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The *Escherichia coli* Heat-Stable Enterotoxin Is a Long-Lived Superagonist of Guanylin

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The mechanism by which bacterial heat-stable enterotoxins (ST I, ST_A) cause diarrhea in humans and animals has been linked to the activation of an intestinal membrane-bound guanylate cyclase. Guanylin, a recently discovered rat intestinal peptide, is homologous in structure to ST I and can activate guanylate cyclase present on the human colonic carcinoma cell line T84. To directly test the mechanistic association of guanylate cyclase activation with diarrhea, we synthesized guanylin and a guanylin analog termed N^9P^{10} guanylin and compared their biological activities with those of a synthetic ST I analog, termed ST Ib(6-18). We report that guanylin is able to inhibit the binding of a radiolabeled ST I analog to rat intestinal cells but causes diarrhea in infant mice only at doses at least 4 orders of magnitude higher than that of ST Ib(6-18). In contrast, N^9P^{10} guanylin was enterotoxic in mice at much lower doses than guanylin but proved to be a weaker inhibitor of radiolabeled ST I than guanylin in the receptor binding assay. The pattern of guanylate cyclase activation observed for ST Ib(6-18) and the two guanylin analogs parallels the results observed in the receptor binding assay rather than those observed in the diarrheal assay. Treatment of guanylin with chymotrypsin or lumenal fluid derived from newborn mouse intestines resulted in a rapid loss of binding activity. Together, these results suggest that ST I enterotoxins may represent a class of long-lived superagonists of guanylin.

The heat-stable enterotoxins are a group of small homologous peptides elaborated by enterotoxigenic strains of bacteria (23, 29, 35). They are collectively responsible for a large proportion of worldwide cases of secretory diarrhea in humans and animals. These enterotoxins, abbreviated ST I (or ST_A), are known to bind to receptors located on the brush border surface of intestinal cells (13, 15, 17, 20, 22) and to cause an elevation of intracellular cyclic GMP (cGMP) levels (11, 16, 19, 26). It is generally believed that the binding of the enterotoxin to its receptor is coupled to the activation of a guanylate cyclase and that cGMP acts as the intracellular second messenger causing the eventual onset of diarrhea. Although more than one class of ST I receptors may exist (20, 24), research efforts in this field have focused on the cloning of one class of guanylate cyclases acting as ST I receptors (7, 8, 31, 33). The evidence linking the ST I-induced elevation of cGMP levels to secretory diarrhea has centered around the administration of a nonhydrolyzable analog of cGMP termed 8-bromo-cGMP to ligated rabbit intestines and to infant mice (16, 19). This treatment resulted in fluid accumulation in both animal models and suggested that an increased production of intracellular cGMP is a necessary step leading to watery diarrhea. Recently, a naturally occurring peptide termed guanylin was isolated from rat jejunum and was found to activate a particulate form of guanylate cyclase present on the human colonic carcinoma cell line T84 (6). This peptide was able to displace the binding of ^{125}I-labeled ST I to receptors on the surface of T84 cells (6). Guanylin is a 15-amino-acid peptide that is highly homologous in sequence to a region of ST I, abbreviated ST Ib(6-18), that codes for its receptor binding and enterotoxigenic properties (4, 14, 32, 39). In particular, identical residues are found at eight positions within the 13-amino-acid sequence of ST Ib(6-18) (Fig. 1). A major difference between the two peptides is that ST Ib(6-18) has six cysteine residues participating in three intramolecular disulfide bridges within its sequence (14, 32) while guanylin has only four cysteines and two disulfide bridges (6) (Fig. 1). As a consequence of structural and functional similarities between guanylin and ST I, one would expect guanylin to cause diarrhea in mammals at a concentration relative to ST I that parallels its ability to inhibit the binding of radiolabeled ST I to intestinal cells. In this study, we report the synthesis of two analogs of guanylin and their ability to (i) inhibit the binding to rat cells of a radiolabeled ST I analog (4, 14), (ii) cause a diarrheal response in infant mice (18), and (iii) stimulate rat intestinal brush border guanylate cyclase (22).

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MATERIALS AND METHODS

Preparation of analogs. Peptides were synthesized on an Applied Biosystems Model 430A automated peptide synthesizer using *tert*-butoxycarbonyl-protected amino acids coupled to a phenylacetamidomethyl resin support by classical solid phase methods (34). The peptides were cleaved from the resin by using anhydrous HF in the presence of anisole, dimethyl sulfide, and *p*-thiocresol. The reduced peptides were dissolved in a dilute aqueous solution (50 μM), pH 8.5, for 5 days to allow disulfide pairing to occur. The oxidized peptides were concentrated on a preparative C_{18} column, eluted with 40% (vol/vol) acetonitrile in water, and lyophilized. The peptides were then purified by high-performance liquid chromatography (HPLC) on a semipreparative C_{18} column with a linear gradient going from 10 to 40% (vol/vol) acetonitrile–0.08% (vol/vol) trifluoroacetic acid in water–0.1% (vol/vol) trifluoroacetic acid over a 60-min period. HPLC peaks were initially screened for their ability to inhibit the binding of a radiolabeled ST Ib analog, ^{125}I-Y^{4}ST Ib(4-18), to rat villus cells (4). Only those peaks which exhibited inhibitory activity in this experiment were further analyzed. The homogeneity, amino acid composition, and

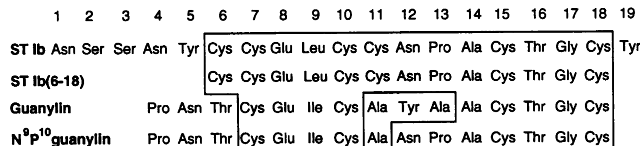

FIG. 1. Amino acid sequences of the *E. coli* heat-stable enterotoxin ST Ib (1, 25) and the three peptides prepared for this study: ST Ib(6-18), guanylin, and N^9P^{10} guanylin. The numbering refers to the position of the amino acids in relation to the ST Ib sequence. ST Ib(6-18) corresponds to the biologically active domain of ST Ib (4, 14, 32, 39). Regions of sequence homology are boxed. The substitution Leu-9 → Ile represents a conversed substitution observed in other ST I sequences (35). Amino acids are identified by their three-letter codes. All peptides were synthesized as described in Materials and Methods.

molecular weight of each analog were respectively confirmed by thin-layer chromatography, amino acid analysis, and fast atom bombardment mass spectrometry.

Displacement assay of ^{125}I-Y^4ST Ib(4-18) binding to rat villus cells. Y^4ST Ib(4-18), a peptide consisting of residues 6 to 18 of ST Ib, in addition to two N-terminal tyrosine residues, was radioiodinated and purified as described previously (4, 14). Displacement assays were performed as follows. Twenty-microliter aliquots of unlabeled test peptide dilutions were combined with 10^5 cpm of ^{125}I-Y^4ST Ib(4-18), 20 μl of phosphate-buffered saline (PBS [0.15 M NaCl, 10 mM NaH_2PO_4, pH 7.4]) containing 5 mM EDTA and 0.02% (wt/vol) sodium azide, and 5 × 10^5 villus cells isolated from the small intestines of adult female Sprague-Dawley rats in polypropylene test tubes. Samples were incubated at 37°C for 30 min, treated with 50 μl of 1% (wt/vol) bovine serum albumin in PBS, and placed on ice for 10 min. The samples were filtered onto Whatman GF/A filters, washed four times with PBS, and counted in a γ counter. Displacement curves were constructed from averaged values of experiments performed in triplicate.

Suckling mouse assay. One hundred microliters of each analog dilution prepared in PBS containing 0.04% (wt/vol) Evans blue dye was administered orally to 4-day-old Swiss-Webster suckling mice (18). After 3 h, the mice were sacrificed and their intestines were excised. The guts and remaining carcasses were weighed, and the resulting gut/carcass ratio (G:C) was calculated. Each G:C represents an average of measurements performed for three infant mice. Two regimens were performed in order to evaluate the ability of guanylin to inhibit the enterotoxic effect of ST Ib(6-18) in infant mice. In the first regimen, a 100-μl aliquot of ST Ib(6-18) (10^{-11} mol [see Fig. 3]) sufficient to cause diarrhea in mice was mixed with increasing amounts of guanylin (10^{-9} to 10^{-11} mol) prepared in PBS containing 0.04% (wt/vol) Evans blue dye. The resulting solutions were administered to suckling mice according to the protocol described previously. In the second regimen, 50-μl doses representing increasing amounts of guanylin were administered to the mice, followed 30 min later by a 50-μl dose of ST Ib(6-18) (10^{-11} mol). After 3 h, the G:C was determined as described above.

Guanylate cyclase assay. Aliquots (10 μl) of each peptide dilution were combined on ice with 42 μl of 50 mM Tris-HCl (pH 7.6), 8 μl of rat intestinal brush border preparation (protein content, 28 μg) (22), and 20 μl of a mixture containing 2.5 mM 3-isobutyl-1-methylxanthine, 38 mM phosphocreatine, and 3.2 U of phosphocreatine kinase. The reaction was initiated by the addition of 20 μl of a substrate solution consisting of 5 mM GTP and 20 mM $MgCl_2$. The samples were incubated at 37°C for 5 min, and the reaction was quenched by the addition of 400 μl of cold sodium acetate buffer (50 mM, pH 4.0), followed by immersion of the sample in a boiling water bath for 3 min (22). The samples were centrifuged at 4°C for 15 min, and the supernatants were immediately removed and stored on ice. cGMP levels in 100-μl aliquots of the supernatants were determined with a commercial radioimmunoassay kit (NEN Du Pont, Mississauga, Ontario, Canada).

Protease digestion experiments. Chymotrypsin was purchased from Sigma (St. Louis, Mo.), and its activity was calibrated with the substrate benzoyl-L-tyrosine ethyl ester (Sigma) (38). Infant mouse lumenal fluid was recovered by excising the small intestines of euthanized newborn suckling mice and flushing their contents with cold PBS. The resulting solution was filtered, and the filtrate was immediately stored at −70°C. The remaining flushed small intestine segments were also collected and stored at −70°C without processing. A homogenate of infant mouse intestinal tissue was prepared from the intestinal segments as follows. The frozen tissue was further cooled with liquid nitrogen and pulverized with a precooled mortar and pestle. The pulverized tissue from one intestine was suspended in 400 μl of 0.1 M Tris-HCl (pH 8.1) and vortexed. The suspension was then spun at 12,000 × *g* in a microcentrifuge for 5 min. The supernatant was removed, filtered, and stored on ice until used. Both intestinal preparations were found to contain significant chymotrypsin-like activity by benzoyl-L-tyrosine ethyl ester assay. Samples of each of the three peptides were prepared in Tris buffer at the following concentrations: ST Ib(6-18), 0.02 mg/ml; guanylin, 0.5 mg/ml; N^9P^{10} guanylin, 1 mg/ml. At zero time, the peptide solutions were treated with either chymotrypsin, lumenal fluid, or filtrate of intestinal homogenate to an enzyme/substrate equivalence ratio of 1:50 (on the basis of the benzoyl-L-tyrosine ethyl ester assay) and were incubated at 37°C. Aliquots were withdrawn from each peptide solution prior to the addition of either protease or intestinal extracts. The digests were then sampled after 15, 60, and 180 min of incubation. Each aliquot was immersed in a boiling water bath for 3 min and treated with the inhibitor phenylmethylsulfonyl fluoride (Sigma) to a final concentration of 1 mM phenylmethylsulfonyl fluoride. Twenty-microliter aliquots of each sample, corresponding to the minimal peptide dose able to completely inhibit the binding of 10^5 cpm of ^{125}I-Y^4ST Ib(4-18) to 5 × 10^5 rat villus cells, were then tested with the displacement assay described above.

RESULTS AND DISCUSSION

Structure of guanylin analogs. The rat intestinal peptide guanylin is structurally homologous to heat-stable enterotoxins (ST I), sharing identical residues at eight positions within the 13-amino-acid-long enterotoxic domain [ST Ib(6-18)] (Fig. 1). More importantly, four of the six cysteines of ST I are conserved in guanylin, suggesting that the intestinal peptide may possess functional properties associated with these enterotoxins (6). Guanylin was initially isolated on the basis of its ability to stimulate guanylate cyclase activity in T84 cells and to bind to receptors on the surface of these cells, two properties which it shares with ST I (6). Guanylin represents the C-terminal region of a larger inactive precursor (a 94-amino-acid-long proguanylin form [9]). Bacterial strains may thus produce ST I enterotoxins in order to exploit a mechanistic pathway associated with the naturally occurring intestinal peptide guanylin. Because diarrhea is

TABLE 1. Biological properties of ST Ib(6-18), guanylin, and N^9P^{10} guanylin

Peptide	IC_{50} (M)[a]	ED_{50} (mol)[b]	GC_{50} (M)[c]
ST Ib(6-18)	3.0×10^{-8}	2.5×10^{-12}	10^{-8}
Guanylin	10^{-6}	$>3.0 \times 10^{-8}$	7.0×10^{-7}
N^9P^{10} guanylin	6.0×10^{-5}	3.2×10^{-10}	6.3×10^{-6}

[a] IC_{50}, 50% inhibitory concentration (peptide concentration required to inhibit 50% of the specific binding of radiolabeled Y^4ST Ib(4-18) to rat villus cells).

[b] ED_{50}, 50% effective dose (peptide dose required to cause a half-maximal increase in G:C after oral administration of the analog to infant mice).

[c] GC_{50}, 50% guanylate cyclase activation (peptide concentration required to cause a half-maximal activation of intestinal brush-border guanylate cyclase).

not a normal physiological event, it was projected that the oral administration of guanylin may not cause a diarrheal response in experimental animals. We thus synthesized guanylin and a second guanylin analog, termed N^9P^{10} guanylin, which is a closer homolog of ST I (Fig. 1), particularly within the central region of the ST I molecule (residues 11 to 14). This turn region was previously found to be the most important region of ST Ib in terms of biological activity and is particularly sensitive to amino acid substitutions (4, 27). The objective of this study was to quantitatively compare the biological activities of guanylin and ST Ib(6-18), using established in vitro and in vivo assays in order to define how differences in the primary structure of guanylin and ST I may result in enterotoxicity.

The two guanylin analogs bind to the most abundant ST I receptor on rat enterocytes. The binding of guanylin analogs to the ST I receptor on rat small intestinal villus cells was assessed by measuring their ability to compete with radiolabeled ST I for receptor sites on enterocytes (4, 14). Binding experiments were performed at pH 7.4 in accordance with the slightly alkaline environment observed in the small intestine of both rat and suckling mice (measured pH of lumenal fluid, 7.5 ± 0.5 pH units [this study]). As shown in Fig. 1, synthetic guanylin displaced, in a concentration-dependent manner, the binding of ^{125}I-Y^4ST Ib(4-18) to receptors on rat intestinal cells with a 50% inhibitory concentration 30-fold higher than that of ST Ib(6-18) (Table 1). Thus, guanylin is an agonist of the ST I receptor on rat villus cells. The peptide N^9P^{10} guanylin, on the other hand, was approximately 2,000-fold less active than ST Ib(6-18) in the binding assay (Fig. 2 and Table 1).

The enterotoxicity of guanylin in suckling mice is low. It is widely believed that binding of ST I to intestinal villus cells and the subsequent elevation of intracellular cGMP levels are necessary and sufficient events leading to the eventual macroscopic episode of diarrhea (11, 16, 19, 26). The capacity of ST I analogs to cause intestinal fluid accumulation in infant mice (18) correlated well with the ability of these peptides to bind to rat villus cells (4, 14). Guanylin was unable to cause diarrhea in mice except at concentrations 4 orders of magnitude higher than that of ST Ib(6-18) (Fig. 3 and Table 1). In contrast, N^9P^{10} guanylin was only 125-fold less active than ST Ib(6-18) in this experiment, despite the fact that it was 2,000-fold less active than ST Ib(6-18) in the binding assay (Fig. 2 and 3 and Table 1). If a single class of ST I receptors existed that was mechanistically linked to the onset of diarrhea, one would expect guanylin to act as an antagonist and block diarrhea induced by ST I. To test this hypothesis, doses of guanylin between 10^{-9} and 10^{-11} mol were administered orally to infant mice in conjunction with a dose of ST Ib(6-18) (10^{-11} mol) sufficient to cause diarrhea.

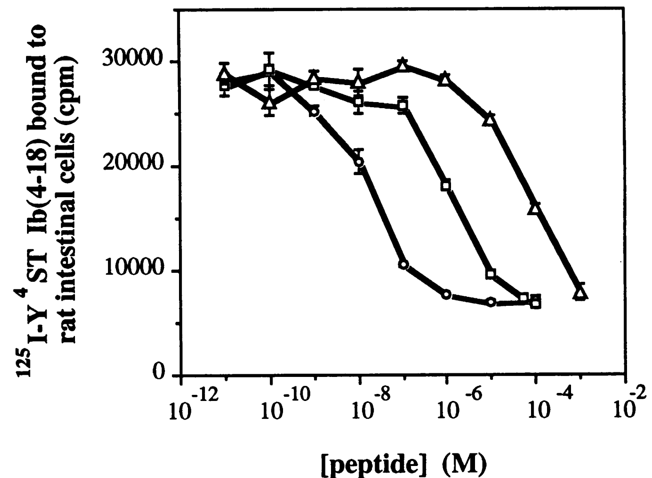

FIG. 2. Displacement curves of the binding of ^{125}I-Y^4ST Ib(4-18) to rat villus cells by ST Ib(6-18) (○), guanylin (□), and N^9P^{10} guanylin (△). The relative affinity of each analog for the ST I receptor was determined by its concentration-dependent capacity to inhibit the binding of ^{125}I-Y^4ST Ib(4-18) to rat enterocytes. Each datum point represents the average of experiments performed in triplicate.

Guanylin was unable to act as an antagonist, because diarrhea was observed in all suckling mice even at a molar ratio of guanylin to ST Ib(6-18) of 10^2 [10^{-9} mol of guanylin to 10^{-11} mol of ST Ib(6-18)]. The results were identical whether guanylin was administered simultaneously to ST Ib(6-18) or 30 min prior to ST Ib(6-18) (data not shown). These results suggest that guanylin lacks the enterotoxic potential of ST I but binds predominantly to the most prevalent class of ST I receptors which are coupled to a guanylate cyclase.

Both guanylin analogs are able to activate a rat intestinal guanylate cyclase. Both guanylin analogs were assayed for their ability to activate rat intestinal brush border guanylate

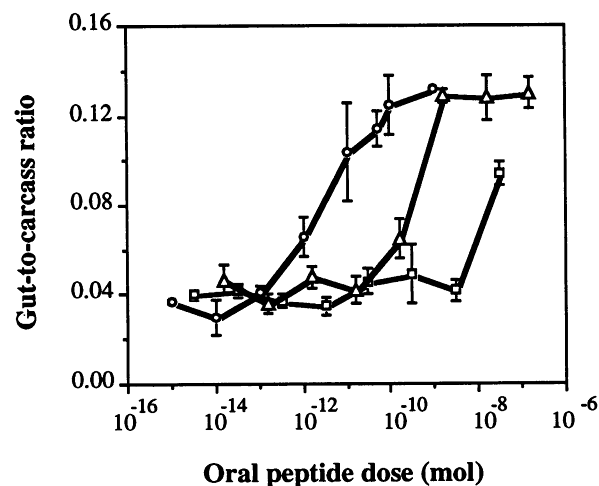

FIG. 3. Enterotoxicity of ST Ib(6-18) (○), guanylin (□), and N^9P^{10} guanylin (△) in suckling mice. The capacity of each analog to cause a diarrheal response in infant mice is quantified according to the increase in G:C resulting from an oral dose of a peptide (18). Each datum point represents the average of experiments performed in triplicate.

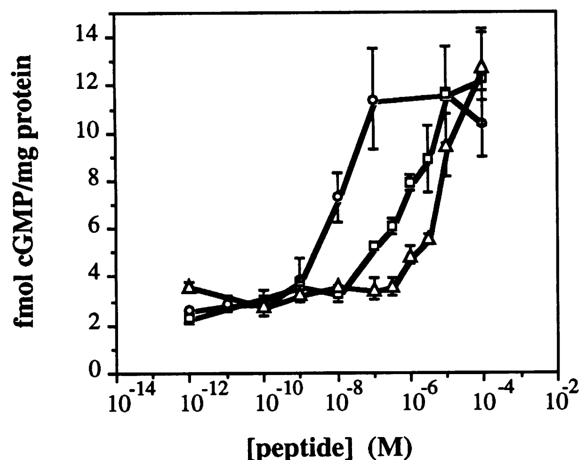

FIG. 4. Concentration-dependent activation of rat brush border membrane guanylate cyclase by ST Ib(6-18) (○), guanylin (□), and N^9P^{10} guanylin (△). Each datum point represents the average quantity of cGMP produced per milligram of protein in experiments performed in duplicate.

cyclase in relation to ST Ib(6-18). A comparison of Fig. 2 and 4 clearly shows that the pattern of activation of guanylate cyclase for the three peptides mirrors their pattern of binding affinities for the ST I receptor. Guanylin is a 30-fold weaker ligand than ST Ib(6-18) in the receptor binding assay and is 70-fold less active in activating membrane-bound guanylate cyclase. In contrast, N^9P^{10} guanylin is 2,000-fold weaker than ST Ib(6-18) in the binding assay and 630-fold less active in the guanylate cyclase assay (Table 1). The binding of ST I to its receptor has been correlated with the elevation of intestinal cGMP levels (10). Recent studies have indicated that the toxin binding site and the cyclase domain reside within the same intestinal membrane protein (7, 31, 33). If the activation of a specific guanylate cyclase was linked to diarrhea, one would have expected the relative pattern of potency for the concentration-dependent activation of guanylate cyclase observed for all three analogs to parallel their dose dependency in causing diarrhea in mice, with the relative order of decreasing potency being ST Ib(6-18) > N^9P^{10} guanylin > guanylin.

Structural and mechanistic implications. Two models can be proposed that reconcile past and present findings in relation to the mechanism of action of ST I. The first model would view ST I as a long-lived superagonist of guanylin and that processing or clearance of ST I from the guanylin receptor (previously referred to as the ST I receptor) is not as efficient as in the case of guanylin causing a chronic activation of the guanylate cyclase. The rapid proteolysis of guanylin would represent the most probable event. The tyrosine-alanine segment present in the sequence of guanylin is the only site absent in ST I enterotoxins which represents a segment potentially susceptible to protease digestion (putative chymotryptic site [Fig. 1]). Figure 5A shows that guanylin is rapidly inactivated by chymotrypsin compared with N^9P^{10} guanylin and ST Ib(6-18). Similar results in terms of loss of receptor binding activity were observed when guanylin was exposed to lumenal fluid recovered for small intestines of suckling mice (Fig. 5B) or treated with filtrates of infant mouse intestinal tissues (Fig. 5C). The replacement of Tyr-Ala with Asn-Pro in N^9P^{10} guanylin restored both properties of enterotoxicity and protease resistance. The

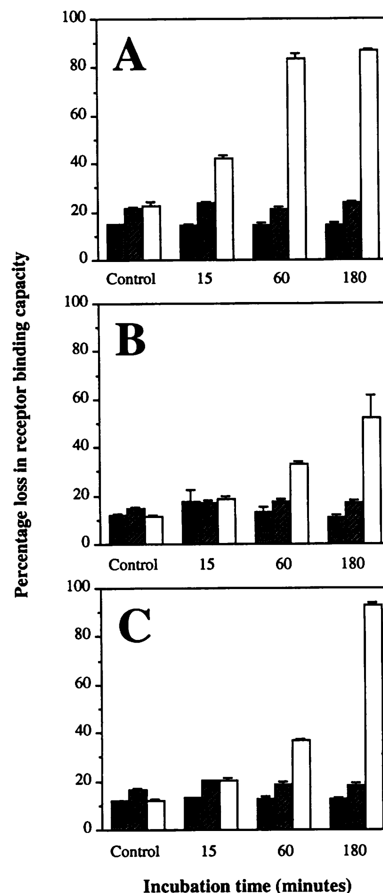

FIG. 5. Susceptibility of ST Ib(6-18) (shaded bars), N^9P^{10} guanylin (hatched bars), and guanylin (open bars) to protease degradation. The ability of treated analogs to inhibit the binding of radiolabeled toxin to rat intestinal cells was measured as a function of time of exposure to either chymotrypsin (A), suckling mouse intestinal fluid (B), or filtrates of infant mouse intestinal tissue homogenates (C). Each histogram bar represents the average of experiments performed in duplicate.

long-lived nature of ST I enterotoxins may thus be partially responsible for their ability to chronically activate the intestinal guanylate cyclase, causing an ensuing diarrheal response. Interestingly, an analog of ST I with a substitution of Asn-12 by tyrosine was shown to be enterotoxic (40). It would thus appear that the presence of a proline residue at position 13 of ST Ib is sufficient to reduce the protease susceptibility of the Tyr-Pro peptide bond. Cohen and Giannella (5) have reported that the susceptibility of immature rat jejunum to ST I action is higher than that of adult rat intestinal tissue. They concluded that the rapid clearance of ST I observed in adult rats and the slow disappearance of the enterotoxin in young rats are predominantly linked to biochemical events other than ST I's exposure to intestinal proteases present in lumenal fluid or to brush border membrane-associated proteases (5). Finally, the recent observations that guanylin and ST Ia(5-17) bind to the same receptor and are able to produce a similar elevation in intracellular cGMP and stimulate Cl^- secretion in T84 cells support the notion that one receptor common to both peptides is probably involved in the diarrheal response (12).

An alternate mechanism of action for ST I enterotoxins

would be to invoke the existence of a low-abundance class of ST I receptors not linked to the activation of guanylate cyclase but potentially acting in conjunction with the activated cyclase, resulting in the net efflux of water, sodium, and chloride ions. The recent observation that a class of high-affinity, low-occupancy receptors for ST I which are not coupled to guanylate cyclase may exist on rat intestinal membranes would provide some support for this hypothesis (20). Similarly, a novel receptor for ST I enterotoxins has been identified on IEC-6 cells (24). The key to the ligand specificity of the two (or more) receptor classes may lie in the central region of the ST I sequence, which is closely homologous to the same region of N^9P^{10} guanylin but is less so to guanylin. Experiments involving amino acid substitutions within the ST I sequence (4, 21, 27) have pointed to the functional importance of the central structural region of ST I. Interestingly, the sequence of the recently discovered *Escherichia coli* enteroaggregative heat-stable enterotoxin is more divergent than that of guanylin when compared with other ST I enterotoxins. Its enterotoxicity may thus be dependent on the structural context of Ala-14 and Cys-15 in particular rather than the entire turn region encompassing residues Asn-12 to Cys-15 (30). A consequence of this hypothesis is that residues such as Asn-12 and Pro-13 may only play an indirect role in the binding and/or signalling event leading to the diarrheal effect. In addition, several signal transduction pathways (linked or not to cGMP as a second messenger) have been implicated in the induction of intestinal secretion by ST I enterotoxins. Specifically, other groups have provided evidence for the activation of a protein kinase C (37) and the production of inositol polyphosphates (2, 3). It is unclear whether these responses are essential steps leading to fluid accumulation or are a consequence of triggering other intracellular second messengers. In this model, the ST I receptor guanylate cyclase remains occupied with either guanylin or ST I, implying that the elevation of intracellular cGMP is an ongoing event that may still play a supportive if not essential role in the onset of diarrhea. Because the overall increase in cGMP is similar between ST Ib(6-18) and the guanylin analogs (Fig. 4), it may be that the potency of ST I causes a downregulation in the expression of the ST I-receptor guanylate cyclase and thus a short-term insensitivity of these cells to the presence of guanylin. One would thus project a drop in intracellular cGMP levels after the initial stimulatory event. This event may also be a key to the action of ST I and was originally observed by Giannella and Drake (16). The physiological role of guanylin remains to be understood.

In summary, it was found that native guanylin competes with radiolabeled ST Ib analog for binding to rat cells but does not cause a diarrheal response in suckling mice except at elevated doses. In contrast, N^9P^{10} guanylin is a weaker competitor than guanylin in displacing radiolabeled ST Ib bound to rat cells, yet in mice it causes diarrhea at much lower concentrations. The pattern of activation of rat intestinal guanylate cyclase for the two guanylin analogs parallels the results of the receptor binding assay but not those of the infant mouse assay. Guanylin is rapidly degraded by components present in the infant mouse intestinal tract, suggesting that ST I enterotoxins may be acting as long-lived superagonists of guanylin.

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