretic peptide receptors A and B (NPR-A and NPR-B) (1–4). Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) have been shown to selectively bind and stimulate these two homologous receptors (for review, see ref. 5). These natriuretic peptides, which share a similar structure with a conserved disulfide bond creating a 17-amino acid ring structure, are involved in the regulation of fluid and electrolyte homeostasis.

More recently, a third member of the receptor-guanylyl cyclase family that is specifically expressed in the intestine has been identified as the heat-stable enterotoxin receptor (STaR) (6, 7). Heat-stable enterotoxins (STa) are small peptides of 18 or 19 amino acids, secreted into the intestine by enterotoxigenic strains of *Escherichia coli* (8). The 13 amino acids necessary for the toxic activity of the peptide include 6 cysteines that form three disulfide bridges (9). Binding of STa to STaR induces a dramatic increase of the cGMP content of the cell (10, 11). This increase, in turn, inhibits salt absorption and stimulates chloride secretion. This imbalance of ions is accompanied by a massive accumulation of water in the gut that gives rise to the diarrhea and dehydration characteristic of enterotoxin activity (10).

The initial identification of a receptor for STa on intestinal brush border membranes (12) suggested the existence of an endogenous activator. Recently, guanylin, a 15-amino acid peptide purified from rat small intestine, was described as a potential ligand for the STaR (13). This peptide shares sequence similarity with STa, including four conserved cysteines. Furthermore, guanylin can compete with ^{125}I-labeled STa (^{125}I-STa) binding and stimulate cGMP production in T84 cells, a human colonic cell line known to express the STaR (14, 15). We report here the molecular cloning of the human and the mouse cDNAs encoding guanylin.¶ Their sequences reveal that guanylin is present at the C-terminal end of a larger precursor protein. Characterization of human proguanylin expressed in mammalian cells indicates that the 94-amino acid proguanylin is inactive. The biologically active guanylin can be released by either chemical or enzymatic treatment of proguanylin. We also show by Northern blot and *in situ* hybridization that the guanylin mRNA is specifically expressed in cells of the intestinal epithelium.

MATERIAL AND METHODS

cDNA Cloning. An oligonucleotide probe (5′-CCCAACACCTGTGA$^{G}_{A}$ATCTG$^{C}_{T}$GCCTATGCTGC$^{C}_{T}$TGCACAGGCTG-3′) was derived from the protein sequence for rat guanylin (13) using human codon bias (16) and was synthesized and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. This probe was used to screen 10^6 clones of a human terminal ileum cDNA library in λgt10. Duplicate filters were hybridized under low stringency conditions at 42°C in 20% formamide, 5× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate), 10× Denhardt's, 0.05 M sodium phosphate (pH 6.5), 0.1% sodium pyrophosphate, 50 μg of sonicated salmon sperm DNA per ml, and 10% dextran sulfate. Filters were rinsed in 2× SSC and then washed once in 1× SSC/0.1% SDS at 42°C. Hybridizing phage were plaque-purified and the cDNA inserts were subcloned into the Bluescript plasmid (Stratagene). Both strands were sequenced by the dideoxy chain-termination procedure (17).

Expression. The full-length guanylin cDNA was subcloned into the mammalian cell expression vector pRK5 (R. Klein and D.V.G., unpublished data), under the control of the cytomegalovirus immediate early promoter. Human embryonic kidney 293 cells, maintained in Dulbecco's modified

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Abbreviations: STa, heat-stable enterotoxin(s); NPR-A, natriuretic peptide receptor A; NPR-B, natriuretic peptide receptor B; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; STaR, STa receptor.

¶The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M95174 (human guanylin) and M95175 (mouse guanylin)].

Eagle's medium (DMEM) supplemented with F-12 nutrient mixture, 20 mM Hepes (pH 7.4), and 10% fetal bovine serum, were transfected by the calcium phosphate method as described (18). Twenty-four hours following transfection the cells were rinsed with serum-free medium and then incubated for 4 hr in the presence of 200 μCi of [^{35}S]cysteine and [^{35}S]methionine per ml. Conditioned medium was collected and loaded on a Phast Hi-Density SDS/polyacrylamide gel (Pharmacia). After electrophoresis, the gel was dried and exposed to a storage phosphor imaging plate for 12 hr and then developed with a Fuji Bas-2000 bio-image analyzer (Fuji Medical Systems, Stamford, CT).

Purification of Guanylin. Fifty milliliters of serum-free medium conditioned for 48 hr was loaded on a Vydac C_{18} 300A column (4.7 × 250 mm) and eluted with a linear gradient of 10–40% acetonitrile in 0.1% trifluoroacetic acid (TFA) in 120 min. Fractions containing proguanylin were pooled and rechromatographed on a Synchrom C_4 4000A column (2 × 100 mm). Purified proguanylin was then digested with Promega modified trypsin using an enzyme-to-substrate ratio of 1:50 in 80 μl of 0.2 M NH_4HCO_3 (pH 8.0) for 1 hr at 37°C. The digest was directly injected on a Synchrom C_4 column and the peptides were separated by a linear gradient of 0.1% TFA to 70% acetonitrile/0.08% TFA in 20 min. The active fraction was rechromatographed on the same column using a linear gradient of 0.1% TFA/10% 1-propanol to 40% 1-propanol in 40 min. The sample was analyzed by electrospray mass spectroscopy, amino acid analysis, and protein sequencing.

Protein Sequencing, Amino Acid Analysis, and Mass Spectroscopy. Automated Edman degradation was performed on a 477 Applied Biosystems sequencer equipped with a 120A phenylthiohydantoin amino acid analyzer. Peaks were integrated with a Chrom Perfect (Justice Innovation, Palo Alto, CA) data system (19). Amino acid composition was determined after hydrolysis with 6 M HCl in a Millipore Pico-Taq workstation. The hydrolysates were dried and applied to a Beckman model 6300 amino acid analyzer equipped with ninhydrin detector. Electrospray spectra were obtained on a Sciex APIII triple quadrapole mass spectrometer. Spectra were obtained by direct infusion at a flow rate of 1.5 μl/min.

Binding and cGMP Assay. Competition of ^{125}I-STa binding and guanylyl cyclase stimulation of 293-STaR cells were measured as described (7, 15).

Northern Blot. Approximately 20 μg of total RNA (intestine) or 2 μg of poly(A)$^+$ RNA from various human tissues (Stratagene) was electrophoresed into a formaldehyde/1.2% agarose gel and blotted onto a nylon membrane. The membrane was hybridized with a ^{32}P-labeled guanylin cDNA fragment. The blot was washed at 65°C in 0.1× SSC/0.1% SDS and then exposed overnight to a storage phosphor imaging plate.

***In Situ* Hybridization.** *In situ* hybridization was performed essentially as described (20). ^{35}S-labeled riboprobes were generated from appropriately linearized plasmids containing a full-length cDNA clone of human guanylin. Hybridization was carried out overnight at 55°C in a hybridization buffer containing 50% formamide, 0.3 M NaCl, 1× Denhardt's, 25 μg of tRNA per ml, 10 mM dithiothreitol, and probe at 5 × 10^5 cpm/μl. After hybridization, slides were washed as follows: 50% formamide, 2× SSC, 1× Denhardt's at 55°C, two washes in 2× SSC at room temperature, RNase A at 37°C in 2× SSC, 2× SSC at 42°C, and finally 0.1× SSC at 55°C. Washed, dehydrated slides were dipped in Kodak NBT emulsion and exposed at 4°C for 6–12 days before developing and counterstaining with hematoxylin/eosin.

RESULTS

cDNA Cloning. A DNA probe was synthesized based on the amino acid sequence of rat guanylin (13) using human codon bias and was used to screen a human terminal ileum cDNA library. Several positive clones were identified and five were found to contain inserts of about 600 base pairs (bp). DNA sequencing indicated that these five clones differed only by the length of their poly(A) tails and 5′ untranslated regions. The presumed initiation codon at position 5–7 in the longest clone (Fig. 1) is within a consensus sequence favorable for eukaryotic translation initiation (21) and defines the beginning of an open reading frame of 115 amino acids. The N terminus of the predicted amino acid sequence is highly hydrophobic and probably corresponds to a signal peptide. The consensus rules described by von Heijne (22) indicate potential cleavage sites at positions 16 and 21, generating prohormones of 99 amino acids (10,712 Da) or 94 amino acids (10,343 Da). The prohormone contains six cysteines, an unusually high number of prolines, 12, as well as one potential N-linked glycosylation site. The C terminus of the protein corresponds to the published rat guanylin sequence except for the glycine at position 102 which is asparagine in the rat sequence. The TAG stop codon is followed by 215 nucleotides of 3′ untranslated sequence. A poly(A) consensus signal AAUAAA (23) is found at position 548. In a homology screen, neither the cDNA nor the predicted amino acid sequence showed any significant homology with sequences present in the Genbank or Dayhoff data bases.

A cDNA fragment corresponding to the coding region of human guanylin was used to screen a mouse small intestine cDNA library of a million clones. Approximately 200 positive plaques were identified and 2 of them were sequenced.

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                              10                           ↓          20   ↓                        30
         MetAsnAlaPheLeuLeuPheAlaLeuCysLeuLeuGlyAlaTrpAlaAlaLeuAlaGlyGlyValThrValGlnAspGlyAsnPheSerPheSer
1     tgccATGAATGCCTTCCTGCTCTTCGCACTGTGCCTCCTTGGGGCCTGGGCCGCCTTGGCAGGAGGGGTCACCGTGCAGGATGGAAATTTCTCCTTTTCT

                        40                            50                           60
      LeuGluSerValLysLysLeuLysAspLeuGlnGluProGlnGluProArgValGlyLysLeuArgAsnPheAlaProIleProGlyGluProValValPro
101   CTGGAGTCAGTGAAGAAGCTCAAAGACCTCCAGGAGCCCCAGGAGCCCAGGGTTGGGAAACTCAGGAACTTTGCACCCATCCCTGGTGAACCTGTGGTTC

          • 70                           80  •                       90
        IleLeuCysSerAsnProAsnPheProGluGluLeuLysProLeuCysLysGluProAsnAlaGlnGluIleLeuGlnArgLeuGluGluIleAlaGlu
201   CCATCCTCTGTAGCAACCCGAACTTTCCAGAAGAACTCAAGCCTCTCTGCAAGGAGCCCAATGCCCAGGAGATACTTCAGAGGCTGGAGGAAATCGCTGA

       100          •        •     110     •     •
       Asp[ProGlyThrCysGluIleCysAlaTyrAlaAlaCysThrGlyCys]
301   GGACCCGGGCACATGTGAAATCTGTGCCTACGCTGCCTGTACCGGATGCTAGgggggcttgcccactgcctgcctcccctccgcagcagggaagctcttt

401   tctcctgcagaaagggccacccatgatactccactcccagcagctcaacctaccctggtccagtcgggaggagcagcccggggaggaactgggtgactgg

501   aggcctcgccccaacactgtccttccctgccacttcaacccccagctaataaaccagattccagagtaaaaaaaaaaaaaaaaa
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of human guanylin. Nucleotides are numbered at the beginning of each line. Numbers above the sequence refer to the amino acid sequence. The predicted signal peptide cleavage sites are indicated by arrows. The potential N-linked glycosylation site is underlined and cysteine residues are indicated by a solid dot above the sequence. The amino acid sequence corresponding to the purified rat guanylin (13) is boxed.

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Hu   1 MNAFLLFALCLLGAWAALAGGVTVQDGNFSFSLESVKKLK
Mu   1 MNACVLSVLCLLGALAVLVEGVTVQDGDLSFPLESVKKLK

Hu  41 DLQEPQEPRVGKLRNFAPIPGEPVVPILC-SNPNFPEELK
Mu  41 GLREVQEPRLVSHKKFAPRLLQPVAPQLCSSHSALPEALR

Hu  80 PLCKEPNAQEILQRLEEIAEDPGTCEICAYAACTGC
Mu  81 PVCEKPNAEEILQRLEAIAQDPNTCEICAYAACTGC
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FIG. 2. The predicted amino acid sequences of human (Hu) and mouse (Mu) guanylin are aligned. A gap introduced into the human sequence for optimal alignment is shown by a dash. Identical amino acids are boxed.

Alignment of the human and the mouse amino acid sequences (Fig. 2) indicates that their C-terminal sequences are highly conserved (87% homology between amino acids 85 and 115). The rest of the molecule is much less conserved, with only 58% identity between the first 84 residues. Both cysteines in the precursor region are conserved as well as 8 of the 11 prolines present in the human prohormone. An 8-amino acid motif (LESVKKLK) is present in both sequences at position 33.

Expression. The full-length human cDNA was inserted in the expression vector pRK5 and transfected into the human embryonic kidney 293 cell line. Twenty-four hours later conditioned medium was analyzed for the presence of novel proteins by metabolic labeling and SDS/PAGE (Fig. 3). A 10-kDa protein was detected in the supernatant of cells transfected with pRK-guanylin but not in those transfected with pRK alone. However, the proguanylin-containing conditioned medium was not able to inhibit the binding of ^{125}I-STa binding to 293-STaR cells.

To further characterize the properties of guanylin, human proguanylin was purified from 50 ml of conditioned medium by reverse-phase HPLC. N-terminal sequence analysis and electrospray mass spectrometric analysis (10,337 Da) indicate that the prohormone sequence starts at residue 22, the second predicted cleavage site for the signal peptide, that it is not glycosylated, and that all six cysteines are involved in disulfide bridges. The proguanylin was digested with trypsin and the peptides generated were separated on a C_4 reverse-phase column. The fraction containing the 22-amino acid (2258 Da) C-terminal fragment was characterized by mass spectrometry. The electrospray mass spectrum contains a single ion signal at m/z 1130 that corresponds to the diprotonated peptide-ion. Amino acid sequencing revealed the presence of a single peptide with the correct sequence. Amino acid analysis indicated a yield of ≈12 nmol/50 ml of conditioned medium.

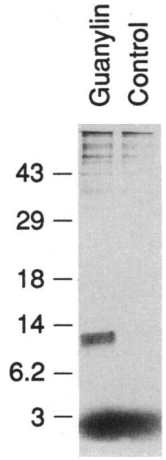

FIG. 3. SDS/PAGE analysis of ^{35}S-labeled proteins from the supernatant of 293 cells that were transiently transfected with pRK or with pRK-guanylin. Molecular mass markers (kDa) are indicated.

The ability of the 22-amino acid peptide to compete with ^{125}I-STa for binding to STaR was studied by incubating intact 293-STaR cells with 25 pM ^{125}I-STa and increasing concentrations of guanylin (Fig. 4*A*). Fifty percent displacement of bound ^{125}I-STa was observed with 100 nM guanylin. To measure the capacity of guanylin to stimulate guanylyl cyclase activity, 293-STaR cells were treated with increasing concentrations of guanylin and the accumulation of intracellular cGMP was measured. In unstimulated cells, 0.6 pmol of cGMP per 10^6 cells was detected. This concentration increased 75-fold to 45 pmol per 10^6 cells in the presence of 0.01 mM guanylin (Fig. 4*B*). With STa, maximal stimulation of 293-STaR cells was obtained at 0.1 μM, where the cGMP

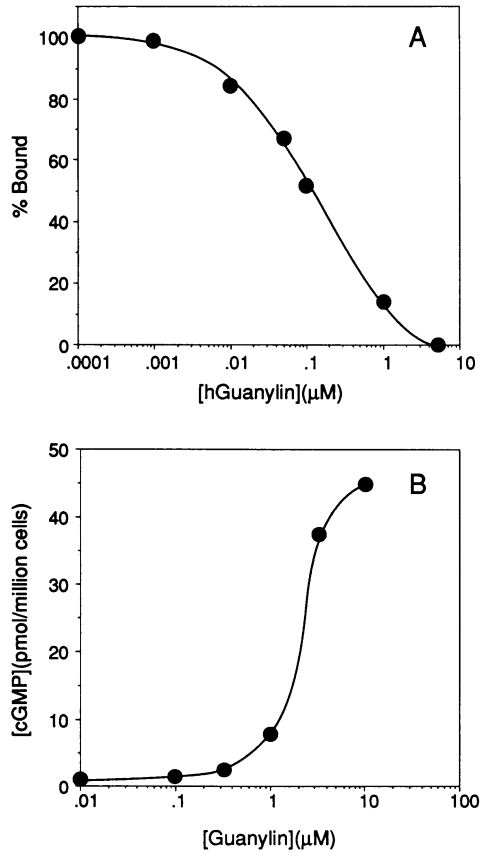

FIG. 4. (*A*) Various concentrations of guanylin were incubated with 25 pM ^{125}I-STa and 2 × 10^5 293-STaR cells. Nonspecific binding was determined in the presence of 5 μM guanylin. The percent specific binding is plotted versus the concentration of STa. Each point represents the mean of duplicate determinations. (*B*) 293-STaR cells were incubated with various concentrations of guanylin for 30 min. Intracellular cGMP accumulation was then determined. Each point represents the mean of duplicate samples assayed in duplicate.

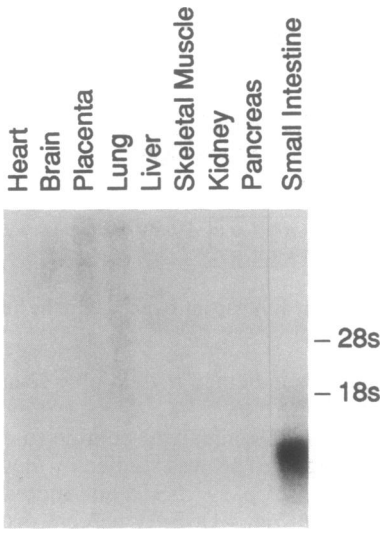

FIG. 5. Northern blot analysis of human guanylin steady-state mRNA levels. RNAs prepared from different human tissues were fractionated on a 1.2% denaturing agarose gel and blotted to nylon. The blot was probed with a 270-bp cDNA fragment corresponding to the coding region of human guanylin.

concentration increased 153-fold to 92 pmol per 10^6 cells (data not shown).

Tissue Distribution of Guanylin. To study the tissue distribution of guanylin, we analyzed RNA from various adult human tissues by Northern blot. A single abundant guanylin transcript of ≈0.7 kilobase was detected only in the small intestine (Fig. 5). Cell-specific expression of guanylin was studied in the small intestine by *in situ* hybridization to sections of human tissue. Strong hybridization of the guanylin antisense probe to cells at the base of the intestinal crypts was observed (Fig. 6*A*). Cells hybridizing to the guanylin probe contained prominent eosinophilic granules in their apical cytoplasm and were located in the same position as cells in parallel sections that hybridized to a human lysozyme probe (data not shown). This indicates that the guanylin-positive cells in the small intestine are Paneth cells (20). Sections hybridized to sense strand riboprobes showed no hybridization signal (Fig. 6*B*).

DISCUSSION

The receptor-guanylyl cyclases are a family of proteins that can be activated by small peptide ligands. The most recent member of the family to be identified is STaR (6, 7), an intestinal receptor for the STa secreted by enterotoxigenic strains of *E. coli*. However, no endogenous ligand able to regulate this receptor had been identified. Recently, a 15-amino acid peptide called guanylin was purified from rat intestine and shown to bind and stimulate the STaR present on T84 cells (13). We report here the cloning of the human and the mouse cDNAs encoding this hormone, confirming that guanylin is of intestinal origin and was not purified from a bacterial contamination of the intestinal extracts. The cDNA sequences revealed that guanylin is the C-terminal end of a larger proguanylin molecule. The natriuretic peptides ANP, BNP, and CNP, which activate the NPR-A and NPR-B receptor-guanylyl cyclases, are also the C-terminal portions of longer precursors. Interestingly, the hormone-containing C-terminal region is highly conserved between human and mouse proguanylin, whereas the N-terminal part of the precursor is much less conserved.

Transfection of the full-length cDNA in human 293 cells leads to the secretion of an inactive 10-kDa protein, indicating that these cells are unable to correctly process the prohormone. The proline previously reported (13) as the first amino acid of the purified guanylin probably does not correspond to the N terminus of the natural hormone. This active peptide was probably generated by cleavage at the Asp-100–Pro-101 bond during the acetic acid boiling step used in the purification process. The only basic residue present in the highly conserved region between the human and the mouse sequences, Arg-93, is more likely to be a physiologic processing site. Therefore we chose to digest proguanylin *in vitro* with trypsin. Cleavage at Arg-93 generates a 22-amino acid C-terminal peptide that is a competitive inhibitor of ^{125}I-STa binding and stimulates cGMP production in 293-STaR cells. However, as for the previously purified 15-amino acid peptide, relatively high concentrations are required for half-maximal displacement of ^{125}I-STa binding (0.1 μM) and

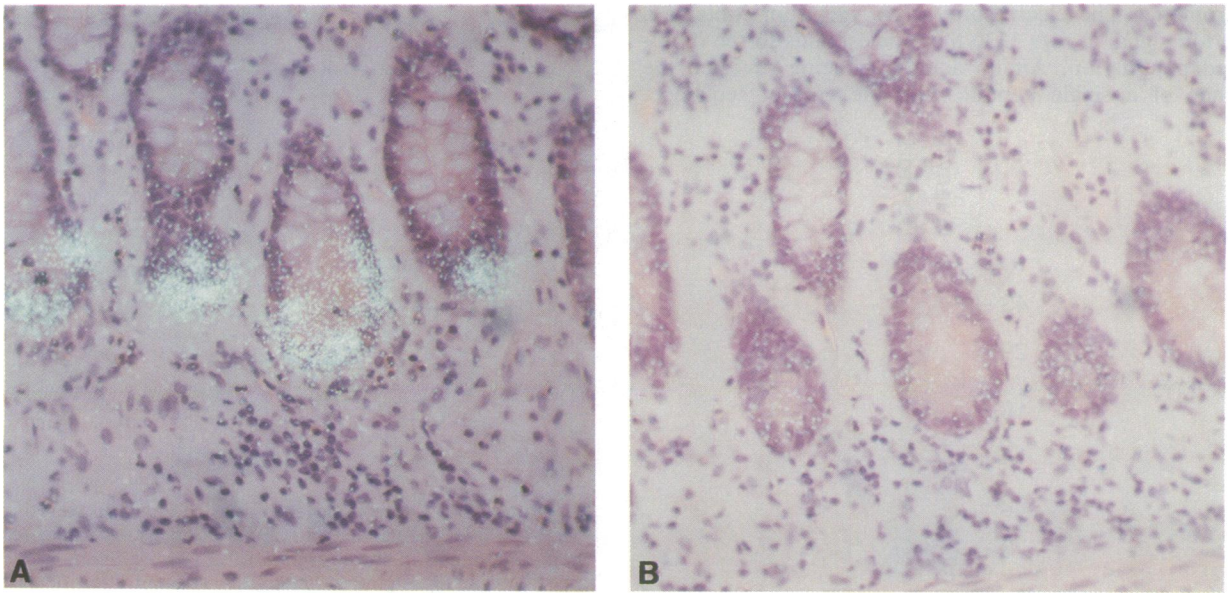

FIG. 6. *In situ* hybridization to human small intestine. ^{35}S-labeled human guanylin antisense (*A*) and sense (*B*) strand riboprobes were hybridized to sections of human small intestine. Sections were exposed for 7 days, counterstained with hematoxylin/eosin, and viewed by epiluminescence and bright-field microscopy. (×60.)

half-maximal cGMP stimulation (2 μM). Even the high levels of guanylin mRNA expression detected in the intestine by Northern blot are unlikely to result in the synthesis of micromolar concentrations of guanylin. Therefore, it may not be possible for the receptor to be maximally stimulated by guanylin *in vivo*. The large amounts of cGMP produced at 0.01 mM guanylin are comparable to those produced at 0.1 μM STa (45 pmol per 10^6 cells versus 92 pmol per 10^6 cells) and might result in diarrhea such as is caused by STa. It is also possible that, *in vivo*, proguanylin is processed at a different site, generating a ligand with greater potency. Dibasic sites are among the most common processing sites in mammals and are cleaved by specific proprotein converting enzymes (24). The Lys-37–Lys-38 dipeptide, which is found within an 8-amino acid motif that is identical in the mouse and human sequences, could represent such a site. However, the use of processing sites upstream from the Arg-93 would generate hormone sequences that would be less well conserved between human and mice and that might exhibit species specificity. Alternatively, there may be another, as yet unidentified, ligand with a higher affinity for STaR. For example, NPR-A has two ligands, ANP and BNP, that bind to the receptor and stimulate cGMP production, but ANP is 10-fold more potent than BNP (4).

Northern blot analysis of human mRNA did not reveal expression in tissues other than the intestine. However, more extensive *in situ* hybridization analysis or the use of more sensitive techniques such as PCR may demonstrate expression in other tissues, as a small amount of guanylin-like activity was detected in the rat kidney (13). Alternatively, study of other tissues may lead to the identification of guanylin-related ligands that signal through the STaR or other related receptor-guanylyl cyclases.

As evidenced by the severe secretory diarrhea resulting from infection with enterotoxin-expressing bacteria, activation of intestinal guanylyl cyclase can have profound effects on fluid balance in the intestine. Guanylin and perhaps related molecules are likely to be the endogenous mediators regulating secretion in the small and large intestine under physiological and pathological conditions. In the latter case, increased intestinal mobility and copious secretion may constitute a defense mechanism against potentially invasive microorganisms. This interpretation is consistent with the expression of guanylin in Paneth cells of the small intestine. As Paneth cell numbers are increased in response to inflammation and bacterial colonization, it is thought they play a significant role in regulating the fauna of the small intestine. Furthermore, Paneth cells synthesize a small group of proteins (lysozyme, cryptdin, defensins) that are primarily microbicidal (25, 26). However, Paneth cells also express other genes not directly involved in microbial killing, such as tumor necrosis factor (20) and epidermal growth factor (27). Thus guanylin may have other effects on epithelial cells that remain to be defined. It will be of great interest to examine the expression of guanylin in human disease and in animal models of intestinal pathology.

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