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A GRADIENT IN EXPRESSION OF THE ESCHERICHIA COLI HEAT-STABLE ENTEROTOXIN RECEPTOR EXISTS ALONG THE VILLUS-TO-CRYPT AXIS OF RAT SMALL INTESTINE[1]

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Binding of Escherichia coli heat-stable enterotoxin to its receptor is critical to the initiation of toxin-induced secretion and diarrheal disease; it is also likely, however, that this receptor binds an endogenous ligand. In order to characterize the expression of the heat-stable enterotoxin receptor in the small intestine, we isolated epithelial cells from villus tip to crypt in rat jejunum and ileum. Binding of radiolabeled toxin was maximal in the villus preparations and gradually decreased along the villus-to-crypt axis, paralleling the decline of sucrase activity. Northern blots of total RNA identified a single heat stable enterotoxin receptor transcript (3.8 kb), predominantly in the villus cell fractions. *In situ* hybridization demonstrated clear signal in the villus cells with no apparent signal in the crypt cells, lamina propria or muscularis. Expression of this receptor was greatest after enterocytes leave the proliferative cycle and enter villi. This pattern of gene and protein expression may reflect a role of this receptor in binding endogenous ligands which in turn may regulate intestinal ion flux along the villus-to-crypt axis.

Enterotoxigenic Escherichia coli elaborate heat labile and/or heat-stable toxins (ST_a) which are both potent intestinal secretagogues. ST_a binds to receptors on intestinal epithelial cells and activates guanylate cyclase, resulting in an increased intracellular concentration of cyclic GMP

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Abbreviations used: Escherichia coli heat-stable enterotoxin=ST_a; Escherichia coli heat stable enterotoxin receptor=ST_aR.

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(1). These events initiate a cascade that leads to intestinal electrolyte and fluid secretion (2). Recently, an intestinal receptor for Escherichia coli heat-stable enterotoxin (ST_aR) was cloned and shown to be a novel transmembrane guanylate cyclase with an extracellular toxin binding domain (3). It seems unlikely that the sole function of this receptor would be to mediate the effects of a bacterial toxin. We therefore postulate that there is an endogenous function for this intestinal receptor separate from its role in mediating diarrheal disease.

[^{125}I]ST_a has previously been shown to bind to rat enterocytes and brush border membranes prepared from both jejunum and ileum, indicating ST_aR expression along the longitudinal axis of the small intestine (1,4). Less is known about ST_aR expression along the crypt/villus unit or vertical axis of the small intestine. Crypt cells are thought to be the site of intestinal secretion. Evidence for this comes from the observations that: 1) net fluid and electrolyte secretion occurs in experimental situations and in disease states where villus cells are lost and crypt cells remain (5-7) and 2) crypt but not villus cells have been shown to secrete chloride (8-10). Proliferating crypt cells which are poorly exposed to luminal contents, continuously migrate up the vertical axis (11,12). When enterocytes reach the base of the villus, proliferation ceases and a new pattern of expression of digestive enzymes (disaccharidases, peptidases and alkaline phosphatase) begins (13). These villus cells, which are exposed to the luminal contents of the intestine, are therefore thought to have a primarily absorptive function.

To better understand the nature of ST_a-mediated intestinal secretion and the possible site of action of an endogenous ligand for this receptor, we determined whether the ST_aR was expressed equally in both mature and proliferating enterocytes, whether the ST_aR was significantly expressed in the lamina propria or muscularis of the intestine and whether the pattern of ST_aR expression was similar in jejunum and ileum.

Methods

PCR Amplification of Rat Guanylyl Cyclase C (GC-C) and Preparation of cDNA Probe:

A 2 μg aliquot of total RNA (14) isolated from adult rat jejunum, was used in a reverse transcription reaction containing 20 pmoles of an antisense primer (5'-GCTCCGATCCGTTCTTGTAA-3') from the rat GC-C sequence (3) and Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). The resulting cDNA was used as a template for a polymerase chain reaction in the presence of an antisense and sense primer (5'-AACCCACGCTGATGTTCTGG-3') and Amplitaq Polymerase (Perkin Elmer Cetus). The product of this reaction, a 548 bp fragment (nucleotides 54-602) of the rat GC-C cDNA (3), was gel purified and cloned into Bluescript (Stratagene) by blunt ligation. The identity of the recombinant clone (p5'GC-C) was confirmed by sequencing using the dideoxy chain termination method (15).

Epithelial Cell Isolation, RNA Extraction, [^{125}I]ST_a Binding, Northern and Slot Blot Analyses:

Sprague-Dawley rats weighing 175-200g were allowed access to standard food and water. Animals were sacrificed by injection with sodium pentobarbital; 20 cm of mid-jejunum and mid-

ileum were excised, flushed with saline containing 1mM dithiothreitol and 5% fetal calf serum in Joklik's Minimal Essential Media as a mucolytic. Intestinal epithelial cells were then isolated with six sequential 10 min incubations using the method described by Weiser (16). Sections of jejunum and ileum were removed after sequential incubations for histological validation.

At the end of each incubation, loops were emptied and flushed to obtain the sequential fractions. Each fraction was divided into two aliquots and centrifuged at 800 x *g* for 5 mins. One aliquot was homogenized in phosphate buffered saline (PBS, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 145 mM NaCl, 4 mM KCl, pH 7.2). Protease inhibitors (leupeptin 10 μg/ml, soybean trypsin inhibitor 0.01%) were added and the aliquot was frozen at -80° for determination of [^{125}I]ST_a binding (4) and sucrase activity (17) as previously described. Total RNA was isolated from the other aliquot by the guanidine isothiocyanate method (14) for use in Northern and slot blot analyses.

For Northern blots, total RNA (20μg) was denatured with glyoxal, fractionated on a 1% agarose gel, and transferred to a nylon membrane by capillary action. For slot blot analyses, 10μg of total RNA was denatured with formaldehyde and bound to a nylon membrane using the Minifold II Slot-Blotter (Schleicher and Schuell). For both analyses, RNA was crosslinked to the membranes with short wave UV light. The p5'GC-C insert was radiolabeled with [α-^{32}P]CTP by random primer DNA synthesis (18), hybridized under stringent conditions (19), and the blots washed with 0.1X SSC, 0.5% SDS at 65°C. Autoradiography was performed with XAR-5 film (Eastman Kodak) using Cronex Lightening Plus intensifying screens (New England Nuclear) at -80°C. Signal intensities were quantitated with an LKB laser densitometer after normalization with the signal obtained using an oligonucleotide probe complementary to 18S ribosomal RNA (20).

In Situ Hybridization:

Sense and antisense riboprobes labeled with [^{35}S]UTP were prepared from a linearized p5'GC-C template in the presence of T7 and T3 RNA polymerase by Lofstrand Laboratories (Gaithersburg, Md). The riboprobes were ethanol precipitated and used in *in situ* hybridization experiments with 12μ sections of fixed rat mid-jejunum and mid-ileum as previously described (21). Slides were developed 1-3 weeks later according to the method of Angerer et. al. (22). Verification on Northern blots of total RNA confirmed the presence of a single transcipt of the correct size (data not shown) with the antisense probe; no signal was seen with the sense probe.

Results

[^{125}I]ST_a Binding and ST_aR mRNA Expression in Isolated Intestinal Epithelial Cells:

Subpopulations of enterocytes were sequentially removed along the villus-to-crypt axis. In order to characterize the populations of cells removed, 1-cm segments were removed from tissue pieces before the first and after the second, fourth and sixth (last) incubations. Histologic examination of these sections demonstrated progressive loss of epithelial cells with loss of villus cells by fraction four and complete loss of crypt epithelium by the sixth incubation (data not shown). Additionally, as shown in Figure 1, sucrase activity, a measure of enterocyte differentiation, progressively declined in jejunum and ileum from villus to crypt.

As shown in Figure 2, binding of [^{125}I]ST_a to ST_aR was maximal in the villus preparations and gradually decreased along the villus-to-crypt axis, paralleling the decline of sucrase activity. Northern blots of total RNA isolated from sequential cell fractions identified

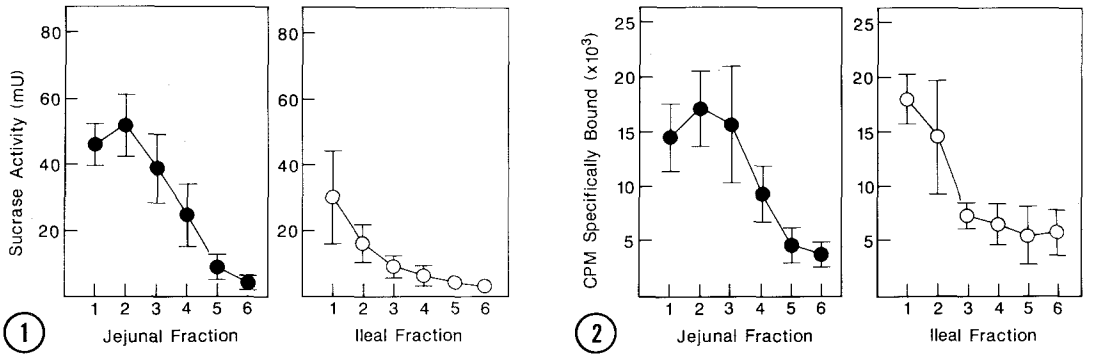

Figure 1. Sucrase activity (mU/mg protein) of membranes prepared from intestinal epithelial cells from villus to crypt (fraction 1 to 6) as described in Methods. Histologic correlation of sequential cell fractions demonstrated denuded villi with crypt cells remaining by fraction 4 and complete loss of crypt cells by fraction 6 (data not shown). Data are mean ± SE of 3 determinations.

Figure 2. [^{125}I]ST_a specific binding activity (counts per minute specifically bound/100 μg membrane protein). Membranes were prepared from intestinal epithelial cells from villus to crypt (fraction 1 to 6) as described in Methods. See legend Figure 1. Data are mean ± SE of 3 determinations.

a single ST_aR species (3.8kb) when hybridized with the 5'GC-C probe (Figure 3). Signal was present in all fractions but was more pronounced in the villus cell fractions, comparable to the pattern of [^{125}I]ST_a binding (Figure 2). More sensitive and quantitative slot blot analyses

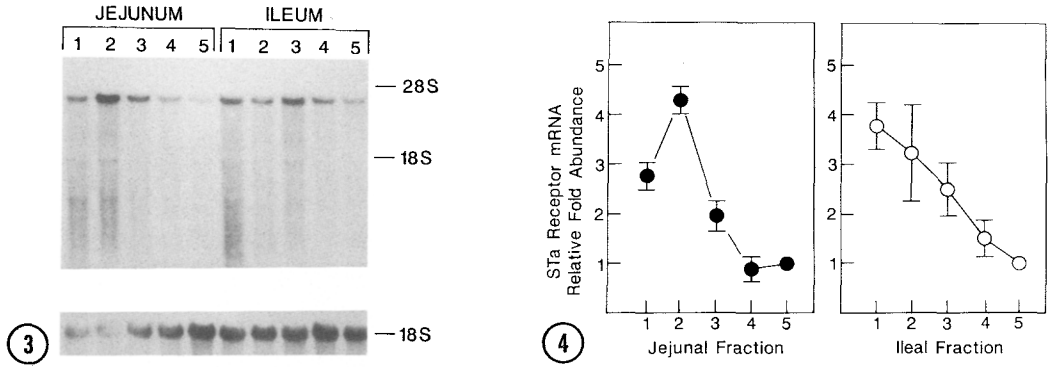

Figure 3. Representative Northern analysis of ST_aR expression. Total RNA from intestinal epithelial cells sequentially eluted from villus to crypt (fraction 1 to 5) was hybridized with radiolabeled 5'GC-C. Migration of 28S and 18S ribosomal RNA is shown for comparison. Hybridization of fraction 6, not shown on this blot, gave similar results to fraction 5 in other analyses. Hybridization with an 18S rRNA probe served as a control (bottom).

Figure 4. Slot blot analysis of ST_aR mRNA expression in rat intestinal epithelial cells sequentially eluted from villus to crypt (fraction 1 to 5). Hybridization intensities were quantitated by densitometry and the values obtained were normalized based on 18S ribosomal RNA content as described in Methods. A value of 1 was arbitrarily assigned to the level of ST_aR mRNA in the crypt cell preparations. Data are mean ± SE of at least 3 densitometric readings from separate blots.

demonstrated a 4.3-fold jejunal and a 3.8-fold ileal villus-to-crypt gradient in relative ST_aR mRNA expression (Figure 4). The maximal absolute signal intensity was also similar in villus cells from jejunum and ileum.

In situ Hybridization:

In situ hybridization of rat jejunal and ileal sections with an antisense RNA probe prepared from the 5'GC-C template demonstrated that autoradiographic grains were concentrated only over villus epithelial cells (Figure 5A and 5B). The greatest signal intensity appeared to be at the mid-villus level. Autoradiographic grain density was similar to background in crypt cells, lamina propria and muscularis. As a control, only background signal was appreciable using a sense probe prepared from the 5'GC-C template (Figure 5C).

Discussion

We have shown that ST_aR expression in the small intestine is much greater after enterocytes leave the proliferative cycle and migrate onto the villi. In both jejunum and ileum, the pattern of ST_aR mRNA expression and [^{125}I]ST_a binding followed the pattern of increasing enterocyte differentiation as measured by sucrase activity. Additionally, there was approximately four-fold greater ST_aR mRNA expression and four-fold greater [^{125}I]ST_a binding in predominantly villus cell fractions compared to crypt cell fractions in both jejunum and ileum. This close correlation between ST_aR mRNA expression and [^{125}I]ST_a binding activity is consistent with the interpretation that steady-state mRNA expression is an important determinant of ST_aR gene expression along this villus-to-crypt axis.

The *in situ* hybridization experiments demonstrated a transition from no signal to clear signal at the villus-crypt junction; there was no signal above background in the lamina propria or the muscularis layers. Although small populations of cells expressing ST_aR might not be identified by *in situ* hybridization, our interpretation of these results is that ST_aR gene expression is primarily, if not exclusively, limited to enterocytes in the small intestine. This is in contrast to the expression of atrial natriuretic peptide receptor. This receptor, which is also a transmembrane guanylate cyclase, appears to be expressed in the lamina propria of the intestine and does not appear to be under the influence of a luminally active ligand (23-26).

The observation of significantly lower, albeit present, [^{125}I]ST_a binding and ST_aR mRNA by Northern and slot blot analyses in crypt cell fractions could represent declining but present ST_aR in crypt cells. This is in contrast to our finding of no signal by *in situ* hybridization. Since the sequential elution technique does not totally separate crypt and villus cell populations (27), the [^{125}I]ST_a binding and ST_aR mRNA expression observed in the predominantly crypt cell fractions could be associated with the small amounts of villus cells contained in these fractions.

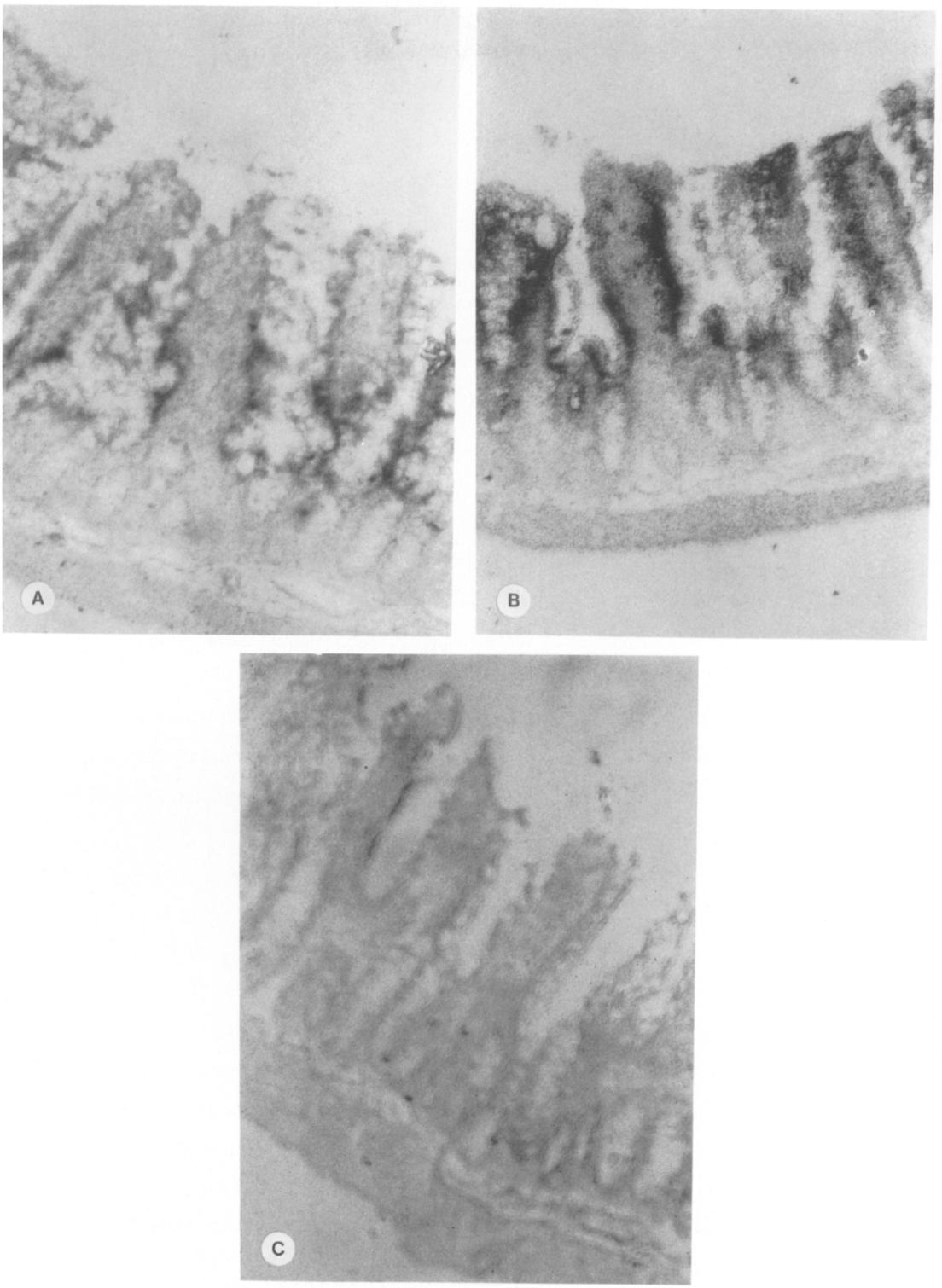

Figure 5. Bright field photomicrographs (30X) of *in situ* hybridization of rat jejunum (A) and ileum (B) with [^{35}S]labeled antisense riboprobe synthesized from the 5′GC-C template and rat ileum (C) hybridized with [^{35}S]labeled sense riboprobe synthesized from the complementary strand of 5′GC-C.

However, in either case, a consistent finding in our studies is the relatively smaller amount of ST_aR in crypt versus villus cells. The ST_a-enterocyte interaction has been shown to result in chloride secretion (2,28,29) which is thought to be primarily a crypt cell function (7-9). To reconcile these observations, we suggest that: 1) mid-villus cells, containing a high proportion of the ST_aR, are capable of significant ST_a-induced chloride secretion, and/or 2) there is communication between villus and crypt cells by an undefined second signal pathway, which leads to crypt cell chloride secretion in response to ST_a binding to villus cell ST_aR, and/or 3) there is a second, as yet undescribed ST_aR receptor, that is linked to secretory pathways and expressed in crypt cells. However, alterations in intestinal ion transport are produced by ST_a independent of the effect of the toxin on small intestinal chloride secretion. Inhibition of sodium and chloride absorption in response to ST_a (2,28), is also an important mechanism in toxin-mediated diarrheal disease; this effect is produced in villus cells (30). Furthermore, there may be a significant role for secretion and impaired absorption in the colon in response to ST_a (31,32).

Binding of ST_a to this receptor is critical to the initiation of toxin-induced secretion. However, the primary role of the ST_aR may be to serve as a receptor for an endogenous ligand rather than as a toxin receptor. Guanylin, a 15 amino acid peptide with approximately 50% homology to ST_a, has recently been identified in rat jejunum as the first putative endogenous ligand for the ST_aR (33). At present, the site of guanylin expression within the intestine is unknown. Based on the levels of ST_aR expression along the villus-to-crypt axis and previous evidence that the ST_aR is localized to the brush border membrane of the enterocyte (34), we suggest that the endogenous receptor-ligand interaction occurs on mature enterocytes and is influenced by luminal contents in both jejunum and ileum.

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