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## Citation

Rodighiero, Chiara, Yukako Fujinaga, Timothy R. Hirst, and Wayne I. Lencer. 2001. "A Cholera Toxin B-Subunit Variant That Binds Ganglioside GM1but Fails to Induce Toxicity." *Journal of Biological Chemistry* 276 (40): 36939–45. <https://doi.org/10.1074/jbc.m104245200>.

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## A Cholera Toxin B-subunit Variant That Binds Ganglioside G<sub>M1</sub> but Fails to Induce Toxicity\*

Received for publication, May 10, 2001, and in revised form, July 11, 2001  
Published, JBC Papers in Press, July 30, 2001, DOI 10.1074/jbc.M104245200

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**Entry of cholera toxin (CT) into target epithelial cells and the induction of toxicity depend on CT binding to the lipid-based receptor ganglioside G<sub>M1</sub> and association with detergent-insoluble membrane microdomains, a function of the toxin's B-subunit. The B-subunits of CT and related *Escherichia coli* toxins exhibit a highly conserved exposed peptide loop (Glu<sup>51</sup>–Ile<sup>58</sup>) that faces the cell membrane upon B-subunit binding to G<sub>M1</sub>. Mutation of His<sup>57</sup> to Ala in this loop resulted in a toxin (CT-H57A) that bound G<sub>M1</sub> with high apparent affinity, but failed to induce toxicity. CT-H57A bound to only a fraction of the cell-surface receptors available to wild-type CT. The bulk of cell-surface receptors inaccessible to CT-H57A localized to detergent-insoluble apical membrane microdomains (lipid rafts). Compared with wild-type toxin, CT-H57A exhibited slightly lower apparent binding affinity for and less stable binding to G<sub>M1</sub> *in vitro*. Rather than being transported into the Golgi apparatus, a process required for toxicity, most of CT-H57A was rapidly released from intact cells at physiologic temperatures or degraded following its internalization. These data indicate that CT action depends on the stable formation of the CT B-subunit-G<sub>M1</sub> complex and provide evidence that G<sub>M1</sub> functions as a necessary sorting motif for the retrograde trafficking of toxin into the secretory pathway of target epithelial cells.**

Cholera toxin (CT)<sup>1</sup> and heat-labile toxin type I (LTI) are responsible for the massive secretory diarrheas caused by infections with *Vibrio cholerae* and toxigenic *Escherichia coli*. To induce disease, both toxins must first bind ganglioside G<sub>M1</sub> at the apical surface of polarized intestinal epithelial cells, enter the cell by endocytosis, and traffic retrograde through the Golgi cisternae to the ER, where the toxin's enzymatic subunit unfolds and translocates to the cytosol (1–3). Recent studies indicate that toxin function depends on binding specifically to G<sub>M1</sub> and on association with detergent-insoluble membrane mi-

crodomains (lipid rafts) (4). Based on these data, we have proposed that toxin binding to G<sub>M1</sub> represents a form of protein-lipid modification that provides the sorting motif for association with lipid rafts and retrograde trafficking into the secretory pathway of target epithelial cells (1, 4).

CT and LTI consist of five identical B-subunits (≈11 kDa) and a single A-subunit with two domains termed the A<sub>1</sub>- and A<sub>2</sub>-peptides. The pentameric B-subunit (≈55 kDa) binds with high affinity to G<sub>M1</sub> at the apical cell surface (5–7) and thus tethers the toxin to the membrane of host cells. The A<sub>1</sub>-peptide (≈22 kDa) is enzymatically active and acts inside the cell to activate adenyl cyclase by catalyzing the ADP-ribosylation of the heterotrimeric GTPase G<sub>αs</sub> (8–10). In the intestine, activation of adenyl cyclase induces Cl<sup>-</sup> secretion, the fundamental transport event responsible for secretory diarrhea.

The B-subunits of CT and LTI isolated from all toxigenic *V. cholerae* and *E. coli* strains contain a highly conserved exposed peptide loop (Glu<sup>51</sup>–Ile<sup>58</sup>) that faces the cell membrane upon B-subunit binding to G<sub>M1</sub>. The peptides in this loop are not essential for B-subunit assembly or secretion (11–13), and the reason for the high degree of evolutionary conservation in this region is not readily apparent. Residues at both “edges” of this loop, however, participate indirectly in binding the oligosaccharide domain of G<sub>M1</sub> (14). We have recently examined the function of this loop by alanine scanning mutagenesis. Substitution of histidine with alanine at position 57 resulted in a toxin (CT-H57A) that bound purified G<sub>M1</sub> with high affinity, nearly equivalent to that of wt CT (15). The H57A substitution, however, rendered the B-subunit unable to induce apoptosis when applied to primary isolates of mouse CD8<sup>+</sup> T cells and the holotoxin functionally inactive when applied to cultured human intestinal T84 cells (15). These data implied that the H57A mutation uncoupled toxin binding to its cell-surface receptor G<sub>M1</sub> from downstream signal transduction, possibly by affecting toxin trafficking into the cell.

In this study, we sought to understand the defect in the cellular processing that rendered the H57A toxin variant inactive when applied to the polarized human intestinal cell line T84. Our data show that CT-H57A bound to only a fraction of the cell-surface receptors available to wt CT. The bulk of cell-surface receptors inaccessible to CT-H57A localized to detergent-insoluble apical membrane microdomains. Compared with wt CT, CT-H57A exhibited less stable binding to G<sub>M1</sub>, and the bulk of CT-H57A was rapidly released from intact cells at physiologic temperatures or degraded intracellularly. CT-H57A was not transported into the Golgi and ER, which is necessary for toxicity. These results indicate that CT action on polarized epithelial cells depends on stable high affinity binding to G<sub>M1</sub> and emphasize the importance of lipid trafficking in CT action.

\* This work was supported by National Institutes of Health Grants DK48106 (to W. I. L.) and DK34854 (to the Harvard Digestive Diseases Center) and by the Medical Research Council (to T. R. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: CT, cholera toxin; CTB, cholera toxin B-subunit; LTI, heat-labile toxin type I; G<sub>M1</sub>, [Gal(β1→3)GalNAc(β1→4)](NeuAc(α2→3))Gal(β1→4)Glc(β1→1)ceramide]; G<sub>D1a</sub>, [NeuAc(α2→3)Gal(β1→3)GalNAc(β1→4)](NeuAc(α2→3))Gal(β1→4)Glc(β1→1)ceramide]; ER, endoplasmic reticulum; wt, wild-type; HBSS, Hanks' balanced salt solution; HRP, horseradish peroxidase; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay.

## EXPERIMENTAL PROCEDURES

**Materials, Cell Culture, and Electrophysiology**—Hanks' balanced salt solution (HBSS) (Sigma) supplemented with 10 mM HEPES and with pH adjusted to 7.4 was used for all assays unless otherwise stated. T84 cells obtained from American Type Culture Collection were cultured and passaged as described previously (16). Measurements of short circuit current (I<sub>sc</sub>) and resistance were performed with 0.33-cm<sup>2</sup> monolayers, whereas biochemical studies were performed with 5- or 45-cm<sup>2</sup> monolayers as described previously (4, 17).

**Preparation of Recombinant Wild-type and Mutant Holotoxins and B-pentamers (Recombinant CT, CT-H57A, CTB, and CTB-H57A)**—Plasmid pATA14, encoding the wild-type cholera toxin operon, was constructed by ligating polymerase chain reaction-amplified fragments of the *ctxA* and *ctxB* genes as previously reported (18). Plasmid pATA14 was used as a template to generate a polymerase chain reaction fragment with a site-directed mutation in the His<sup>57</sup> codon of *ctxB*, which was then substituted for the wild-type *ctxB* cistron of pATA14 to generate plasmid pATA21 as described by Aman *et al.* (15). *V. cholerae* O395NT cells (19), harboring pATA14 and pATA21, were cultured in 3 liters of NZCYM medium (18) supplemented with 200 μg/ml ampicillin at 37 °C on a rotary shaker overnight in the presence of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The toxin was co-precipitated from the clarified supernatant with sodium hexametaphosphate as previously reported (18), and the sample was dialyzed against two changes of 20 mM Na<sub>2</sub>HPO<sub>4</sub> and 0.5 M NaCl (pH 7.5) (buffer A). After clarification and filtration, this was applied to a HiTrap<sup>®</sup> affinity column (5 ml; Amersham Pharmacia Biotech) pre-charged with 0.1 M NiCl<sub>2</sub> and equilibrated with buffer A, and the bound material was eluted using a linear gradient of imidazole (0–0.5 M) in buffer A. Fractions containing the toxin were pooled; desalted by overnight dialysis against 20 mM Tris base and 20 mM NaCl (pH 8.75) at 4 °C; and then applied to a Resource Q ion-exchange column (6 ml; Amersham Pharmacia Biotech). Samples were eluted with a linear gradient of NaCl (20 mM to 0.5 M) in 20 mM of Tris base (pH 8.75). Fractions containing the toxin were added to a NAP-10 column (Amersham Pharmacia Biotech) to replace the buffer with phosphate-buffered saline (pH 7.2), aliquoted, and stored at –80 °C. Toxin concentration was determined by UV absorbance at λ = 280 nm using the theoretical molar extinction coefficient for the holotoxin. Recombinant wt and mutant H57A cholera toxin B-subunits (CTB and CTB-H57A, respectively) were purified by the method reported by Richards *et al.* (20).

**Competition for Cholera Toxin Binding to Non-polarized T84 Cells and Immobilized G<sub>M1</sub> on Plates with Excess B-subunit**—T84 cells grown on 96-well plates (Costar Corp., New Bedford, MA) or 96-well plates coated with 2 μg/ml G<sub>M1</sub> (Matreya, Inc., Pleasant Gap, PA) were incubated with 3 nM HRP-labeled cholera toxin B-subunit (Sigma) alone or in combination with increasing concentrations of recombinant wild-type or mutant cholera toxin B-subunit as a competitor in HBSS containing 0.5% BSA for 45 min at 4 or 37 °C, respectively. Plates were washed with HBSS and developed at 37 °C using standard conditions for enzyme-linked immunosorbent assays (4).

**Toxin Binding to Mixed Liposomes of G<sub>M1</sub> and G<sub>D1a</sub>**—Liposomes containing pure G<sub>M1</sub>, G<sub>D1a</sub> (Matreya, Inc.), or the indicated combinations of the two were prepared by dissolving the gangliosides in methanol, followed by evaporation of the solvent using a vacuum pump. Pellets were dissolved in phosphate-buffered saline (pH 7.4) (2 μg/ml final concentration), immediately sonicated, and used for coating 96-well plates. Toxin binding affinity was assessed as described above.

**Steady-state Binding of Toxin to Non-polarized T84 Cells**—Binding isotherms for wild-type CTB and mutant CTB-H57A were determined by incubating at 4 °C increasing amount of toxins in HBSS and 0.5% BSA with T84 cells grown to confluence on 96-well plates. After 45 min, the plates were washed with HBSS, and the presence of specifically bound CTB or CTB-H57A was assayed using rabbit polyclonal antiserum raised against the cholera toxin B-subunit (1:1000 in HBSS and 0.5% BSA). The antibody recognized both CTB and CTB-H57A equally as assessed by ELISA and Western blotting. Binding curves were analyzed for mass action binding to fit one or two classes of receptor as previously reported (21). ATP depletion of glucose-starved T84 cell monolayers was achieved using antimycin A (10 μM) and 2-deoxyglucose (10 mM) following the method described by Bacallao *et al.* (22).

**Preparation of Lipid Rafts**—Wild-type or mutant cholera toxin B-subunits (20 nM) were applied to apical membranes of confluent monolayers of T84 cells in 45-cm<sup>2</sup> inserts at 4 °C. Cells were washed of any unbound toxins and used for isolation of detergent-insoluble membranes (lipid rafts) by sucrose equilibrium density centrifugation as

previously described (23). Representative fractions of the floating membranes and the soluble fractions were collected as needed.

**Endocytosis**—Wild-type or mutant cholera toxin B-subunits (20 nM) were applied at 4 °C for 45 min to confluent T84 cells grown on 96-well plates and washed three times with ice-cooled HBSS and once with warm HBSS (37 °C). Plates were then moved to 37 °C for the indicated times and back to 4 °C prior to immunolabeling. Binding of surface-bound toxin was visualized using rabbit polyclonal antiserum raised against the cholera toxin B-subunit (1:1000 in HBSS and 0.5% fish gelatin).

**Immunoprecipitation, SDS-polyacrylamide Gel Electrophoresis, and Western Blotting**—For studies of cell-associated toxin at 4 °C and after endocytosis, 20 nM CTB or CTB-H57A was applied apically to confluent monolayers of T84 cells in 5-cm<sup>2</sup> inserts at 4 °C for 45 min. Cells were washed of unbound toxins with ice-cooled HBSS and moved to 37 °C for the indicated times or kept at 4 °C until lysis in 400 μl of 5% SDS in water. Samples were boiled for 5 min, and the DNA was removed by hard vortexing and centrifugation (12,000 × g for 10 min). Samples were run on 10–20% denaturing Tris-HCl-polyacrylamide gels (Bio-Rad), transferred to nitrocellulose by electroblotting, Western-blotted using rabbit polyclonal antiserum raised against the cholera toxin B-subunit (1:1000 in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20) and HRP-conjugated goat secondary antibody, and detected with an enhanced chemiluminescence reagent (Pierce).

For immunoprecipitation, cells were treated with toxins as described above (both apically and basolaterally); scraped; and washed with buffer containing 0.34 M sucrose, 10 mM HEPES, 1 mM EDTA, and 0.1 mM MgCl<sub>2</sub> at 4 °C. Pellets were then extracted at 4 °C with 2 ml of buffer composed of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1.25% n-octyl β-D-glucopyranoside, 0.25% SDS, and 0.1% BSA with the addition of a complete protease inhibitor mixture (Roche Molecular Biochemicals). After solubilization of the cellular and cell-associated proteins, samples were clarified (10,000 × g for 10 min), and the B-subunit of CT was immunoprecipitated using specific antibodies covalently coupled to polystyrene beads (Polyscience) prepared according to the manufacturer's instructions. Immune complexes formed overnight at 4 °C were recovered by centrifugation (6000 × g for 6 min) and washed three times with water. Immunoprecipitated proteins and co-immunoprecipitated gangliosides were released from the beads by heating in SDS-polyacrylamide gel electrophoresis sample buffer at 100 °C for 5 min. For the detection of G<sub>M1</sub>, samples were run on polyacrylamide gels (any ganglioside will run with the dye front), transferred to nitrocellulose using a transfer buffer without methanol, and blotted with HRP-labeled cholera toxin B-subunit (3 nM in 1% BSA in Tris-buffered saline containing 0.1% Tween 20).

**Retrograde Transport Assay**—Wild-type and mutant H57A holotoxins were engineered to contain the sulfation consensus motif SAEDYEYEPES at the C terminus of their respective B-subunits.<sup>2</sup> Recombinant toxins were produced and purified as described above. For *in vivo* sulfation experiments, T84 cells were first washed three times with sulfate-free HBSS, kept at 37 °C for 1.5 h in sulfate-free HBSS, and then incubated with 0.5 mCi/ml Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> in the same buffer for 30 min. The sulfation toxin variants were added to T84 cells both apically and basolaterally to a final concentration of 20 nM, further incubated for 50 min at 37 °C, and then washed twice with ice-cold HBSS. Following total cell lysis, immunoprecipitation using rabbit anti-CTB antibodies as described previously (17) was performed. Samples were run on 10–20% denaturing Tris-HCl-polyacrylamide gels and analyzed with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

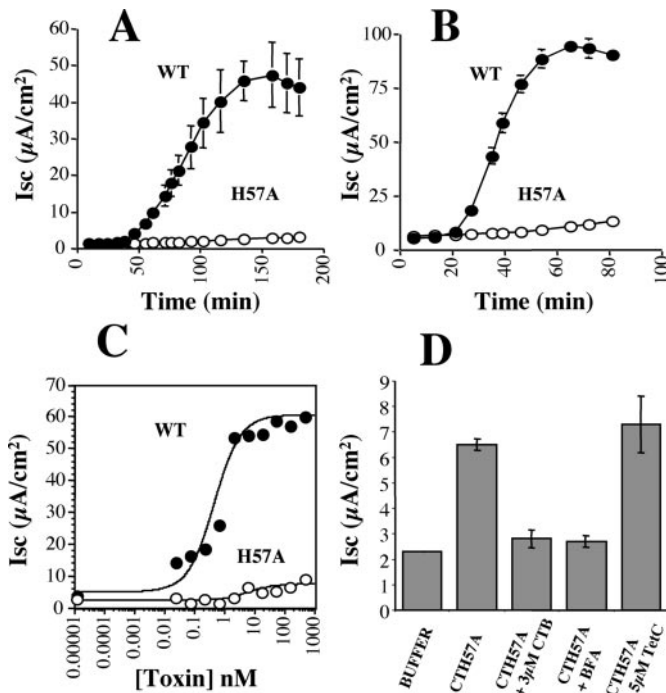
## RESULTS

**Production and Characterization of Wild-type and Mutant CT and CTB**—Both wt CT and the H57A variant (CT-H57A) were expressed and purified from *V. cholerae* O395NT supernatants. Expression and assembly into holotoxin of CT-H57A were equal to those observed with wt CT (data not shown). The purified CT B-subunit containing the H57A substitution exhibited stability in protein folding and high affinity binding to purified G<sub>M1</sub> as assessed by surface plasmon resonance (BIAcore; apparent  $K_d$  = 46 and 249 pM for wt CTB versus CTB-H57A when tested at 20 nM) (15) and modified G<sub>M1</sub> ELISA (apparent  $K_d$  ≈ 1 nM for both toxins).

Initial studies showed that the H57A substitution strongly

<sup>2</sup> Y. Fujinaga and W. I. Lencer, unpublished data.

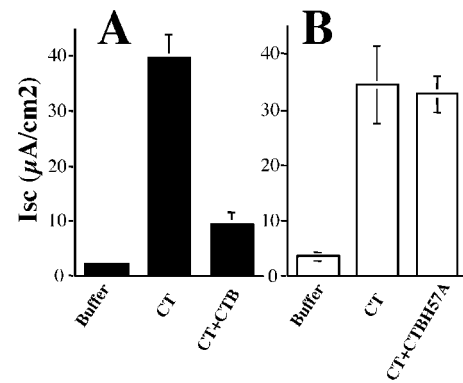




**FIG. 1. Mutant CT-H57A fails to induce a secretory response from T84 cells.** Shown is a time course of electrogenic Cl<sup>-</sup> secretion (*I*<sub>sc</sub>) induced by the addition of 20 nM wt CT (●) or mutant CT-H57A (○) to the apical (A) or basolateral (B) surface of T84 cell monolayers (with data points representing the means ± S.D. of two independent monolayers). Three independent experiments gave similar results. In C, the dose response induced by wt CT (●) or CT-H57A (○) was measured as electrogenic Cl<sup>-</sup> secretion (*I*<sub>sc</sub>) induced 110 min after the addition of toxin to the apical surfaces of polarized T84 cells. D shows that the Cl<sup>-</sup> secretion induced by the addition of 100 nM CT-H57A to the apical surfaces of polarized T84 cells could be inhibited by co-incubation of the toxin with 3 μM recombinant CTB or treatment with 5 μM brefeldin A (BFA), but not 5 μM tetanus toxin C fragment (*TetC*).

attenuated the toxicity of the holotoxin when applied to human intestinal T84 cell monolayers (Fig. 1). Both apical (Fig. 1A) and basolateral (Fig. 1B) cell surfaces were equally resistant to toxin application. Dose-response experiments showed that CT-H57A displayed an almost complete lack of toxicity (Fig. 1C). A small secretory response was, however, detected with CT-H57A concentrations >50 nM. This activity was completely inhibited by competition with excess wt CT B-subunit, but not tetanus toxin C fragment, which binds only ganglioside G<sub>D1b</sub> (Fig. 1D). Moreover, brefeldin A, a fungal metabolite that inhibits vesicular transport in the secretory pathway and vesicular exchange between endosomes and Golgi cisternae/ER in most eukaryotic cells (24, 25), including T84 cells (26), also inhibited CT-H57A-induced Cl<sup>-</sup> secretion (Fig. 1D). Thus, the small secretory response induced by high concentrations of CT-H57A was specific to toxin binding to G<sub>M1</sub> and sensitive to inhibition of retrograde vesicular transport into the ER. These characteristics are consistent with the mechanism of action described for wt CT (1, 27). Nonetheless, the H57A substitution rendered CT completely inactive when applied at toxin concentrations (20 nM) that far exceeded the apparent *K<sub>d</sub>* for binding G<sub>M1</sub> and were predicted to saturate 100% of available receptors at steady state (Fig. 1, A and B). These initial data implied that the H57A mutation uncoupled the CT-G<sub>M1</sub> complex from downstream signal transduction, presumably by affecting toxin trafficking into the cell.

Given the discrepancy between the *in vitro* binding studies and the *in vivo* toxicity data, we asked if the H57A toxin variant could bind G<sub>M1</sub> on T84 cell membranes with wild-type affinity as seen with immobilized G<sub>M1</sub> on plates. In initial



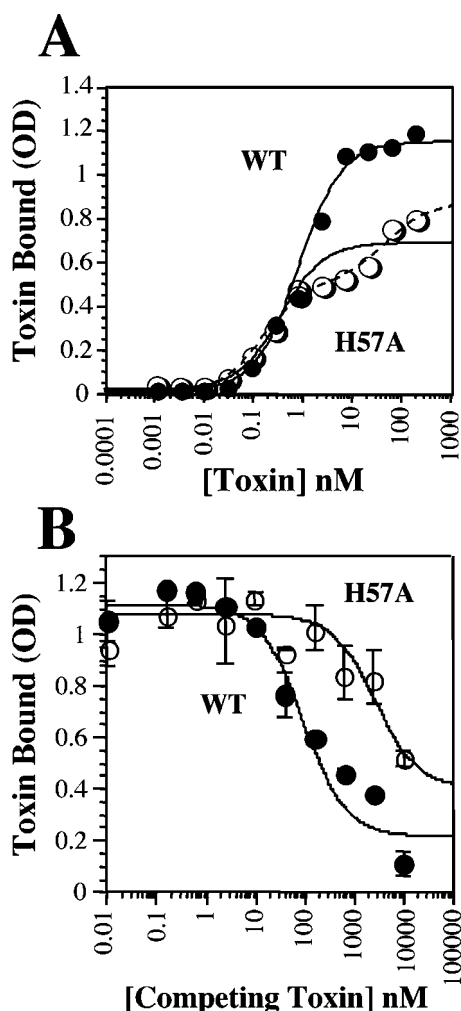
**FIG. 2. Mutant CTB-H57A fails to compete with CT for induction of Cl<sup>-</sup> secretion.** The secretory response (*I*<sub>sc</sub>) induced by 0.5 nM CT applied apically to polarized T84 cells was measured in the presence or absence of 500 nM competitor CTB (A) or CTB-H57A (B). Data points represent the means ± S.D. of two independent monolayers, with four independent experiments giving similar results.

studies, we compared the ability of wt and H57A variant B-subunits to act as competitive inhibitors of CT action on T84 cells. Fig. 2A shows that excess wt CT B-subunit effectively blocked the Cl<sup>-</sup> secretory response to wt holotoxin (0.5 nM applied apically), but excess B-subunit containing the H57A substitution had no effect (Fig. 2B). These results were not predicted based on the high affinity binding of CT-H57A to G<sub>M1</sub> as assessed *in vitro* (15). Rather, they indicated that CT-H57A exhibited a lower affinity binding to receptors on T84 cell membranes and failed to compete with wt CT for entry into the cell. To test this idea, toxin binding to confluent monolayers of T84 cells was assessed directly.

**Toxin Binding to Cell-surface Receptors**—Steady-state binding isotherms showed that CTB-H57A exhibited high affinity binding to T84 monolayers (apparent *K<sub>d</sub>* equal to that of wt CT as assessed in this assay), but did not recognize all binding sites available to wt toxin (Fig. 3A). Even at the highest doses tested (2.5 logs higher than that required for wt toxin to elicit maximal binding and a maximal physiologic response), still ~30–40% less CTB-H57A was bound to T84 cell membranes. These data were confirmed by Western blotting of total cell-associated toxin (data shown below in Fig. 6) and reproduced at 37 °C on T84 monolayers depleted of ATP to inhibit endocytosis. We also found that unlike wt CT, the binding isotherms for CTB-H57A fit only to a Michaelis-Menten model for two classes of receptors. In contrast, binding of wt CTB to T84 cells fit closely to a mass action model for a single class of receptor sites.

Apparent binding affinities for cell-surface receptors were also assessed for CTB and CTB-H57A by competition against wt CTB labeled with HRP. These studies indicated that CTB-H57A exhibited a notable change in binding affinity for native membrane receptors. Fig. 3B shows the binding isotherm for HRP-labeled CT B-subunit (3 nM) incubated with increasing doses of recombinant wt or H57A variant B-subunits as competitive ligands. Compared with wt CTB, CTB-H57A exhibited 30-fold lower affinity for binding T84 cell membranes. At the highest dose tested (10 μM), CTB-H57A displaced less than the full complement (~60%) of wt toxin from the cell surface.

When assessed for binding to purified G<sub>M1</sub> immobilized on plastic, the apparent ID<sub>50</sub> for CTB-H57A competition against HRP-labeled CTB was shifted 3–6-fold to the right (Fig. 4A), comparable to that found for toxin binding to G<sub>M1</sub> as assessed by surface plasmon resonance. These data implied that the H57A substitution affected the lectin activity of the B-subunit. In support of this idea, we found that, in contrast to wt CT, CTB-H57A failed to bind the closely related ganglioside G<sub>D1a</sub>

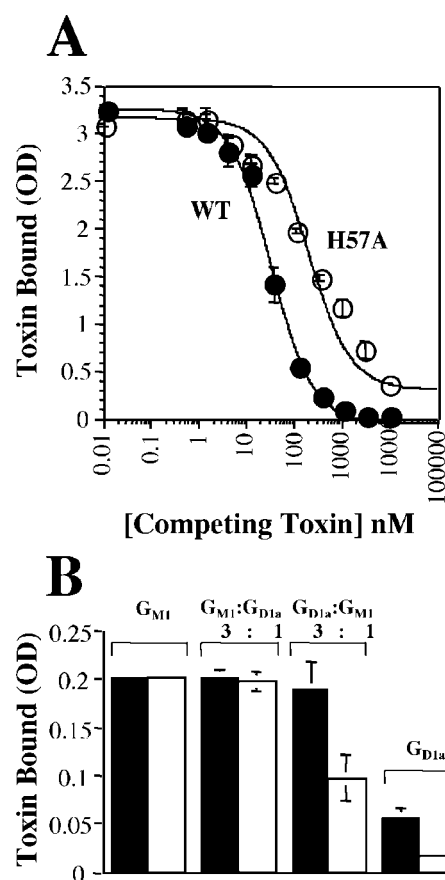


**FIG. 3. Binding of CTB-H57A to T84 cell membranes.** *A*, steady-state binding of wt CTB (●) and CTB-H57A (○) to the cell-surface membranes of T84 cells at 4 °C by modified ELISA, with data points representing the means  $\pm$  S.E. of four independent monolayers. *Solid lines* represent curve fits to a mass action model for binding a single class of receptors. The *dashed line* represents curve fit to a mass action model for binding two classes of receptors. Three independent experiments gave similar results. *B*, binding of 4 nM HRP-labeled CTB competed with increasing concentrations of wt CTB (●) or CTB-H57A (○) on the cell surface of T84 cells at 4 °C.

(Fig. 4*B*).  $G_{D1a}$  does not function as a receptor for CT in T84 cells (4), but can be used *in vitro* as an alternative lower affinity receptor for CT that differs structurally from  $G_{M1}$  by the addition of an extra sialic acid after the terminal galactose in the oligosaccharide head group. Thus, the H57A substitution had a direct but subtle effect on the lectin function of the B-subunit, as CTB-H57A exhibited a small but reproducible decrease in apparent binding affinity for the oligosaccharide domain of  $G_{M1}$  and failed to bind the alternative receptor  $G_{D1a}$ .

The H57A substitution disproportionately affected toxin binding to receptors localized within lipid rafts (Fig. 5, *A* and *B*). Compared with wt CTB, CTB-H57A did not fractionate preferentially with detergent-insoluble membrane microdomains (compare *first* and *third* lanes for CTB and *second* and *fourth* lanes for CTB-H57A). In these studies, both soluble and raft-associated toxins were recovered in their pentameric form (data not shown), indicating that Triton X-100 extraction had no effect on the stability of the wt or H57A B-pentamer.

Taken together, these data provide evidence for functional heterogeneity in receptor sites for CT on T84 cells that was evident only when assessed using B-subunits containing the



**FIG. 4. Binding of CTB-H57A to purified gangliosides.** *A*, binding of 4 nM HRP-labeled CTB competed with increasing concentrations of wt CTB (●) or CTB-H57A (○) on  $G_{M1}$  plates at 37 °C; *B*, direct binding of 5 ng of wt CTB (closed bars) or CTB-H57A (open bars) to wells coated with pure or a mixed ratio of  $G_{M1}$  and  $G_{D1a}$  liposomes (with data points representing the means  $\pm$  S.E. of three independent measurements). For each panel, three independent experiments gave similar results.

H57A mutation. Even at the highest toxin concentrations, CT-H57A bound at steady state to only a subset of receptors available to wt CT, and CT-H57A specifically failed to bind those receptors associated with detergent-insoluble membrane microdomains that are thought to be the functional binding sites. Still, at 4 °C, a significant fraction of CTB-H57A bound stably to T84 cell membranes and co-fractionated with apical membrane lipid rafts. At 20 nM CT-H57A, with 40–50% of the available receptors occupied, we would have predicted a functional response. However, this was not observed.

**Endocytosis of Wild-type and Mutant Toxins**—To explain why CT-H57A bound to cell-surface receptors, but failed to induce wild-type toxicity, we examined whether the toxin entered the cell. CTB or CTB-H57A was bound to T84 cell monolayers at 4 °C for 45 min, washed free of unbound toxin, and then shifted to 37 °C for the indicated times. Immunoreactive toxin remaining at the cell surface was then detected by modified cell-surface ELISA. Fig. 6*A* shows that after <1 min of incubation at 37 °C, the immunoreactive mass of CTB-H57A located at the cell surface was >3-fold less than that of wt CTB. Fig. 6*A* also shows that both toxins disappeared from the cell surface over time, consistent with the idea that both CTB and CTB-H57A were internalized by endocytosis. Data shown in Fig. 6*B* partially confirmed this interpretation. Total cell-associated toxin was assessed after incubations at 4 and 37 °C by extraction in SDS and analysis by Western blotting. As previously described, 3-fold more wt CTB than CTB-H57A bound to the cell surface at 4 °C, and lanes labeled *H57A(3X)* were

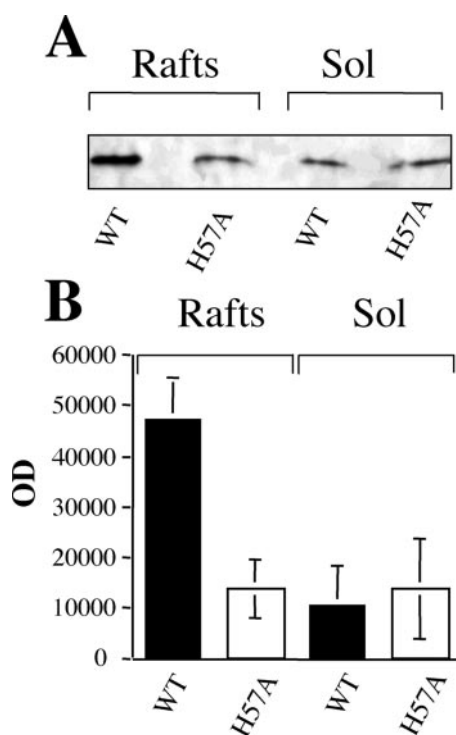


FIG. 5. Association of toxin-G<sub>M1</sub> complexes with detergent-insoluble membrane microdomains. A, Western blot for the toxin B-subunit contained in the lipid rafts or detergent-soluble fractions (Sol) of T84 cell monolayers exposed to 20 nM wt CTB or CTB-H57A; B, densitometry of three independent experiments as described in A (means  $\pm$  S.D.).

loaded with 3-fold more cell extracts (v/v). When shifted to 37 °C, the bulk of cell-associated CT-H57A declined rapidly compared with wt CT, with very little CT-H57A detected after 5-min incubations and none detected after 2-h incubations.

Nearly identical results were obtained when cell-associated toxin was assessed by immunoprecipitation of CTB from total cell extracts (Fig. 6C, second through fifth lanes (wt CTB) versus seventh through tenth lanes (CTB-H57A)). In these studies, we also tested for co-immunoprecipitation of the toxin-G<sub>M1</sub> complex by Western or ligand blotting. The data show that wt CTB co-immunoprecipitated with G<sub>M1</sub> under all experimental conditions, but CTB-H57A did not (Fig. 6C, first versus sixth lane). These results implied that the H57A substitution rendered the association between the B-subunit and G<sub>M1</sub> less stable after homogenizing the monolayers in *n*-octyl  $\beta$ -D-glucopyranoside, which dissolved lipid rafts and solubilized the ganglioside.

Thus, compared with wt CTB, nearly undetectable amounts of CTB-H57A remained cell-associated after incubations at 37 °C. Although at high toxin concentrations, a fraction of CT-H57A entered the cell and moved retrograde into the ER as evidenced by the induction of a small Cl<sup>-</sup> secretory response (Fig. 1, C and D), the bulk of CT-H57A somehow dissociated from the cell. However, these data do not rule out the alternative possibility that CT-H57A underwent rapid intracellular degradation after endocytosis.

To confirm this idea, that CT-H57A trafficked into the cell differently from wt toxin, we utilized a toxin variant engineered to contain the sulfation consensus motif SAEDYEYPS (28, 29). Tyrosine sulfation at this site is catalyzed by an enzyme localized to the *trans*-Golgi network, and this reaction can be used to directly measure toxin trafficking into this compartment.<sup>2</sup> Fig. 6D shows that when T84 monolayers were incubated for 50 min with Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> and CT containing the

SAEDYEYPS motif, sulfation at this site was readily observed. In contrast, when incubated under identical conditions, sulfation of the same toxin containing the H57A substitution was not detected. Thus, under physiologic conditions, wt CT trafficked from the cell surface into the Golgi apparatus of T84 cells, but the H57A variant did not.

#### DISCUSSION

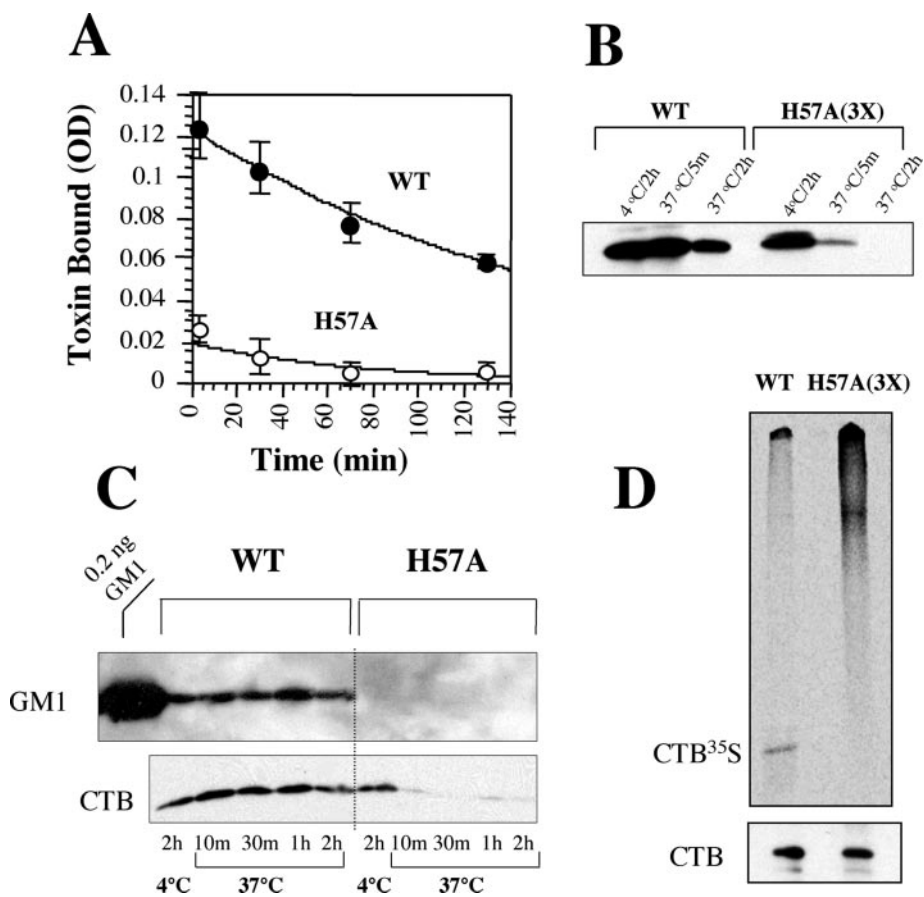
The results of this study indicate that CT requires stable high affinity binding to its glycolipid receptor, ganglioside G<sub>M1</sub>, for transport into the Golgi apparatus of target epithelial cells and the induction of toxicity. These data emphasize the importance of lipid trafficking in CT action. We also found that specific high affinity binding to G<sub>M1</sub> as assessed *in vitro* does not necessarily predict stable binding to receptors on membranes of intact cells or a functional toxin. Previous studies have demonstrated the absolute dependence on binding to G<sub>M1</sub> for toxin action, as inactivating mutations in the G<sub>M1</sub>-binding site of the CT B-subunit (30) or the construction of a chimeric toxin that switched the specificity for toxin binding from G<sub>M1</sub> to ganglioside G<sub>D1a</sub> (4) renders CT non-functional when applied to the polarized human intestinal cell line T84. Here, we defined the defect in cellular processing that caused the H57A toxin variant, which exhibits nearly full binding capacity for G<sub>M1</sub> (and carries a fully functional enzymatic subunit), to be almost completely inactive when applied to polarized monolayers of T84 cells.

Our data show that a subtle attenuation in the lectin function of the B-subunit caused by the H57A substitution correlated with a significant defect in toxin binding to receptors displayed on native cell membranes and almost a complete loss of toxin function. Both the apparent binding affinity and the total number of receptors on T84 cells accessible for toxin binding were reduced by the H57A substitution. We found evidence for heterogeneity in structure and presumably function of G<sub>M1</sub>-containing receptor sites on T84 cell surfaces, and the bulk of membrane receptors restricted from access to CT-H57A co-localized with lipid rafts that are thought to be the functional receptor sites for CT action (4, 31). The H57A variant exhibited less stable binding to G<sub>M1</sub> *in vitro*, and when tested on intact cells, very little CT-H57A remained cell-associated after incubations at physiologic temperature. In contrast to wt CT, no detectable toxin containing the H57A substitution was transported into the Golgi apparatus. These data support the view that G<sub>M1</sub> functions as the essential sorting motif, although perhaps not fully sufficient, for retrograde trafficking into the Golgi cisternae and ER of target epithelial cells.

Two structural explanations for the subtle change in binding to the G<sub>M1</sub> oligosaccharide exhibited by the H57A toxin variant are readily apparent from solution of the crystal structures for wt CT and LTI. First, in the wt proteins, histidine 57 in the B-subunit makes van der Waals contact with the terminal galactose of G<sub>M1</sub> (11, 14). Substitution at this site removes this contact and may plausibly reduce the stability of toxin binding. Second, the crystal structure of CTB-H57A bound to galactose (15) shows that the H57A substitution displaces almost the entire loop (residues 51–58) toward the central pore of the B-pentamer and away from the oligosaccharide domain of G<sub>M1</sub>, with all subunits of the pentamer equally effected. Such a shift in the position of the Glu<sup>51</sup>–Ile<sup>58</sup> loop may interfere with the initial interactions between the toxin and cell-surface G<sub>M1</sub> or, alternatively, may reduce the flexibility of this loop in accommodating to or interacting with cell-surface components surrounding or competing for the G<sub>M1</sub> oligosaccharide. In addition, Glu<sup>51</sup> (which forms direct hydrogen bonds) and Gln<sup>56</sup>, His<sup>57</sup>, and Ile<sup>58</sup> (which normally make a network of solvent-mediated hydrogen bonds to the G<sub>M1</sub> oligosaccharide) are displaced, and



**FIG. 6. CTB-H57A fails to enter T84 cells.** *A*, rate of endocytosis for wt CTB (●) and CTB-H57A (○) measured as the disappearance of cell surface-bound toxin. T84 cells preincubated at 4 °C with 20 nM concentrations of the respective toxins were moved to 37 °C for the indicated times, and the specific surface-exposed toxin was measured by modified ELISA. *B*, total cell-associated CTB before and after endocytosis as assessed by Western blotting of total cell lysates. Lanes labeled H57A(3X) were loaded with 3-fold more cell extracts. *C*, immunoprecipitation of CTB from total T84 cell extracts before and after endocytosis and co-immunoprecipitation of the CT B-subunit with ganglioside G<sub>M1</sub>. Toxin B-subunits were detected by Western blotting, and G<sub>M1</sub> was detected by ligand blotting using HRP-labeled CTB as ligand. *D*, sulfation of wt CTB and CTB-H57A containing the sulfation motif SAEDYEYPS appended to the C terminus of the B-subunit after 50 min of incubation with T84 cells at 37 °C. The lower panel shows that equal amounts of cell-associated toxin were loaded in each lane after immunoprecipitation from total T84 cell extracts. Immunoprecipitation of CTB-H57A was performed on a 3-fold greater mass of T84 cell lysates.



this may also contribute to the observed decrease in lectin activity.

The differential kinetics of binding of CTB-H57A to T84 cell membranes compared with those of wt CTB indicate that all G<sub>M1</sub>-binding sites on host cells cannot be equal. Such functional heterogeneity in receptor sites could be due to differences in the structure of the ceramide domain of G<sub>M1</sub> itself. A similar observation has been made for Shiga toxin-glycolipid globotriaose interaction (32). Alternatively, differences in the cell-surface display of G<sub>M1</sub> on target cell membranes may affect the density or accessibility of the G<sub>M1</sub> oligosaccharide for toxin binding. It is also formally possible that certain binding sites contain other molecules that interact with the Glu<sup>51</sup>-Ile<sup>58</sup> loop to stabilize the CT-G<sub>M1</sub> complex. Such a proposed diversity in structure of membrane microdomains that contain G<sub>M1</sub> can be readily found in T84 cells. For example, both raft and non-raft membrane microdomains contain G<sub>M1</sub>,<sup>3</sup> and this alone may explain the observed heterogeneity in receptor function. We have also found direct evidence for diversity in lipid raft structure by differential fractionation of CT-containing RAFTs with the caveola-associated protein caveolin-1 (23), and other groups have shown that a subset of lipid rafts from the same cell form specific “ganglioside signaling domains” (33).

The 30-fold lower apparent binding affinity of CT-H57A for T84 cell membranes was not predicted from our *in vitro* studies on toxin binding to G<sub>M1</sub> immobilized on plastic or BIAcore chips. In recent studies on *E. coli* LTIIa, we have also found that despite the apparent high affinity of LTIIa for G<sub>M1</sub> immobilized on plastic, this toxin cannot bind G<sub>M1</sub> displayed on the surface of T84 cells.<sup>4</sup> Such results emphasize the dependence of

toxin binding on the context in which ganglioside receptors are displayed on native cell membranes together with other cell-surface components. Thus, it is worth restating the point that, although entirely speculative, the stability of wt CT binding to membrane receptors may depend not only on the lectin activity of the B-subunit, but also on the presence of a functional (or flexible) Glu<sup>51</sup>-Ile<sup>58</sup> loop, which may interact with other membrane components, as recently proposed by Aman *et al.* (15). Such cooperative interactions by wt toxin, among other possibilities, could enhance the affinity and stability of binding to cell-surface receptors or increase the efficiency of toxin action, or both.

When applied to polarized human intestinal T84 cells, CT-H57A exhibited almost no toxicity and was not detected in Golgi cisternae or in total cell lysates 50 min or 2 h after uptake, respectively. Although at high toxin concentrations, a fraction of CT-H57A clearly entered the cell by binding cell-surface receptors and moved retrograde into the ER as evidenced by the induction of a Cl<sup>-</sup> secretory response, these data imply that the bulk of CT-H57A disassociated from the cell or was degraded rapidly. Thus, very little CT-H57A reached the Golgi apparatus, which is necessary for retrograde trafficking into the ER, where the A-subunit unfolds (3) and presumably translocates to the cytosol. The wild-type CT B-subunit does not efficiently enter a degradative pathway and typically exhibits a long half-life after internalization into host cells, presumably because the B-subunit resides in Golgi stacks or recycles between the ER and Golgi at steady state. We have been able to detect the B-subunit in T84 cells 48 h after initial exposure.<sup>5</sup> Given these findings, it is therefore all the more surprising that CT-H57A failed to remain associated with T84

<sup>3</sup> A. A. Wolf, P. Fredman, and W. I. Lencer, unpublished data.

<sup>4</sup> S. Wimer-Mackin, R. K. Holmes, A. A. Wolf, W. I. Lencer, and M. G. Jobling, submitted for publication.

<sup>5</sup> C. Rodighiero and W. I. Lencer, unpublished data.

cells after only 5 min of incubation at physiologic temperatures.

Based on the altered apparent stability of CT-H57A for binding  $G_{M1}$ , we propose that CT-H57A fails to induce toxicity in T84 cells because the bulk of the mutant toxin dissociates from  $G_{M1}$ -containing receptor sites before or after endocytosis. In the absence of binding stably to membrane receptors, CT-H57A would be handled as any other fluid-phase cargo that inefficiently enters the endocytic compartment and sorts into late endosomes and lysosomes for intracellular degradation (34). In contrast, wt CT, which remains stably tethered to the membrane by high affinity binding to  $G_{M1}$ , moves efficiently into the endosome by receptor-mediated endocytosis and then traffics into the Golgi and ER, which is required for toxicity. Thus, toxin action depends on stable formation of the CT B-subunit- $G_{M1}$  complex. These data explain the critical function of the toxin's B-subunit to stably cluster a pentameric ring of a specific glycolipid that anchors the toxin to the membrane. This unique protein-lipid complex acts as a necessary sorting motif for retrograde trafficking into the cell.

*Acknowledgments*—We thank Dr. T. A. Aman for providing the pATA21 plasmid containing the CTB-H57A construct, Dr. M. Kenny for purifying the wild-type and mutant B-subunits, Dr. S. Wimer-Mackin for assistance with studies using tetanus toxin C fragment, Dr. R. S. Pitman for critical reading of the manuscript, and M. Ferguson-Maltzman for expert technical assistance.

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## **A Cholera Toxin B-subunit Variant That Binds Ganglioside G<sub>M1</sub> but Fails to Induce Toxicity**

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*J. Biol. Chem.* 2001, 276:36939-36945.

doi: 10.1074/jbc.M104245200 originally published online July 30, 2001

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Access the most updated version of this article at doi: [10.1074/jbc.M104245200](https://doi.org/10.1074/jbc.M104245200)

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