

## Design of a photoreactive analogue of the *Escherichia coli* heat-stable enterotoxin STIb: Use in identifying its receptor on rat brush border membranes

(photoaffinity labeling/synthetic peptide)

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**ABSTRACT** The *Escherichia coli* heat-stable enterotoxin, STIb was prepared by solid-phase peptide synthesis and purified to homogeneity by high-pressure liquid chromatography. This analogue was iodinated and shown to bind specifically to rat intestinal membranes. The radiolabeled peptide was derivatized at the amino terminus with the photoreactive heterobifunctional crosslinking agent *N*-hydroxysuccinimidyl *p*-benzoylbenzoate. This photoreactive probe also exhibited binding specificity. It was mixed with rat intestinal brush border membranes and photolyzed in the presence or absence of excess unlabeled STIb. Polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulfate and 2-mercaptoethanol indicated that the peptide probe was cross-linked specifically to two molecular species of 57 and 75 kDa. One or both of these molecules appear to constitute the enterotoxin receptor or to be in close proximity to it.

Diarrhea is a life-threatening dehydrating illness of malnourished children in developing countries. Enterotoxigenic *Escherichia coli* strains are a major cause of diarrhea and elaborate at least three classes of enterotoxins that differ widely in their mechanism of action. One of these toxins, abbreviated LT, is a heat-labile cholera-like toxin that activates intestinal adenylate cyclase through the ADP-ribosylation of one of the cyclase regulatory subunits (1). The two remaining classes of enterotoxins are nonhomologous low molecular weight peptides termed STI (or ST<sub>1</sub>) and STII (or ST<sub>2</sub>) (2-6). STI activity is assayed in suckling mice (7), whereas STII activity is monitored in weaned-pig intestinal loops (8, 9). The mode of action of STII is unknown (10). STI activates a membrane-bound intestinal guanylate cyclase (11-15). The subsequent elevation of intracellular cGMP leads to a profound secretory diarrhea. STI enterotoxins from human, bovine, and porcine enterotoxigenic *E. coli* strains have been purified and sequenced (4-6, 16-18). Although their N-terminal amino acid sequences are heterogeneous, the enterotoxic domain is confined to a highly conserved C-terminal segment of 13 amino acids (ref. 19; unpublished results). Recently, a detergent-solubilized brush border protein has been reported to bind radiolabeled STI (20). We have synthesized a 19-amino acid enterotoxic analogue of STI, termed STIb (5, 6) (see Fig. 2) and report in this communication the design of a photoreactive radiolabeled derivative of this analogue that was used to specifically crosslink two rat intestinal brush border membrane molecules that may constitute the STI toxin receptor.

### EXPERIMENTAL PROCEDURES

**Materials.** All chemicals and solvents were reagent grade unless otherwise stated. All protected amino acids and resins

were purchased from Peninsula Laboratories (Belmont, CA). HPLC-grade CH<sub>3</sub>CN was obtained from Baker, while dioxane and CF<sub>3</sub>COOH were purchased from Aldrich. Na<sup>125</sup>I was obtained from New England Nuclear. Bovine gamma globulin and polyethylene glycol (*M<sub>r</sub>* 8000) were purchased from Sigma. *N*-hydroxysuccinimidyl *p*-benzoylbenzoate was a gift from Robert Parker and Robert Hodges (Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada).

**Synthesis of STIb.** STIb was synthesized by conventional solid-phase peptide synthesis (21) on a Beckman 990B peptide synthesizer.

**Purification of STIb.** The peptide was air-oxidized (22), lyophilized, and purified by reverse-phase chromatography on an Altex Ultrasphere ODS semipreparative (10-mm i.d. × 250 mm) C<sub>18</sub> column (Altex, Berkeley, CA). The peptide peaks were well resolved by a 30-min, 10-30% linear gradient of CH<sub>3</sub>CN in water containing 0.1% (vol/vol) CF<sub>3</sub>COOH. The enterotoxigenic peak [by suckling mouse assay (7, 23)] was rechromatographed on an analytical Hibar LiChrosorb RP-18 reverse-phase (4-mm i.d. × 250 mm) C<sub>18</sub> column (Merck). The peptide was eluted as a sharp peak by using a 10-40% linear gradient of CH<sub>3</sub>CN in water containing 0.1% CF<sub>3</sub>COOH for 30 min. Less than 5 ng of purified STIb produced diarrhea in infant mice (result not shown). The amino acid composition of the peptide was confirmed by amino acid analysis.

**Radiolabeling of STIb.** One microgram of STIb was iodinated enzymatically (Enzymobeads, Bio-Rad) in the presence of 1 mCi (1 Ci = 37 GBq) of Na<sup>125</sup>I and D-glucose. The radiolabeled peptide (<sup>125</sup>I-STIb) was rapidly separated from the unincorporated iodide on a Sep-Pak C<sub>18</sub> cartridge (Waters Associates) by eluting the peptide with 40% CH<sub>3</sub>CN in water (24). <sup>125</sup>I-STIb was concentrated *in vacuo*, and the specific activity was determined to be 300-500 Ci/mmol (130-220 μCi/μg). The receptor binding properties of <sup>125</sup>I-STIb peptide were stable for 10 days.

**Coupling of Crosslinking Agent to Radiolabeled STIb.** A 100-ng sample of <sup>125</sup>I-STIb was dissolved in 50 μl of 0.5 M NaHCO<sub>3</sub> (pH 7.5) and mixed with 100 μl of a dioxane solution of *N*-hydroxysuccinimidyl *p*-benzoylbenzoate (NHS-BB; 1 mg/ml). The resulting solution was stirred at room temperature for 18 hr. A 50-μl aliquot of 0.2 M glycine (pH 8.0) was then added to the reaction vial to block the excess unreacted NHS-BB, and the reactants were mixed for 5 hr at room temperature. The photoprobe solution (<sup>125</sup>I-STIb-BB) was then diluted in phosphate-buffered saline (10 mM sodium

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Abbreviations: STIb, a 19-amino-acid heat-stable enterotoxin isolated from a human enterotoxigenic *E. coli* strain; <sup>125</sup>I-STIb-BB, radiolabeled STIb derivatized at its amino terminus with a benzoylbenzoate photoreactive group; STIb-(6-18), synthetic analogue of STIb comprising residues 6-18.

phosphate/0.15 M NaCl, pH 7.2; PBS) and was used immediately for the crosslinking experiment.

**Crosslinking of  $^{125}\text{I}$ -STIb-BB to Brush Border Membranes.** Proper precautions were taken to avoid any exposure of the samples to light before the photolysis step. Typically, 40- $\mu\text{l}$  aliquots of brush border membranes in PBS (2.7 mg of protein per ml) were mixed with either 20  $\mu\text{l}$  of PBS or 20  $\mu\text{l}$  of unlabeled STIb in PBS (2  $\mu\text{g}$  of STIb). A 20- $\mu\text{l}$  aliquot of either  $^{125}\text{I}$ -STIb-BB (100,000 cpm) or  $^{125}\text{I}$ -STIb (100,000 cpm) diluted in PBS was added to each membrane sample, and the reactants were incubated at 37°C for 30 min to allow the binding of the radiolabeled STI analogues to the brush border membranes. Photolysis was performed at 4°C for 45 min by using a UVGL-25 Mineralight lamp (Ultraviolet Products, San Gabriel, CA) set at 366 nm, with the samples located 1 cm from the lamp.

**Gel Electrophoresis of the Photolyzed Membranes.** Gel electrophoresis was performed as described by Laemmli (25). The photolyzed samples were immediately solubilized under reducing conditions by adding 40  $\mu\text{l}$  of a sample boiling solution (62.5 mM Tris/4 mM EDTA/10% glycerol/2% NaDodSO<sub>4</sub>, pH 6.8) and 10  $\mu\text{l}$  of 2-mercaptoethanol to each tube, boiled for 10 min, and loaded on a 15% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue to visualize the molecular weight standards and was submitted to radioautography on XAR-5 film (Eastman, Rochester, NY).

**Preparation of Intestinal Membranes.** Intestinal villus cells were isolated by using a modification of the method described by Weiser (26). All buffers and solutions contained 1 mM phenyl methylsulfonyl fluoride and 1  $\mu\text{M}$  pepstatin. Six Sprague-Dawley rats were sacrificed, and their small intestines were excised and flushed rapidly with cold saline containing 1 mM dithiothreitol. The epithelial cells were dissociated from the lining of the intestine by incubating it in citrate buffer (27 mM sodium citrate/1.5 mM KCl/96 mM NaCl/8 mM KH<sub>2</sub>PO<sub>4</sub>/5.6 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.3) for 20 min at 4°C. The intestines were flushed, filled with PBS containing 1.5 mM EDTA and 0.5 mM dithiothreitol, and incubated at 4°C for 10 min, and the cells were dispensed into plastic conical tubes. This step was repeated four times, and the cells were pooled, collected by centrifugation, washed twice in PBS containing 1.5 mM EDTA, and counted in a hemacytometer. Usually, 1–2  $\times 10^9$  cells are recovered from six intestines by this procedure. Intestinal membranes were prepared from these cells by the method of Ahnen *et al.* (27). The cells were suspended in 20 ml of 20 mM Tris/5 mM EDTA, pH 7.0 and disrupted in a Dounce homogenizer (10

strokes, type B pestle). The homogenate was then centrifuged at 55,000  $\times g$  for 20 min to remove cytosolic components. The pellet was suspended in 20 ml of 20 mM Tris/250 mM sucrose/1 mM EDTA/12.5 mM NaCl, pH 7.0 and rehomogenized (Dounce; 15 strokes; type B pestle). The homogenate was centrifuged at 1400  $\times g$  for 10 min to produce a microsomal supernatant (S<sub>1</sub>) and a partially purified brush border pellet (P<sub>1</sub>). The P<sub>1</sub> pellet was resuspended in 15 ml of 10 mM Tris/50 mM mannitol, pH 7.0 and was rehomogenized with two 30-sec bursts of a Polytron homogenizer (Brinkmann) set at position 4. Calcium chloride (100 mM in the above buffer) was then added to the homogenate to a final concentration of 10 mM. The mixture was stirred for 3 hr at 4°C and centrifuged at 2000  $\times g$  for 10 min to remove the aggregated non-brush-border membranes. The resulting supernatant was then centrifuged at 20,000  $\times g$  for 20 min to pellet the brush border membranes. Samples of P<sub>1</sub> and brush border membranes were resuspended in PBS and kept frozen at –20°C until needed.

**Intestinal Membrane Binding Assay.** The intestinal membrane binding assay was performed by the method of Kuno *et al.* (28). Briefly, suspensions of intestinal membranes (P<sub>1</sub>) in PBS (20  $\mu\text{l}$ ; 5–10 mg of protein per ml) were dispensed into 12  $\times$  75 mm polypropylene tubes. In order to study the kinetics of membrane binding by  $^{125}\text{I}$ -STIb, either 40  $\mu\text{l}$  of PBS or 40  $\mu\text{l}$  of unlabeled STIb in PBS (1  $\mu\text{g}$ ) was added to each sample, followed by 20  $\mu\text{l}$  of  $^{125}\text{I}$ -STIb (100,000 cpm). The tubes were incubated at 37°C for various time intervals and then placed on ice. A 300- $\mu\text{l}$  volume of PBS containing 1% (wt/vol) bovine gamma globulin and 20% (wt/vol) polyethylene glycol in PBS was immediately added to each tube. The samples were mixed and left on ice for 10 min. The membranes in each sample were recovered on Whatman GF/B glass filters (2.4 cm) and assayed for radioactivity. Each data point represents the average of experiments performed in triplicate. The capacity of  $^{125}\text{I}$ -STIb to saturate intestinal membrane toxin receptors was assessed as described above except that the concentration of  $^{125}\text{I}$ -STIb was varied from 0 to 85 nM, and the incubation period at 37°C was held constant at 45 min. Competition experiments with  $^{125}\text{I}$ -STIb and unlabeled STIb were conducted with increasing amounts of unlabeled STIb from 0 to 1  $\mu\text{g}$ , which were added to the membrane suspensions together with 20- $\mu\text{l}$  aliquots of  $^{125}\text{I}$ -STIb (100,000 cpm). The samples were incubated at 37°C for 45 min to ensure close to 100% specific binding of  $^{125}\text{I}$ -STIb to the membranes.

**Protein Determination.** The protein content of each intes-

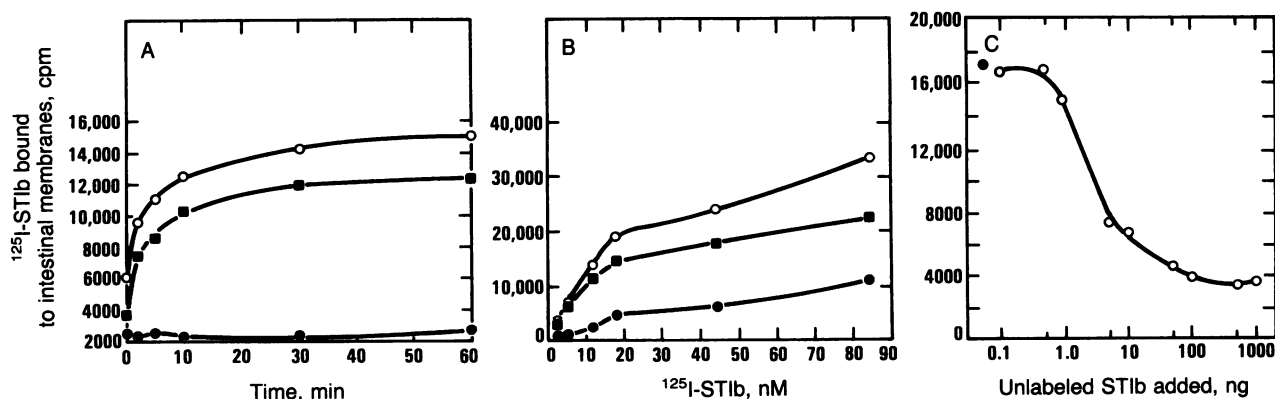


FIG. 1. Binding characteristics of  $^{125}\text{I}$ -STIb to intestinal membranes. (A) Rate of binding of  $^{125}\text{I}$ -STIb (100,000 cpm) to intestinal membranes at 37°C. Specific binding (■) represents the difference in cpm of membrane-bound radioactivity in the absence (○) and presence (●) of unlabeled STIb. (B) Saturation curve for the binding of  $^{125}\text{I}$ -STIb to intestinal membranes. The symbol nomenclature is described in A. (C) Competitive inhibition of binding of  $^{125}\text{I}$ -STIb (100,000 cpm) to intestinal membranes in the presence of increasing amounts of unlabeled STIb. ●, Amount of radioactivity bound when no unlabeled STIb was added. Each point represents an average value from experiments performed in triplicate.

tinal membrane preparation was determined by the Lowry method (29).

## RESULTS AND DISCUSSION

The initial step in the mechanism of action of STI involves a receptor binding event as inferred from binding studies of radiolabeled STI to cells and membranes (30–33). Radiolabeled native toxin associates with a brush border molecule that migrates with an apparent mass of 100-kDa when analyzed by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis under nonreducing conditions (20). Treatment of cells or intestinal membranes with Pronase and trypsin results in a reduction of toxin binding, suggesting that the receptor is a protein (20, 31). In this paper, we report the synthesis of a radiolabeled, photoreactive analogue of STI, termed <sup>125</sup>I-STIb-BB, and its use in identifying specific STI receptor molecules.

**Binding of Synthetic <sup>125</sup>I-STIb to Rat Intestinal Membranes Is Rapid, Specific, and Saturable.** A 19-amino acid analogue corresponding to the entire sequence of STIb from an enterotoxigenic *E. coli* strain of human origin was prepared by solid-phase peptide synthesis (5, 6). Less than 5 ng of the

purified peptide induced diarrhea in suckling mice (results not shown). The sequence (see Fig. 2) includes two tyrosine residues, not involved in the enterotoxicity of the peptide (ref. 19; unpublished results), that can be iodinated.

The binding of <sup>125</sup>I-STIb to intestinal membranes was examined. "Specific binding" was defined as the difference between the cpm of bound <sup>125</sup>I-STIb in the presence and absence of a 400-molar excess of unlabeled STIb. <sup>125</sup>I-STIb bound to intestinal membranes rapidly at 37°C; 50% maximal binding was achieved in 2 min (Fig. 1A). Specifically bound <sup>125</sup>I-STIb reached an asymptote at a toxin concentration of 30 nM, indicating that saturation of the toxin binding sites had occurred (Fig. 1B).

Competitive inhibition of binding by increasing amounts of unlabeled toxin from 0 to 1 μg was conducted with 100,000 cpm of <sup>125</sup>I-STIb and intestinal membranes (Fig. 1C). A linear curve of inhibition is evident between 1.0 and 10 ng of unlabeled toxin; 50% binding inhibition was produced by 2.7 ng of unlabeled STIb. STIb and STIb-(6–18), a synthetic toxin analogue comprising residues 6–18 of STIb, were equally active in competition experiments (data not shown), indicating that the receptor binding domain of STIb is encoded by a peptide as small as 13 amino acids. Unrelated

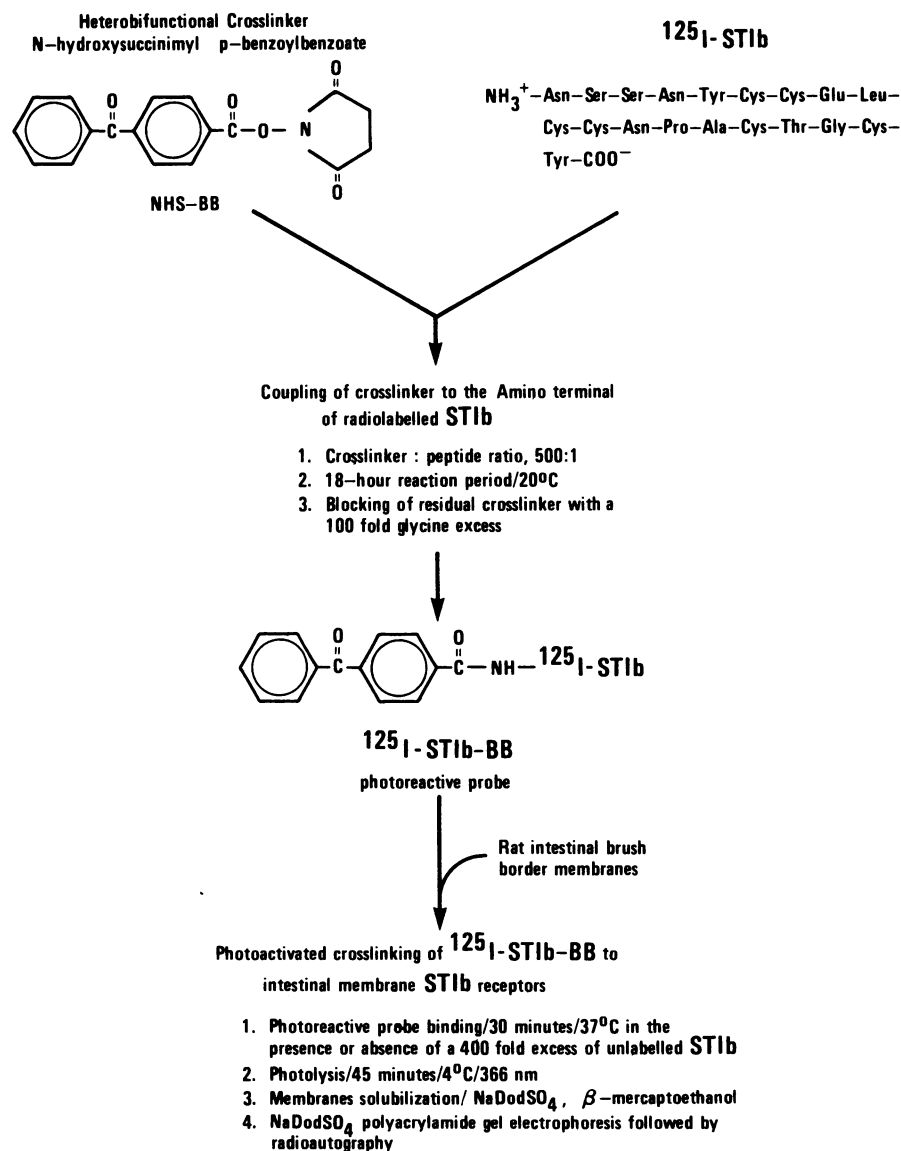


FIG. 2. Scheme summarizing the construction of the photoreactive radiolabeled analogue of STIb and its use to identify the toxin receptor on rat brush border membranes.

Table 1. Binding of  $^{125}\text{I}$ -STIb\* and  $^{125}\text{I}$ -STIb-BB†

Synthetic toxin analogue	Membrane-bound radiolabeled analogue,‡ cpm			% specifically bound
	Total bound	Nonspecifically bound	Specifically bound	
$^{125}\text{I}$ -STIb	16,826 (292)§	3523 (248)	13,303	79
$^{125}\text{I}$ -STIb-BB	15,502 (1240)	3855 (357)	11,647	75

\*Synthetic  $^{125}\text{I}$ -STIb.†Photoreactive synthetic  $^{125}\text{I}$ -STIb.‡ $^{125}\text{I}$ -STIb or  $^{125}\text{I}$ -STIb-BB (100,000 cpm) was incubated with intestinal membranes in the presence and absence of a 400-fold molar excess of unlabeled STIb. The total, nonspecifically bound and specifically bound toxin analogue was determined as described.§Values represent the radiolabeled analogue (in cpm) bound to 20  $\mu\text{l}$  of intestinal membranes (7 mg of protein per ml)  $\pm$  the standard deviation (in parentheses) from experiments performed in triplicate.

proteins such as ovalbumin (50  $\mu\text{g}$ ) were tested as negative controls and did not cause binding inhibition, providing additional evidence that the interaction of synthetic STIb and STIb-(6–18) with rat intestinal membranes is specific. If the intestinal membranes and  $^{125}\text{I}$ -STIb were incubated for 1 hr at 37°C prior to the addition of a 400-molar excess of unlabeled STIb, only half of the specifically bound radioactivity was eluted from the membranes. Thus, the binding of  $^{125}\text{I}$ -STIb to its receptor may not be entirely reversible once the toxin is receptor bound, suggesting that a conformational or covalent modification of the receptor-peptide complex occurs. A mixed disulfide reaction between toxin and receptor has been proposed (34).

**Preparation and Binding Properties of a Photoreactive, Labeled Analogue of STIb.** The N-terminal amino group of  $^{125}\text{I}$ -STIb was derivatized with the heterobifunctional cross-linking agent *N*-hydroxysuccinimidyl *p*-benzoylbenzoate. The structure of the photoreactive analogue,  $^{125}\text{I}$ -STIb-BB, is depicted in Fig. 2. The binding of  $^{125}\text{I}$ -STIb-BB and  $^{125}\text{I}$ -STIb to rat intestinal membranes was compared (Table 1) and found to be similar; 75% and 79% were specifically bound respectively. This result was expected since this N-terminal modification of this 19-amino acid analogue is 5 amino acids away from the C-terminal toxic domain (residues 6–18). In addition, the specificity of the  $^{125}\text{I}$ -STIb-BB photoprobe for the STIb receptor was confirmed by the NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis experiment described below in which the receptor labeling by the photoprobe is completely abolished by the addition of unlabeled STIb to the brush border membranes prior to photolysis (Fig. 3, lane 3).

**$^{125}\text{I}$ -STIb-BB Specifically Labels Two Intestinal Brush Border Membrane Molecules.**  $^{125}\text{I}$ -STIb-BB and brush border membranes were incubated in the presence or absence of excess unlabeled STIb and subjected to photolysis. The reactants were heated in the presence of NaDodSO<sub>4</sub> and 2-mercaptoethanol, and the membrane moieties bound by the photoprobe were identified by polyacrylamide gel electrophoresis and radioautography (Fig. 3). Two major molecular species of approximately 57 and 75 kDa were specifically labeled (Fig. 3, lane 1). Complete inhibition of binding occurred in the presence of excess unlabeled STIb, substantiating the specificity of the reaction (Fig. 3, lane 3). In contrast, no major component was labeled by photolysis when  $^{125}\text{I}$ -STIb was substituted for  $^{125}\text{I}$ -STIb-BB, indicating that the toxin-receptor complex is dissociated by the conditions under which NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis is ordinarily conducted (Fig. 3, lanes 2 and 4).

We have observed that radiolabeled STIb specifically binds a molecular species of about 140-kDa when a detergent-solubilized extract of these intestinal membranes is used and the STIb-receptor complex is analyzed by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol and sample boiling (results not shown). Although this latter result differs from the observation of Dreyfus and

Robertson (20), the accuracy of molecular weight estimates by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis is dependent on achieving a complete denaturation of individual proteins in the presence of NaDodSO<sub>4</sub> (35, 36), which may not occur under nonreducing conditions. Three explanations may account for the specific labeling of at least two molecular species: (i) the STIb receptor may constitute only one of the bound molecules but may be in close proximity or associated with another membrane molecule; (ii) the actual toxin binding site may be formed by closely associated regions contributed by each of these molecules; and (iii) the 75-kDa species may be partially degraded during the preparation of intestinal membranes to a 57-kDa species. Sample proteolysis was mitigated by the use of protease inhibitors (EDTA, phenylmethylsulfonyl fluoride, and pepstatin) during the isolation and storage of intestinal membranes. In support of the first two possibilities, the putative receptor complex has

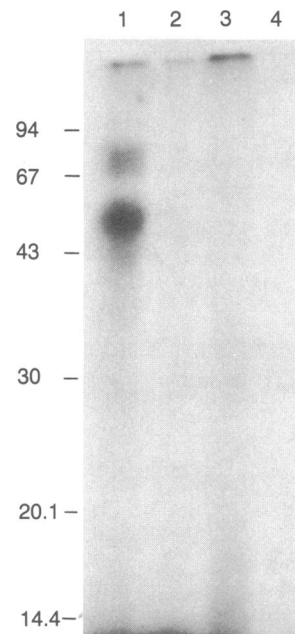


FIG. 3. Radioautogram of a 15% polyacrylamide gel depicting the specific binding of  $^{125}\text{I}$ -STIb-BB to two rat intestinal membrane molecular species. Brush border membrane samples were incubated at 37°C in the presence of STIb analogues, photolyzed, treated with NaDodSO<sub>4</sub> and 2-mercaptoethanol, and then boiled prior to the electrophoresis step. The addition of  $^{125}\text{I}$ -STIb-BB in the absence (lane 1) and presence (lane 3) of a 400-molar excess of unlabeled STIb indicates that a 57-kDa and a 75-kDa membrane component are crosslinked specifically by the photoreactive analogue. In contrast, the addition of  $^{125}\text{I}$ -STIb in the absence (lane 2) and presence (lane 4) of unlabeled STIb resulted in no labeling of brush border molecules. Sizes are shown in kDa.

been solubilized and partially purified by Kuno *et al.* (30) with 0.1% LUBROL-PX. This preparation retains its ability to bind radiolabeled STI and, when crosslinked with disuccinimidyl suberate and dissociated in the presence of NaDodSO<sub>4</sub> and 2-mercaptoethanol, generates an electrophoretic pattern similar to Fig. 3. The broad band at 75 kDa (Fig. 3) could be resolved into two bands of about 68 and 80 kDa.

In summary, we have synthesized an analogue of the *E. coli* enterotoxin STI that is toxigenic and can be radiolabeled and used in an intestinal membrane receptor binding assay. The analogue was coupled to a heterobifunctional photoactivated crosslinking agent. The photoreactive probe bound two molecular species that appear to constitute the STI receptor or are in close spatial proximity to it on rat brush border membranes. We are now purifying these molecules to further understand the mechanism of action of STI.

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1. Gill, D. M. & Richardson, S. H. (1980) *J. Infect. Dis.* **141**, 64–70.
2. Picken, R. N., Mazaitis, A. J., Maas, W. K., Rey, M. & Heyneker, H. (1983) *Infect. Immun.* **42**, 269–275.
3. Lee, C. H., Moseley, S. L., Moon, H. W., Whipp, S. C., Gyles, C. L. & So, M. (1983) *Infect. Immun.* **42**, 264–268.
4. So, M. & McCarthy, B. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4011–4015.
5. Moseley, S. L., Hardy, J. W., Imdadul Huq, M., Echeverria, P. & Falkow, S. (1983) *Infect. Immun.* **39**, 1167–1174.
6. Aimoto, S., Takao, T., Shimonishi, Y., Hara, S., Takeda, T., Takeda, Y. & Miwatani, T. (1982) *Eur. J. Biochem.* **129**, 257–263.
7. Dean, A. G., Ching, Y. C., Williams, R. G. & Harden, L. B. (1972) *J. Infect. Dis.* **125**, 407–411.
8. Burgess, M. N., Bywater, R. J., Cowley, C. M., Mullan, N. A. & Newsome, P. M. (1978) *Infect. Immun.* **21**, 526–531.
9. Gyles, C. L. (1979) *Can. J. Comp. Med.* **43**, 371–379.
10. Kennedy, D. J., Greenberg, R. N., Dunn, J. A., Abernathy, R., Ryerse, J. S. & Guerrant, R. L. (1984) *Infect. Immun.* **46**, 639–643.
11. Field, M., Graf, L. H., Jr., Laird, W. J. & Smith, P. L. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2800–2804.
12. Hughes, J. M., Murad, F., Chang, B. & Guerrant, R. L. (1978) *Nature (London)* **271**, 755–756.
13. Giannella, R. A. & Drake, K. W. (1979) *Infect. Immun.* **24**, 19–23.
14. Guerrant, R. L., Hughes, J. M., Chang, B., Robertson, D. C. & Murad, F. (1980) *J. Infect. Dis.* **142**, 220–228.
15. Rao, M. C., Guandalini, S., Smith, P. L. & Field, M. (1980) *Biochim. Biophys. Acta* **632**, 35–46.
16. Lallier, R., Bernard, F., Gendreau, M., Lazure, C., Seidah, N. G., Chrétien, M. & St-Pierre, S. A. (1982) *Anal. Biochem.* **127**, 267–275.
17. Ronnberg, G., Wadstrom, T. & Jornvall, H. (1983) *FEBS Lett.* **155**, 183–186.
18. Thompson, M. R. & Giannella, R. A. (1985) *Infect. Immun.* **47**, 834–836.
19. Yoshimura, S., Ikemura, H., Wanatabe, H., Aimoto, S., Shimonishi, Y., Saburo, H., Takeda, T., Miwatani, T. & Takeda, Y. (1985) *FEBS Lett.* **181**, 138–142.
20. Dreyfus, L. A. & Robertson, D. C. (1984) *Infect. Immun.* **46**, 537–543.
21. Erickson, B. W. & Merrifield, R. B. (1976) in *The Proteins*, eds. Neurath, H. & Hill, R. L. (Academic, New York), Vol. 2, pp. 255–527.
22. Gariépy, J., O'Hanley, P., Waldman, S. A., Murad, F. & Schoolnik, G. K. (1984) *J. Exp. Med.* **160**, 1253–1258.
23. Waldman, S. A., O'Hanley, P., Falkow, S., Schoolnik, G. K. & Murad, F. (1984) *J. Infect. Dis.* **149**, 83–89.
24. Miller, J. J., Schultz, G. S. & Levy, R. S. (1984) *Int. J. Pept. Protein Res.* **24**, 112–122.
25. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
26. Weiser, M. M. (1973) *J. Biol. Chem.* **248**, 2536–2541.
27. Ahnen, D. J., Santiago, N. A., Cezard, J.-P. & Gray, G. M. (1982) *J. Biol. Chem.* **257**, 12129–12135.
28. Kuno, T., Saijoh, K. & Tanaka, C. (1983) *J. Neurochem.* **41**, 841–847.
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
30. Kuno, T., Kamisaki, Y., Waldman, S. A., Gariépy, J., Schoolnik, G. & Murad, F. (1986) *J. Biol. Chem.*, in press.
31. Thomas, D. D. & Knoop, F. C. (1983) *J. Infect. Dis.* **147**, 450–459.
32. Giannella, R. A., Luttrell, M. & Thompson, M. (1983) *Am. J. Physiol.* **245**, G492–G498.
33. Frantz, J. C., Jaso-Friedmann, L. & Robertson, D. C. (1984) *Infect. Immun.* **43**, 622–630.
34. Dreyfus, L. A., Jaso-Friedmann, L. & Robertson, D. C. (1984) *Infect. Immun.* **44**, 493–501.
35. Shapiro, A. L., Vinuela, E. & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815–820.
36. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.