Cholera Toxin Entry into Pig Enterocytes Occurs via a Lipid Raft- and Clathrin-Dependent Mechanism†

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ABSTRACT: The small intestinal brush border is composed of lipid raft microdomains, but little is known about their role in the mechanism whereby cholera toxin gains entry into the enterocyte. The present work characterized the binding of cholera toxin B subunit (CTB) to the brush border and its internalization. CTB binding and endocytosis were performed in organ-cultured pig mucosal explants and studied by fluorescence microscopy, immunogold electron microscopy, and biochemical fractionation. By fluorescence microscopy CTB, bound to the microvillar membrane at 4 °C, was rapidly internalized after the temperature was raised to 37 °C. By immunogold electron microscopy CTB was seen within 5 min at 37 °C to induce the formation of numerous clathrin-coated pits and vesicles between adjacent microvilli and to appear in an endosomal subapical compartment. A marked shortening of the microvilli accompanied the toxin internalization whereas no formation of caveolae was observed. CTB was strongly associated with the buoyant, detergent-insoluble fraction of microvillar membranes. Neither CTB's raft association nor uptake via clathrin-coated pits was affected by methyl-β-cyclodextrin, indicating that membrane cholesterol is not required for toxin binding and entry. The ganglioside GM_1 is known as the receptor for CTB, but surprisingly the toxin also bound to sucrase−isomaltase and coclustered with this glycosidase in apical membrane pits. CTB binds to lipid rafts of the brush border and is internalized by a cholesterol-independent but clathrin-dependent endocytosis. In addition to GM_1, sucrase−isomaltase may act as a receptor for CTB.

Bacterial toxins employ different strategies to intoxicate their target cells, and in particular the endocytic trafficking pathways whereby toxins enter cells have been the focus of many studies in recent years (*1*, *2*). Clathrin-dependent endocytosis, where ligands bound to cell-surface receptors are recruited to coated membrane invaginations and subsequently pinched off in vesicles, remains the best studied mechanism (*3*, *4*). However, clathrin-independent pathways exist as well and in many cases appear to be the preferred route whereby toxins gain access to their target cells (*2*, *5*, *6*). These pathways instead rely on the presence of lipid rafts at the cell surface, i.e., microdomains enriched in cholesterol and sphingolipids (*7*−*9*). Lipid rafts have been implicated in a number of physiologically important cell membrane-related processes, including signal transduction (*10*, *11*), cholesterol transport (*12*, *13*), and pathogen invasion (*14*−*16*), but their general ability to act as lateral sorting platforms also makes them exploitable by toxins as entry points into target cells (*2*, *5*). Morphologically, lipid rafts may appear as caveolae, small flask-shaped invaginations of the cell membrane implicated in endocytosis (*17*, *18*), but over the past few years it has become increasingly clear that raft microdomains are dynamic structures that vary in both shape and size (*19*, *20*).

The exotoxin of *Vibrio cholera* (cholera toxin, CT)[1] belongs to the family of structurally related AB_5 proteins consisting of five B subunits that mediate binding to the cell surface and a single A subunit that exerts the toxic effect (*21*−*23*). The ganglioside GM_1 [Galβ3GalNAcβ-(NeuNAcα2,3)4Galβ4Glcβcer], a widespread membrane lipid, is generally considered to be the main natural receptor for CT (*24*, *25*), and uptake mechanisms of CT in different cell types have been studied by a number of investigators (*2*, *5*). Unfortunately, no clear picture has yet emerged concerning the question whether CT is taken up by clathrin-dependent or -independent pathways. Thus, although its glycolipid receptor is frequently used as a marker for lipid rafts and caveolae, clathrin-dependent endocytosis of CT has been reported to occur in Caco-2 cells (*26*) as well as in neuronal cells (*27*). On the other hand, GM_1-containing lipid rafts reportedly are depleted within clathrin-coated pits in COS-7 cells (*28*), and CT is found within caveolin-positive regions of the cell surface of a number of cell types (*29*). Consequently, a caveolae/raft-dependent endocytosis, defined

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[1] Abbreviations: CT, cholera toxin; CTB, cholera toxin B subunit; HRP, horseradish peroxidase; SAC, subapical compartment.

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by clathrin independence and sensitivity to cholesterol depletion, has been proposed as an alternative pathway for toxin internalization (*30*, *31*).

Although the small intestinal epithelium is the prime natural target of *V. cholera*, little is known about the entry mechanism of CT into enterocytes. Unlike other types of cell surfaces, the enterocyte brush border membrane is an extremely effective diffusion barrier that normally is nonpermissive with regard to endocytosis of macromolecules by any mechanism (*32*). The brush border contains a high amount of glycolipids (*33*), and several of its major digestive enzymes, including sucrase−isomaltase, are known to reside in lipid raft microdomains (*34*−*37*). However, unlike lipid rafts from most other cell membranes, those of the brush border largely resist cholesterol depletion, and they are organized as structures that seem to be stabilized mainly by interactions between the glycolipids and the galactosyl-binding lectin galectin-4 (*38*, *39*).

In the present work we studied the internalization of cholera toxin B subunit (CTB) across the brush border of enterocytes. The toxin rapidly induced a massive formation of apical clathrin-coated pits and vesicles, and although strongly associated with lipid rafts, CTB was internalized via coated pits/vesicles into an endosomal subapical compartment. Interestingly, CTB internalization was resistant to treatment with methyl-β-cyclodextrin, indicating that its lipid raft association is cholesterol-independent. In addition to GM_1, CTB bound to lipid raft-associated sucrase−isomaltase at the microvillar cell surface, and coclustering of toxin and glycosidase was observed in pits between adjacent microvilli, suggesting an internalization of the receptor−ligand complex.

MATERIALS AND METHODS

Materials. Cholera toxin B subunit (CTB) from *V. cholerae*, rabbit anti-cholera toxin, horseradish peroxidase (HRP), methyl-β-cyclodextrin, 2-methylbutane, and gold-labeled goat anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin were obtained through Sigma. The cholera toxin B subunit−Alexa Fluor 488 conjugate was obtained from Molecular Probes (Copenhagen, Denmark). A mouse monoclonal antibody to clathrin heavy chain was from Transduction Laboratories (Lexington, KY). The rabbit polyclonal antibodies to porcine sucrase−isomaltase and galectin-4 used were previously described (*40*). Antifade mounting media, HRP-coupled swine anti-rabbit IgG, and rabbit anti-mouse IgG were from DAKO (Glostrup, Denmark).

Jejunal segments of pig small intestine, taken 1−2 m from the pylorus of anesthetized animals, were kindly provided by Letty Klarskov and Mette Olesen from the Department of Experimental Medicine, The Panum Institute.

Organ Culture of Intestinal Mucosal Explants. After a rinse of the intestinal segments in Hanks' buffered salt solution, mucosal explants were excised and cultured in NEM medium as described previously (*41*). Toxin binding to the explants was performed for 30 min at 4 °C by addition of 5 μg/mL CTB or the CTB−Alexa Fluor 488 conjugate to the medium. For toxin internalization, the explants were quickly washed in cold medium, transferred to 37 °C, and cultured for periods of 5−15 min. In some experiments, the mucosal explants were preincubated with 1% (w/v) methyl-β-cyclodextrin for 1 h for cholesterol extraction. In experiments with HRP, the fluid phase marker (5 mg/mL) was present during the entire culture period.

Subcellular Fractionation. Microvillar and Mg^{2+}-precipitated (basolateral plus intracellular) membranes were prepared from intestinal mucosa by the divalent cation method (*42*), as previously described (*43*). Briefly, the tissue was homogenized in a Potter-Elvehjem homogenizer. The homogenate was cleared by centrifugation at 500*g* for 10 min, and $MgCl_2$ was added to a final concentration of 10 mM. After incubation for 10 min on ice, the preparation was centrifuged at 1500*g* for 10 min to pellet intracellular and basolateral membranes. Finally, the supernatant was centrifuged at 48000*g* for 30 min to obtain a pellet of microvillar membrane vesicles and a supernatant of soluble proteins.

Lipid Raft Analysis. Lipid raft analysis by detergent extraction followed by sucrose gradient centrifugation (*44*) was performed essentially as described previously (*34*) with the modification that the extract was placed in a cushion of 60% sucrose with a gradient of 50−25% sucrose on top. After centrifugation overnight in a swinging bucket rotor at 35000 rpm (g_{max} = 217000), the gradients were collected in 12 1-mL fractions for subsequent analysis by SDS−PAGE and immunoblotting. In some experiments, the floating fractions were carefully collected by a pipet, diluted five times with 25 mM HEPES−HCl and 150 mM NaCl, pH 7.1, and centrifuged at 48000*g* for 1 h to obtain a pellet of lipid rafts for further biochemical analysis.

A "superraft" analysis was performed essentially as previously described (*38*). Briefly, microvillar membrane vesicles, prepared as described above, were suspended in 25 mM HEPES−HCl and 150 mM NaCl, pH 7.1, and incubated with 2 μg/mL CTB on ice for 30 min. The membranes were then pelleted by centrifugation at 20000*g* for 10 min, washed once in the same buffer, resuspended, and extracted with 1% Triton X-100 on ice for 10 min. After centrifugation at 20000*g* for 10 min, the supernatant (S_0, containing nonraft proteins) and pellet were collected. The pellet was resuspended in the above buffer and reextracted with 1% Triton X-100 at 20 °C. After centrifugation, the supernatant (S_{20}) and pellet fractions were collected again. Finally, the detergent extraction and centrifugation steps were repeated at 37 °C to obtain a supernatant (S_{37}) and pellet (P_{37}, containing superrafts and insoluble cytoskeletal proteins).

Electrophoresis and Immunoblotting. SDS−PAGE in 10% or 15% polyacrylamide gels was performed according to Laemmli (*45*). After electrophoresis and electrotransfer onto Immobilon, immunoblotting was performed with primary antibodies to either cholera toxin, sucrase−isomaltase, galectin-4, or clathrin heavy chain, followed by incubation with HRP-coupled secondary anti-immunoglobulin antibodies. Blots were developed by an electrochemiluminescence detection reagent kit according to the protocol supplied by the manufacturer (Amersham Biosciences).

Fluorescence Microscopy. The mucosal explants were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 2 h at 4 °C. After a rinse in 0.1 M sodium phosphate buffer, pH 7.2, the mucosal explants were frozen in precooled 2-methylbutane and mounted on a precooled cryostat table. Ten micrometer thick sections were cut in a Leitz cryostat at −20 °C and collected on glass slides. The

sections were mounted in antifade mounting medium and examined in a Leica DM 4000 B microscope equipped with a Leica DC 300 FX camera.

Electron Microscopy. For ultrastructural analysis the mucosal explants were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 2 h at 4 °C. The explants were postfixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.2, for 1 h at 4 °C, dehydrated in acetone, and finally embedded in Epon according to standard procedures. Ultrathin sections were cut on an LKB Ultrotome III ultramicrotome, stained in 1% uranyl acetate in water and lead citrate, and finally examined in a Zeiss EM 900 electron microscope equipped with a Mega View camera system. For single and double immunogold labeling, identical mucosal explants were fixed in 2% paraformaldehyde/0.1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 2 h at 4 °C. After a rinse in the above buffer the explants were infused with 2.3 M sucrose containing 1% paraformaldehyde overnight, then mounted on top of a metal pin, and frozen in liquid nitrogen. Ultrathin sections were cut in an RMC 6000 XL ultracryomicrotome, and immunogold labeling using antibodies against CTB, HRP, clathrin heavy chain, and sucrase−isomaltase was performed as previously described (*46*). Control experiments were performed by omission of the primary antibody, and in immunogold double labeling experiments the second primary antibody was omitted.

Morphometric Analysis. The length of the microvilli was estimated by measuring 50 randomly chosen microvilli cut at a perpendicular angle. The number of coated pits/coated vesicles in the apical area (up to 1 μm below the brush border) was counted in 25 randomly chosen tight junction-to-tight junction sections of enterocytes.

RESULTS

Internalization of CTB Bound to Mucosal Explants. The CTB−Alexa Fluor 488 conjugate distinctly stained the entire brush border of enterocytes after culture for 30 min at 4 °C but was not detectable inside the cells or at the basolateral side of the cells, indicating that the toxin did not leak through the brush border or the tight junctions (Figure 1A). However, when excess fluorescent CTB was removed by a brief wash with medium and the temperature shifted to 37 °C for 15 min, the toxin was rapidly internalized into the enterocytes (Figure 1B). This experiment thus shows that the mucosal explant system is suitable for studying binding and internalization of CTB.

CTB Induces Rapid Formation of Apical Coated Pits and Vesicles. The enterocyte glycocalyx-coated brush border is a digestive/absorptive surface that normally acts as an efficient diffusion barrier preventing luminal macromolecules from entering the cell. In agreement with this function, Figure 2A shows electron micrographs of the apical brush border where, in the absence of CTB, coated pits are rarely seen between adjacent microvilli. However, when added, CTB rapidly reduced the microvillar length and induced the formation of numerous clathrin heavy chain−positive coated pits and vesicles below the apical cell surface (Figure 2B−F; Table 1). To visualize the induction of endocytosis, HRP, a marker for fluid phase uptake, was added to the medium. In the absence of CTB, HRP only marginally increased the number of coated pits/vesicles after 15 min of culture at 37 °C (Table 1), and only modest amounts of HRP

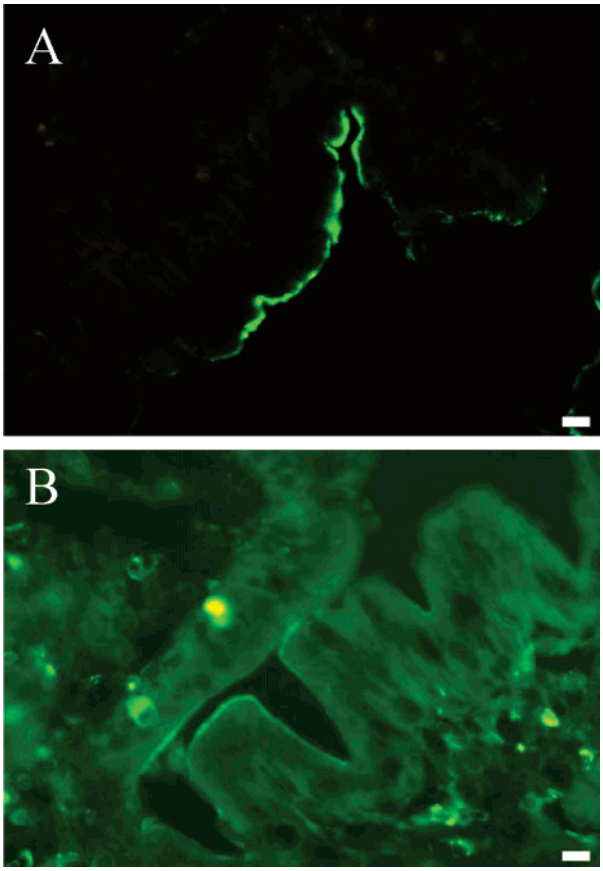

FIGURE 1: CTB binding and entry into enterocytes. Mucosal explants were cultured in the presence of the CTB−Alexa Fluor 488 conjugate for 30 min at 4 °C, followed by culture at 37 °C for 15 min, as described in Materials and Methods. (A) Binding of fluorescent CTB to the brush border at 4 °C. (B) Internalization of fluorescent CTB into enterocytes after 15 min at 37 °C. Notice that the cytoplasm and the basolateral plasme membrane of the enterocytes are devoid of labeling at 4 °C. Bars = 10 μm.

Table 1: CTB Induces Formation of Coated Pits and Vesicles[a]

treatment	total no. of coated pits/ 25 cell sections	total no. of coated vesicles/ 25 cell sections	microvillus length (μm)
CTB + HRP, 0 min	11	4	1.57 ± 0.38
CTB + HRP, 5 min	36	49	1.08 ± 0.29
CTB + HRP, 15 min	46	83	1.03 ± 0.39
CTB + HRP + MβCD, 0 min	10	7	1.39 ± 0.18
CTB + HRP + MβCD, 5 min	45	69	1.01 ± 0.19
CTB + HRP + MβCD, 15 min	36	110	0.95 ± 0.27
HRP, 15 min	17	15	1.45 ± 0.26

[a] Mucosal explants, preincubated in the absence or presence of methyl-β-cyclodextrin (MβCD), were cultured for 30 min at 4 °C in the presence of CTB, followed by culture for the indicated periods of time at 37 °C in the presence of HRP. As a control experiment, mucosal explants were cultured in the presence of HRP alone. After culture, the explants were analyzed by electron microscopy, the number of coated pits/vesicles in the apical region was counted, and the length of the microvilli was measured, as described in Materials and Methods. The data listed for the microvillus length are the mean values ± SD.

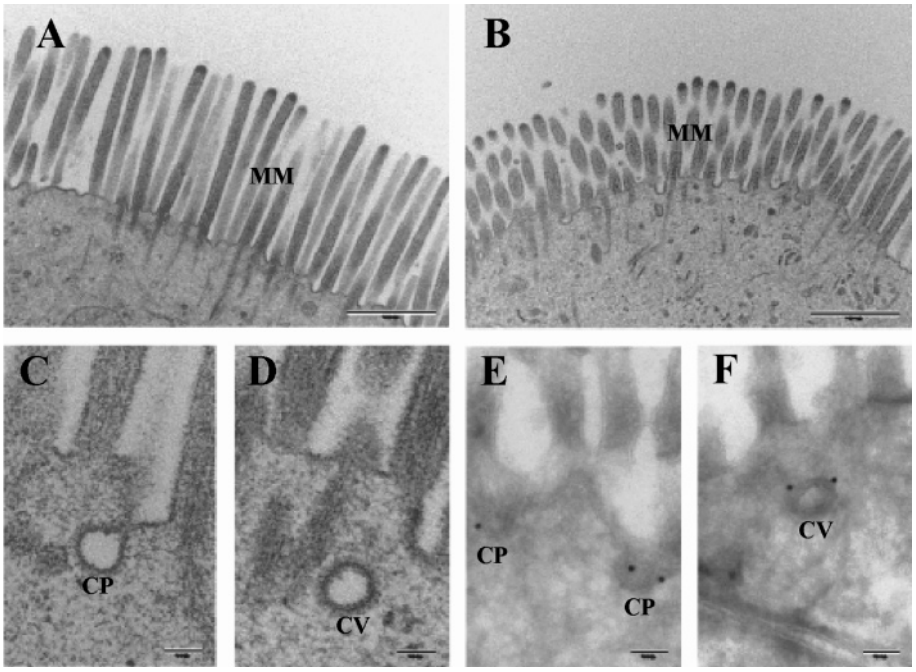

FIGURE 2: CTB induces formation of apical coated pits and vesicles. Electron micrographs of the microvillar membrane of mucosal explants incubated at 4 °C in the absence (A) or presence (B−F) of CTB and shifted to culture at 37 °C for 15 min. Notice the increased number of coated pits in (B). (C and D) Higher magnification images of a coated pit (CP) and coated vesicle (CV), respectively. (E and F) In ultracryosections immunogold labeling for clathrin heavy chain is seen in coated pits and coated vesicles. Bars = 1 μm (A and B) and 0.1 μm (C−F).

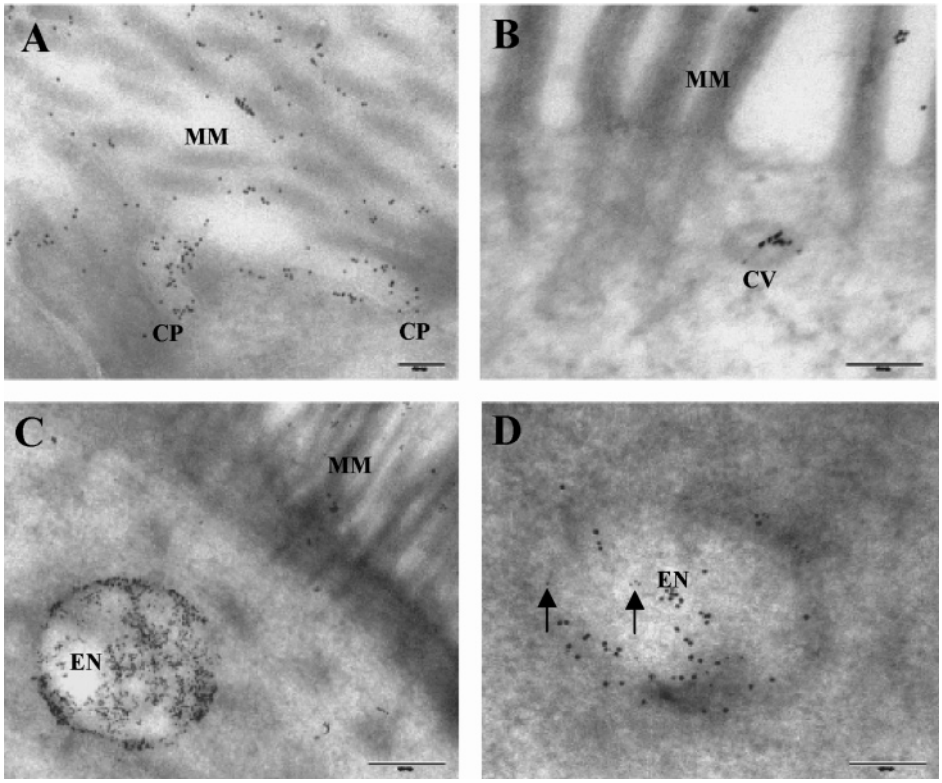

FIGURE 3: CTB induces apical endocytosis of HRP. Mucosal explants were cultured in the presence of CTB for 30 min at 4 °C and then shifted to culture at 37 °C for 5 min in the presence of HRP. (A) Immunogold labeling for HRP along microvilli (MM) and in coated pits (CP). (B) Immunogold double labeling for HRP (large particles) and clathrin heavy chain (small particles). Notice HRP in the lumen and clathrin heavy chain in the membrane of a coated vesicle (CV). (C) HRP labeling accumulated in an apical endosome (EN). (D) Immunogold double labeling for HRP (large particles) and CTB (small particles, indicated by arrowheads) in an apical endosome. Bars = 500 nm (A and C) and 200 nm (B and D).

were taken up into the endosomal subapical compartment (SAC) of enterocytes (data not shown). In the presence of CTB, however, HRP concentrated in apical coated pits, and massive amounts of the marker accumulated together with CTB in the SAC within 5 min at 37 °C (Figure 3). In addition, HRP was seen in clathrin heavy chain−positive

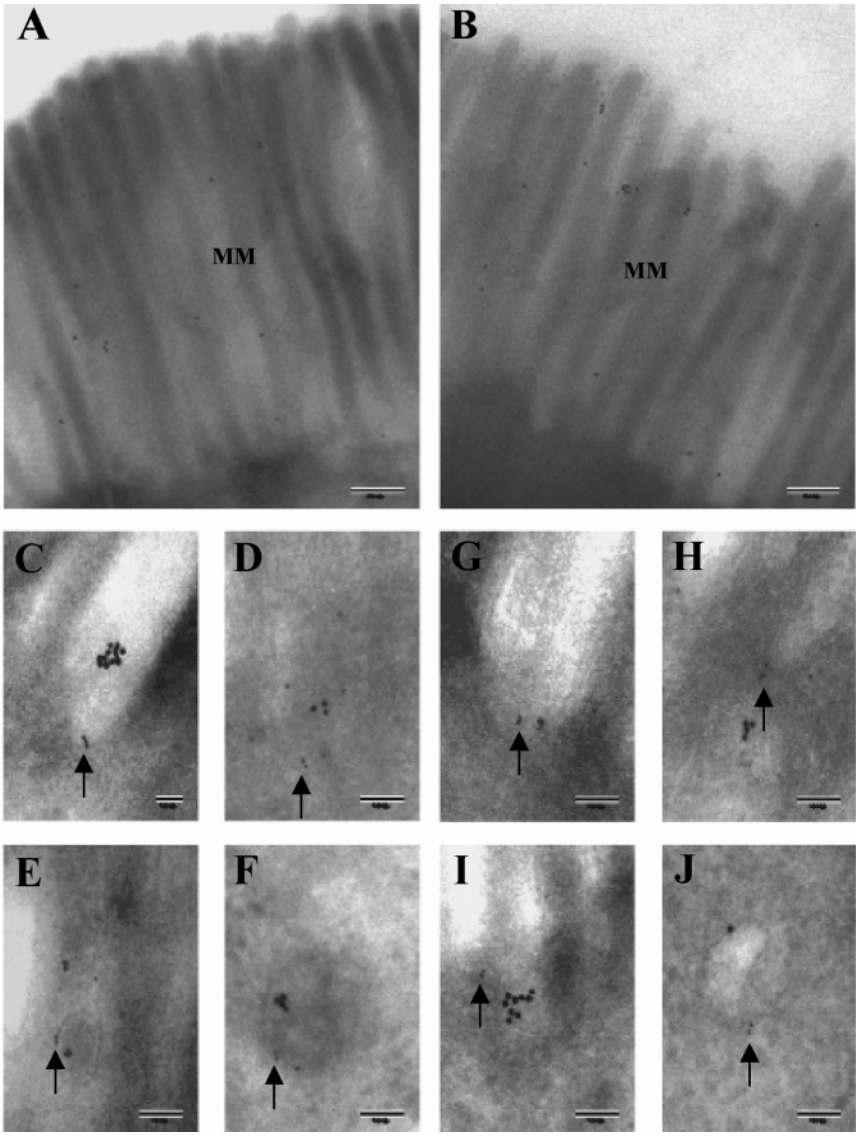

FIGURE 4: Cholesterol independence of CTB binding and internalization. Immunogold labeling electron micrographs of the apical region of mucosal explants cultured in the absence (A, C−F) or presence (B, G−J) of methyl-β-cyclodextrin for 1 h as described in Materials and Methods. (A and B) At 4 °C, CTB binds to the microvilli (MM) regardless of the pretreament with methyl-β-cyclodextrin. (C−F and G−J) After the temperature is raised to 37 °C for 5 min, CTB (large particles) appears in coated pits and vesicles (CV) where clathrin heavy chain (small particles, arrows) is also seen. Bars = 500 nm (A and B), 200 nm (C), and 100 nm (D−J).

small apical vesicles (Figure 3B). At the apical surface labeling for CTB itself was seen over the microvilli, and like HRP, it colocalized with clathrin heavy chain in coated pits and vesicles (Figure 4).

To summarize, the data presented above show that CTB induces the formation of clathrin-coated pits at the enterocyte brush border and employs the clathrin-dependent endocytic pathway to enter the cell. In contrast, CTB induced no formation of morphologically recognizable caveolae at the brush border. Although sparse labeling for caveolin-1 has been reported in the brush border region of enterocytes (*47*, *48*), flask-shaped caveolae are hard to detect between adjacent microvili. Deep apical tubules, a recently described lipid raft-containing region of the brush border membrane present between adjacent microvilli (*47*), were rarely CTB-positive (data not shown). Therefore, although other mechanisms of internalization cannot be entirely excluded, these results indicate that CTB is taken up into enterocytes mainly by a rapid induction of clathrin-mediated endocytosis.

The cholesterol dependence of binding and uptake of CTB was studied by preincubation of the mucosal explants for 1 h with methyl-β-cyclodextrin, a drug previously shown to extract >60% of the microvillar cholesterol in the organ culture system (*49*). By immunogold electron microscopy, cholesterol depletion had no visible effect on the binding of CTB to the microvilli and the accumulation of toxin in clathrin-coated pits and vesicles (Figure 4, Table 1). Thus, CTB binding to the brush border and its subsequent entry into the cell are not critically dependent on the concentration of membrane cholesterol.

Lipid Raft Analysis of CTB Binding to Microvillar Membrane Vesicles. Figure 5 shows a lipid raft analysis by sucrose gradient centrifugation of microvillar membrane vesicles following a short incubation with CTB. The bulk of microvillar proteins was detergent soluble, but some components, including the major brush border enzyme sucrase−isomaltase, were partially associated with lipid rafts, as previously described (*34*−*36*). CTB bound to the mi-

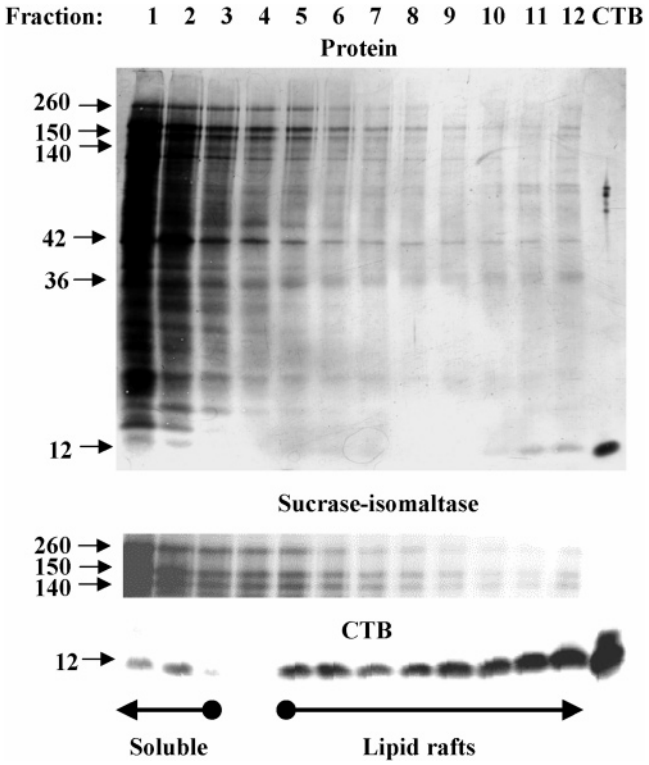

FIGURE 5: CTB binds to microvillar lipid rafts. Microvillar membrane vesicles suspended in 25 mM HEPES−HCl and 150 mM NaCl, pH 7.1, were incubated for 30 min on ice with 5 μg/mL CTB. After incubation, the membranes were pelleted by centrifugation at 20000*g* for 10 min and washed once in the same buffer. The membranes were then resuspended in the above buffer, extracted with 1% Triton X-100 on ice for 10 min, and subjected to sucrose gradient centrifugation as described in Materials and Methods. Samples of equal volume from the fractions of the gradient and a sample of CTB were analyzed by SDS−PAGE in a 15% gel, followed by immunoblotting for CTB and sucrase−isomaltase and finally staining for protein with Coomassie Brilliant blue. Molecular mass values (kDa) and fractions of soluble proteins and lipid rafts are indicated.

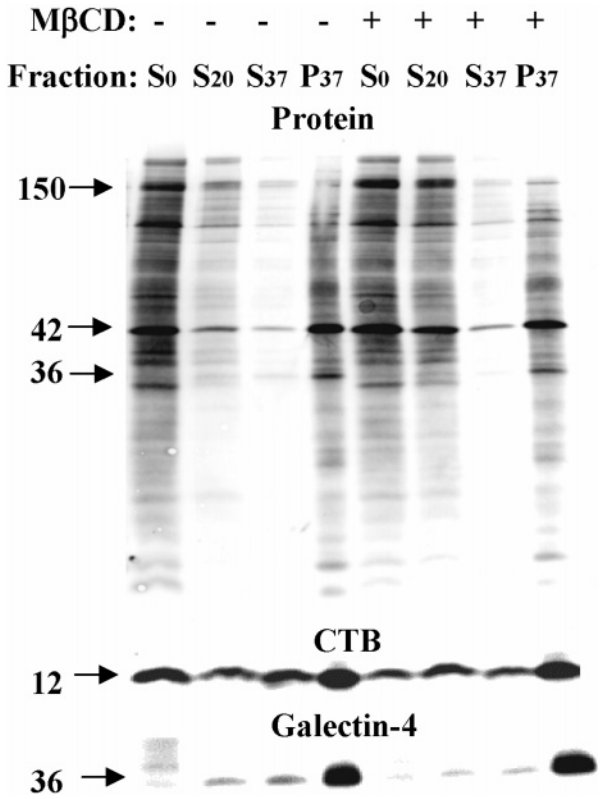

FIGURE 6: Superraft analysis of CTB associated with microvillar membrane vesicles. Microvillar membrane vesicles were resuspended in 25 mM HEPES−HCl and 150 mM NaCl, pH 7.1, and incubated at room temperature for 30 min in the presence or absence of 1% methyl-β-cyclodextrin (MβCD), followed by an incubation for 30 min on ice in the presence of 2 μg/mL CTB. The membranes were then extracted repeatedly with 1% Triton X-100 on ice at 20 °C and finally at 37 °C as described in Materials and Methods. The resulting fractions S_0 (containing nonraft proteins), S_{20}, S_{37}, and P_{37} (containing superraft proteins) were analyzed by SDS−PAGE in a 15% gel by immunoblotting for CTB and galectin-4. Total protein was visualized by staining with Coomassie Brilliant blue. Molecular mass values (kDa) are indicated.

crovillar membranes was almost exclusively associated with the lipid raft fractions, reflecting the microdomain localization of GM_1, its glycolipid receptor.

To assess further the relative strength of CTB's association with microvillar lipid rafts, its extractibility with Triton X-100 in subsequent steps at increasing temperature was studied by a superraft analysis (*38*). As shown in Figure 6, a major part of the toxin partitioned in the superraft fraction (P_{37}) following extraction at 37 °C. The microvillar lectin galectin-4, which like CTB probably binds to the terminal galactosyl residue of GM_1, was likewise strongly enriched in the superraft fraction and has previously been proposed to function as a core raft stabilizer/organizer for more loosely raft-associated proteins (*38*). Therefore, the presence of CTB in this fraction indicates a strong association with the glycolipid-based core of microvillar membrane microdomains. In addition, Figure 6 shows that preincubation with methyl-β-cyclodextrin did not reduce the lipid raft association of CTB. Overall, these results agree well with the data shown in Figure 5 and Table 1 and with our previous observation that although methyl-β-cyclodextrin removes most of the microvillar cholesterol, the lipid raft organization of the membrane is largely unaffected by this treatment (*39*).

Lipid Raft Analysis of CTB Binding to Mucosal Explants. Figure 7 shows a lipid raft analysis of CTB associated with the microvillar- and Mg^{2+}-precipitated (basolateral and intracellular) membranes of mucosal explants, following incubation with the toxin at 4 °C for 30 min and subsequent culture at 37 °C for 10 min. In the microvillar fraction, CTB was mainly seen in the lipid raft fractions, similar to the binding of the toxin to lipid rafts of microvillar membrane vesicles in vitro (Figure 5). In the same membrane fraction, clathrin heavy chain was mainly detergent soluble, but a small part could be detected in the lipid raft fractions. A substantial amount of CTB was present in the Mg^{2+}-precipitated membranes, representing the fraction of the toxin internalized by 10 min. Here, the major part of CTB was detergent soluble and thus no longer associated with lipid rafts.

Sucrase−Isomaltase as a Cholera Toxin Receptor. Figure 8A is an overlay/blotting experiment, showing distinct binding of CTB to major polypeptides of a microvillar membrane fraction with molecular mass values of 260, 150, and 140 kDa. Since prominent polypeptides of this size represent the proform (260 kDa) of pig brush border sucrase−isomaltase and the constituent isomaltase (150 kDa) and sucrase (140 kDa) subunits of the cleaved proform,

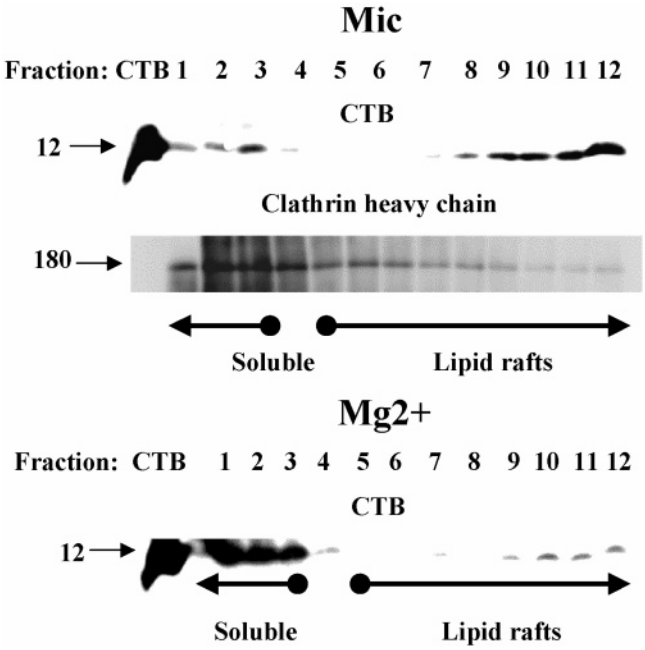

FIGURE 7: CTB internalization in mucosal explants and dissociation from lipid rafts. Mucosal explants were cultured for 30 min at 4 °C in the presence of 5 μg/mL CTB. The explants were then briefly washed with medium to remove unbound CTB and then cultured for 10 min at 37 °C in CTB-free medium. After culture, the microvillar (Mic) and Mg^{2+}-precipitated (Mg^{2+}) fractions were isolated and subjected to lipid raft analysis as described in Materials and Methods. Finally, the gradient fractions were analyzed by SDS−PAGE in 15% gels together with samples of CTB. CTB and clathrin heavy chain were visualized by immunoblotting. Molecular mass values (kDa) and fractions of soluble proteins and lipid rafts are indicated.

respectively (*40*), this binding suggests that the glycosidase might act as a receptor for CTB. To test this hypothesis, microvillar membranes, solubilized by Triton X-100 at 37 °C, were incubated with protein A−Sepharose beads coated with CTB absorbed to anti-CTB antibodies. As shown in Figure 8B, sucrase−isomaltase specifically bound to the beads, confirming that this brush border enzyme indeed functions as a receptor for the toxin. In addition, CTB and sucrase−isomaltase were frequently seen by immunogold double labeling to colocalize along the microvilli and in membrane pits between adjacent microvilli (Figure 9), suggesting that the glycosidase may act as an internalization receptor for the toxin. However, Figure 5 shows that, after binding to microvillar membranes and Triton X-100 extraction, CTB was predominantly distributed in the lighter raft fractions whereas sucrase−isomaltase predominantly localized in the soluble fractions and heavier raft fractions. Therefore, the glycosidase can only account for a minor part of the microvillar binding sites for CTB.

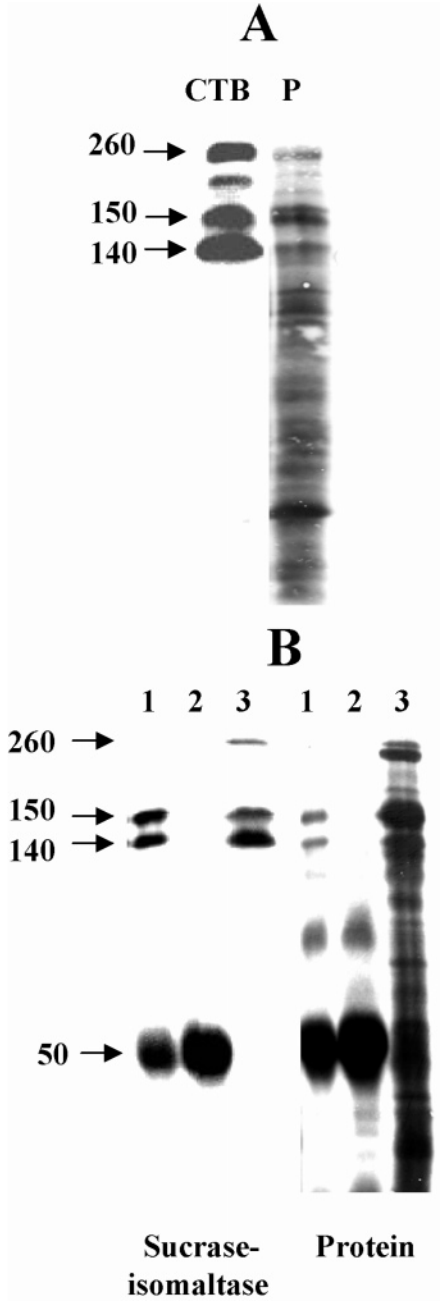

FIGURE 8: CTB binds to sucrase−isomaltase. (A) SDS−PAGE of a microvillar membrane fraction in a 10% gel. After electrophoresis and electrotransfer onto Immobilon, the membrane was incubated with 1 μg/mL CTB in 25 mM HEPES−HCl and 150 mM NaCl, pH 7.1, for 2 h. After a wash, bound CTB was visualized by immunoblotting and total protein (P) by staining with Coomassie Brilliant blue. (B) Microvillar membranes suspended in 25 mM HEPES−HCl and 150 mM NaCl, pH 7.1, were solubilized by 1% Triton X-100 for 10 min at 37 °C. The extracts were cleared by centrifugation at 20000*g* for 10 min and incubated with either protein A−Sepharose coated with anti-CTB antibodies alone (2) or protein A−Sepharose coated with anti-CTB antibodies preincubated with CTB (1). After incubation for 1 h at room temperature, the protein A−Sepharose beads were washed twice in the above buffer and analyzed by SDS−PAGE in a 10% gel together with a sample of solubilized microvillar membranes (3). After electrophoresis, sucrase−isomaltase was visualized by immunoblotting and total protein by staining with Coomassie Brilliant blue. Molecular mass values (kDa) are indicated. The broad bands of 50 kDa represent the IgG heavy chain of the anti-CTB antibodies.

DISCUSSION

The main conclusion of the present work was the observation that CTB entry into enterocytes is mediated by a mechanism that includes *both* binding to lipid raft components and induction of a clathrin-mediated endocytosis. The latter process is otherwise commonly considered to be lipid raft-independent, the receptors for transferrin and LDL being the typical detergent-soluble model cargo proteins for this process. Concerning the lipid requirement for clathrin-dependent endocytosis, phosphoinositides at the cytosolic leaflet of the bilayer have been proposed to play a role by

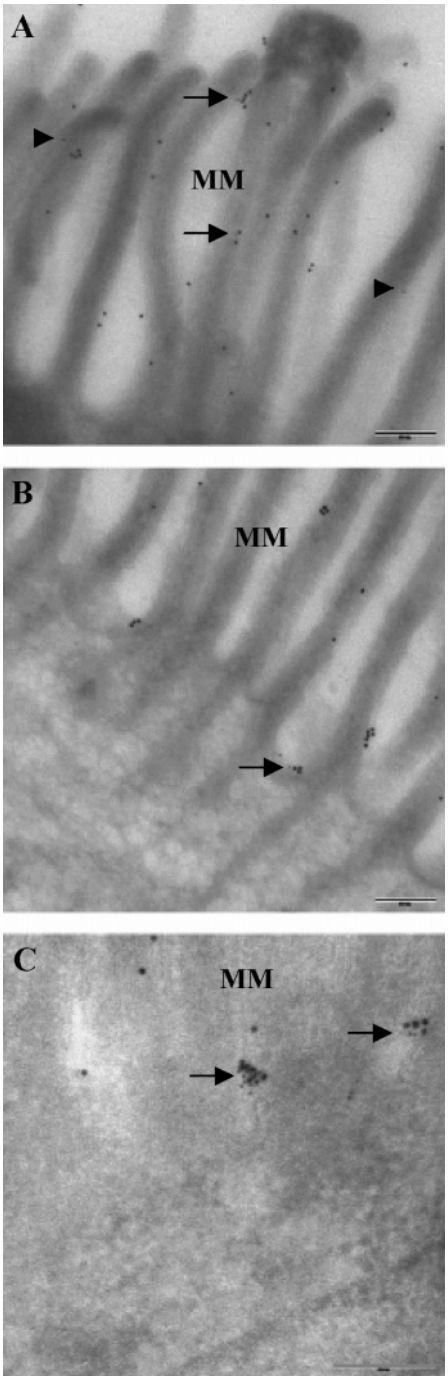

FIGURE 9: Coclustering of CTB and sucrase−isomaltase. Immunogold double labeling electron micrographs of sucrase−isomaltase (large particles) and CTB (small particles) in the apical region of mucosal explants, treated as described in Materials and Methods. (A) Some CTB and sucrase−isomaltase are colocalized in the microvillar membrane (arrows), but CTB is also seen unassociated with the brush border enzyme (arrowheads). (B and C) Coclustering of CTB and sucrase−isomaltase in pits between adjacent microvilli (arrows). Bars = 200 nm.

interacting specifically with various proteins during coated pit nucleation, including synaptotagmin and the adaptor complex AP-2 (*4*). Although uncommon, other examples of lipid raft association combined with clathrin-mediated endocytosis have been reported. Thus, the B-cell receptor clusters into lipid rafts upon ligand binding (*50*) but is subsequently internalized by a clathrin-mediated endocytosis (*51*). Here, induction of the endocytic process required a lipid raft-dependent tyrosine phosphorylation of clathrin heavy chain by a member of the Src family of kinases. In addition, a similar sequence of events has been proposed for entry into target cells of the anthrax toxin produced by *Bacillus anthracis* (*52*). Interestingly, the lipid raft-associated glycosylphosphatidylinositol-anchored prion protein is constitutively endocytosed by coated pits but has been shown to leave the lipid raft domain into a nonraft membrane prior to entry into coated pits (*53*). Although microvillar CTB in the present work seemed to be predominantly associated with lipid rafts, the small amounts of detergent-soluble toxin might represent a subfraction enriched in coated pits. Clearly, more work is needed to define in greater detail the fate of lipid raft components during entry by clathrin-dependent endocytosis.

The dense brush border architecture normally prevents endocytic uptake of macromolecules by any mechanism, and the way in which CTB binding triggers the significant increase in the number of apical coated pits and vesicles is not known. The strong cross-linking of GM_1 ganglioside by the pentameric toxin, reflected in its superraft localization, might provide a change in membrane curvature that favors entry into coated pits. Thus, in a recent study, cargo capture was proposed to stabilize newly formed coated pits and prevent them from collapsing (*54*).

The pig intestinal brush border is highly enriched in glycosphingolipids, including GM_1 (*33*), which in other cell types selectively stimulate caveolar endocytosis (*55*, *56*). We cannot exclude the possibility of a caveolar uptake taking place simultaneously with the clathrin-mediated endocytosis, but the absence of morphologically recognizable caveolae at the apical cell surface, regardless of the presence of CTB, argues against this. In addition, we observed no accumulation of the toxin in the deep apical tubules between adjacent microvillar rootlets which are likely centers for apical membrane trafficking (*47*). Furthermore, both caveolae and deep apical tubules are sensitive to cholesterol depletion, and methyl-β-cyclodextrin would therefore be expected to affect their functioning. By their strategic location, extending up to 1 μm into the terminal web region, these caveolin-1-positive tubules may instead, as previously suggested (*47*), play a role in trans- and exocytotic trafficking.

A characteristic property of CTB entry into enterocytes was that cholesterol depletion of the membrane had no detectable bearing on the process. In other cell types, sensitivity to this treatment is otherwise considered a hallmark of caveolae/lipid raft-dependent membrane traffic (*30*, *31*). However, that cholesterol is not essential for lipid raft formation and stability in the enterocyte microvillar membrane has previously been observed for a number of resident proteins (*38*, *39*) and is likely to reflect the existence of atypical, stable microdomains, the core of which consists of glycolipids cross-linked by galectin-4 rather than cholesterol (*37*). The prominent presence of CTB in the superraft fraction observed in the present work indicates that it associates strongly with these core microdomains. A similar lack of cholesterol dependence of CTB internalization via clathrin-mediated endocytosis was previously reported to occur in hippocampal neurons (*27*), and together these observations underline the notion that lipid raft association per se is neither clear evidence *for* endocytosis via caveolae nor evidence *against* a clathrin-mediated mechanism. Clearly, the relationship between lipid raft microdomains and different types of

endocytosis mechanisms is more complex than commonly thought, and further studies are required to define more precisely the parameters that decide which endocytic pathway a given cell is likely to employ.

Rabbit sucrase−isomaltase has previously been shown to contain a functional receptor for toxin A of *Clostridium difficile* (*57*), and the heat-labile toxin of *Escherichia coli*, a protein structurally related to CTB, has earlier been shown to bind to this major brush border enzyme from rat and rabbit (*58*, *59*). In these studies, CTB itself showed either no or little affinity for sucrase−isomaltase, and it has been concluded that the receptor GM_1 saccharide is restricted to glycolipids and has not been convincingly detected in glycoproteins (*60*). In addition, a study using generation of GM_1−neoganglioproteins has indicated that these proteins appear to behave as nonfunctional receptors for cholera toxin (*61*). Nevertheless, a specific association between CTB and pig sucrase−isomaltase was shown in the present work biochemically as well as by immunogold double labeling electron microscopy. Since detergent-solubilized sucrase−isomaltase could be captured by CTB coated to beads, the brush border enzyme seems capable of functioning independently as a CTB receptor. In addition, the detectable coclustering of toxin and glycosidase in pits between adjacent microvilli suggests that at least some CTB enters the cell as a receptor−ligand complex with sucrase−isomaltase. Clearly, further work is needed to evaluate the potential role of this major brush border enzyme as a toxin receptor.

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