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Uroguanylin gene expression in the alimentary tract and extra-gastrointestinal tissues

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Abstract Uroguanylin, a member of the guanylin peptide family, is a novel peptide regulator for intestinal salt and water transport. A cDNA encoding a precursor for rat uroguanylin was cloned from a rat jejunum cDNA library and sequenced. The precursor was 106 amino acids long and included a 21 residue putative signal peptide at the N-terminus. Rat uroguanylin consisted of 15 amino acids similar to, but distinct from human uroguanylin; the C-terminal leucine residue was deleted and 3 residues were substituted compared to those in the human peptide. Synthetic rat uroguanylin-15 dose-dependently increased the cyclic GMP level in cultured T84 cells. RNA blot analysis showed that rat uroguanylin mRNA is expressed not only in the gastrointestinal tract but also in the lung, pancreas and kidney. Evidence for uroguanylin expression in extra-gastrointestinal tissues indicates the possible existence of a novel system for water and electrolyte homeostasis, and a more global effect of uroguanylin on epithelial cell function.

Key words: Uroguanylin; Guanylin; Guanylate cyclase C; cDNA cloning

1. Introduction

Guanylin and uroguanylin are novel peptide regulators for intestinal salt and water transport [1–5]. The two peptides have 50% amino acid sequence homology and two intramolecular disulfide bonds in common. Human and opossum uroguanylins have been isolated and sequenced, and their bioactivities have been analyzed [6–9]. Human uroguanylin, a 16-amino acid peptide isolated from urine, stimulates cyclic GMP (cGMP) production in enterocytes by activating an apical membrane, receptor-guanylate cyclase C (GC-C) [7]. In addition, the peptide increased the cGMP level in T84 human colon cancer cells that express GC-C, displaced heat-stable enterotoxin (ST) binding to GC-C, and stimulated Cl^- secretion [7]. Uroguanylin has also been shown to cause natriuretic effects in the perfused rat kidney [10,11]. Uroguanylin is speculated to be a potent physiological regulator of intestinal and/or renal fluid and electrolyte transport.

To understand better its physiological implications and pathophysiological significance in water and electrolyte homeostasis, detailed analysis of its cell and tissue distribution is required. We report the cloning and sequence analysis of rat uroguanylin cDNA. We also used RNA blot analysis to determined the expression of the uroguanylin gene.

2. Materials and methods

2.1. Tissue preparation

The stomach, duodenum, jejunum, ileum, colon, liver, pancreas, kidney, lung, heart and brain were resected immediately after decapitation of five 8-week-old male Sprague Dawley rats that had fasted overnight. The jejunum was resected 40–50 cm from the pyloric ring, the ileum 10–20 cm above the terminal ileum and the colon 5–15 cm below the terminal ileum. All the rats used were kept in an air-conditioned room and fed with standard laboratory chow and water ad libitum.

2.2. PCR amplification of rat uroguanylin cDNA probe

Complementary DNA prepared from rat jejunum RNA was used in each of the series of PCR experiments performed with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM of each dNTP, 1.25 units of Taq DNA polymerase (Perkin-Elmer Corp.), and 10 μM of each PCR primer. PCR conditions were 35 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 45 s, and extension at 72°C for 1 min. The primers were a series of degenerative oligonucleotides based on the amino acid sequence of human uroguanylin [7]. Each of two sense primers was used in the PCR reactions with each of four antisense primers. The sense primers had the following sequences;

S1: AA(T/C)GA(T/C)GA(T/C)TG(T/C)GA(A/G)TT(A/G)TG
S2: AA(T/C)GA(T/C)GA(T/C)TG(T/C)GA(A/G)CTNTG

and the antisense primers;

AS1: TANAG(A/G)CANCC(T/C)GT(A/G)CA(T/C)GC
AS2: TANAG(A/G)CANCC(T/C)GT(A/G)CA(A/G)GC
AS3: TANAG(A/G)CANCC(A/G)GT(A/G)CA(T/C)GC
AS4: TANAG(A/G)CANCC(A/G)GT(A/G)CA(A/G)GC
N=A, G, C, T

An amplified product of the expected size (50 bp) was obtained only by the use of the S2 and AS2 primer pair. After polyacrylamide gel purification, the amplified product was ligated directly to the pT7Blue T-Vector (Novagen). The insert was cut from the amplified vectors and used as the screening probe for the rat jejunum cDNA library.

2.3. cDNA library construction

Total RNA was extracted from rat jejunum by the acid-guanidinium thiocyanate-phenol-chloroform (AGPC) method [12]. Poly(A)$^+$ RNA was isolated using Oligo(dT)-Latex (TaKaRa). Double-stranded DNA was synthesized from 5 μg of rat jejunum poly(A)$^+$ RNA by the method of Gubler and Hoffman [13] using a cDNA synthesis kit (Pharmacia). The cDNA was ligated to *Eco*RI adaptors and size-fractionated in a Sepharose CL-4B column (Pharmacia). The size-fractionated cDNA was ligated to 1 μg of λZAP II vector arms and packaged in vitro using Gigapack III Gold (Stratagene).

2.4. cDNA library screening and sequence analysis

Hybridization was carried out at 37°C in a solution of 40% formamide, 6×SSPE (0.9 M NaCl, 50 mM NaH_2PO_4 (pH 7.4), 5 mM EDTA), 5×Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 μg/ml denatured salmon sperm DNA. Approx.

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The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL DNA database with accession number U41322.

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3×10^5 recombinant phages were transferred to nylon membranes and screened using a 50-bp cDNA fragment encoding rat uroguanylin as the probe, prepared as described above. The membranes were washed twice in 2×SSC (0.3 M NaCl, 30 mM sodium citrate), 0.1% SDS at 50°C for 30 min. A pBluescript with positive DNA inserts was recovered from the λZAP II phage by the in vivo excision method with the ExAssist helper phage (Stratagene) [14]. The clone (λRUG23) that carried the longest cDNA insert was sequenced by the dyeprimer cycle sequencing method in an automated DNA sequencer, Model 373A (Applied Biosystems).

2.5. Peptide synthesis

Rat uroguanylin-15 (TDECELCINVACTGC), located at the C-terminal end of rat prepprouroguanylin, was synthesized by solid-phase techniques in a peptide synthesizer, Model 430 (Applied Biosystems), then purified by reverse-phase high-performance liquid chromatography (RP-HPLC). The correct synthesis was confirmed by amino acid analysis and sequencing. Two disulfide bonds at positions 4 and 12, and at 7 and 15 were successively linked by a two-step selective forming reaction.

2.6. Bioassay for uroguanylin

cGMP production in T84 cells by synthetic rat uroguanylin was investigated. T84 cells were obtained from American Type Culture Collection. The cells were grown to confluence in 24-well culture dishes at 37°C in a humidified atmosphere containing 5% CO_2 with a 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). A confluent monolayer of T84 cells was incubated first for 30 min at 37°C in DMEM containing 25 mM HEPES (pH 7.4) and 0.1 mM isobutylmethylxanthine (IBMX) then for 30 min in the same medium containing 0.1% bovine serum albumin (BSA) and rat uroguanylin-15, rat guanylin-15, human uroguanylin-16 or *E. coli* ST (Sigma). After incubation, the cGMP concentration in the medium was measured with an RIA kit (Yamasa).

2.7. RNA blot analysis

Poly(A)$^+$ RNA (10 μg) or total RNA (20 μg) was fractionated on a 1% agarose-formaldehyde gel. After electrophoresis, the RNA was transferred to a nylon membrane (Zeta Probe, Bio-Rad) and fixed by ultraviolet irradiation (Stratalinker, Stratagene). The membrane first was prehybridized at 37°C for 2 h in 50% formamide, 6×SSPE, 5×Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA, then hybridized at 42°C for 18 h in the same buffer containing a ^{32}P-labeled cDNA insert of λRUG23. The blot was washed twice at 60°C in 2×SSC, 0.1% SDS for 30 min, after which it was exposed to X-ray film at −80°C. The same membrane was used for sequential hybridizations with probes specific for rat guanylin and β-actin.

3. Results

3.1. cDNA and precursor structure of rat uroguanylin

Because the amino acid sequence of rat uroguanylin had not been determined, we synthesized primer mixtures representing all possible sequences on the basis of the established amino acid sequence of human uroguanylin [7] in order to amplify the cDNA fragment of rat uroguanylin. A 50-bp PCR product was obtained by the use of only one PCR primer pair (S2/AS2) when rat jejunum cDNA was the template.

When the 50-bp PCR product was used as the probe, RNA blot analysis revealed that the rat uroguanylin gene was highly expressed in the jejunum (data not shown). Therefore, a cDNA library for the isolation of clones encoding a uroguanylin precursor was constructed with poly(A)$^+$ RNA extracted from rat jejunum. A portion of the library (approx. 3×10^5 recombinant phages) was screened with the amplified

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-36                                  GCAGAAACCCAGAGGTGTGAGCTGGGAAGCCGGGCC  -1
  1 ATGTCAGGAAGCCAACTGTGGGCTGCTGTACTCCTGCTGCTGGTGCTGCAGAGTGCCCAG  60
  1 MetSerGlySerGlnLeuTrpAlaAlaValLeuLeuLeuLeuValLeuGlnSerAlaGln  20

 61 GGTGTCTACATCAAGTACCATGGCTTCCAAGTCCAGCTAGAATCGGTGAAGAAGCTGAAT 120
 21 GlyValTyrIleLysTyrHisGlyPheGlnValGlnLeuGluSerValLysLysLeuAsn  40

121 GAGTTGGAAGAGAAGCAGATGTCCGATCCCCAGCAGCAGAAAAGTGGCCTCCTCCCCGAT 180
 41 GluLeuGluGluLysGlnMetSerAspProGlnGlnGlnLysSerGlyLeuLeuProAsp  60

181 GTGTGCTACAACCCCGCCTTGCCCCTGGACCTCCAGCCTGTTTGTGCATCCCAGGAAGCT 240
 61 ValCysTyrAsnProAlaLeuProLeuAspLeuGlnProValCysAlaSerGlnGluAla  80

241 GCCAGCACCTTCAAGGCCTTGAGGACCATTGCCACTGATGAATGTGAGCTGTGTATAAAT 300
 81 AlaSerThrPheLysAlaLeuArgThrIleAlaThrAspGluCysGluLeuCysIleAsn 100

301 GTTGCCTGTACGGGCTGCTGATGAAATGACTCCAGACACCTACCCCCACAGCCTACCCTG 360
101 ValAlaCysThrGlyCys***                                        106

361 CCCATACTTAGGTACCATTGACATAATTACCACCCTCCCAGCACAAATGGATCCATAGCA 420
421 AGACAATATGGATGCAGAGCCGCCATATTTGGTCCCCAGGCAGCTGCACCGGAATAAAAA 480
481 TGTTACCCGC(poly A)                                           490
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Fig. 1. Nucleotide sequence of rat uroguanylin cDNA. The termination codon is marked with three consecutive asterisks. The uroguanylin sequence is double underlined. The possible signal peptide is single underlined. The dashed line indicates the polyadenylation signal.

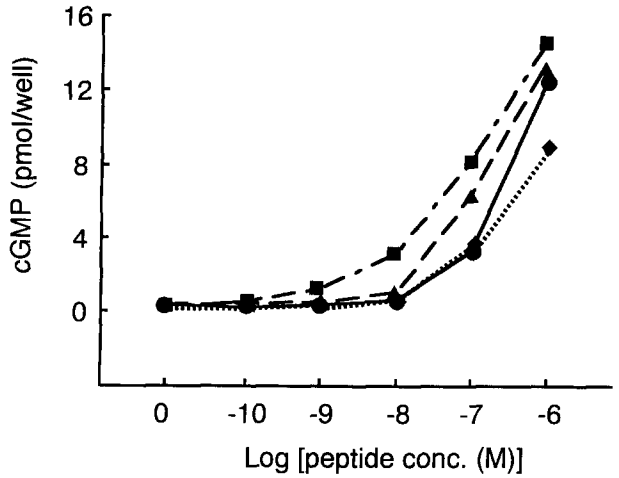

Fig. 2. cGMP production in T84 cells by rat uroguanylin-15 (●), human uroguanylin-16 (▲), rat guanylin-15 (◆) and *E. coli* ST (■). Representative experiment that was repeated twice with the same results.

probe, 6 positive clones being isolated. A second screening of these clones gave 3 positive clones. The clone designated λRUG23, which harbored the longest cDNA insert (approx. 550 bp), was sequenced. Fig. 1 shows the complete nucleotide sequence of the cDNA which is 526 bp long (excluding the poly(A) tail). Two putative initiation methionine codons are located in a long open reading frame (nucleotides 1–3, and 139–141). The initiation codon ATG probably is located at nucleotides 1–3 because a highly hydrophobic structure (most likely a signal peptide) follows the first methionine. A termination codon TGA is present 106 codons later at nucleotides 319–321, and a consensus polyadenylation signal, AATAAA, at nucleotides 467–478.

The deduced amino acid sequence encoded in the open reading frame is shown in Fig. 1, indicating that rat uroguanylin mRNA encodes a 106-residue protein. The nucleotide sequence of bases 274–318 corresponds to the amino acid sequence of the mature uroguanylin (rat uroguanylin-15), which was confirmed by the bioassay using T84 cells. The first 21-residue peptide, starting from the initial methionine, is thought to be a signal peptide because of its characteristic hydrophobic features [15]. The first processing of the precursor probably takes place between Gly^{21} and Val^{22} and generates a 85-residue prouroguanylin.

3.2. cGMP assay for uroguanylin

We examined the cGMP production of rat uroguanylin-15 and guanylin-15, human uroguanylin-16 and *E. coli* ST in cultured T84 cells. The four peptides showed a dose-dependent stimulation of cGMP production between 10^{-9} and 10^{-6} M (Fig. 2). The cGMP-stimulating activity of rat uroguanylin-15 at 10^{-7} M was almost the same as when rat guanylin-15 was used as the cGMP stimulant, but less than those of human uroguanylin-16 and ST.

3.3. Distribution of uroguanylin mRNA

Fig. 3 shows RNA blot analysis of rat uroguanylin. Rat uroguanylin mRNA of approx. 600 bases, almost as long as that of human uroguanylin [16], was detected in the stomach to the colon. A moderate level of rat uroguanylin mRNA was observed in the kidney whereas guanylin mRNA was detected faintly, and low levels of uroguanylin mRNA in the lung and pancreas where no guanylin mRNA was detected (Fig. 3A). Rat uroguanylin mRNA is abundant in the upper small intestine, whereas guanylin mRNA shows a gradual increase along the duodenum-to-colon axis (Fig. 3B).

4. Discussion

Uroguanylin and guanylin are potent physiological regulators of intestinal fluid and electrolyte transport [1–5]. Their structural homologies and similar bioactivities indicate that they are members of the same peptide family. In the present study, we report the isolation of clones encoding a precursor

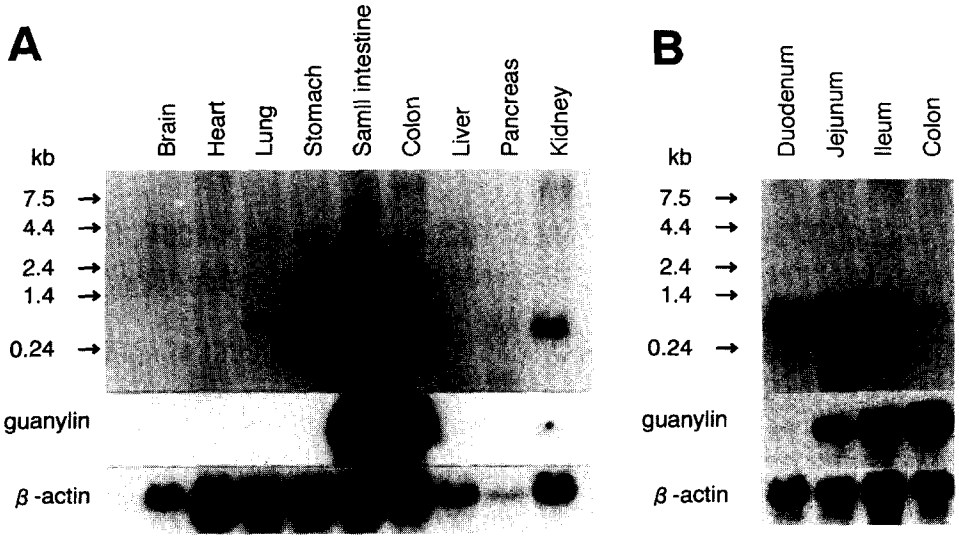

Fig. 3. RNA blot analysis of the uroguanylin transcript in rat tissues. Each lane contained (A) 10 μg of poly(A)$^+$ RNA and (B) 20 μg of total RNA. Numbers on the left are kilobases as determined from the RNA size markers. The same membrane was used for sequential hybridizations with probes for rat guanylin and β-actin.

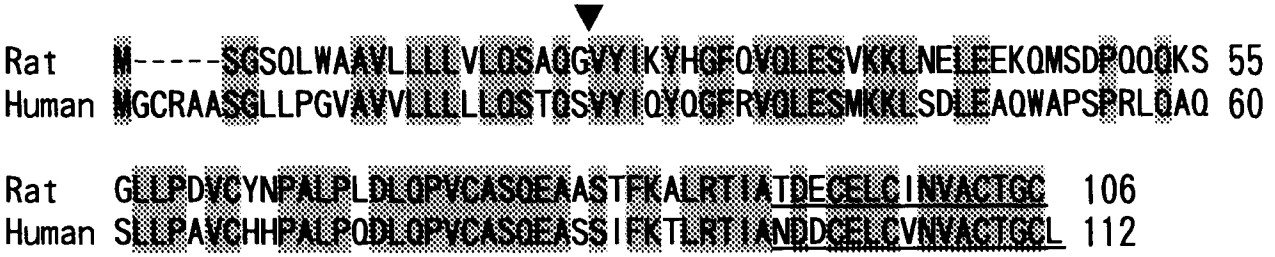

Fig. 4. Comparison of the amino acid sequences of rat and human uroguanylin precursors. Identical residues are shaded. The putative signal peptide cleavage site is indicated by the arrowhead. The uroguanylin sequences are underlined.

for rat uroguanylin. The deduced amino acid sequence of rat uroguanylin precursor was a 106-residue protein. Fig. 4 shows the alignment of amino acid sequences of rat and human uroguanylin precursors. To maximize the alignment of these sequences, five amino acid gaps had to be added to the rat sequence. The amino acid sequence homology of rat and human uroguanylin precursors is approx. 63%. On the basis of the established amino acid and cDNA sequences of human uroguanylin [7,16,17], the mature form of rat uroguanylin is predicted to be a 15-amino-acid peptide (rat uroguanylin-15) located at the end of long prepro uroguanylin. In fact, synthetic rat uroguanylin-15 increased the cGMP level in T84 cells. However, its cGMP-stimulating activity is less than that of human uroguanylin-16. We found that two conformationally distinct isoforms of human uroguanylin-16 are produced during its synthesis; one isoform stimulates cGMP production but another does not [18,19]. In our bioassay, we used the bioactive form of human uroguanylin-16, and the mixture of bioactive and inactive forms of rat uroguanylin-15. This might explain why rat uroguanylin-15 showed almost half of the cGMP production compared with human uroguanylin-16.

Guanylin gene expression is nearly restricted to the intestine, whereas uroguanylin mRNA is present in the stomach, kidney, lung and pancreas in addition to the intestine. Furthermore, uroguanylin mRNA is most abundant in the upper small intestine, but guanylin mRNA in the colon. These results indicate that the two peptides may have different roles in the regulation of epithelial function associated with water and salt balance. Fan et al. [9] recently reported that opossum uroguanylin gene was expressed in the heart, but not in the stomach or kidney. We did not detect uroguanylin mRNA in rat heart. Species-specific differences in tissue expression of uroguanylin gene may explain these disparate data. However, the expression level of uroguanylin mRNA in these tissues is thought to be much lower than that in the intestine. In situ hybridization analysis or the use of more sensitive techniques such as PCR could demonstrate gene expression in these tissues. Evidence for uroguanylin expression in the extra-gastrointestinal tissues such as lung, kidney and heart indicates that uroguanylin may play a pivotal role in the regulation of the circulatory system.

Uroguanylin, as well as guanylin, might be a potent physiological regulator of intestinal fluid and electrolyte transport. However, uroguanylin mRNA was detected not only in the intestine but also in the kidney. Intravenous administration of *E. coli* ST elicited large increases in urine cGMP excretion and urine Na^+ secretion [20]. These results suggest that uroguanylin activates GC-C in the kidney and regulates renal fluid and electrolyte transport. An intestinal natriuretic factor has been sought because oral ingestion of sodium chloride causes a dramatic increase in urine salt excretion, whereas the same amount of sodium chloride administered intravenously has little effect on renal salt excretion [4,21]. The active form of uroguanylin is abundant in normal human urine [18], which suggests that this peptide links the intestine and kidney in an endocrine pathway that controls renal salt metabolism. Further investigations of the guanylin family (guanylin and uroguanylin) should furnish new insights into the regulation of water and electrolyte homeostasis.

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