

Rabbit Sucrase-Isomaltase Contains a Functional Intestinal Receptor for *Clostridium difficile* Toxin A

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Abstract

The intestinal effects of *Clostridium difficile* toxin A are initiated by toxin binding to luminal enterocyte receptors. We reported previously that the rabbit ileal brush border (BB) receptor is a glycoprotein with an α -D-galactose containing trisaccharide in the toxin-binding domain (1991. *J. Clin. Invest.* 88:119–125). In this study we characterized the rabbit ileal BB receptor for this toxin. Purified toxin receptor peptides of 19 and 24 amino acids showed 100% homology with rabbit sucrase-isomaltase (SI). Guinea pig receptor antiserum reacted in Western blots with rabbit SI and with the purified toxin receptor. Antireceptor IgG blocked in vitro binding of toxin A to rabbit ileal villus cell BB. Furthermore, anti-SI IgG inhibited toxin A–induced secretion (by 78.1%, $P < 0.01$), intestinal permeability (by 80.8%, $P < 0.01$), and histologic injury ($P < 0.01$) in rabbit ileal loops in vivo. Chinese hamster ovary cells transfected with SI cDNA showed increased intracellular calcium increase in response to native toxin (holotoxin) or to a recombinant 873–amino acid peptide representing the receptor binding domain of toxin A. These data suggest that toxin A binds specifically to carbohydrate domains on rabbit ileal SI, and that such binding is relevant to signal transduction mechanisms that mediate in vitro and in vivo toxicity. (*J. Clin. Invest.* 1996; 98:641–649.) Key words: *Clostridium difficile* • enterotoxin • toxin receptor • sucrase-isomaltase • lectin binding

Introduction

Toxigenic strains of *Clostridium difficile* are the major cause of antibiotic-associated colitis in humans and animals (1). *C. difficile* produces two protein exotoxins: toxin A, an enterotoxin which also possesses mild cytotoxic activity, and toxin B, a cytotoxin which does not elicit enterotoxicity in animals (2, 3). Experiments in our laboratory (3) and elsewhere (2, 4) indicate that the primary pathogenic factor in *C. difficile*–associ-

ated disease in experimental animals is toxin A, a 308-kD protein (5) which inactivates the intracellular GTP binding proteins Rho A and B (6, 7). Sequence analysis of toxin A (5) indicates that the receptor binding domain resides in a series of tandem repeats which occupy the carboxyl-terminal 868 amino acids, or approximately one-third of the molecule. A necessary first step for enterotoxicity is binding of toxin A to luminal enterocyte receptors. For example, a monoclonal antibody directed against this binding domain of toxin A blocks the enterotoxic activity of toxin A in mouse intestine by inhibiting toxin binding to its receptor (8). Moreover, vaccination of hamsters with a recombinant peptide from the receptor binding portion of toxin A protects against *C. difficile* (9).

Specific receptor binding activity for toxin A has been demonstrated on rabbit, hamster, and human enterocyte membranes (10–13) as well as on rabbit erythrocytes (14) and neutrophils (15), but thus far these receptors have not been identified. A single class of toxin A glycoprotein receptors on rabbit intestinal brush border (BB)¹ appears to be linked to a pertussis toxin–sensitive G protein(s) (10). We also observed that toxin A receptor binding activity is not expressed in newborn rabbit BB but slowly increases after weaning to adult levels (16), a phenomenon which might explain the low level of biologic responsiveness of newborn rabbit intestine to toxin A (16).

The purpose of this study was to characterize the toxin A membrane receptor from rabbit ileal BB. Our purification strategy was based on prior reports that toxin A interacted with its receptor via lectin-like binding to a specific trisaccharide Gal α ₁₋₃ Gal β ₁₋₄ GlcNAc (11, 14). The plant lectin BS-1 derived from the seeds of *Bandeirea simplicifolia* also binds specifically to glycoconjugates bearing terminal α -D-galactose residues (11). Using a BS-1 lectin affinity column and a toxin A affinity column we were able to partially purify and identify a specific toxin A binding glycoprotein on rabbit BB. We report here that the rabbit ileal BB exoenzyme sucrase-isomaltase (SI), a complex of sucrose α -D-glucohydrolase, EC 3. 2. 1. 48, and isomaltase, EC 3. 2. 1. 10, specifically binds toxin A on its carbohydrate side chains and mediates toxin-related effects in cultured cells and in rabbit intestine in vivo.

Methods

Toxin purification and radiolabeling. Toxin A was purified from culture supernatants of *C. difficile* strain 10463 and tritiated and biotinylated as described previously (10, 16). Enterotoxicity of labeled and

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1. Abbreviations used in this paper: BB, brush border; CHO, Chinese hamster ovary; SI, sucrase-isomaltase; WGA, wheat germ agglutinin.

unlabeled toxin A preparations was determined in rat and rabbit intestinal loops (15, 17–20). Stock solutions of purified toxin A contained between 400 and 1,400 µg/ml protein as measured by the bicinchoninic acid protein assay reagent (Pierce Laboratories, Rockford, IL).

Expression and purification of recombinant toxin A carboxyl-terminal peptides. Plasmid pCDtoxA.03, containing the *C. difficile* toxin A gene (21), was digested with Sau3A, and the resulting restriction fragments were separated by electrophoresis in 1% agarose. An ~3.5-kb restriction fragment was excised and purified from the agarose gel using Sephaglas Prep Kit (Pharmacia Biotech, Piscataway, NJ). This fragment contained the carboxyl-terminal 2,604 bp of the toxin A gene encoding the entire carbohydrate binding repeating unit of toxin A as well as five upstream amino acids. The 3' recessed ends were filled in using exonuclease-free Klenow fragment (United States Biochemical Corp., Cleveland, OH), and the toxin A gene fragment was subcloned at the SmaI site of the glutathione *S*-transferase expression vector pGEX-3X (Pharmacia Biotech). A recombinant plasmid containing the fragment in the correct orientation for expression was determined by restriction analysis and designated p3X-ARU. *Escherichia coli* DH5α harboring p3X-ARU was grown in 1 liter of Terrific Broth (22) containing 0.1 mg/ml of ampicillin to an optical density of 0.6 at 600 nm. Recombinant protein expression was then induced by addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. After 5 h, cells were pelleted by centrifugation and suspended in 20 ml of phosphate-buffered saline, pH 7.4. Cells were disrupted by sonication, and cellular debris was removed by centrifugation. Lysates were filtered through 0.45 µm pore size membranes. The recombinant glutathione *S*-transferase fusion protein containing the receptor binding domain of toxin A was purified from lysates by affinity chromatography with glutathione-Sepharose 4B.

Purity of the recombinant peptide was analyzed by SDS-PAGE, using the discontinuous system of Laemmli (23). The purified peptide contained the toxin A repeating units as determined by immunoblot analysis using the monoclonal antibody PCG-4 (24).

Toxin A affinity column. Toxin A-Sepharose was prepared by coupling activated CH-Sepharose 4B (Pharmacia Biotech) to purified toxin A. Briefly, 1 gram of CH-Sepharose was first suspended in 200 ml of 1 M HCl for 15 min, then centrifuged (1,000 g × 10 min at 4°C) and suspended in 3 ml of bicarbonate buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.0). 3 ml (0.5–1 mg/ml) of purified toxin A was dialyzed against bicarbonate buffer (1 liter × 24 h) and incubated with the Sepharose suspension for 16 h at 4°C. The toxin A-Sepharose mixture was introduced into a 1 × 2-cm glass column, washed with bicarbonate buffer, and any remaining active groups were blocked by washing with 0.1 M Tris-HCl buffer (pH 8.0) for 1 h. The column was washed twice with 0.1 M sodium acetate buffer containing 0.5 M NaCl (pH 6.0) and 0.1 M Tris-HCl buffer containing 0.5 M NaCl before use.

Receptor purification. Rabbit ileal BB membranes were solubilized in 2% CHAPS (3-[cholamidopropyl dimethylammonio]-1-propanesulfonate; Pierce Laboratories) as previously described (10). All purification steps were performed at 4°C using a working buffer of 10 mM Tris (pH 7.4) containing 0.2% CHAPS. Solubilized BB (50–100 mg) were loaded onto a 10 × 2-cm *B. simplicifolia* (BS-1) Sepharose column (10 ml) (E-Y Laboratories, Inc., San Mateo, CA) equilibrated with working buffer. The column was washed with 40 ml of working buffer and 30 ml of buffer containing 0.3 M NaCl to remove unbound BB proteins. Proteins containing α-D-galactose were specifically eluted with buffer containing 0.2 M of α-D-galactose. ³H-Toxin A binding activity of eluted proteins was measured by immobilization of solubilized receptor proteins on wheat germ agglutinin (WGA)-lectin-agarose (E-Y Laboratories, Inc.) as described previously by us (10). The fraction with the highest toxin A binding activity from the BS-1 column was then further purified on the toxin A affinity gel (see above). Final toxin A receptor purification was achieved by HPLC on a 7.5 mm × 30 cm G400 SW molecular sieve column (Phenomenex Inc., Torrance, CA). Affinity-purified receptor (0.2 ml) was injected into the column which was then eluted at 1 ml/min with 10 mM Tris

buffer containing 0.2% CHAPS. 1-ml fractions were monitored for protein by absorbance at 230 nm and for ³H-toxin A binding activity by the WGA-agarose soluble binding assay (10). SDS gel electrophoresis of the purified receptor was performed as described by Laemmli (23) on 7.5% separating slab gels, 1 mm in thickness.

³H-Toxin A binding to solubilized BB receptor. Binding of ³H-toxin A to solubilized receptor fractions immobilized on WGA-agarose was measured as described previously (10). The apparent dissociation constant of binding (*K*_d) was calculated by Scatchard plot analysis using the program "Ligand" as described by Munson (25). In some experiments purified SI (50 ng–10 µg) was absorbed to WGA-agarose and ³H-toxin A binding was then estimated (10). In another series of experiments purified SI (5 µg) was preincubated (37°C) for 0–8 h with 0.5 U of α-galactosidase (Sigma Immunochemicals, St. Louis, MO) in 0.4 ml of 10 mM Tris buffer (pH 7.4) or buffer alone (8 h, control). The mixture was then absorbed to WGA-agarose and ³H-toxin A specific binding was then estimated (10). Two such experiments were performed, with triplicate determinations per time point.

Amino acid sequencing of receptor tryptic peptides. 20 µg of the toxin A affinity-purified receptor was electrophoresed onto a 7.5% SDS-polyacrylamide gel and the proteins were electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). The membrane was immersed for 60 s in 0.1% Ponceau S dye (Sigma Immunochemicals) in 1% acetic acid. Excess stain was removed by washing the membrane in 1% acetic acid for 1–2 min, and the major protein band containing the toxin A binding activity at between 120 and 140 kD was excised, transferred to an Eppendorf tube, and destained by washing with 200 µM NaOH and distilled water. The nitrocellulose membrane was then incubated (30 min at 37°C) in 1.2 ml of polyvinylpyrrolidone (PVP-40; Sigma Immunochemicals) dissolved in 100 mM acetic acid followed by extensive washing with distilled water. The membrane was then cut into small (1 mm × 1 mm) pieces which were incubated in situ with trypsin (Worthington Biochemical Corp., Freehold, NJ) in 0.2 ml of 100 mM NaHCO₃ buffer (pH 8.2) for 16 h at 37°C. Eluted tryptic peptides were immediately loaded onto an HPLC C18 reverse-phase column (VYDAC, Hesperia, CA) that was acidified with 10 µl of trifluoroacetic acid (TFA; Pierce Laboratories)/H₂O 10:90 (vol/vol). Tryptic fragments were eluted with a linear gradient of 0.1% TFA in double distilled water to 0.09% TFA in acetonitrile at 0.1 ml/min. 1-ml fractions were monitored for protein by optical density at 215 nm. Amino acid sequences of peptides were determined with a protein sequencer (model 477A; Applied Biosystems, Inc., Foster City, CA) with a 120A on-line PTA-AA Analyzer.

Receptor antibodies. Adult male guinea pigs (Charles River Breeding Laboratories, Wilmington, MA) were immunized with the 120–140-kD toxin A receptor BB protein eluted from SDS-PAGE of the toxin A affinity column-purified receptor (10 µg). A 250-µl aliquot of the eluted receptor protein was then mixed 1:1 with adjuvant (Ribi Adjuvant System; Ribi ImmunoChem Research, Inc., Hamilton, MT) in distilled water following the manufacturer's recommendations. Animals were injected both subcutaneously (0.4 ml) and intraperitoneally (0.1 ml) with the receptor-adjuvant mixtures every 2 wk for 4 wk, and then every month for 2 mo. Two guinea pigs were immunized with vehicle plus adjuvant alone (control). Sera were collected 7 d after the last two injections and the IgG fraction was purified on a Protein A-Sepharose column (Pharmacia Biotech). Two other guinea pigs were immunized with 0.5 mg each of rabbit ileal SI purified by the method of Hunziker et al. (26). Pooled sera from both animals in each group were used in all experiments.

Western blotting of the toxin A receptor and purified SI with antireceptor antibodies. Toxin A BB receptor (0.5 µg) purified by toxin A affinity chromatography or purified SI were subjected to 7.5% SDS-PAGE, transferred to nitrocellulose sheets, and a Western blot was performed using antireceptor IgG or control guinea pig IgG.

Effect of antireceptor IgG on binding of biotinylated toxin A to rabbit ileum. Binding of biotinylated toxin A to rabbit ileum was determined on 5-µm sections of formalin-fixed, paraffin-embedded il-

Table I. Purification of Toxin A Receptor from Rabbit Ileal BB

Step	Total protein*	Specific activity	Purification
	mg	pmol ³ H-toxin/mg	
Native BB	320	0.3	1
Solubilized BB	115	0.3	1.1
BS-1 affinity	3.4	13.6	45.3
Toxin A affinity	0.1	41.9	140
HPLC (fraction 7)	0.06	47.9	160

*Protein was estimated by the BCA method (see Methods).

eum of normal 80-d-old rabbits using previously described methods (16). Slides were incubated with either anti-toxin A receptor antibody or control antibody (50–200 µg/ml) for 1 h followed by three washes with PBS. Biotinylated toxin A (200 µg/ml) was then added for 1 h followed by three washes with PBS followed by the addition of streptavidin-peroxidase (10 µg/ml) (Sigma Immunochemicals) for 30 min. Control positive slides were made by incubating biotinylated toxin A with normal rabbit ileum, and control negative slides were made by using nonbiotinylated toxin A to normal rabbit ileum. Slides were examined by a histopathologist (H.P. Desai) who was unaware of the antibody used.

Effect of anti-SI antibodies on toxin A-mediated enterotoxicity in rabbit ileal loops. Ileal loops were constructed in fasting anesthetized New Zealand rabbits (two 10-cm loops per animal) (Hare-Marland Laboratories, Hewit, NJ) as described previously by us (15, 17). Before injection of loops, both renal pedicles were tied and a bolus of ³H-mannitol (100 µCi) was injected intravenously. Loops were then injected with purified toxin A (20 µg) in 1 ml of 50 mM Tris buffer (pH 7.4) or 1 ml of buffer alone. In some animals ileal loops were first injected with either guinea pig anti-SI polyclonal IgG (1 mg/loop) or the same amount of control guinea pig IgG 15 min before toxin A administration. We measured fluid secretion at 5 h as loop weight to length ratio (mg/cm) and intestinal mucosal permeability by dpm of ³H-mannitol in luminal fluid (dpm/cm loop). Full thickness ileal tissue samples were fixed in formalin, paraffin embedded, and sections were stained with hematoxylin and eosin. Coded sections were examined and graded for (1) epithelial cell damage, (2) congestion and edema of the mucosa, and (3) neutrophil margination and tissue inflammation. A score of 0–3, denoting increasingly severe abnormality was assigned to each of these three parameters as described previously (15, 19). An overall histologic score was obtained by adding the individual scores for each of the three parameters.

Cell transfections. The cDNA clone for SI (kindly provided by Dr. Walter Hunziker, Department of Biochemistry, Swiss Federal Institute of Technology, Zurich, Switzerland) was cloned into the expression vector pcDNA1-amp (Invitrogen, San Diego, CA). Chinese hamster ovary (CHO) cells were grown in DMEM with 10% FCS in a humidified CO₂ atmosphere. To generate permanent cell lines 50–60% confluent 60-mm dishes were transfected using the calcium-phosphate DNA coprecipitation method (27). The transfection mixture contained 16 µg of plasmid DNA (pcDNA-SI) and 1 µg of pRSV-hygroplasmid which confers resistance to hygromycin D (28, 29). Plasmid pRSV-hygro contains the hygromycin B phosphotransferase gene (30) upstream of the Rous sarcoma virus promoter. After 48 h of incubation at 37°C the cultures were split 1:5 and plated in DMEM medium supplemented with 10% FCS and 500 µg/ml hygromycin B. Cells were refed every 3 d until discrete colonies resistant to hygromycin B appeared. Isolated colonies collected using cloning cylinders were grown to confluency and further characterized for production of SI by Northern blot analysis and SI enzymatic activity. For Northern analysis RNA was prepared by the method of Chomczynski

and Sacchi (31) and was electrophoresed on 1% agarose-formaldehyde gel blotted onto nitrocellulose and hybridized with a uniformly labeled rabbit SI cDNA probe. SI enzymatic activity was estimated by the method of Dahlqvist (32). Briefly, cells were washed twice with ice-cold PBS and then scraped off the culture dishes with a rubber policeman, and centrifuged at 11,000 g for 5 min. Cell pellets were resuspended in 10 mM Tris buffer (pH 7.5) containing 0.5% Triton X-100 (Sigma Immunochemicals) and kept at 4°C for 1 h. The lysate was centrifuged at 11,000 g for 20 min and aliquots of the supernatants were tested for SI activity using sucrose as a substrate as described by Dahlqvist (32). Isolated clones expressing SI were stored in liquid nitrogen.

Fluorescence imaging of intracellular calcium. For fluorescence microscopy, transfected and nontransfected CHO cells were seeded onto coverslips and allowed to attach and spread for 24–48 h before study. Cells were loaded with fura-2 AM as described previously (33). Cells with visible sequestration of dye into cytoplasmic compartments were excluded. Intracellular calcium was determined by dual fluorescence microscopy on a Zeiss Axiovert inverted microscope equipped for epifluorescence. Calcium concentration was estimated from the following equation: $Ca^{++} = K_d \times B \times (R - R_{min}) / (R_{max} - R)$; where K_d is equal to 224 nM and B is equal to the ratio of raw fluorescence signals of calcium-free fura-2 at an excitation wavelength of 380 nm (34). Toxin A or recombinant toxin A peptide was delivered to CHO cells by pressure ejection through micropipettes (approximate tip diameter 1.0 µm) prepared on a Brown-Flaming Micropipette puller (model P80PC; Sutter Instrument Co., Novato, CA). The micropipette was positioned via a Narashige three-dimensional hydraulic micromanipulator at a magnification of 1,000 and toxin was expelled by the delivery of a 10-psi pulse of nitrogen. Results represent the mean magnitude of calcium response derived only from cells which responded. A significant calcium response to whole or recombinant toxin A was defined as a change in fluorescence which corresponded to > 5 nM cytosolic calcium.

Results

Purification of the toxin A BB receptor. We purified the toxin A receptor ~160-fold by a combination of BS-1, toxin A affinity chromatography, and molecular sieve HPLC (Table I). As expected from previous reports (10, 11) that the toxin A receptor contained α-D-galactose in its ligand binding domain, the majority of the toxin A binding activity was specifically eluted from the BS-1 Sepharose column with α-D-galactose providing a 44-fold purification (Table I). Further purification was

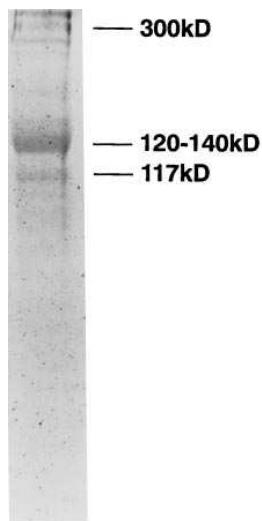


Figure 1. Gel electrophoresis of the toxin A receptor. 10 µg of the HPLC-purified receptor (see Methods) was electrophoresed in the presence of SDS according to the method of Laemmli (23). The sample was incubated for 30 min with 2-mercaptoethanol and 1.25% SDS and then heated to 100°C for 2 min. Electrophoresis was performed on a 1-mm thick 7.5% acrylamide gel at 200 V for 45 min. Calculated molecular masses are shown on the right estimated by the migration of molecular mass standards run simultaneously.

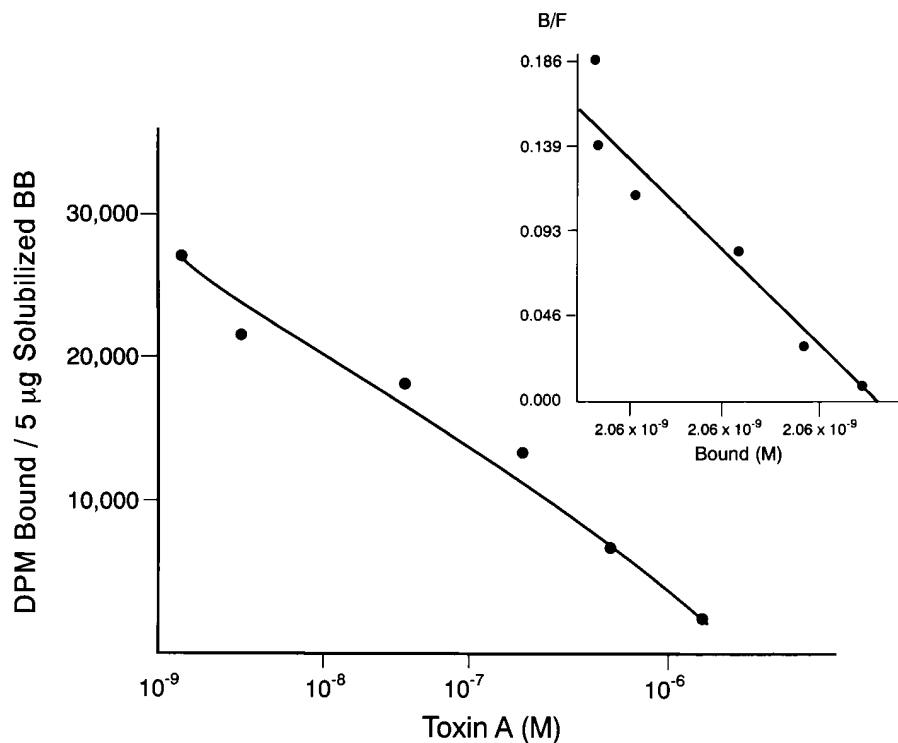


Figure 2. Competitive inhibition of ³H-toxin A binding to partially purified toxin A receptor. 5 µg of the toxin A affinity partially purified receptor was adsorbed to WGA-agarose and then incubated with 66 ng of ³H-toxin A (190,000 dpm) and increasing doses of unlabeled toxin A for 60 min at 22°C in 10 mM Tris (pH 7.4) containing 0.2% CHAPS (*n* = 3 per group). The final concentration of ³H-toxin A was 1.4 nM. Specific binding was determined as described in Methods. (Inset) Scatchard plot of binding data.

achieved by toxin A-Sepharose affinity chromatography. The majority of bound receptor activity on this column was eluted with 0.5 M NaCl yielding a further fivefold purification. A 1-ml fraction from the toxin A affinity column containing high ligand binding activity was further purified by HPLC on a G4000 SW molecular sieve column. Most of the toxin A binding activity was eluted as a broad peak in the void volume with the fraction containing the highest binding activity at 7 min. When rechromatographed under identical conditions, fraction 7 migrated as a single peak at 7 min (not shown) with a purification of ~160-fold and a yield of 2.9% (Table I). SDS-PAGE of this fraction revealed a major protein band at 120–140 kD, a less prominent band at 117 kD, and several minor bands at ~300 kD (Fig. 1).

Binding of ³H-toxin A to purified receptor. ³H-Toxin A binding to purified BB receptor was linear between 500 ng and 5 µg of BB protein, saturable at 5 µg of solubilized receptor, and maximal at 60 min (data not shown). As shown in Fig. 2, binding of ³H-toxin A to purified receptor was inhibited by ~50% in the presence of an 80-fold molar excess of unlabeled toxin A. Based on binding experiments from two separate BB preparations, each derived from five animals, we calculated an apparent *K_d* of 6×10^{-8} M, similar to the *K_d* of 5.4×10^{-8} M reported earlier for toxin A binding to intact BB (10). Computer analysis of the binding data revealed a best fit to a linear Scatchard plot (Fig. 2, inset) suggesting the presence of a single class of receptors.

Antireceptor IgG inhibits toxin A binding to ileal BB. Guinea pigs were immunized with the 120–140-kD protein fraction eluted from polyacrylamide gels of affinity-purified toxin A receptor preparations (Fig. 1). Western blot analysis indicated that antireceptor IgG, but not control IgG, recognized the 120–140-kD BB protein (Fig. 3). Binding of biotinylated toxin A to rabbit ileal BB was completely blocked by preincubation of rabbit ileal sections with 100 µg/ml of antire-

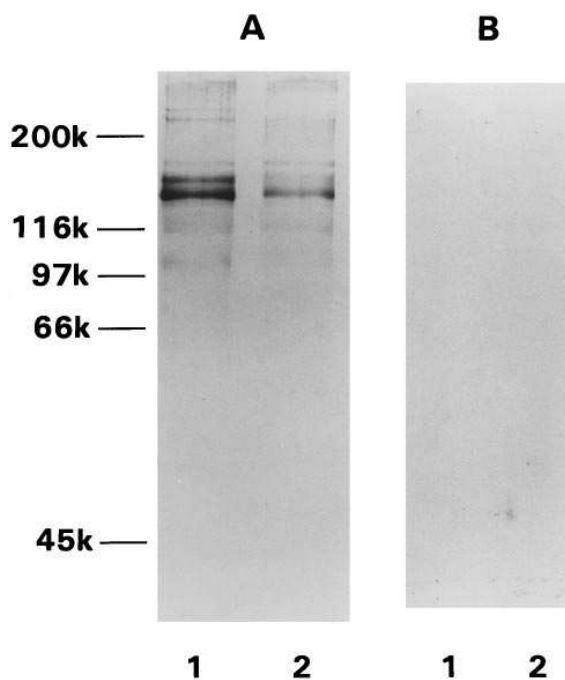


Figure 3. Western blot of the toxin A receptor using antireceptor antibodies. Toxin A receptor (0.5 µg) purified from either BS-1 affinity chromatography (lane 1) or toxin A affinity gel (lane 2) was subjected to SDS-PAGE as described in the legend to Fig. 1 and blotted to nitrocellulose sheets. Blots were incubated with anti-toxin A receptor (A) or control IgG (B). Identification of reactive proteins was determined by incubation with anti-guinea pig IgG linked to avidin-alkaline phosphatase as described in Methods. Numbers on the left represent the migration of prestained molecular weight standards: egg albumin (45 k, where k represents 1,000), bovine serum albumin (66 k), phosphorylase B (97 k), β-galactosidase (116 k), and myosin (200 k).

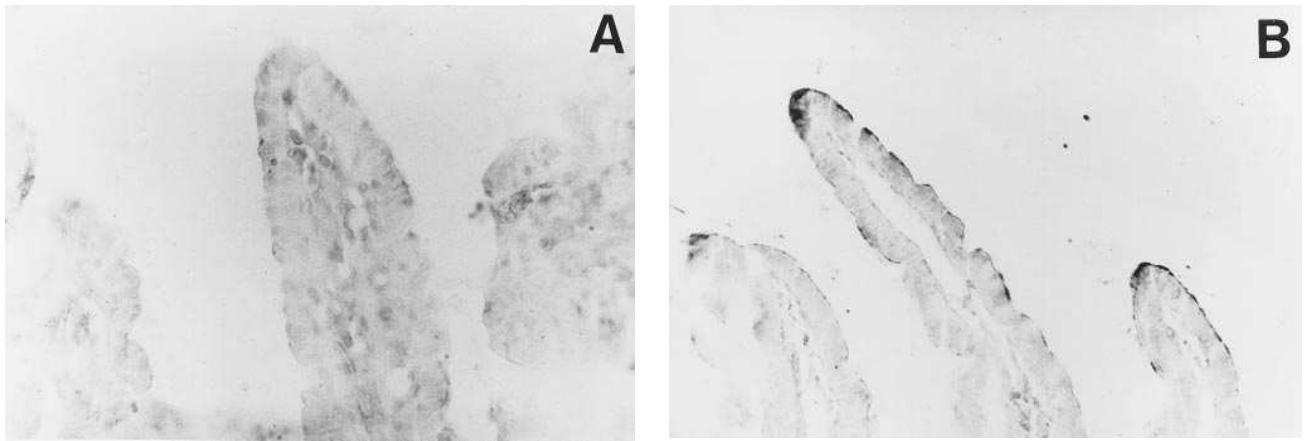


Figure 4. Inhibition of biotinylated-toxin A reactivity by anti-toxin A receptor antibody. Fixed sections of adult rabbit ileum were stained with biotinylated toxin A. Sections were first incubated (1 h at 37°C) with either 100 µg/ml of anti-toxin A receptor IgG (A) or 100 µg/ml of control IgG (B) before staining with biotinylated toxin A.

ceptor IgG (Fig. 4 A). Binding of biotinylated toxin A was clearly seen when control IgG from vehicle-immunized guinea pig was used (Fig. 4 B), giving a staining pattern similar to that seen in the absence of added immunoglobulin (data not shown).

Amino acid sequence of receptor peptides. Two major and several minor peptide peaks were identified by reverse-phase HPLC of a tryptic digest of the 120–140-kD toxin A receptor band (Fig. 1). Amino acid sequences of the two major peptides were: peptide 1: R-Y-E-V-P-H-Q-F-V-T-E-F-A-G-P-A-A-T-E, and peptide 2: T-D-N-P-Y-S-V-S-S-T-Q-Y-S-P-T-G-I-T-A-D-L-Q-L-N. Computer-based comparison of these peptide sequences with known proteins revealed that peptide 1 was identical to amino acid residues 169–187 of rabbit SI and peptide 2

was identical to amino acid residues 979–1001 of the same protein (26). Moreover, the anti-toxin A receptor antiserum, but not control antiserum, reacted strongly with purified SI (Fig. 5).

Toxin A binding to purified SI. Using the soluble binding assay as described in Methods, we demonstrated that binding of ³H-toxin A to purified rabbit SI (Fig. 6) was completely inhibited by a 90-fold molar excess of unlabeled toxin A (not shown). Removal of α-galactose residues from purified SI with α-galactosidase significantly inhibited ³H-toxin A-specific binding (Fig. 6), indicating that α-galactosyl residues on purified SI were probably involved in toxin A binding, as previously reported for the endogenous rabbit BB receptor (10, 16).

Anti-SI antiserum inhibits toxin A enterotoxicity in vivo. Injection of 20 µg of purified toxin A into rabbit ileal loops stimulated fluid secretion, caused a 27-fold increase in blood-to-lumen mannitol permeability (Table II), and caused substantial mucosal injury as determined by histologic score (3, 15, 17). Each of these effects was significantly inhibited by prein-

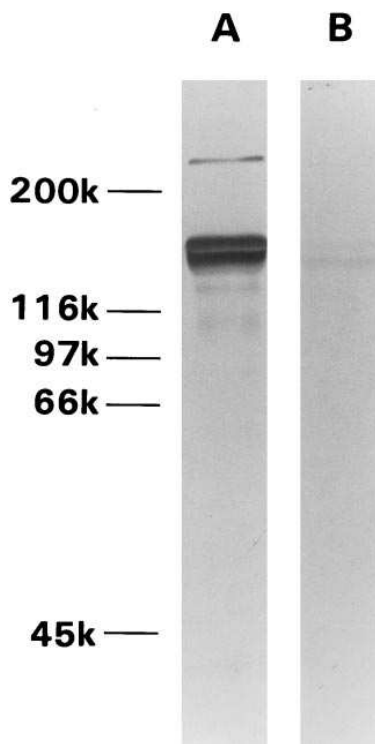


Figure 5. Western blot of purified SI using anti-toxin A receptor antibodies. Purified SI (5 µg) was subjected to SDS-PAGE as described in the legend to Fig. 1 and blotted to nitrocellulose sheets. Blots were incubated with either anti-toxin A receptor IgG (A) or control IgG (B) and identification of reacted proteins was performed as described above (Fig. 3). Numbers on the left represent prestained molecular mass standards as described in the legend to Fig. 3.

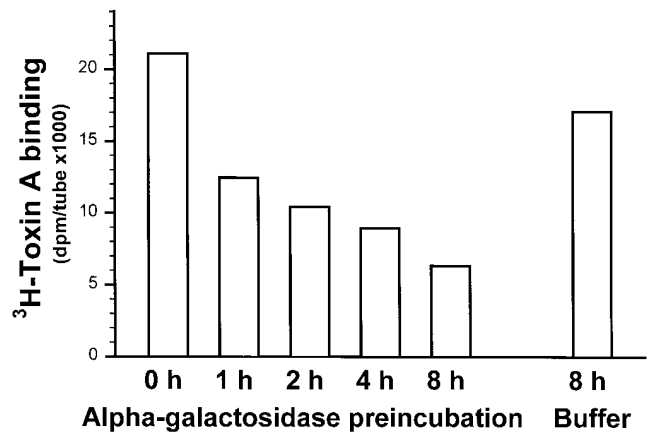


Figure 6. α-galactosidase inhibits toxin A-specific binding to rabbit SI. Purified rabbit SI (5 µg) was preincubated (37°C) for the indicated time points with either 10 mM Tris buffer (pH 7.4) alone (control) or with buffer containing 0.5 U of α-galactosidase. Samples were then absorbed to WGA-agarose and ³H-toxin A specific binding was determined as described previously (10). Each data point represents the mean of triplicate determinations.

Table II. Anti-SI Antiserum Inhibits Toxin A-mediated Secretion and Mannitol Permeability

	Fluid secretion	³ H-Mannitol permeability	Histologic score
	mg/cm	dpm/cm loop	
Buffer	400±20	2300±880	0.75±0.25
Toxin A	984±81*	58780±6400*	6.40±0.49*
Toxin A + anti-SI IgG	564±35**	13280±3600**	3.39±0.60**
Toxin A + control IgG	985±115*	43630±6300*	7.8±0.58*

Rabbit ileal loops were injected with 1 ml either buffer or buffer containing 1 mg of anti-SI antiserum or control guinea pig antiserum 15 min before administration of 20 mg of toxin A or buffer into the loops (*n* = 12 per group). After 5 h, loops were excised and fluid secretion was measured by loop weight (mg)/length (cm) ratio. Intestinal permeability to ³H-mannitol was estimated by scintillation counting of aliquots of loop samples. Histologic score was determined in five to eight loops per group as described in Methods. Results are expressed as mean±SE. **P* < 0.01 vs. buffer, and ***P* < 0.01 vs. toxin A alone.

jection of the loops with anti-SI antiserum (1 mg/loop). Control guinea pig antiserum had no statistically significant effects.

Transfection of CHO cells with rabbit SI cDNA. Exposure of target cells to toxins A and B is followed by a nearly immediate rise in intracellular calcium (33, 35). This rise in calcium was used to assess toxin A binding to SI in CHO cells transfected with rabbit SI cDNA. CHO cells transfected with rabbit SI cDNA contained SI mRNA and protein by Northern blot analysis, as well as SI enzymatic activity, whereas no SI activity was detected in untransfected CHO cells (Fig. 7). As shown in Fig. 8 (left) exposure of wild-type CHO cells to toxin A (holotoxin) or recombinant toxin A receptor binding peptide caused calcium elevations in 6.2 and 11.8% of cells, respectively. However, when CHO cells transfected with rabbit SI cDNA were exposed to holotoxin or a recombinant toxin A receptor binding domain, the percentage of cells exhibiting an increase in intracellular calcium was 66.7 and 69.2, respectively (*P* < 0.01). Response amplitude of the calcium signal in SI-transfected cells was increased by approximately twofold compared with untransfected CHO cells (Fig. 8, right).

Discussion

We report here that the BB exoenzyme SI specifically binds toxin A of *C. difficile* with a *K_d* similar to those reported previously for rabbit BB membranes (10). Additionally, we provide evidence that binding of toxin A to CHO cells expressing SI elicits a rise in intracellular calcium and that IgG antibody to SI partially blocks enterotoxicity in rabbit ileum *in vivo* (Table II). We conclude from these observations that SI contains a functional receptor for toxin A on rabbit enterocytes.

The SI complex, an intrinsic BB disaccharidase expressed almost exclusively on the luminal surface of small intestinal villus enterocytes (36, 37), is synthesized as a 260-kD precursor (pro SI) that is split by pancreatic proteases into two subunits, isomaltase (~140 kD) and sucrase (~120 kD), that are non-covalently linked. SI has five functional/structural domains. The enzymes sucrase and isomaltase are attached by a short connecting segment or stalk to the outer leaflet of the entero-

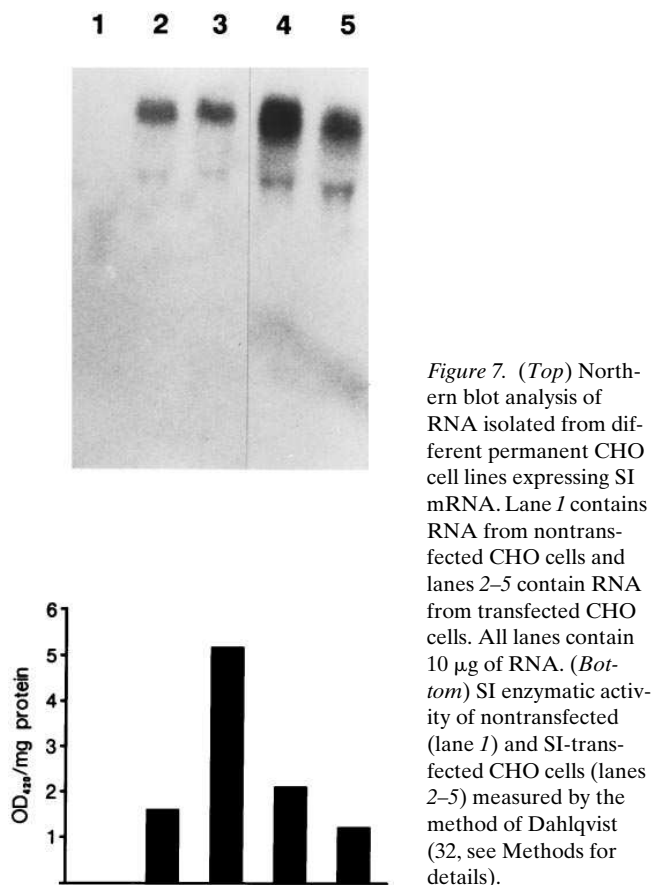


Figure 7. (Top) Northern blot analysis of RNA isolated from different permanent CHO cell lines expressing SI mRNA. Lane 1 contains RNA from nontransfected CHO cells and lanes 2–5 contain RNA from transfected CHO cells. All lanes contain 10 µg of RNA. (Bottom) SI enzymatic activity of nontransfected (lane 1) and SI-transfected CHO cells (lanes 2–5) measured by the method of Dahlqvist (32, see Methods for details).

cyte BB. A hydrophobic transmembrane segment spans the lipid bilayer and a short cytoplasmic domain of 12 amino acids extends into the cytoplasm (26). The solubilized SI heterodimer produces two patterns on SDS-PAGE; a 120–140-kD doublet (as in Fig. 3), or, more frequently, a broad band (similar to the major band in Fig. 1). The latter is generated when the amino-terminal stalk of the isomaltase subunit is proteolytically cleaved (37). Because toxin A binding to SI was dramatically inhibited by enzymatic removal of α-galactose residues (Fig. 6), we conclude that toxin A binds to a terminal α-galactose residue on one or more of the potential extracellular glycosylation sites on SI. Our data do not exclude the possibility that other BB glycoproteins bearing the same trisaccharide might also function as toxin A receptors. Antibody to SI significantly inhibited toxin A secretion, permeability, and mucosal injury (Table II) but inhibition was not complete. This raises the possibility that membrane glycoproteins other than SI can bind the toxin, perhaps via the same trisaccharide group proposed to bind toxin A on SI.

Occupancy of the toxin A receptor in human neutrophils is associated with a rapid rise in intracellular calcium that can be inhibited by pretreatment of these cells with pertussis toxin (35). These findings are typically associated with membrane receptors belonging to the large class of G-protein-associated receptors with seven membrane-spanning domains, a family of proteins whose structure is unrelated to SI. However, SI does have certain structural features in common with a second family of G-protein-associated receptors with single spanning transmembrane domains (38–40). An interesting member of this family of receptors is the sperm receptor protein β-1,4 ga-

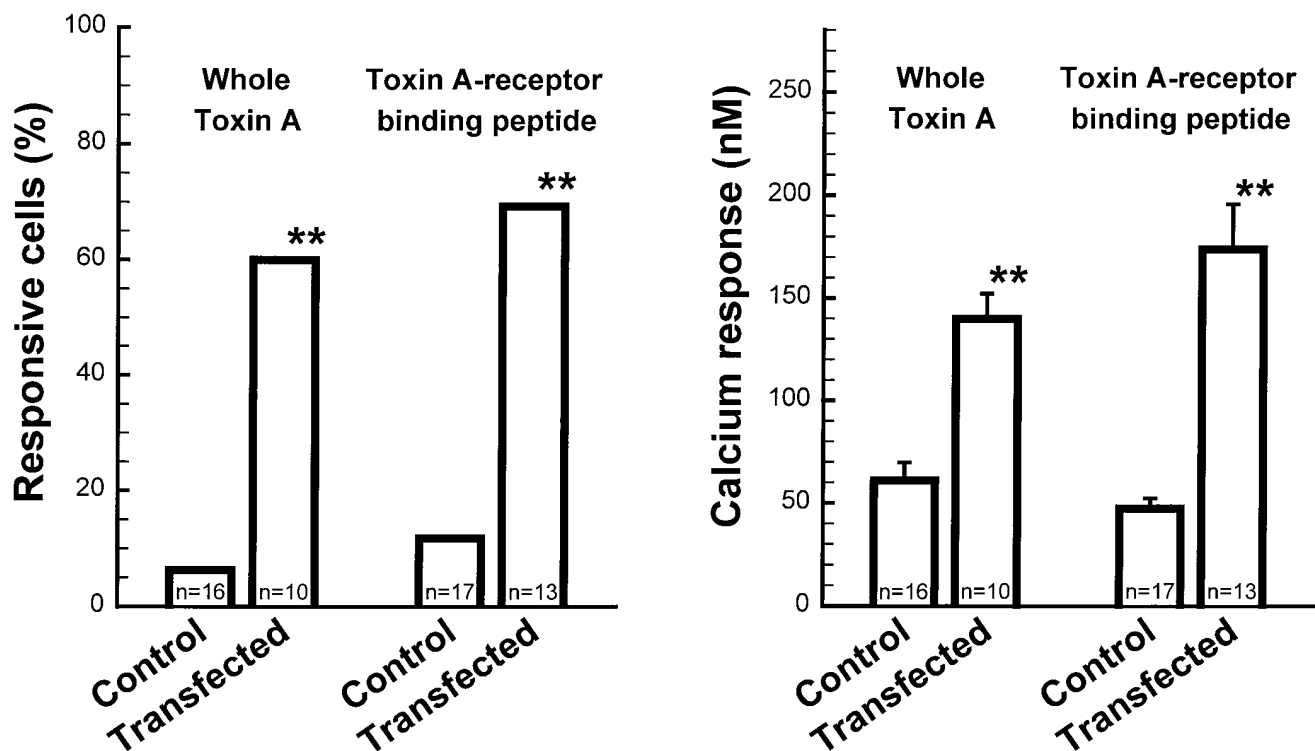


Figure 8. Effect of toxin A (holotoxin) and of recombinant toxin A binding peptide on intracellular calcium responses of CHO cells transfected with SI cDNA. Untransfected and SI-transfected CHO cells were loaded with fura-2 and imaged for intracellular calcium by fluorescence microscopy. Holotoxin or toxin A binding peptide was delivered by pressure ejection adjacent to the cell membrane. A significant calcium response to whole or recombinant toxin was defined as a change of fluorescence which corresponded to > 5 nM cytosolic calcium. (Left) Percentage of cells which were responsive (number of cells demonstrating calcium response/number of cells exposed to holotoxin or toxin A binding peptide). (Right) Amplitude of intracellular calcium response, represented as a change of intracellular calcium concentration (nM). Results represent the mean per group. *n* indicates the number of cells studied. ** $P < 0.01$ vs. control nontransfected cells.

lactosyltransferase which binds O-linked oligosaccharides on the glycoprotein ZP3 on the egg zona pellucida (41). Binding induces a signal transduction cascade culminating in the acrosome reaction that allows the sperm to penetrate the zona pellucida. The cytoplasmic domain of sperm galactosyltransferase consists of a 24-amino acid peptide with serine and threonine residues that are phosphorylated in response to ligand binding (41). Recently the 12-amino acid cytoplasmic domain of SI was shown to contain a serine at position 6 that can be phosphorylated by protein kinase A *in vivo* and *in vitro* (42). The physiologic significance of SI phosphorylation is not known, but these observations raise the possibility that toxin A binding to SI on the BB membrane may trigger phosphorylation of serine 6 in the cytoplasmic domain that in turn enhances its interaction with heterotrimeric G-proteins.

A major function of toxin receptors is to facilitate the entry of these large proteins into target cells. Our data do not allow us to speculate how the SI-toxin A complex is internalized. However, aminopeptidase N, another intestinal BB exoenzyme, was shown recently to be a major receptor for transmissible gastroenteritis virus, a pathogenic corona virus in newborn pigs (43). Like SI, aminopeptidase N is highly expressed in mature villus enterocytes and lacks structural homology with other viral receptors or with seven membrane-spanning domain receptors. Intestinal damage from this virus in piglets is highly correlated with the tissue distribution of aminopeptidase N (43). Similarly, certain biologic effects of toxin A corre-

late with the known intestinal distribution of SI (36, 44). We reported earlier that biotinylated toxin A bound exclusively to rabbit villus cells but not to crypts (16). Moreover, exposure of rat small intestine to toxin A results predominantly in villus cell damage and sparing of crypts (45). Finally the lack of toxin A binding observed in newborn rabbit ileum (16) is consistent with the lack of expression of SI on small intestinal villus cells in infant rabbits (46).

Our data indicate that the catalytic portion of toxin A that possesses glucosyltransferase activity for rho proteins (47) is not required for binding to or activation of the receptor. Recombinant toxin A binding domain was able to elicit a three-fold increase in intracellular calcium in CHO cells transfected with rabbit SI (Fig. 8), indicating that modification of Rho is not required for this effect. This ability of toxin A recombinant peptide to activate a receptor-mediated cascade finds parallel in other microbial receptor-ligand systems. For example, the binding subunit of pertussis toxin is a potent mitogen for T lymphocytes (48). Adenovirus attachment of mammalian cells is mediated by a 400-kD protein pentamer, the penton base. Cells exposed to soluble penton base undergo morphologic changes similar to cells exposed to whole virus (49), an effect mediated by binding of the penton base to cell surface integrin receptors (50).

The tissue distribution of SI is limited to the small intestine. Thus, our demonstration here that SI is a major binding protein for toxin A in rabbit ileum cannot explain the ability of

toxin A to cause damage in other cells or organs. Rat, rabbit, and human colon as well as fibroblasts, monocytes, and neutrophils are quite sensitive to toxin A (2, 10, 15, 35, 45, 51, 52), yet none expresses SI. Binding of toxin to these cells apparently involves other glycoproteins or glycolipids with the appropriate glycosyl residues required for binding. Moreover, the trisaccharide Gal α ₁₋₃Gal β ₁₋₄GlcNAc mediates toxin A binding in rodents, but this trisaccharide is not expressed in human cells because they lack the appropriate α -galactosyltransferase (53). We conclude from our observations that different receptors with various carbohydrate prosthetic groups can function as toxin A binding proteins in various cells. In support of this, Tucker and Wilkins (54) reported that the purified carbohydrate antigens I, X, and Y expressed on various human epithelial cells are capable of binding toxin A.

In conclusion, the rabbit small intestinal BB membrane enzyme SI contains specific binding sites for *C. difficile* toxin A. Our data suggest that SI is the major toxin A binding site in rabbit ileum, but we cannot exclude the possibility of additional binding sites. Binding of toxin to α -galactosyl residues on this enzyme produces biologic effects in the ileum and in CHO cells expressing rabbit SI. Toxin A binding to carbohydrates on SI is analogous to the binding of cholera toxin to the BB glycolipid monosialoganglioside (GM1) (55), or the binding of thyroid-stimulating hormone to its glycoprotein receptor via an α -galactose-containing trisaccharide (56). Given the limited tissue expression of SI, it appears likely that multiple glycoproteins or glycolipids can serve as toxin A receptors, with considerable organ and species specificity. Our studies should allow further elucidation of the signal transduction processes, particularly calcium mobilization and G-protein activation, that follow binding of *C. difficile* toxin A to its receptor.

Acknowledgments

This work is dedicated to the memory of our colleague and friend, Christos Cladaras, Ph.D., who died in August 1994.

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