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Human guanylin: cDNA isolation, structure, and activity

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Guanylin is a mammalian peptide homologue of heat-stable enterotoxins that acts on intestinal guanylate cyclase to elicit an increase in cyclic GMP. We have isolated a cDNA encoding an apparent precursor of guanylin from a human intestinal cDNA library. The mRNA is expressed at high levels in human ileum and colon. Human guanylin stimulated increases in T84 cell cyclic GMP levels, displaced ^{125}I-labelled heat-stable enterotoxin (STa) binding to this cell line, and stimulated increases in short-circuit current (Isc) of isolated rat proximal colonic mucosa. This peptide may play a role in regulating fluid and electrolyte absorption in human intestines.

Guanylin; Heat-stable enterotoxin; Guanylate cyclase; Cyclic GMP

1. INTRODUCTION

The recent discovery of the intestinal peptide, guanylin, from the rat jejunum has provided evidence for a new intestinal paracrine/endocrine system that acts, in part, through the second messenger, cyclic GMP [1]. Guanylin apparently acts on the isoform of guanylate cyclase, termed GC-C or intestinal guanylate cyclase, to increase intestinal epithelial cell cyclic GMP levels [1] and thus regulate intestinal fluid and electrolyte absorption through the second messenger, cyclic GMP. Intestinal guanylate cyclase has for many years been known to be a target for the heat-stable enterotoxins (STs) [2,3]. The peptide sequence of rat guanylin is similar to that of the STs, and it competes for the same intestinal binding sites [1]. Purified rat guanylin is an acidic peptide 15 amino acids in length. The peptide is characterized by the presence of 4 cysteines that require oxidation and disulfide bridge formation for full expression of activity [1]. Cloning of rat guanylin indicated that this peptide is derived from the processing of a 115 amino acid containing precursor encoded on a 600 base mRNA [4,5].

The mechanism of STs' actions on intestinal fluid and electrolyte transport is thought to occur through activation of the guanylin receptor (intestinal guanylate cyclase) by binding of the toxin. This elicits an increase in cyclic GMP that mediates the increase in chloride secretion and the decrease in sodium and water absorption [2,3,6]. The net effect of these actions in the intestine is to cause a secretory diarrhea [7]. In developing countries, the diarrhea caused by the family of heat-stable enterotoxins is thought to be a major cause of death in the infant population [7,8]. Heat-stable enterotoxins appear to mimic guanylin to activate intestinal guanylate cyclase and produce their pathogenic effect.

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2. MATERIALS AND METHODS

2.1. *Gene isolation and mRNA analysis*

A human duodenum cDNA library in λgt10 obtained from Clontech (Palo Alto, CA) was screened under low stringency hybridization conditions [9]. The filters were probed using purified insert from pMON9022, the rat guanylin cDNA [4], labeled with ^{32}P by random priming. Adult human intestinal RNAs were the generous gift of Dr. Burton Wice and were described in [10]. Northern blots [9] were hybridized with a full-length human guanylin cDNA insert which had been labeled by random priming.

2.2 *Peptide synthesis*

Human guanylin$_{101-115}$ was synthesized by the solid-phase method with an Applied Biosystems 430A peptide synthesizer essentially as described previously [1]. The structures and purity of the synthetic peptides were verified by thermospray/mass spectroscopy, amino acid analysis, and gas-phase sequence analysis.

2.3. *Biological assays*

Cyclic GMP and binding assays were done as described previously [1]. For binding, the results are expressed as the percentage of [^{125}I]STa$_{5-18}$ specifically bound. For short circuit current measurements, proximal colon tissue, consisting of only mucosa and submucosa, was mounted between two Ussing half-chambers and bathed on both sides. Electrical measurements were monitored with an automatic voltage clamp and direct connecting voltage- and current-passing electrodes were utilized to measure trans-epithelial potential difference and short-circuit current. Tissues were equilibrated under short-circuit conditions until short-circuit current had stabilized (usually 30–45 min). Basal tissue resistance values averaged 44 ± 4 Y/cm^2 ($n = 10$) 30 min after mounting.

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3. RESULTS

We have used relaxed stringency hybridization to isolate a human homologue of the rat guanylin gene. A full-length rat cDNA fragment was used as a probe to screen a cDNA library made from human duodenum RNA. Thirty-six positive phages were identified and plaque purified by two subsequent rounds of plating and screening. DNA was prepared from each of these positive clones and analyzed by restriction mapping and Southern blotting. Eleven of the clones remained positive on the Southern blots when probed with the rat guanylin cDNA insert, as described above, and washed at high stringency. When labeled insert from one of these 11 clones was used to probe all 36 λ DNA isolates on a Southern blot under high stringency the same 11 clones hybridized. Inserts from four of these clones were subcloned into pGEM7 for sequencing, whilst the end points of the rest were sequenced directly in the λ DNA. The 589 bp sequence obtained from these clones is shown in Fig. 1. The cDNAs obtained are large enough to be near full-length clones of the mRNA (see below). The complete sequence given was determined in three non-sibling clones.

By Northern blot analysis, the human guanylin mRNA ran as a single, somewhat diffuse, species of about 600 bases in length. Like rat guanylin, the human guanylin mRNA is expressed preferentially in the distal regions of the cephalocaudal axis of the gut (Fig. 2). The highest levels of expression are observed in colon and ileum, lower levels in jejunum and duodenum, and the message is undetectable in stomach.

To confirm the identity of this gene and assess its biological activity we synthesized the homologue

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1   TCGCTGCCATGAATGCCTTCCTGCTCTTCGCACTGTGCCTCCTTGGGGCCTGGGCCGCC
              MetAsnAlaPheLeuLeuPheAlaLeuCysLeuLeuGlyAlaTrpAlaAla

60  TTGGCAGGAGGGGTCACCGTGCAGGATGGAAATTTCTCCTTTTCTCTGGAGTCAGTGAAG
    LeuAlaGlyGlyValThrValGlnAspGlyAsnPheSerPheSerLeuGluSerValLys

120 AAGCTCAAAGACCTCCAGGAGCCCCAGGAGCCCAGGGTTGGGAAACTCAGGAACTTTGCA
    LysLeuLysAspLeuGlnGluProGlnGluProArgValGlyLysLeuArgAsnPheAla

180 CCCATCCCTGGTGAACCTGTGGTTCCCATCCTCTGTAGCAACCCGAACTTTCCAGAAGAA
    ProIleProGlyGluProValValProIleLeuCysSerAsnProAsnPheProGluGlu

240 CTCAAGCCTCTCTGCAAGGAGCCCAATGCCCAGGAGATACTTCAGAGGCTGGAGGAAATC
    LeuLysProLeuCysLysGluProAsnAlaGlnGluIleLeuGlnArgLeuGluGluIle

300 GCTGAGGACCCGGGCACATGTGAAATCTGTGCCTACGCTGCCTGTACCGGATGCTAGGGG
    AlaGluAspProGlyThrCysGluIleCysAlaTyrAlaAlaCysThrGlyCysEnd

360 GGCTTGCCCACTGCCTGCCTCCCCTCCGCAGCAGGGAAGCTCTTTTCTCCTGCAGAAAGG

420 GCCACCCATGATACTCCACTCCCAGCAGCTCAACCTACCCTGGTCCAGTCGGGAGGAGCA

480 GCCCGGGGAGGAACTGGGTGACTGGAGGCCTCGCCCCAACACTGTCCTTCCCTGCCACTT

540 CAACCCCCAGCTAATAAACCAGATTCCAGAGTAAAAAAAAAAAAAAAAAA
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Fig. 1. Sequence of the human guanylin cDNA. The guanylin sequence is indicated by a double underline; the possible leader sequence, the Lys–Lys site and the polyadenylation signals are all indicated by single underlines. The GenBank accession number for this sequence is M97496.

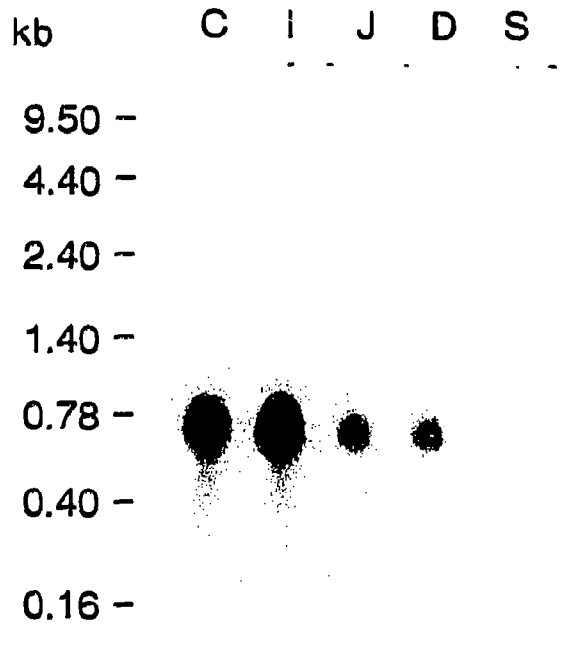

Fig. 2. Guanylin steady-state mRNA levels in human gastrointestinal tract by Northern blot analysis. The RNA in the respective lanes (10 μg) are: colon (C), ileum (I), jejunum (J), duodenum (D) and stomach (S).

of the 15 amino acid rat guanylin peptide, human guanylin$_{101-115}$. Chemical synthesis of the bioactive species in high yield required sequential deprotection and oxidation of the cysteine disulfide pairs to force pairing of Cys-4 with Cys-12 and Cys-7 with Cys-15. Air oxidation of a peptide with all four cysteines unprotected produced a mixture of isomers, with the correctly folded form representing only a small fraction of the total material (data not shown).

Synthetic human guanylin caused a concentration-dependent increase in T84 cell cyclic GMP (Fig. 3A). Human guanylin and rat guanylin had a similar relative potency for activation of intestinal guanylate cyclase. Both were about one order of magnitude less potent than STa. A similar profile of relative potency for displacing [^{125}I]-STa$_{5-18}$ specific binding from T84 cells was also observed with STa the most potent, and human and rat guanylin somewhat less potent (Fig. 3B). The data indicate that while heat-stable enterotoxin is more potent at stimulating intestinal guanylate cyclase, all of these peptides share common binding sites.

To assess the effects of human guanylin on intestinal function it was tested on isolated rat proximal colon mounted in Ussing chambers. In these experiments, the measurement of short-circuit current (Isc) is used as an indicator of chloride secretion. Previous studies have indicated that the change in Isc elicited by STa and rat guanylin is, for the most part, accounted for by a stimulation of chloride secretion [2,3,11]. Human guanylin

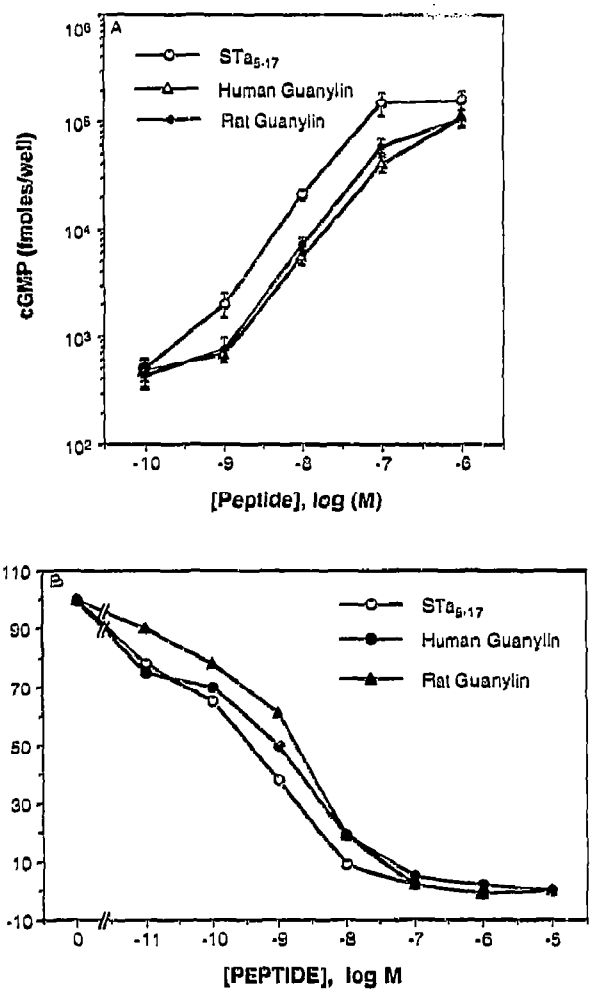

Fig. 3. Comparison of the effects of STa$_{5-17}$, human guanylin and rat guanylin on T84 cyclic GMP levels and [^{125}I]STa$_{5-18}$ binding. (A) Effect of increasing concentrations of STa, human guanylin and rat guanylin on intracellular cyclic GMP levels in T84 cells. Values represent means ± S.E.M. (n = 6). (B) Competitive inhibition of [^{125}I]STa$_{5-18}$ binding by unlabeled STa$_{5-17}$, (○), human guanylin (●) or rat guanylin (▲) in T84 cells. Each point represents the mean of triplicate determinations. Similar results were obtained in two additional experiments.

was added to the mucosal side in increasing concentrations in a cumulative manner. Human guanylin stimulated an increase in Isc with an EC$_{50}$ of 30 nM (Fig. 4), which is similar to that recently reported for rat guanylin and STa [11]. The maximal increase in Isc of 49±4 μA/cm^2 is similar to maximal effects elicited by rat guanylin and STa in the proximal colon [11].

4. DISCUSSION

The cDNA sequence encodes an apparent preprohormone of 115 amino acids. The open reading frame, beginning with the only in-frame methionine in the cDNA, encodes a peptide that shares 14 of 15 amino acids with rat guanylin at its C-terminal end. The rat and human cDNAs share a striking 65–70% identity

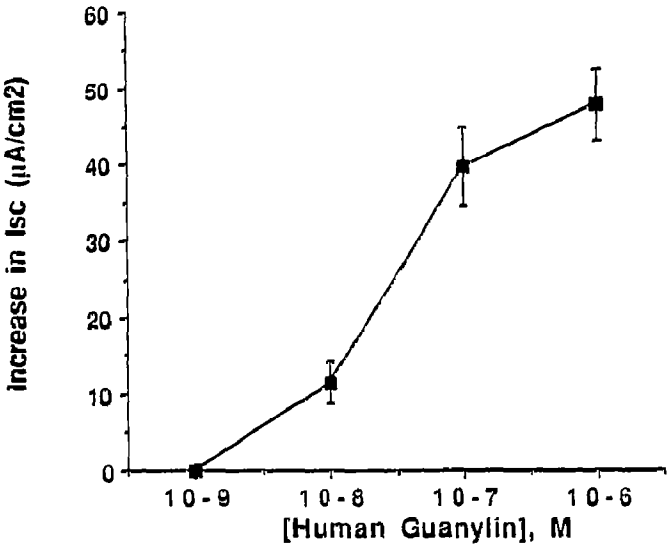

Fig. 4. Effects of increasing concentrations of human guanylin on short-circuit current (Isc) across rat proximal colonic mucosa. All additions were made from the mucosal side and the values represent the mean ± S.E.M of 8 determinations from 4 animals.

overall in both the DNA and protein sequences (Fig. 5) and have their major structural features in common. The N-terminus is hydrophobic, which is consistent with a leader sequence, and there is a polyadenylation signal and poly-A tail at the 3′ end of the mRNA. There is one dibasic pair (Lys–Lys) which might be used for processing, although the location of this sequence is not conserved between rat and human. A 15 amino acid peptide analogous to rat guanylin would result from cleavage between Asp and Pro at position 100 of the precursor, just as it would in the rat.

Interestingly, the guanylin precursors also share some structural similarity with the STa precursors identified in *E. coli* [12,13]. Both STa and guanylin are encoded on relatively small precursor proteins, 72 amino acids for STa vs. 115 for guanylin, which contain a typical secretion leader. In each case the identified active peptide is encoded in the C-terminal residues of the precursor. Both precursors contain Lys–Lys dibasic sites, though in neither case is there yet evidence for processing at those sites. The highest sequence conservation exists between the active peptides and, most importantly, the positions of the cysteine residues and the pattern of disulfide linkage are conserved between the STa and guanylin peptides.

We found that human guanylin stimulates an increase in cyclic GMP in T84 cells in a manner similar to that of rat guanylin and heat-stable enterotoxin. Human and rat guanylin also displaced the specific binding of ST to T84 cells. Potency comparisons indicate little difference between human and rat guanylin in regard to their effect on T84 cyclic GMP and displacement of ST binding. These results also indicated that the toxin is more potent than these synthetic 15 amino acid forms of guanylin. Human guanylin stimulated a concentration-dependent increase in short-circuit current in the proximal rat colon mounted in a Ussing chamber. This change in short circuit current in the proximal rat colon is thought to be due, in large part, to a stimulation of chloride secretion [2,3,11]. These studies indicate that guanylin acts in a manner similar to ST, and therefore would be expected to cause the secretion of chloride and fluids through a cyclic GMP-dependent mechanism.

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1   MNAFLLFALCLLGAWAALAGGVTVQDGNFSFSLESVKKLKDLQEPQEPRV 50
    ||| ||  |||||| | |  |||||||. || ||||| || | | |||
1   MNAWLLSVLCLLGALAVLVEGVTVQDGDLSFPLESVKQLKHLREVQEPTL 50

51  GKLRNFAPIPGEPVVPILCSNPNFPEELKPLCKEPNAQEILQRLEEIAED 100
      ||    || | |||   ||| | |||  ||| |||||||  || |
51  MSHKKFALRLPKPVAPELCSQSAFPEALRPLCEKPNAEEILQRLEAIAQD 100

101 PGTCEICAYAACTGC 115
    | ||||||||||||||
101 PNTCEICAYAACTGC 115
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Fig. 5. Comparison of human (upper) and rat (lower) guanylin prohormone sequences. This comparison was made with the GCG program Bestfit [14] using the default settings.

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