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Purification, cDNA sequence, and tissue distribution of rat uroguanylin

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Abstract

Guanylin, a peptide purified from rat jejunum, is thought to regulate water and electrolyte balance in the intestine. We show here, using a combination of Northern blots, Western blots, and functional assays, that guanylin and its receptor (GCC) are not distributed in parallel within the rat intestine. To investigate the possibility that there might be a second intestinal peptide that serves as a ligand for GCC, we assayed tissue extracts for the ability to stimulate cyclic GMP synthesis in a GCC-expressing cell line. Duodenal extracts display a peak of biological activity that is not present in colon and that does not comigrate with guanylin or proguanylin. The activity co-purifies with a novel peptide (TIATDECELCINVACTGC) that has high homology with uroguanylin, a peptide initially purified from human and opossum urine. A rat uroguanylin cDNA clone was found to encode a propeptide whose C-terminus corresponds to our purified peptide. Northern blots with probes generated from this clone reveal that prouroguanylin mRNA is strongly expressed in proximal small intestine, but virtually absent from colon, corroborating our biochemical measurements. Taken together, these studies demonstrate an intestinal origin for uroguanylin, and show that within the intestine its distribution is complementary to that of guanylin. © 1997 Elsevier Science B.V.

Keywords: GCC; STa receptor; CFTR; Guanylin; Uroguanylin

1. Introduction

A considerable body of evidence supports a role for the cyclic GMP pathway in the control of ion transport in the gastrointestinal tract. Elevation of intracellular cyclic GMP levels in intestinal epithelial cells enhances secretion of chloride into the intestinal lumen [1], and diminishes absorption of sodium and chloride [2]. The combination of increased secretion and decreased absorption elevates the osmolarity of the lumen, and drives the luminal accumulation of water. This mechanism was initially identified because it can be induced by heat-stable enterotoxin (STa)[1], an 18 amino acid peptide secreted by pathogenic strains of *Escherichia coli* [3–5]. Exposure to high levels of toxin, as occurs during acute bacterial infections, triggers non-physiological movement of electrolytes, and produces a watery diarrhea that can lead to dehydration and death.

When the STa receptor was cloned from a small intestinal cDNA library [6], it was found to belong to a family of receptors that contain endogenous guanylate cyclase (GC) activity. Two other members of this family are the natriuretic peptide receptors, GCA and GCB [7]. Because the STa receptor was the third such receptor cloned, it was named GCC. All members of this family contain: (a) an intracellular catalytic domain responsible for the conversion of GTP to cyclic GMP, (b) an intracellular regulatory domain that controls the activity of the catalytic domain, (c) a single transmembrane domain, and (d) an extracellular receptor domain that provides an agonist binding site [7].

These findings led to the hypothesis that GCC serves as a receptor for one or more endogenous ligands in the GI

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[1]The abbreviations used are: STa, heat-stable enterotoxin; GCA, guanylate cyclase type A; GCB, guanylate cyclase type B; GCC, guanylate cyclase type C; HBSS, Hanks' buffered salt solution; IBMX, 3-isobutyl-1-methylxanthine; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; BSA, bovine serum albumin; RIA, radioimmunoassay.

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tract, by analogy to the way that the other membrane-associated guanylate cyclases (GCA and GCB) serve as receptors for peptide ligands produced by tissues like the heart and brain [7]. Recently, a candidate intestinal peptide, called guanylin, was purified from jejunal extracts [8]. Guanylin is believed to be a natural ligand for GCC because it (a) elevates intracellular cyclic GMP levels in GCC-expressing cells [8], (b) competes with STa for binding to GCC [8–10], and (c) stimulates the secretion of chloride by intestinal epithelial cells [9,11–15]. One key feature shared by guanylin and STa is a set of four conserved cysteines connected by specific disulfide bonds; this provides the secondary structure required for biological activity [8,16].

The mRNA encoding GCC is strongly expressed in the intestine [6,17], exclusively in epithelial cells [18,19]. No other tissue tested displays significant levels. However, binding studies indicate that receptors for guanylin are present in the kidney [20,21]. Furthermore, STa and guanylin can stimulate sodium and potassium excretion by the isolated, perfused kidney [22,23] and can elevate cyclic GMP in organ cultured kidney slices [21,24]. These observations suggest that guanylin, or a guanylin-like peptide, may play a role in regulating kidney function. One possibility is that guanylin is produced locally in the kidney. Indeed, low levels of guanylin probe hybridization have been reported on Northern blots of mRNA isolated from rat kidney [25]. However, this observation was not confirmed in comparable studies with mouse [26] or human [10] kidney, and, furthermore, only small amounts of guanylin-like bioactivity are present in rat kidney extracts [8,27].

A second possibility is that guanylin or a related peptide is delivered to the kidney from some other source. In an effort to identify such a peptide, Hamra et al. [28] and Kita et al. [29] analyzed opossum and human urine for the presence of guanylin-like peptides. They found small amounts of guanylin, and larger amounts of a second, structurally-similar peptide, which they named uroguanylin. Uroguanylin can bind to the STa/guanylin receptor: its EC_{50} for activating cyclic GMP synthesis in GCC-expressing cells is intermediate between that of guanylin and STa [28,29], and it can competitively displace ^{125}I-STa binding [28,29]. Uroguanylin is similar in amino acid sequence to guanylin and STa, and it retains their characteristic disulfide bond structure. Thus, guanylin and uroguanylin define a family of naturally occurring peptides that are structurally and functionally related.

These studies leave open the question of what tissue serves as the biological source of uroguanylin found in the urine. In our current study, we show that small intestine is potentially one such source: (a) Rat duodenal extracts contain a peptide that displays guanylin-like bioactivity, but is chromatographically distinct from guanylin. (b) This second peptide is much more closely related to uroguanylin (80% amino acid identity with human and opossum uroguanylin) than to guanylin (50% amino acid identity with rat guanylin). (c) Cloning of a rat uroguanylin cDNA confirms that this second peptide represents the rat isoform of uroguanylin. (d) Northern blots show relatively selective expression of uroguanylin mRNA in proximal small intestine.

2. Experimental procedures

2.1. Tissue and Extract Preparation

Tissues were removed from Sprague–Dawley rats (250–275 g) under urethane anesthesia (1.6 g urethane/kg administered via i.p. injection). For RNA isolation, the whole intestinal tract was removed and put in ice cold Ringer's-glucose (130 mM Na^+, 120 mM Cl^-, 25 mM HCO_3^-, 1.2 mM Mg^{2+}, 1.2 mM Ca^{2+}, 2.4 mM K_2HPO_4, 0.4 mM KH_2PO_4, 10 mM glucose). Tissues were then isolated as rapidly as possible, frozen on dry ice, and stored at −80°C until used for RNA purification.

For the preparation of peptide-containing extracts for Western blots, the mucosal layer of each tissue was stripped free of the muscle layers, and homogenized in a buffer containing protease inhibitors (25 mM HEPES pH = 7.4, 1 mM phenylmethylsulfonylfluoride, 10 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM benzamidine). After homogenization, the extracts were centrifuged at 10 000 × g for 20 min and insoluble material was discarded. The protein concentration was determined by the bicinchoninic acid method (BCA kit obtained from Pierce).

For the preparation of peptide-containing extracts for HPLC fractionation, rat intestines were frozen on dry ice, and kept at −80°C until used. After thawing, the intestines were divided into regions corresponding to duodenum and colon. Duodenal tissue was taken as the 4 to 5 cm segment of intestine immediately distal to the stomach. Colon was taken to include both proximal and distal segments of the large bowel (posterior to the caecum and anterior to the sigmoid colon). Tissue pieces were split lengthwise and rinsed with normal saline. The tissue was minced in 10 volumes 1 M acetic acid, placed in a boiling water bath for 5 min, and then homogenized. Boiled extracts were centrifuged at 4°C for 20 min at 230 000 × g. The resulting supernatant fractions were filtered through Whatman No. 2 paper and applied to Waters C_{18} Sep-Pak cartridges. Unbound and weakly bound material was washed through with a solution of 10% acetonitrile–0.1% trifluoroacetic acid (TFA) in water. Tightly bound material was eluted with 60% acetonitrile–0.1% TFA. The eluted material was dried under vacuum and reconstituted either in bioassay medium (Hanks' buffered salt solution containing 1 mM IBMX) for bioassay on T84 cells, or in 10% acetonitrile/0.1% TFA for HPLC analysis (see below).

For measuring region-specific responses to STa, segments of tissue approximately 4 cm in length were placed in a dissecting pan filled with 37°C Ringer's-glucose bubbled continuously with 95% O_2–5% CO_2. Tubular sections were cut longitudinally, exposing a flat luminal surface, and luminal contents were discarded. Individual pieces of tissue (0.5 cm × 0.5 cm) were excised, placed in shell vials containing standard Ringer's-glucose solution at 37°C, and exposed to test solutions (see below).

2.2. Northern blots

Selected regions of the uroguanylin, guanylin, and GCC genes were amplified by PCR and subcloned into plasmid vectors (pBS, Stratagene), as described below and in a previous publication [18]. The cDNA inserts were isolated and used as templates for the synthesis of randomly-primed [^{32}P]-labeled cDNA probes (DECAprime II kit, Ambion).

Total RNA was isolated by standard techniques [30], fractionated on a 1% agarose formaldehyde gel (2.2 M formaldehyde), and transferred to a nylon membrane (ICN). Membranes were treated with 10 ml prehybridization solution (50% formamide, 5X SSPE, 5X Denhardt's, 0.25mg/ml sperm DNA, 0.5% SDS) for 3 h and then hybridized in 10 ml Northern blot hybridization solution (50% formamide, 5X SSPE, 1X Denhardt's, 0.1 mg/ml sperm DNA, 0.1% SDS, 10% dextran sulfate with 10^6 cpm/ml of each [^{32}P]-labeled probe) at 42°C for 24 h (SSPE = 150 mM NaCl, 11.5 mM NaH_2PO_4, 1 mM EDTA, pH 7.4). Membranes were then washed twice (15 min each) at room temperature in 2X SSC with 0.1% SDS (SSC = 150 mM NaCl, 15 mM Na citrate, pH 7), followed by two 30 min washes at 55°C in 0.1X SSC with 0.1% SDS.

2.3. Western blots

Samples of tissue extracts or HPLC fractions were dried under vacuum, boiled in electrophoresis sample buffer, and fractionated on 15% SDS–polyacrylamide gels made according to standard procedures [31] except for the composition of the electrode buffers (upper = 100 mM Tris-OH, 100mM tricine, 0.1% SDS, pH 8.3; lower = 200mM Tris-Cl, pH 8.9). After electrophoresis, samples were transferred to 0.1 μm nitrocellulose membranes (Schleicher and Schuell) using a TE 22 transphor apparatus (Hoefer Scientific). The membranes were blocked with 3% BSA, washed, and incubated for 1 h at room temperature with a 1:500 dilution of antiserum 2538, a polyclonal antiserum that was raised against a 14 amino acid synthetic peptide whose sequence appears near the amino terminus of the rat guanylin prohormone [32]. The membranes were then washed and incubated with a secondary antibody (horseradish peroxidase-conjugated sheep-antirabbit IgG diluted 1:10 000, Boehringer-Mannheim) for 60 min at room temperature. After an additional wash, the membranes were treated with chemiluminescence reagent as specified by the manufacturer (Boehringer-Mannheim), and exposed to Kodak XAR-5 film.

2.4. Bioassay

The studies described below have made use of two different bioassay procedures. The first procedure was used to evaluate the relative ability of colon and duodenum to respond to STa (Fig. 1b). Shell vials containing 1 ml standard Ringer's-glucose solution with 0.5 mM IBMX were placed in a 37°C water bath. Vials were continuously bubbled with 95% O_2–5% CO_2 throughout the experiment. During the period of temperature and gas equilibration, 100 units/ml STa (Sigma) was added to the appropriate vials. Pieces of tissue from each region of the gut were then placed in the vials for 30 min. Reactions were stopped by removing the tissues from the vials and quick freezing them on a metal plate resting on a bed of dry ice. The frozen tissue was then homogenized in 6% trichloroacetic acid (TCA) and centrifuged to separate TCA-insoluble protein from TCA-soluble cyclic GMP. The protein pellet was dissolved by heating (37°C) in 1 M NaOH. Cyclic GMP was quantitated by RIA [33,34] and protein was determined using a dye-binding assay (Bio-Rad Bradford Assay) with BSA as standard. Results are reported as pmol cyclic GMP/mg protein.

The second procedure was used to evaluate HPLC fractions for guanylin-like activity (Figs. 2–5). This bioassay, based on the method of Currie et al. [8], employs a reporter cell line (T84 cells) to detect the presence of GCC ligands, as manifested by an increase in intracellular cyclic GMP levels. T84 cells are derived from a human colon carcinoma, and retain many properties of crypt epithelium, including expression of high levels of GCC [35]. In addition, because T84 cells express very low levels of the other known membrane cyclases [8,36], they provide a relatively specific bioassay system for ligands that target GCC. The cells were grown to confluency in 12- or 24-well plastic trays. Cells were then washed two times with HBSS and incubated for 10 min at 37°C in HBSS containing 1 mM IBMX. HPLC fractions were reconstituted in bioassay medium, then applied to the cells for an additional 30 min. The reaction was stopped by removing the incubation solution and replacing it with 0.5 ml ice cold 6% TCA. Cells were scraped and transferred to plastic microcentrifuge tubes and the wells were rinsed with an additional 0.5 ml ice cold 6% TCA. Protein and cyclic GMP content were determined as above. Because the protein content per well is quite uniform for T84 cell cultures within a single plating, experimental results are reported as pmol cyclic GMP/well rather than pmol cyclic GMP/mg protein.

Activation of HPLC fractions: In our initial studies, the biological activity of our HPLC fractions was quite low.

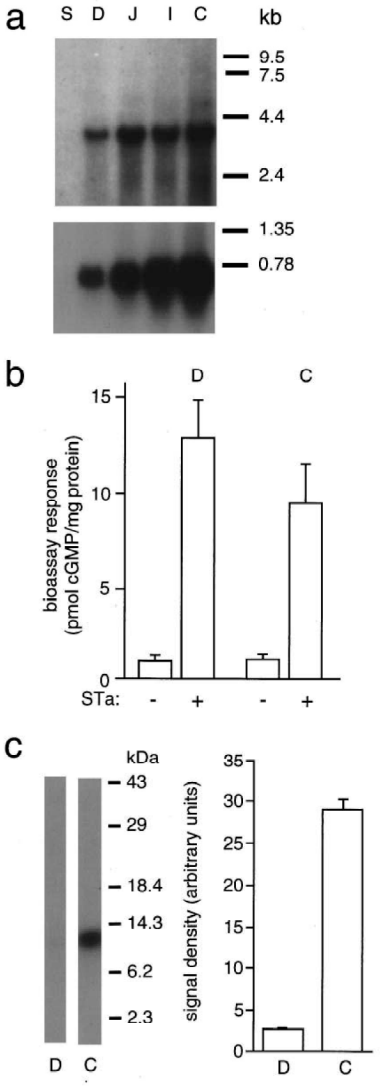

Fig. 1. Distribution of guanylin and GCC along the rostrocaudal axis of the GI tract. (a) Northern blot of poly A^+ RNA (10 μg/lane) hybridized with radiolabeled guanylin and GCC probes. RNA was isolated from rat stomach (S), duodenum (D), jejunum (J), ileum (I), or colon (C). All lanes are from a single membrane hybridized simultaneously with probes for GCC (upper), and guanylin (lower). Because the guanylin transcript is much more abundant than the GCC transcript, the lower half of the blot is shown after a 14 h exposure, and the upper half after a 4 day exposure. Size standards, in kb, are indicated on the right. Tissue was pooled from three animals to obtain the RNA for this blot. Comparable results (not shown) have been obtained with RNA isolated from individual animals. (b) Cyclic GMP levels in duodenum (D) and colon (C) after incubation with (+) or without (−) STa at 100 units/ml. Each bar indicates the mean and standard error of nine determinations. (c) Western blot of tissue extracts performed with an antibody raised against an amino terminal domain of the guanylin prohormone. The autoradiograms (left) are of representative duodenal (D) and colonic (C) samples obtained from a single animal, with the positions of molecular weight standards as indicated. The bar graph (right) presents the densitometrically-determined mean (±SEM) of data obtained from four separate animals.

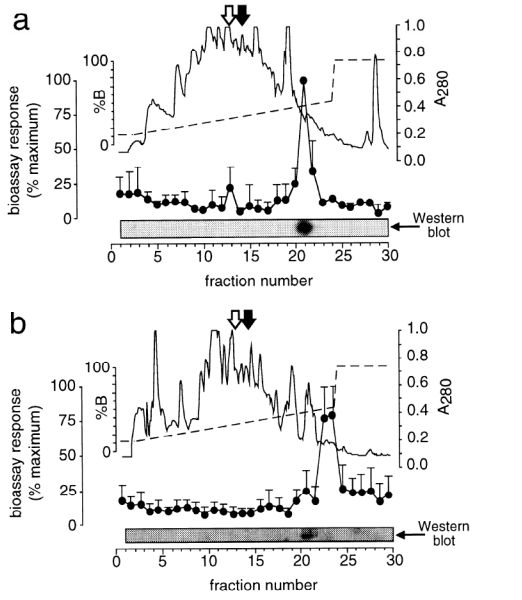

Fig. 2. HPLC comparison of colonic and duodenal extracts. (a) Two extracts (generated from 15 and 30 colons, respectively) were independently analyzed by reverse phase HPLC, as described in Section 2. Upper trace: representative UV absorbance profile from one of the extracts. Middle trace (dashed line): the gradient of acetonitrile used to elute the column. Lower trace: bioassay responses to individual column fractions (mean±range from the two column runs, normalized as a percentage of the maximum response to correct for differences in the potencies of the extracts). Fractions were preincubated at 37°C before the bioassay was performed, in order to enhance their activity (see Section 2). At the bottom of the panel are Western blot data for HPLC fractions from one column run (pooled in pairs, except for fractions 19–26, which were analyzed individually) using an antibody that recognizes the N-terminus of the guanylin prohormone. An abbreviated region of the gel containing the immunoreactive proguanylin band is shown. The black and white arrows indicate the retention times of synthetic rat guanylin and opossum uroguanylin standards, respectively. (b) Three extracts (each generated from 15–30 duodena) were independently chromatographed under conditions identical to those employed for colonic extracts. UV absorbance, elution profile, and bioassay responses are plotted as in panel (a).

Therefore, in order to obtain duplicate bioassay measurements, we dried each HPLC fraction, reconstituted it in bioassay buffer, applied the entire fraction to one well of T84 cells for 30 min, and then transferred it to a second well for 30 min. We noticed that the response of the cells in the second well was always significantly greater than the response of the cells in the first well, suggesting that something was happening during the first incubation to activate the sample. Further experiments showed that samples could be activated simply by incubating them at 37°C for 30 min. As described in Section 3, we believe that this is due to proteolytic cleavage by a contaminating protease. We have used this activation procedure to evaluate samples in all of the HPLC analyses presented below; in some cases the activation was achieved by incubating the sample on a well of T84 cells at 37°C, in others by

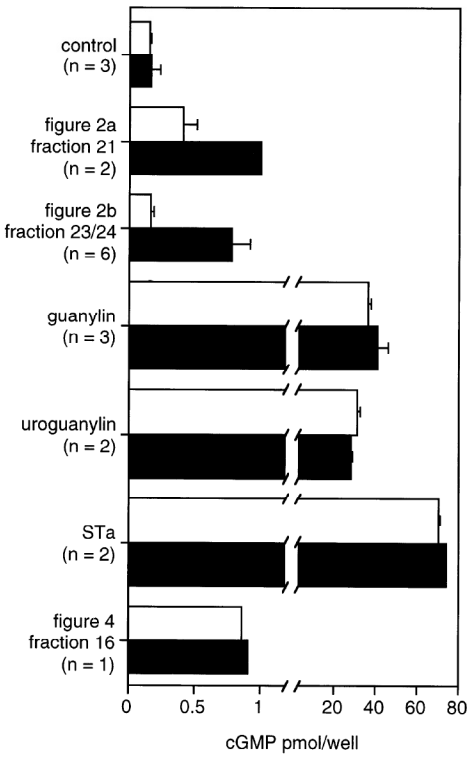

Fig. 3. Preincubation at 37°C enhances the activity of HPLC column fractions, but not of synthetic guanylin or uroguanylin. The bars show the mean level of cyclic GMP (±SEM or range) in T84 cells after exposure to the indicated stimuli. Each stimulus was either held at 4°C (□) or incubated at 37°C (■) prior to applying it to the cells. The numbers used to identify the HPLC fractions correspond to (i) the figure in which the pertinent HPLC run is shown and (ii) the appropriate fraction number(s) from that column run.

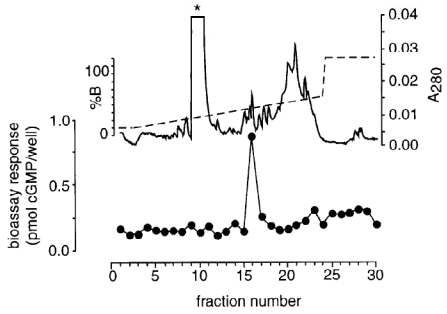

Fig. 4. Preincubation at 37°C leads to a shift in the retention time of the duodenal peptide. An extract of 30 duodena was fractionated as in Fig. 2b. Fractions 23 and 24 were pooled, dried under vacuum (to remove HPLC solvents), resuspended in bioassay medium, incubated for 60 min at 37°C, and rechromatographed on the same column using the same elution profile. The upper trace shows UV absorbance; the dashed line shows the elution profile; the lower trace shows bioassay responses to individual fractions. Biological activity now elutes in fraction 16, while most of the UV absorbing material continues to elute in fractions 23 and 24. The large peak marked with the asterisk is due to IBMX, which was present in the bioassay medium.

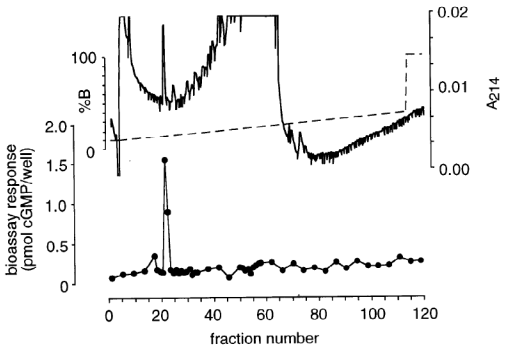

Fig. 5. Final step in purification of the duodenal peptide. Fraction 16 from the column run illustrated in Figure 4 was dried under vacuum, resuspended in 10% acetonitrile + 0.1% TFA, and rechromatographed as described in Section 2. The upper trace shows UV absorbance; the dashed line shows the elution profile; the lower trace shows bioassay responses to individual fractions.

incubating it without cells at 37°C. We have the impression that the activation process occurs more efficiently if T84 cells are present, but we have not compared the two procedures in enough detail to be certain of this.

2.5. HPLC purification

2.5.1. Step 1

Reconstituted Sep-Pak fractions were fractionated on a PepRPC HR5/5 (C_{18}) column (Pharmacia), pre-equilibrated with 10% buffer B (buffer A = 0.1% TFA; buffer B = 99.9% acetonitrile + 0.1% TFA). After sample application, the column was eluted isocratically for 5 min with 10% buffer B, followed by a linear gradient to 50% buffer B over 43 min, and a final 12 min elution at 100% buffer B. One ml fractions were collected at a flow rate of 0.5 ml/min, while absorbance was monitored at 214 and 280 nm. After chromatography, a portion of each fraction was lyophilized (to eliminate the acetonitrile and TFA), resuspended in bioassay medium, incubated at 37°C for 30 min to activate the peptide (see above), and tested for activity on T84 cells. Typical examples of such chromatograms are shown in Fig. 2.

2.5.2. Step 2

Duodenal fractions with activity were combined for further purification. The pooled fractions were incubated at 37°C for 60 min, then reapplied to the PepRPC HR5/5 column and eluted without modification of the protocol described in step 1. The duodenal peptide now elutes earlier than in step 1, whereas most contaminants retain their original retention time. The fractions containing activity were identified by bioassay, as shown in Fig. 4.

2.5.3. Step 3

Active fractions from step 2 were pooled and applied to a C_{18} analytical column (Vydac), pre-equilibrated with 10% buffer B (buffer A, 10 mM ammonium acetate pH 6.2; buffer B, 99% acetonitrile + 10 mM ammonium acetate pH 6.2). The column was eluted isocratically for 10 min with 10% buffer B, followed by a linear gradient to 40% buffer B over 160 min, and a final 10 min elution at 100% buffer B. Fractions (1.5 ml) were collected at a flow rate of 1 ml/min, and analyzed by bioassay, as shown in Fig. 5.

Synthetic 15 amino acid rat guanylin and opossum uroguanylin (used as HPLC standards) were generously provided by Drs. Ding Chang (Peninsula Labs) and Leonard Forte (University of Missouri), respectively.

2.6. Peptide characterization

Sequencing was performed on a sample of peptide adsorbed to a 0.22 micron PVDF membrane, using a Perkin Elmer/ABI model 491 sequencer with on-line PTH amino acid detection.

2.7. cDNA analysis

Rat duodenal RNA was prepared as described previously [18], and reverse transcribed with SuperScript II using an oligo dT primer (Gibco BRL). The resulting cDNA was then subjected to PCR, using degenerate primers designed from areas of high homology in published sequences of human and opossum uroguanylin [37,38]. The sense primer, TACATCCAGTA(CT)(GC)A(AG)GCCTTCC, and antisense primer, GCAGCC(GT)GTACA(GC)GC(AC)-ACGTT, correspond, respectively, to base pairs 104–124 and 334–354 of the human transcript [38]. PCR was performed for 40 cycles (denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension at 72°C for 2 min) followed by a final extension for 10 min at 72° C using Taq polymerase (Boehringer Mannheim). A 250 bp product was amplified, as expected. This PCR product was subcloned into the pBluescript II SK^- vector using the T/A cloning method [39]. DNA was sequenced at the UNC-CH Automated DNA Sequencing Facility on a Model 373A DNA Sequencer (Applied Biosystems) using the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems). Sequence analysis revealed a high degree of homology at the nucleotide level to the human and opossum forms of uroguanylin (72 and 60%, respectively), confirming that we had amplified the appropriate target sequence.

To determine the full-length uroguanylin cDNA sequence, we used the PCR product to produce a random-primed probe for screening a rat duodenal cDNA library constructed in λGT-11 [40] (a gift from Dr. Andrew Leiter). Phage plaques were adsorbed onto nitrocellulose filters, hybridized with the probe in 50% formamide, 0.8 M NaCl, 20 mM PIPES pH 6.5, 0.5% SDS and 100 μg/ml salmon sperm DNA at 42°C, and washed in 0.1X SSC/0.1% SDS at 55°C. Positive plaques were purified by sequential low-density plating and bacteriophage DNA was isolated with a Qiagen Lambda kit (Qiagen). The cDNA insert was excised with *EcoRI* and subcloned into the pBluescript II SK^- vector for sequencing, as described above.

3. Results

3.1. Guanylin and its receptor are not distributed in parallel in the GI tract

Previous studies have shown that guanylin mRNA is expressed in a rostrocaudal gradient, ranging from quite low in duodenum to quite high in colon [9,32,41,42]. In contrast, as shown in Fig. 1a, the mRNA encoding the guanylin receptor (GCC) is expressed at high levels throughout the GI tract. These data reveal a nonparallel distribution of guanylin and its receptor, with the mismatch particularly evident in duodenum.

To verify that the high level of GCC transcript in duodenum corresponds to a high level of functional receptor, we applied a sub-saturating dose of a GCC-specific ligand (STa) to excised pieces of tissue in organ culture, and measured intracellular levels of cyclic GMP after 30 min of exposure (a time at which the response is still proceeding linearly). This experiment was performed in the presence of a phosphodiesterase inhibitor, isobutyl methylxanthine (IBMX), to minimize the effects of phosphodiesterase enzymes on cyclic GMP metabolism. The agonist-dependent increase in cyclic GMP levels (the amount of cyclic GMP in STa-stimulated tissue minus the amount in unstimulated control tissue) is slightly greater in duodenum than it is in colon (Fig. 1b), indicating that duodenum does express substantial levels of functional receptor.

To verify that the low level of guanylin transcript in duodenum corresponds to a low level of guanylin propeptide, we performed Western blots on extracts of duodenum and colon, using an antibody that recognizes the N-terminus of the propeptide [32]. Extracts were prepared from four separate animals; in each case the level of proguanylin in duodenum, as measured densitometrically, was less than 11% of the level in colon (mean = 8.7% ±1.0% (SEM)) (Fig. 1c).

3.2. HPLC analysis of guanylin-like peptides in intestinal extracts

The ligand/receptor mismatch described above suggests the possibility that duodenum might produce a ligand that resembles guanylin in its receptor specificity, but is biochemically distinct. In order to compare guanylin-like

peptides expressed in duodenum to those expressed in colon, we prepared aqueous extracts of each tissue, fractionated the extracts by HPLC, and assayed an aliquot of each fraction for its ability to stimulate cyclic GMP synthesis in cultured T84 cells, as described in the Section 2. In parallel with these bioassays, we performed Western blots on a sample of each HPLC fraction, using the antibody described in Fig. 1c. For convenience, only the region of the blot containing immunoreactive proguanylin is shown in the figures below.

Colonic extracts contain both guanylin and proguanylin. Fig. 2a illustrates the HPLC analysis of a colonic extract. The Western blot inset at the bottom of the figure shows that proguanylin elutes in fraction 21. When the fractions are bioassayed, a peak of cyclic GMP-promoting activity is also seen in fraction 21. In the course of characterizing this peak, we noticed that its biological activity increases with time if it is incubated at 37°C (Fig. 3). Such an increase is not observed when synthetic guanylin, synthetic uroguanylin, or commercially-purified STa are incubated under similar conditions (Fig. 3). We do not yet know the mechanism by which this time-dependent enhancement of activity occurs; however, as discussed below, we consider it likely that proguanylin, which is biologically relatively inactive [10,25,42,43], has co-eluted with a protease capable of converting it to a smaller, more active peptide. Hamra et al. have previously demonstrated that specific proteases enhance the activity of proguanylin [44].

Duodenal extracts contain biologically-active material that is distinct from both guanylin and proguanylin. When duodenal extracts are analyzed by HPLC, proguanylin-like immunoreactivity is again observed in fraction 21 (Western blot inset, Fig. 2b), though, as expected, the amount is much less than can be seen in comparable colonic extracts (Western blot inset, Fig. 2a). The T84 cell assay confirms that this duodenal proguanylin is associated with a small peak of biological activity, which, as above, can be enhanced by preincubation at 37°C. However, the most conspicuous aspect of the chromatogram is the presence of a second, much larger peak of activity in fractions 23 and 24. This material differs from proguanylin in two ways: it is retained more tightly by the column, and it fails to react with our proguanylin-specific antibody. In addition, the retention time of the material is significantly different from that of synthetic rat guanylin (fraction 13) or synthetic opossum uroguanylin (fraction 15). The novel duodenal peak does, however, share one property with proguanylin: its activity is also enhanced by preincubation at 37°C (Fig. 3). If an intestinal protease is indeed responsible for this activation phenomenon, then the data suggest that such a protease is likely to come off the HPLC column in a broad peak that overlaps both proguanylin (fraction 21) and the material in fractions 23–24.

In order to test whether the duodenal material is a peptide, we incubated it with a mixture of exogenously-added proteases (trypsin, chymotrypsin, elastase, aminopeptidase, and *S. aureus* V8 protease). After a 2 h incubation, its biological activity was completely destroyed (data not shown). This, in conjunction with the purification and sequencing studies described below, confirms the peptide nature of the active material in fractions 23–24.

3.3. Preincubation alters the chromatographic properties of the duodenal peptide

If a proteolytic mechanism is responsible for the activation of the duodenal peptide, then the size of the molecule should be altered once it has been activated, and this should be reflected by a change in its chromatographic properties. To test this prediction, we partially purified the duodenal peptide on our standard reverse phase HPLC column (as in Fig. 2b), allowed it to preincubate at 37°C, rechromatographed it on the same HPLC column, and bioassayed the resulting fractions. Although biological activity is still quite evident (Fig. 4), it now elutes from the column much earlier (fraction 16), consistent with cleavage to a smaller (less hydrophobic) peptide. Furthermore, the material in fraction 16 has become fully active: no enhancement of the T84 cell response can be induced by additional preincubation at 37°C (Fig. 3).

3.4. Purification of the duodenal peptide

In order to achieve further purification, the material in fraction 16 (Fig. 4) was again applied to a C-18 column, but now subjected to a new set of elution parameters using a different ion pairing reagent and a different pH (see Section 2). Activity was recovered as a single, sharp peak that aligns precisely with a major peak of UV absorbance, and appears well separated from other contaminants (Fig. 5).

This material was submitted for amino acid sequencing, revealing the presence of approximately 15–18 pmol of the peptide shown at the top of Fig. 6a. During the sequencing reaction, no amino acid could be identified in cycles 7, 10, 15, and 18, indicating that the amino acid at each of those positions is most likely cysteine. This is consistent with the observation that the blank cycles align exactly with a set of four cysteines that are absolutely required for biological activity in all known guanylin-like peptides (guanylin, uroguanylin, and STa-see Fig. 6a). Also, in the first sequencing cycle, both threonine and glutamate were found in approximately equal abundance and thus the residue at this position is ambiguous. The sequence of the purified peptide suggests that it may be the rat homolog of uroguanylin.

3.5. Sequence and tissue distribution of the rat uroguanylin transcript

In order to confirm and extend our peptide sequence analysis, we used PCR to generate a 250 bp nucleotide

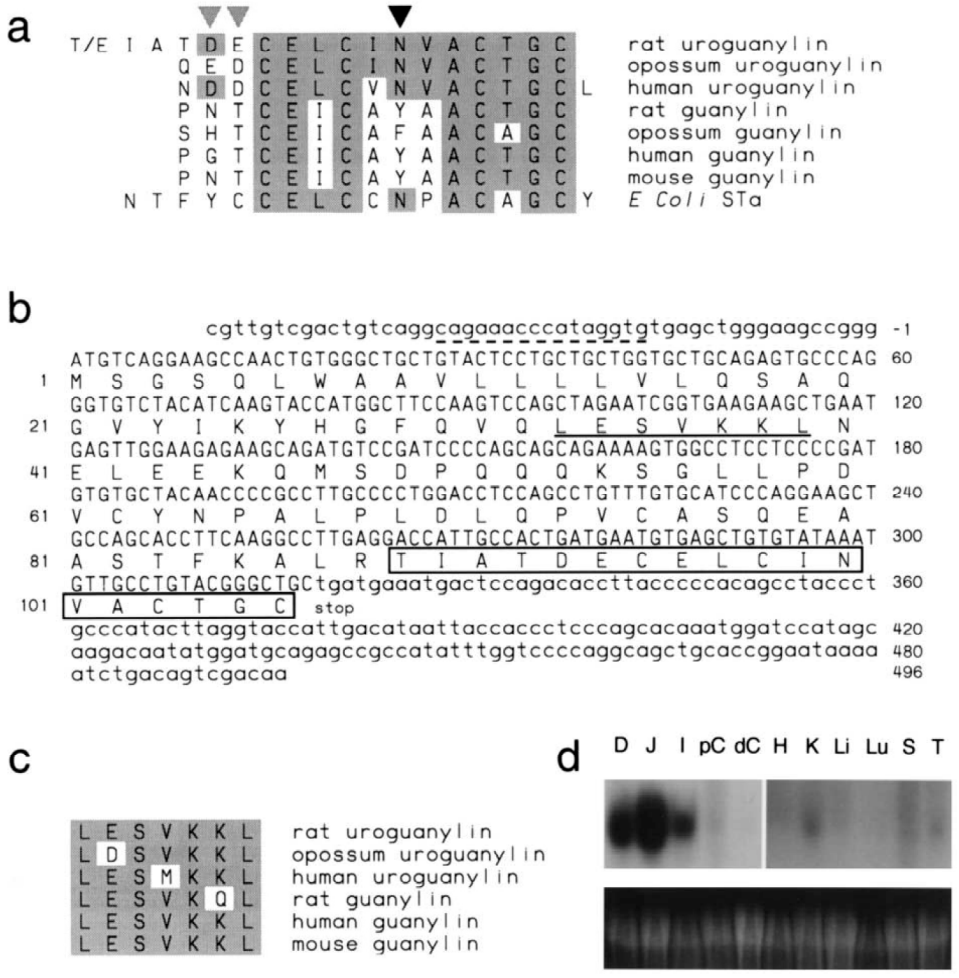

Fig. 6. Amino acid and cDNA sequences of rat uroguanylin. (a) Alignment of the amino acid sequence of the purified duodenal peptide (top) with published sequences of guanylin, uroguanylin, and STa. Amino acid identities are indicated by shading. The arrowheads denote structural features described in the text. (b) The nucleotide and translated amino acid sequences of the cloned rat prepprouroguanylin cDNA. The coding portion of the sequence is given in upper case letters. The box encloses the region of the precursor that corresponds to the peptide purified in our biochemical studies. Solid underlining indicates a second region of the propeptide whose amino acid sequence is conserved in both proguanylin and prouroguanylin [alignment of this region with the comparable portion of the proguanylin sequence is shown in (c)]. Dashed underlining indicates a region present in the 5′ untranslated region of one clone but not the other. (c) Cross-species comparison of the sequence underlined in (b). (d) The upper panel shows the appropriate region from a Northern blot of total RNA (40 μg/lane) hybridized with a radiolabeled uroguanylin probe. The size of the labeled transcript is approximately 600 bp. All lanes are from a single membrane, but the left half of the blot is shown after a 16 h exposure, and the right half after a 14 day exposure. The lower panel shows the ethidium bromide staining of 28S ribosomal RNA prior to transfer to the blotting membrane. RNA was isolated from rat duodenum (D), jejunum (J), ileum (I), proximal colon (pC), distal colon (dC), heart (H), kidney (K), liver (Li), lung (Lu), spleen (S), and testis (T).

probe specific for the rat form of uroguanylin (as described in Section 2) and then used the probe to screen a duodenal cDNA library. We sequenced two positive clones, each containing the entire coding region of the uroguanylin propeptide (Fig. 6b-GenBank accession number U73898). These two clones are identical to each other, except for a 16 nucleotide deletion in the 5′ untranslated region of one of them (underlined with dashes in Fig. 6b). The first in-frame methionine residue was assigned as the translation start site, and is homologous to the start sites proposed for the opossum and human cDNAs. A stop codon defines the C-terminal cysteine residue. The amino acid sequence corresponding to the peptide isolated in our biochemical studies is found at the 3′ end of the coding portion of the prouroguanylin cDNA (boxed in the figure). This reveals that threonine is the correct amino acid at position 1 of the peptide, and confirms that the blank cycles (7, 10, 15, and 18) correspond to cysteines.

The sequence that we have established for rat prouroguanylin is quite homologous to the sequences

previously established for human and opossum prouroguanylin (70% and 73% identity at the nucleotide level, and 67% and 66% identity at the amino acid level, respectively). In addition, as has been noted in these other species [37,38], there are two discrete regions of homology between rat prouroguanylin and rat proguanylin. The first region (boxed in Fig. 6b, and compared across all species for which sequences are available in Fig. 6a) comprises the biologically active C-terminus of each propeptide. The second region (underlined in Fig. 6b, and compared across all species for which sequences are available in Fig. 6c) is found closer to the N-terminus, separated from the conserved C-terminal domain by about 50 residues. The significance of this second conserved motif is not clear, but its retention across multiple species suggests that it has some as-yet unknown biological function.

The tissue distribution of the uroguanylin transcript is shown in Fig. 6d. The size of the uroguanylin transcript is approximately 600 bp, just slightly smaller than the size of the guanylin transcript. It is clear that, like guanylin, uroguanylin mRNA is expressed primarily in the intestine. However, within the intestine, its distribution is quite distinct from that of the guanylin transcript (compare Fig. 6d to Fig. 1a): uroguanylin is most prominent in proximal small intestine, while guanylin is most prominent in distal large intestine. This confirms the distribution of guanylin and uroguanylin peptides determined in our biochemical studies (Fig. 2).

In addition, there is a limited amount of uroguanylin expression outside the intestine, in kidney, testis, and possibly spleen (the exposure time shown in Fig. 6d for non-intestinal tissues is 20 times longer than the exposure time for intestinal tissues).

4. Discussion

Guanylin was initially discovered in a search for endogenous agonists for the STa receptor. Guanylin fulfils several of the criteria expected of such an agonist: it is present in the intestine, it competes with STa for binding to the receptor, and it activates cyclic GMP synthesis when applied to cells that express the receptor. Therefore, in a simplest-case scenario, guanylin expression might be expected to be high in tissues where receptor expression is high, and low in tissues where receptor expression is low. However, within the intestine the tissue distributions of guanylin and its receptor are surprisingly non-parallel. We were particularly struck by the fact that duodenum expresses high levels of receptor but low levels of peptide. In the experiments described above, we have confirmed this mismatch at both the mRNA and polypeptide levels.

These observations led us to consider the possibility that duodenum might produce its own endogenous ligand that resembles guanylin in its target specificity. Our HPLC analysis of duodenal extracts has confirmed this idea, and allowed us to purify a second peptide with guanylin-like biological activity. Interestingly, guanylin and the new peptide have complementary distributions: duodenum has low levels of guanylin and high levels of the new peptide, while colon has high levels of guanylin and little or none of the new peptide. Alignment of the sequence of the duodenal peptide with the appropriate regions of rat guanylin and uroguanylin (Fig. 6a) reveals that the new peptide is more closely related to uroguanylin (80% identity when compared across species, with nearly all differences representing conservative amino acid substitutions) than it is to guanylin (47–53% identity, with few of the differences representing conservative amino acid substitutions). These observations suggest that the duodenal peptide represents the rat isoform of uroguanylin, whose sequence has not yet been determined.

In a previous study, Hamra et al. [28] analyzed extracts of opossum intestinal mucosa by preparative isoelectric focusing, and found two distinct peaks of guanylin-like activity. Purification and sequencing of one of the peaks (pI = 5.2) showed that it was the opossum form of guanylin. The other peak was not purified, but its isoelectric point (pI = 3.0) was consistent with the idea that it could be uroguanylin. In a subsequent publication, Hamra et al. [44] showed that opossum intestine contains both proguanylin and a second inactive propeptide whose biological activity could be enhanced by proteolysis with chymotrypsin. Purification and C-terminal sequencing of this second peptide revealed that it was distinct from proguanylin, and that it had properties more closely related to those of uroguanylin than to those of guanylin. Our present study provides a direct biochemical demonstration that the rat intestine produces a peptide with an N-terminal sequence corresponding to uroguanylin. These results, together with the analysis of rat uroguanylin mRNA expression presented in Fig. 6d, provide convincing evidence that uroguanylin is produced by the rat intestine. In addition, recent cloning studies have identified human [38] and opossum [37] cDNAs encoding a uroguanylin propeptide, and have demonstrated expression of a transcript encoding this propeptide in the human and opossum intestine.

In the course of identifying and purifying uroguanylin, we learned that the biological activity of the partially purified material could be enhanced by incubating it at 37°C. This enhancement was accompanied by a shift in its HPLC retention time. We have observed a similar phenomenon with partially purified proguanylin extracted from the colon. As it is unlikely that this is a spontaneous process, we believe it is most likely that a co-eluting protease is converting inactive or weakly active precursors into active products. As of yet, the peptide processing sites responsible for generating biologically active guanylin or uroguanylin in vivo have not been identified. Thus, the N-terminally extended form of uroguanylin that we have

identified may actually be the form that is active in the intestine.

Interestingly, an N-terminally extended form of guanylin (containing an extra aspartate residue) has recently been detected in extracts of rat ileum and colon, using an RIA directed against the proguanylin C-terminus [45]. This N-terminally extended peptide accounts for about 50% of the total guanylin-like immunoreactivity present in the colon. We have not detected this peptide in our present studies, nor should we have for two reasons: (a) it is inactive when assayed on T84 cells, and thus would fail to show up in our bioassays, and (b) it lacks the proguanylin N-terminus, and thus would fail to show up on our Western blots.

The affinity of GCC for uroguanylin (opossum or human) is about 10-fold higher than its affinity for guanylin (rat or human) [28,29]. Thus, features that are found in uroguanylin, but not in guanylin, offer information about structural elements that specify the strength of the ligand/receptor interaction. Of particular interest are two residues that are basic or uncharged in guanylin but acidic in uroguanylin (stippled arrowheads), and one residue that contains an aromatic ring in guanylin but an acid amide in uroguanylin (solid arrowhead). At all three positions, our duodenal peptide follows the consensus sequence of uroguanylin rather than that of guanylin, and thus we would expect its affinity to be comparable to that of opossum or human uroguanylin. Dose/response curves with synthetic rat peptide will be required to test this idea directly. It will be particularly of interest to determine whether the three extra N-terminal amino acids that distinguish our purified rat peptide from all previously-purified uroguanylins have a significant effect on binding affinity.

In addition to structural similarities at the C-terminus, the guanylin and uroguanylin propeptides share homology across a stretch of seven amino acids near the N-terminus (Fig. 6c). The significance of this second region of conserved primary structure is completely unknown. Potentially, it could play a role in specifying peptide targeting, folding, or processing. Alternatively, it could represent a second biologically active peptide released when the propeptide is cleaved; if so, this peptide must target a receptor other than GCC, since it lacks all the structural features required to interact with the GCC ligand binding domain.

GCC is a member of a family of hormone-regulated receptor guanylate cyclases that has been identified in mammals [46]. Two other cyclases in this family, type-A and type-B (called GCA and GCB), are targeted by a group of structurally related natriuretic peptides, including ANP, BNP, and CNP. Interestingly, the binding specificities of GCA and GCB allow them to discriminate among the natriuretic peptides: ANP is the preferred ligand for GCA and CNP is the preferred ligand for GCB. Guanylin and uroguanylin define a group of ligands that target GCC. By analogy with the natriuretic peptides, the existence of two distinct but related peptides with different affinities for GCC suggests that there could be additional members of the receptor/cyclase family whose binding specificities discriminate among guanylin-like ligands. Indeed, preliminary studies with OK cells (an opossum kidney proximal tubule-like cell line) have revealed the presence of a guanylate cyclase/receptor that can be stimulated by guanylin-like peptides, but that has a different ligand selectivity from GCC [47]. Furthermore, PCR studies with cDNA derived from rat intestinal mRNA have resulted in the amplification of a guanylate cyclase-like sequence (SIM3) that is related to, but distinct from, GCA, GCB, and GCC [6]. The presence in the intestine of multiple guanylin-like peptides, and perhaps multiple receptors for these peptides, suggests that this signaling pathway plays a complex role in GI physiology. In this regard, it will be particularly important to identify the cells responsible for the synthesis of uroguanylin and to compare them to published descriptions of guanylin-synthesizing cells [32,48,49].

In summary, we have purified a guanylin-like peptide from the rat intestine that activates cyclic GMP synthesis in T84 cells. This peptide is much more abundant in duodenum than in colon. Peptide and nucleic acid sequence analysis indicates that we have identified the rat isoform of uroguanylin, a peptide originally isolated from urine. It is possible that uroguanylin is synthesized by the duodenum and released into the circulation as an endocrine agent. Its presence in the urine would therefore be a natural consequence of plasma filtration by the kidney, and the existence of guanylin/uroguanylin receptors in the kidney would imply that kidney is one of the targets of this peptide signaling pathway. It is also reasonable to suggest, however, that some or all of the duodenal uroguanylin may be released locally into the lumen of the GI tract in order to interact with the abundant, luminally-oriented GCC that is expressed in the duodenum. Thus, a view of the guanylin/uroguanylin peptide family is beginning to emerge in which these peptides play a complex role in integrating the responses of diverse target tissues.

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References

[1] Forte, L.R. and Currie, M.G., Guanylin: A peptide regulator of epithelial transport, FASEB J., 9 (1995) 643–650.

[2] Donowitz, M. and Welsh, M.J., Regulation of mammalian small intestinal electrolyte secretion, in L.R. Johnson (Ed.), Physiology of the Gastrointestinal Tract, Raven Press, New York, 1987, pp. 1351–1379.

[3] Hughes, J.M., Murad, F., Chang, B. and Guerrant, R.L., The role of cyclic GMP in the action of heat-stable enterotoxin of *E. coli*, Nature, 271 (1978) 755–756.

[4] Newsome, P.M., Burgess, M.N. and Mullan, N.A., Effect of *Escherichia coli* heat-stable enterotoxin on cyclic GMP levels in mouse intestine, in press, 22 (1978) 290–291.

[5] Field, M., Graf, L.H., Laird, W.J. and Smith, P.L., Heat stable enterotoxin of *E. coli*: in vitro effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine, Proc. Natl. Acad. Sci. U.S.A., 75 (1978) 2800–2804.

[6] Schulz, S., Green, C.K., Yuen, P.S.T. and Garbers, D.L., Guanylyl cyclase is a heat-stable enterotoxin receptor, Cell, 63 (1990) 941–948.

[7] Drewett, J.G. and Garbers, D.L., The family of guanylyl cyclase receptors and their ligands, Endocrine Rev., 15 (1994) 135–162.

[8] Currie, M.G., Fok, K.F., Kato, J., et al., Guanylin: an endogenous activator of intestinal guanylate cyclase, Proc. Natl. Acad. Sci. U.S.A., 89 (1992) 947–951.

[9] Wiegand, R.C., Kato, J., Huang, M.D., Fok, K.F., Kachur, J.F. and Currie, M.G., Human guanylin: cDNA isolation, structure, and activity, FEBS Lett., 311 (1992) 150–154.

[10] De Sauvage, F.J., Keshav, S., Kuang, W.-J., Gillett, N., Henzel, W. and Goeddel, D.V., Precursor structure, expression, and tissue distribution of human guanylin, Proc. Natl. Acad. Sci. U.S.A., 89 (1992) 9089–9093.

[11] Forte, L.R., Krause, W.J. and Freeman, R.H., Guanylin bioactivity in human intestinal and opossum kidney cells, Adv. Second Messenger Phosphoprotein Res., 28 (1993) 133–138.

[12] Forte, L.R., Eber, S.L., Turner, J.T., Freeman, R.H., Fok, K.F. and Currie, M.G., Guanylin stimulation of Cl^- secretion in human intestinal T_{84} cells via cyclic guanosine monophosphate, J. Clin. Invest., 91 (1993) 2423–2428.

[13] Carpick, B.W. and Gariépy, J., The *Escherichia coli* heat-stable enterotoxin is a long-lived superagonist of guanylin, Infect. Immun., 61 (1993) 4710–4715.

[14] Chao, A.C., De Sauvage, F.J., Dong, Y.-J., Wagner, J.A., Goeddel, D.V. and Gardner, P., Activation of intestinal CFTR Cl^- channel by heat-stable enterotoxin and guanylin via cAMP-dependent protein kinase, EMBO J., 13 (1994) 1065–1072.

[15] Cuthbert, A.W., Hickman, M.E., MacVinish, L.J., et al., Chloride secretion in response to guanylin in colonic epithelia from normal and transgenic cystic fibrosis mice, Br. J. Pharmacol., 112 (1994) 31–36.

[16] Houghten, R.A., Ostresh, J.M. and Klipstein, F.A., Chemical synthesis of an octapeptide with the biological and immunological properties of human heat-stable *Escherichia coli* enterotoxin, Eur. J. Biochem., 145 (1984) 157–162.

[17] Laney, Jr., D.W., Mann, E.A., Dellon, S.C., Perkins, D.R., Giannella, R.A. and Cohen, M.B., Novel sites for expression of an *Escherichia coli* heat-stable enterotoxin receptor in the developing rat, Am. J. Physiol., 263 (1992) G816–G821.

[18] Li, Z. and Goy, M.F., Peptide-regulated guanylate cyclase pathways in rat colon: In situ localization of GCA, GCC, and guanylin mRNA, Am. J. Physiol., 265 (1993) G394–G402.

[19] Cohen, M.B., Mann, E.A., Lau, C., Henning, S. and Giannella, R.A., A gradient in expression of the *Escherichia coli* heat-stable enterotoxin receptor exists along the villus-to-crypt axis of rat small intestine, Biochem. Biophys. Res. Comm., 186 (1992) 483–490.

[20] White, A.A., Krause, W.J., Turner, J.T. and Forte, L.R., Opossum kidney contains a functional receptor for the *Escherichia Coli* heat-stable enterotoxin, Biochem. Biophys. Res. Commun., 159 (1989) 363–367.

[21] Forte, L.R., Krause, W.J. and Freeman, R.H., *Escherichia coli* enterotoxin receptors: localization in opossum kidney, intestine, and testis, Am. J. Physiol., 257 (1989) F874–F881.

[22] Fonteles, M.C., Greenberg, R.N., Currie, M.G. and Forte, L.R., Guanylin and uroguanylin are GC-C binding intestinal peptide hormones with natriuretic and kaliuretic activity, J. Invest. Med., 43 (1995) 395A.

[23] Lima, A.A.M., Monteiro, H.S.A. and Fonteles, M.C., The effects of *Escherichia coli* heat-stable enterotoxin in renal sodium tubular transport, Pharmacol. Toxicol., 70 (1992) 163–167.

[24] Forte, L.R., Krause, W.J. and Freeman, R.H., Receptors and cGMP signalling mechanism for *E.coli* enterotoxin in opossum kidney, Am. J. Physiol., 255 (1988) F1040–F1046.

[25] Schulz, S., Chrisman, T.D. and Garbers, D.L., Cloning and expression of guanylin, J. Biol. Chem., 267 (1992) 16019–16021.

[26] Sciaky, D., Jenkins, N.A., Gilbert, D.J., et al., Mapping of guanylin to murine chromosome 4 and human chromosome 1p34–p35, Genomics, 26 (1995) 427–429.

[27] Fonteles, M.C., Villar-Palasi, C., Fang, G., Larner, J. and Guerrant, R.L., Partial characterization of an ANF/urodilatin-like substance released from perfused rabbit kidney under hypoxia, Braz. J. Med. Biol. Res., 26 (1993) 75–79.

[28] Hamra, F.K., Forte, L.R., Eber, S.L., et al., Uroguanylin: Structure and activity of a second endogenous peptide that stimulates intestinal guanylate cyclase, Proc. Natl. Acad. Sci. U.S.A., 90 (1993) 10464–10468.

[29] Kita, T., Smith, C.E., Fok, K.F., et al., Characterization of human uroguanylin: a member of the guanylin peptide family, Am. J. Physiol., 266 (1994) F342–F348.

[30] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J., Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease, Biochemistry, 18 (1979) 5294–5299.

[31] Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 227 (1970) 680–685.

[32] Li, Z., Taylor-Blake, B., Light, A.R. and Goy, M.F., Guanylin, an endogenous ligand for C-type guanylate cyclase, is produced by goblet cells in the rat intestine, Gastroenterology, 109 (1995) 1863–1875.

[33] Harper, J.F. and Brooker, G., Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′O acetylation by acetic anhydride in aqueous solution, J. Cyc. Nuc. Res., 1 (1975) 207–218.

[34] Steiner, A.L., Parker, C.W. and Kipnis, D.M., Radioimmunoassay for cyclic nucleotides, J. Biol. Chem., 247 (1972) 1106–1113.

[35] Huott, P.A., Lin, W., McRoberts, J.A., Giannella, R.A. and Dharmsathaphorn, K., Mechanism of action of *Escherichia coli* heat stable enterotoxin in a human colonic cell line, J. Clin. Invest., 82 (1988) 514–523.

[36] Chrisman, T.D., Schulz, S., Potter, L.R. and Garbers, D.L., Seminal plasma factors that cause large elevations in cellular cyclic GMP are C-type natriuretic peptides, J. Biol. Chem., 268 (1993) 3698–3703.

[37] Fan, X.H., Hamra, F.K., Freeman, R.H., et al., Uroguanylin: Cloning of preprouroguanylin cDNA, mRNA expression in the intestine and heart and isolation of uroguanylin and prouroguanylin from plasma, Biochem. Biophys. Res. Commun., 219 (1996) 457–462.

[38] Hill, O., Cetin, Y., Cieslak, A., Mägert, H.J. and Forssmann, W.G., A new human guanylate cyclase activating peptide (GCAP-II, uroguanylin): precursor cDNA and colonic expression, BBA, 1253 (1995) 146–149.

[39] Hadjeb, N. and Berkowitz, G.A., Preparation of T-overhang vectors with high PCR product cloning efficiency, Biotechniques, 20 (1996) 20–22.

[40] Kopin, A.S., Wheeler, M.B. and Leiter, A.B., Secretin: structure of the precursor and tissue distribution of the mRNA, Proc. Natl. Acad. Sci. U.S.A., 87 (1990) 2299–2303.

[41] Wiegand, R.C., Kato, J. and Currie, M.G., Rat guanylin cDNA: characterization of the precursor of an endogenous activator of intestinal guanylate cyclase, Biochem. Biophys. Res. Comm., 185 (1992) 812–817.
[42] Kato, J., Wiegand, R.C. and Currie, M.G., Characterization of the structure of preproguanylin, Adv. Second Messenger Phosphoprotein Res., 28 (1993) 139–142.
[43] Garcia, K.C., De Sauvage, F.J., Struble, M., Henzel, W., Reilly, D. and Goeddel, D.V., Processing and characterization of human proguanylin expressed in *Escherichia coli*, J. Biol. Chem., 268 (1993) 22397–22401.
[44] Hamra, F.K., Fan, X.H., Krause, W.J., et al., Prouroguanylin and proguanylin: Purification from colon, structure, and modulation of bioactivity by proteases, Endocrinology, 137 (1996) 257–265.
[45] Yamaguchi, H., Nakazato, M., Miyazato, M., Kangawa, K., Matsuo, H. and Matsukura, S., Two novel rat guanylin molecules, guanylin-94 and guanylin-16, do not increase cyclic GMP production in T84 cells, Biochem. Biophys. Res. Commun., 214 (1995) 1204–1210.
[46] Yuen, P.S.T. and Garbers, D.L., Guanylyl cyclase-linked receptors, Ann. Rev. Neurosci., 15 (1992) 193–225.
[47] Landon, L.A., Hamra, F.K., Eber, S. and Forte, L.R., Evidence for a new guanylate cyclase receptor subtype in opossum kidney (OK) cells, FASEB J., 9 (1995) A6.
[48] Cohen, M.B., Witte, D.P., Hawkins, J.A. and Currie, M.G., Immunohistochemical localization of guanylin in the rat small intestine and colon, Biochem. Biophys. Res. Commun., 209 (1995) 803–808.
[49] Cetin, Y., Kuhn, M., Kulaksiz, H., et al., Enterochromaffin cells of the digestive system: Cellular source of guanylin, a guanylate cyclase-activating peptide, Proc. Natl. Acad. Sci. U.S.A., 91 (1994) 2935–2939.