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Review

Membrane traffic and the cellular uptake of cholera toxin

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Abstract

In nature, cholera toxin (CT) and the structurally related *E. coli* heat labile toxin type I (LTI) must breech the epithelial barrier of the intestine to cause the massive diarrhea seen in cholera. This requires endocytosis of toxin-receptor complexes into the apical endosome, retrograde transport into Golgi cisternae or endoplasmic reticulum (ER), and finally transport of toxin across the cell to its site of action on the basolateral membrane. Targeting into this pathway depends on toxin binding ganglioside GM1 and association with caveolae-like membrane domains. Thus to cause disease, both CT and LTI co-opt the molecular machinery used by the host cell to sort, move, and organize their cellular membranes and substituent components.

Keywords: Cholera toxin; *E. coli* heat labile toxin; Ganglioside GM1; Retrograde vesicular traffic; Caveolae

Contents

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1. Introduction

Colonization of the small intestine by *Vibrio cholerae* and toxigenic *Escherichia coli* results in diarrhea due to massive salt and water secretion without epithelial damage [1]. The secretory diarrhea is induced in large part by the direct action of cholera toxin (CT) or *E. coli* type I heat labile toxin (LTI) on polarized intestinal epithelial cells and is mediated by an increase in intracellular cAMP generated by adenylate cyclase [2,3]. Movement of toxin into the cell by endocytosis and vesicular transport is required for bioactivity. This depends exclusively on interactions of toxin with the target cell as neither *V. cholerae* nor toxigenic *E. coli* invade the epithelium or assist the delivery of toxin into the cell by other mechanisms.

The human intestine is lined with a continuous monolayer of polarized epithelial cells that exhibit circumferential tight intercellular junctions sealing one cell to another and preventing passive diffusion. This single-cell thick monolayer forms the rate-limiting barrier against solute transport and microbial invasion, both of which are essential for intestinal function. To gain entry into the epithelial barrier, both CT and LTI co-opt the molecular machinery used by the host epithelial cell to sort, move, and organize their cellular membranes and substituent components. This depends on the ability of CT and LTI to opportunistically bind a specific cell surface lipid, ganglioside GM1, for entry and navigation through the cell. For *V. cholerae*, this was a critical evolutionary choice as binding to other endogenous or experimentally created cell surface receptors, whether protein or lipid, renders the toxin inactive [4–6].

Over the last 30 years, studies on the biology of cholera toxin have impacted significantly on diverse fields in the life sciences including signal transduction, mucosal immunology, lipid and protein biochemistry, and more recently membrane dynamics. This review will focus on membrane dynamics. We attempt to summarize the evidence that CT and LTI must utilize endogenous mechanisms of vesicular transport to enter the cell and induce disease. The membrane systems engaged by CT and LTI are fundamental to the structure and function of eukaryotic cells themselves. In transporting epithelia, membrane traffic accounts ultimately for the formation and maintenance of cell polarity and thus the ability of the epithelium to separate distinct compartments and regulate the transport of water, solutes, and microorganisms between the outside world and the interstitium.

2. Toxin structure

CT and LTI are closely related toxin's whose A- and B-subunits have been defined structurally at 1.25–2.3 Å resolution [7–10] (Fig. 1). The B-subunit is comprised of five identical polypeptides (11.5 kDa) that assemble into a highly stable pentameric ring. The A-subunit is a single polypeptide (28 kDa) comprised of two major domains (A_1 and A_2). The two domains are linked by a surface exposed loop which contains a site for proteolytic cleavage and a single disulfide bond which bridges the cleavage site [8,9,11].

The assembled pentameric B-subunit (55 kDa) binds stoichiometrically to five GM1 molecules on the host cell plasma membrane with high affinity (K_d and < 1 nM) and specificity. The A-subunit exhibits enzymatic activity as a combined ADP-ribosyltransferase and NAD glycohydrolase [12,13]. Proteolytic cleavage within the exposed loop connecting the two domains of the A-subunit and reduction generates the enzymatically more active A_1-peptide (≈ 22 kDa) [14]. The A_1-peptide must enter the cytoplasm to activate adenylyl cyclase by catalyzing the ADP-ribosylation of the α-subunit of the heterotrimeric GTPase Gs (reviewed in [11]). The A_2-peptide (≈ 5 kDa) interacts extensively, but non-covalently, with the central core of the B-subunit pentameric ring

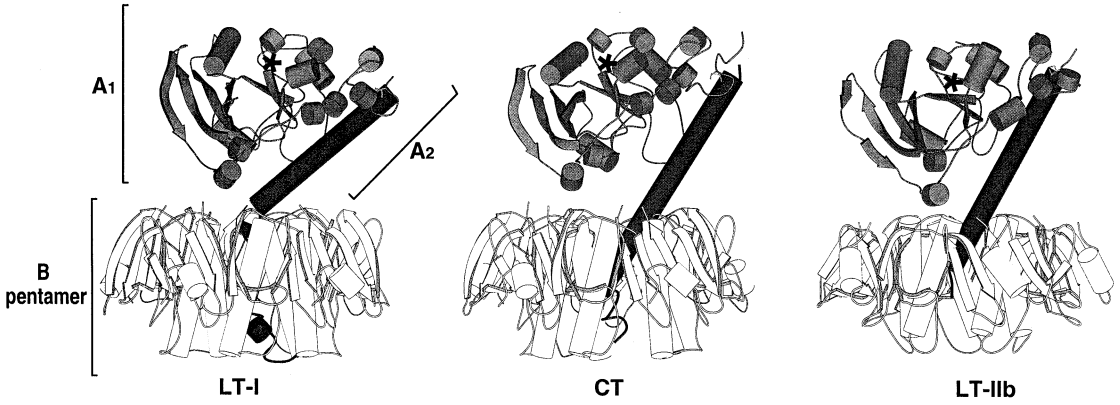

Fig. 1. Crystal structures of CT, LTI, and LTIIb are shown viewed from the side so that the base of the B-pentamers would face the plasma membrane of target cells and the A-subunits would face away from the membrane. B-pentamers are shown in white, A_1-peptides in light gray, and A_2-peptides in dark gray. The * identifies the catalytic site in the A_1-peptides. These figures were kindly provided by Ethan Merritt and Wim Hol, University of Washington, Seattle, WA.

[9,10] and forms the scaffolding which tethers the A_1-peptide and B-subunits together. The A_2-peptide also exhibits a C-terminal ER-targeting motif K(R)DEL. The K(R)DEL residues protrude from the pentameric B-subunit on the side that binds GM1 at the cell surface, where they may plausibly interact with membrane receptors [8].

3. Toxin action: the view prior to 1990

Elegant and persuasive studies over the last 30 years have defined the A-subunits of CT and LTI as potent ADP-ribosyltransferases and NAD-glycohydrolases in vivo and the small GTP-binding protein family of ADP-ribosylating factors (ARF) as allosteric co-factors for this reaction in vitro [15–17]. In both intact and broken cells, the A-subunits catalyze the ADP-ribosylation of the regulatory heterotrimeric GTPase Gsα. The reaction inactivates the intrinsic GTPase activity of the G-protein and thus induces a prolonged activation of adenylyl cyclase with associated rise in intracellular cAMP and concomitant cellular response(s) [18–20].

The B-subunits of both toxins have been defined clearly as pentameric lectins with high affinity and specificity for ganglioside GM1. GM1 was identified as the sole functional receptor for CT (reviewed recently in [11,21,22]), but the B-subunit of LTI also displays affinity for other gangliosides in the GM1 series and unidentified cell surface galactoproteins exhibiting polylactosylaminoglycan carbohydrates [23,24]. Binding of LTI to a galactoprotein receptor also results in a cellular response [25,26]. While the A-subunit alone can activate adenylyl cyclase in broken or permeabilized cells, the B-subunit is essential for toxin action on intact cells.

When studied in vitro using isolated or solubilized membrane fractions, CT was found to act immediately on its substrates in an apparent diffusion limited reaction. When applied to intact cells, however, toxin-induced activation of adenylyl cyclase was not detected until at least 10–40 min after CT bound to the cell surface [27,28]. This characteristic delay in CT and LTI action was termed the 'lag phase' and was thought to represent the time required for the A-subunit to cross the plasma membrane and interact with Gs inside the cell [29]. The time between toxin binding at the cell surface and toxin-induced activation of adenylyl cyclase in the cell interior corresponded closely to the time required for reduction of the A-subunit to the A_1-peptide [28]. Reduction of the A-subunit was thought to occur in the cytosol. Only the A-subunit was found to penetrate biologic membranes [30,31], and translocation of the A-subunit into the cytosol was considered the rate-limiting step in toxin action. All subsequent steps leading to ADP-ribosylation of Gs and activation of adenylyl cyclase in the cell interior were presumed to be diffusion limited. On the other hand, CT was shown by light and electron microscopy to enter endosomes and the *trans*-Golgi compartment of host cells

[32,33], and it was suggested that endocytosis and vesicular transport may play a physiologic role in toxin function [34,35]. This role, however, remained undefined.

4. Toxin action: dependence on membrane dynamics

The current view that toxin action depends on entry into host cells by membrane traffic rests on evidence obtained from studies performed within the last 10 years. Correlations between toxin action and endocytosis were first made in cell fractionation studies on rat hepatocytes intoxicated with CT in vivo. These experiments showed that reduction of the A-subunit and activation of adenylyl cyclase were associated with the appearance of the A_1-peptide in intracellular, but not plasma membrane fractions [36,37]. In rat hepatocytes, CT-induced activation of adenylyl cyclase appeared to depend on endosome acidification, but these data were not confirmed in other studies using different models of toxin action [38–40].

The idea that CT entered the host cell by membrane traffic was given further support by our studies on polarized cells. In polarized intestinal epithelial cells, the physiologic site of toxin binding on the apical membrane is separated spatially from adenylyl cyclase on the cytoplasmic surface of the basolateral membrane by circumferential tight junctions. Thus, simply translocating across the apical membrane of polarized cells would not necessarily be sufficient for bioactivity because in polarized cells the A-subunit (or another signaling molecule) would still require movement across the cell to activate its target effector [41]. The action of CT on polarized epithelial cells was modeled using the human intestinal cell line T84. T84 cells grown on permeable supports form confluent monolayers of columnar epithelia that exhibit high transepithelial resistance, polarized apical and basolateral membranes, and a cAMP-regulated Cl^- secretory pathway analogous to that found in intact intestine [42]. This system is particularly relevant because the model requires that CT enter the cell and transduce a signal from the apical membrane as must occur in vivo.

Studies on polarized T84 cell monolayers showed that signal transduction by CT applied apically depended on entry into the cell by membrane traffic. As assessed by direct epifluorescence microscopy, the kinetics and temperature dependencies of CT-induced Cl^- secretion correlated closely with endocytosis and movement of toxin inside the cell to a position consistent in location with apical recycling and late endosomes and Golgi cisternae (Fig. 2) [41,43]. The strength of these correlations were enhanced by parallel assessment of toxin action by entry into the cell through the contralateral basolateral membrane. Basolaterally applied CT also elicited a Cl^- secretory response, but with shorter lag phase and faster kinetics. Like apically applied CT, the time course and temperature dependencies of basolaterally applied CT also correlated with toxin movement inside the cell to apical recycling endosomes and Golgi cisternae. However, the mechanisms of toxin entry via apical or basolateral cell surfaces were shown to be distinct as evidenced by the clear differences in time course and temperature dependencies [41]. The parallel assessment of toxin action by entry into the cell through apical or basolateral membranes provided robust internal controls for these and subsequent studies.

Further evidence in support of the endocytosis hypothesis resulted from the discovery and use of brefeldin A in studies on membrane dynamics. Brefeldin A inhibits vesicular transport in the endosomal [44,45], transcytotic [46], and exocytotic [47] pathways of most eukaryotic cells [48,49]. Four laboratory groups showed that CT-induced cellular responses were dependent on brefeldin A-sensitive membrane traffic [38,50–52]. In all studies, brefeldin A inhibited reversibly CT-induced activation of adenylyl cyclase. Brefeldin A had no detectable effect on the enzymatic activity of the A-subunit in vitro providing evidence that inhibition of toxin action in intact cells was due to the known effects of brefeldin A on ARF-1 mediated membrane transport [48,53]. In two studies, brefeldin A was found to inhibit the movement of toxin into an intracellular compartment requisite for reduction of the A-subunit to the enzymatically active A_1-peptide [38,50]. The transport block occurred early in the lag phase but after endocytosis of toxin containing membranes. In T84 cells, two sequential and distinct membrane transport steps were identified. Both 'events' occurred after toxin binding to the apical cell surface and after entry into the early endosome. The first event was BFA-

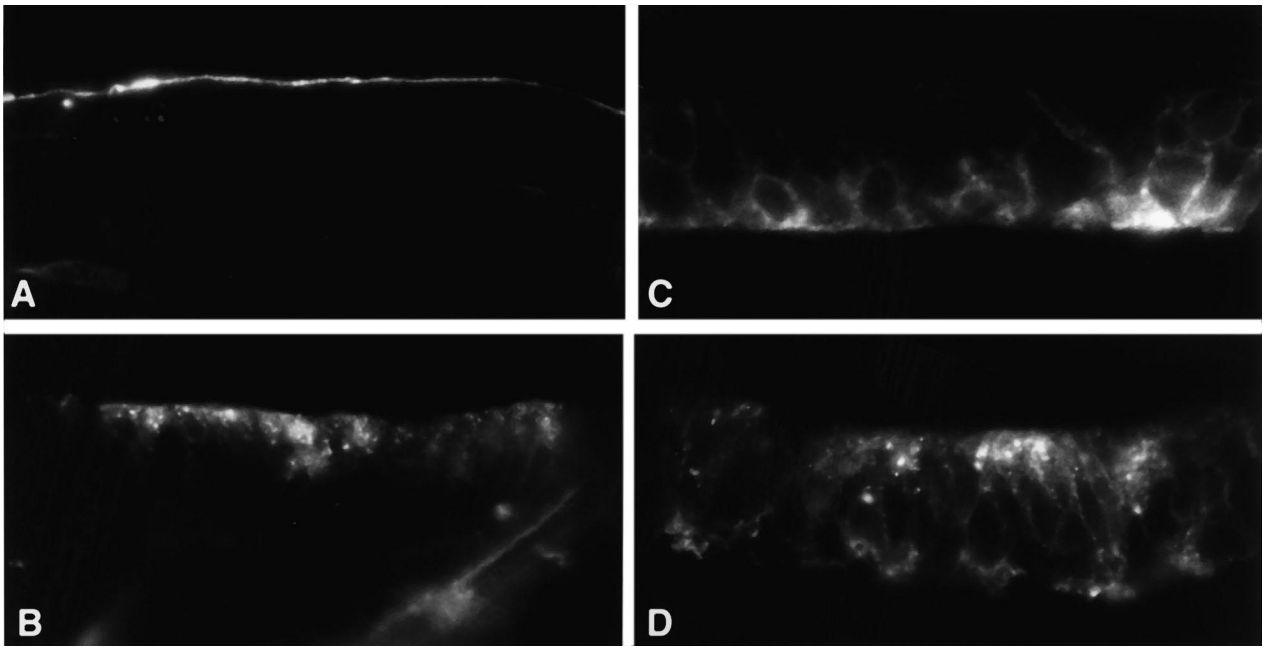

Fig. 2. Entry of CT into polarized T84 cells by endocytosis. Epifluorescence micrographs of frozen 5-μm sections of T84 monolayers exposed apically (A,B) or basolaterally (C,D) to 20 nM rhodamine-labeled CT for 2 h at 4°C (A,C), or 1 h at 37°C (B,D). CT is internalized and located within apical structures consistent in location with late or recycling endosomes and Golgi cisternae at 37°C but not at 4°C. Reproduced from [41] by copyright permission of The Rockefeller University Press.

sensitive and essential for movement of the toxin into a compartment where reduction and formation of the A_1-peptide occurred. The second event was temperature sensitive and required for transduction of a signal, presumably by movement of the A_1-peptide to its site of action on the basolateral membrane [41]. These studies showed that multi-compartmental vesicular transport was required for toxin action.

5. Dependence on retrograde traffic into Golgi cisternae and ER

Given that in all toxin-sensitive cells, inhibition of toxin action correlated closely with BFA-induced disruption of Golgi structure and function [48,49], these data suggested to most investigators that CT may require entry into a functional Golgi complex for bioactivity [38,50–52]. This idea was given further credence by solution of the crystal structures for LTI and CT [7–9] and by the discovery and characterization of the KDEL-dependent ER retrieval system in eukaryotic cells [54–57]. The KDEL receptor [58] is a six- [59] or seven-transmembrane-domain protein localized predominantly to the ER, intermediate compartment, and the Golgi complex with graded distribution from *cis* to *trans* [60,61]. The receptor mediates retrieval of KDEL-containing soluble ER-resident proteins by retrograde vesicular transport from multiple sites within Golgi cisternae including *trans*-Golgi [62–66]. Crystal structures of both CT and LTI showed that the K(R)DEL motif at the C-terminus of the A_2-peptide extended below the B-pentamer in a position where it could plausibly interact with membrane bound KDEL receptors.

The idea that CT and LTI may actually move backwards from plasma membrane to ER of target cells was given further credence by studies on *Pseudomonas* exotoxin A (ETxA) and shiga toxin (ST). Both toxins appeared to require entry into the ER of host cells for bioactivity. This was shown for ETxA in 3T3 fibroblasts to depend on the five C-terminal amino acids REDLK, which when removed ablated toxicity. Toxin action was rescued by replacement with the ER-targeting motif KDEL [67]. Similarly, the bioactivity of shiga toxin (another AB5 toxin structurally related to CT and LTI, [68]) was shown to correlate with movement of toxin from plasma membrane to ER in A431 epidermoid cells [69,70].

That CT and LTI followed a similar retrograde

pathway into Golgi and then ER was provided by four independent lines of evidence. First, two laboratory groups tested the effects of inactivating or removing the K(R)DEL motif on toxin function [71,72]. In polarized intestinal T84 cells, inactivating mutations of K(R)DEL in CT or LTI attenuated the efficiency of toxin action greater than 10-fold without affecting toxin binding to cell surface receptors, endocytosis, or the rate of intracellular degradation [72]. These studies showed that CT and LTI interacted directly with KDEL receptors and implied that both toxins may require retrograde movement into Golgi cisternae and ER for efficient and maximal biologic activity. These data were not reproduced, however, in studies on Chinese hamster ovary (CHO) cells [71]. The discrepancies between these data sets were likely due to differences in the sensitivities of the experimental approaches and cell systems utilized.

In a second line of evidence, wild-type CT A-subunits were localized within the ER of monkey kidney Vero cells by light microscopy and subcellular fractionation. Movement of the A-subunit into ER was observed only after a 30–60 min lag phase. This correlated temporally with toxin action and displayed a dependence on intact microtubules similar to that required for toxin-induced activation of adenylyl cyclase in this cell type [73]. In Vero cells, the A-subunit was found to dissociate physically from the B-subunit before entry into the ER [73,74]. In contrast, the B-subunit moved from plasma membrane into Golgi cisternae, but not further even after 90-min incubations. These data implied that the entire A-subunit (A_1- and A_2-peptides together) may pull out of the central core in the B-subunit to traffic independently into the ER. While plausible in concept and possibly correct, the idea that A- and B-subunits separate physically before entry into the ER does not fit all available evidence and remains to be confirmed in other cell systems (discussed in greater detail in Sections 7 and 8).

In a third line of evidence, reduction of the A-subunit was examined in human intestinal Caco-2 cells and shown to depend on catalysis by protein-disulfide isomerase [75]. The interaction between CT and protein-disulfide isomerase occurred within the lumen of an intracellular compartment identified as ER by subcellular fractionation. Finally, in a fourth line of evidence, ultrastructural studies localized CT B-subunits within the ER of A431 cells treated with the endoplasmic reticulum Ca^{2+} pump (SERCA pump) inhibitor thapsigargin [76] and of murine hepatocyte BNL CL.2 cells [77]. In addition, movement of B-subunit from plasma membrane to ER in thapsigargin-treated cells was inhibited by pretreatment with brefeldin A and this correlated with loss of toxin function [76].

All four independent experimental approaches described above are internally and conjointly consistent with the idea that CT A-subunit must move retrograde into the ER for bioactivity. Such a convergence of evidence supporting the requirement for retrograde transport is also true for shiga- and shiga-like toxins [69,70,78] (which are structurally similar to CT and LTI), *Pseudomonas* exotoxin A [67], and ricin [79,80]. If trafficking by shiga toxin into HeLa cells can be a guide [81], we anticipate that CT and LTI will be found to move directly from recycling endosomes to *trans*-Golgi, bypassing late endosomes en route from plasma membrane to ER. Recent studies show that in Vero cells retrograde transport of CT A-subunits from Golgi cisternae to ER, like retrograde transport of endogenous cellular proteins [82], depends on sorting into COPI-coated vesicles [83]. Specifically, movement of CT A-subunits into ER of Vero cells (assessed by epifluorescence microscopy) was blocked by microinjection of inhibitory Fab fragments against β-COP, an essential component of COPI coats. Furthermore, sorting into this pathway likely depends on the p24 family proteins that are sites for COPI coatomer binding [84] as inhibitory Fab fragments against a member of this family (p23) blocked movement of CT A-subunit into the ER. Thus, like membrane proteins that exhibit dilysine motifs in their cytoplasmic tails [82,85], and soluble ER-resident proteins that exhibit C-terminal KDEL signals [86–88], CT appears to move retrograde from Golgi to ER in COPI-coated vesicles.

6. The site and mechanism of toxin translocation across cellular membranes

While the bulk of evidence supports the view that CT and LTI must move retrograde into Golgi cister-

nae and ER to elicit an efficient and maximal physiologic response, we still do not know exactly where or how the A-subunit translocates across biologic membranes. Unlike anthrax or diphtheria toxins, which mediate translocation of their own toxic components across cellular membranes [89–91], there is little evidence that the B-subunits of CT or LTI can function as conductive pores for their respective A-subunits [92]. As detailed above, the available data indicate instead that CT and LTI rely on membrane systems endogenous to the host cell to enter a subcellular compartment with structure and function favorable for A-subunit reduction and membrane translocation. Based on available evidence, two plausible mechanisms have been envisioned. Each hypothesis emerges from the current view that CT and LTI require retrograde transport into ER or *cis*-Golgi for bioactivity and implicate unique structural and functional components of these compartments to explain peptide translocation. Neither idea, however, has been tested experimentally.

The first hypothesis arises from recent evidence that the biosynthetic pathways of eukaryotic cells (including yeast) are endowed with the ability to identify and eliminate misfolded membrane and soluble proteins by proteosome-dependent degradation in the cytosol [93]. Some misfolded proteins including MHC class I [94], the cystic fibrosis transmembrane regulator (CFTR) [95], yeast vacuolar protease carboxypeptidase Y, yeast pheromone α-factor [96], and others (see [97] for review) have been shown to exit the ER via reverse translocation through the Sec 61p complex. The sec61p complex is the central component of the protein translocation channel in the ER. Based on these data, Hazes and Read [98] proposed that CT and LTI (and other toxins which require entry into ER for bioactivity such as ST and ST-like toxins, pertussis toxin, EtxA, and ricin) may opportunistically utilize Sec 61p for translocation into the cytosol. They further proposed that once in the ER lumen, the A_1-peptide masquerades as a misfolded protein to interact with the required molecular machinery for reverse transport through the translocon. Once exposed to the cytosol, escape from degradation by the proteosome is achieved by the absence or near absence of lysines in the toxic peptide. Lysine residues are targets for covalent addition of ubiquitin, a peptide tag required typically for proteosome-dependent degradation. The exceptionally low content of lysines in each of the toxins mentioned above suggest that they may have evolved in part to escape degradation by the proteosome.

The second hypothesis arises from evidence that the A_1-peptides of CT and LTI exhibit hydrophobic behavior in aqueous solution and when membrane bound [99,100]. These data provide reason to believe the A_1-peptide may release from the A_2-peptide and B-subunit and partition spontaneously into the hydrophobic core of biologic membranes [101]. Only some membranes, however, may be able to accept the A_1-peptide. Thus, *cis*-Golgi or ER membranes may exhibit unique lipid [102] and protein structure permissive for membrane integration of the A_1-peptide, while plasma and endosome membranes may not.

7. Mechanism(s) of signal transduction after translocation

The actual cellular events following reduction and translocation of the A-subunit in both polarized and non-polarized cells are not known. It has largely been assumed that after translocation the A_1-peptide can break free of cell membranes to diffuse in the cytoplasm. This idea seems most attractive given the A/B-subunit structure of the toxin, the clear enzymatic activity of the A_1-peptide in cell free systems in vitro, and the simplicity of the proposed mechanism.

On the other hand, several lines of evidence indicate that in intact cells, the A_1-peptide may remain membrane associated and yet enzymatically active following translocation. First, the A_1-peptide is hydrophobic and when reconstituted into lipid vesicles behaves as an integral membrane protein [99,100]. Further evidence in support of this view is supplied by studies on human fibroblasts [103]. When CT was applied to cell homogenates, the toxin ADP-ribosylated multiple membrane and cytosolic proteins. In contrast, when CT was applied to intact cells, CT ribosylated only a specific subset of potential substrates. This suggests that the enzymatic A_1-peptide does not have access to all cytoplasmic proteins as one would predict for a freely diffusible cytoplasmic enzyme [103].

Studies on protease-resistant toxin variants of CT and LTI also support the idea that the A_1-peptide may not break free of the membrane after translocation. For these studies, toxin variants were prepared with an inactivating mutation in the serine–protease site of the C-loop which tethers the A_1- and A_2-peptides together. Thus, the A_1- and A_2-peptides were predicted to remain covalently associated after reduction in the ER. Nonetheless, when studied in human T84 [104], Caco-2, and CHO cells [105] these protease-resistant toxins displayed clear though diminished activity. While we cannot dismiss the possibility that in all three cell lines the protease resistant toxins were in fact cleaved at alternative sites in very small amounts, these data indicate that physical dissociation of A_1- and A_2-peptides may not be required for bioactivity and raise the possibility that the A_1- and A_2-peptides of native toxins may never fully dissociate after entry into the cell. Since the A_2-peptides of both CT and LT exhibit abundant noncovalent interactions with the B-pentamer [9,14], we have considered the possibility that domain A_1 may translocate into the membrane and exhibit enzymatic activity in the cytosol while still tethered via the A_2-domain to the B-subunit on the contralateral membrane surface. We acknowledge, however, that proteolytic cleavage of the A-subunit may not be required for complete dissociation of A-subunit from the B-pentamer. In support of this possibility, the entire CT A-subunit including the A_2-peptide appeared to separate from the pentameric B-subunit after toxin entry into Vero cells [73,74]. It has also been proposed that CT-activated Gsα, instead of the A_1-peptide, may separate from cell membranes to activate adenylyl cyclase at remote sites in the cell [106], but this view is not supported by available experimental evidence on the mechanism(s) of G-protein signal transduction [107,108].

8. An alternative hypothesis: signal transduction by anterograde vesicular transport

We have proposed that after translocation in the ER, the A_1-peptide may remain membrane associated and regain access to adenylyl cyclase on the plasma membrane by simply moving back out the

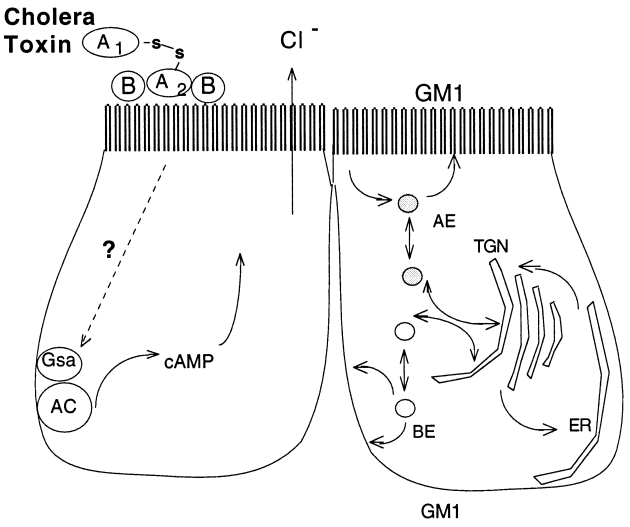

Fig. 3. Proposed model of toxin action on polarized epithelia. Left panel: in polarized cells, CT must bind to GM1 on the apical membrane and activate adenylyl cyclase on the cytosolic surface of the basolateral membrane. In vivo, the toxin is restricted from access to basolateral membranes by circumferential tight junctions and the integrity of the target cell. Toxin-induced activation of adenylyl cyclase leads to elevations in intracellular cAMP which in epithelial cells of the intestinal crypt elicits electrogenic Cl^- secretion – the primary transport event responsible for the massive secretory diarrhea seen in cholera. Right panel: proposed model of toxin action on polarized epithelia. Toxin enters polarized cells by binding GM1 within caveolae-like membrane domains at the apical cell surface. The CT–GM1 complex moves via apical endosomes (AE, shaded) into *trans*-Golgi (TGN). In *trans*-Golgi, the C-terminal K(R)DEL sorting signal on the A-subunit facilitates retrograde movement of the CT–GM1 complex through Golgi cisternae to ER. Reduction and translocation of the A-subunit occurs in the ER. The A_1-peptide may separate from the ER membrane after translocation and diffuse through the cytosol to its site of action on the basolateral membrane. Alternatively, the A_1-peptide may remain membrane associated (or reassociate with the membrane) after translocation and move to the basolateral membrane by entering anterograde transport vesicles and moving back out the secretory pathway. Fusion of basolaterally targeted vesicles carrying the CT–GM1 complex delivers the translocated A-subunit to a site near the Gs/adenylyl cyclase complex on the cytosolic surface and the B-subunit to the exocytoplasmic surface of the basolateral membrane (termed 'indirect transcytosis'). AE, apical endosome; TGN, *trans*-Golgi network and Golgi cisternae; ER, endoplasmic reticulum; BE, basolateral endosome; AC, adenylate cyclase; Gsa, heterotrimeric GTPase Gs.

biosynthetic pathway in anterograde-directed transport vesicles [72,109] (Fig. 3). Evidence for this idea comes from studies on polarized T84 cells.

In nature, CT binds initially to GM1 on the apical membrane of polarized intestinal epithelial cells, but acts on adenylyl cyclase at the cytoplasmic surface of the basolateral membrane [110,111]. One way for proteins to move from apical to basolateral surfaces of polarized epithelia is to move across the cell by vesicular traffic in a process termed transcytosis. When tested experimentally, we found that CT entered a basolaterally directed transcytotic pathway in T84 cells [109] and that transcellular transport correlated closely with toxin function [39,72,109]. Unlike the pathway for transcytosis of pIgA [112,113], however, movement of CT across the cell was not direct as both CT and LT required transit through Golgi cisternae and possibly ER en route to the basolateral membrane [72]. We have termed this process 'indirect transcytosis'.

Several lines of evidence support this hypothesis. First, inactivating mutations in the ER targeting signal K(R)DEL on CT diminished the efficiency and magnitude of both transcytosis and toxin-induced signal transduction [72]. The two were closely correlated, suggesting that interaction with KDEL receptors (and by implication retrograde transport into Golgi and ER) was required for transcytosis of toxin subunits. Second, since only the B-subunit was readily detected on the exocytoplasmic surface of the basolateral membrane after transcytosis, these data provided evidence that the CT A-subunit had translocated to the cytoplasm (or to the cytosolic membrane surface) during transport across the cell [109]. Transcytosis of CT B-subunits was correlated with a functional response. In cells pretreated with brefeldin A, however, CT was delivered to the basolateral membrane in a non-functional configuration with the A-subunit remaining tethered to the B-subunit on the outside rather than inside the cell. Thus, brefeldin A appeared to alter the route and mechanism of transcytosis so that entry of CT into the Golgi cisternae or ER was inhibited, and the A_1-peptide was unable to translocate into the cell. This was correlated with a complete block in toxin action. These data identified Golgi cisternae and ER as intermediary compartments through which CT must traffic en route to the basolateral membrane and strengthened the view that indirect transcytosis and toxin action may be mechanistically related.

Further evidence that indirect transcytosis may account for CT action was provided by kinetic studies. These data showed that indirect transcytosis of CT B-subunits displayed identical physical characteristics (lag time, time course, and temperature-dependency) with that of CT-induced signal transduction [109]. In aggregate, these data suggested to us that both A- and B-subunits of CT may move together from apical receptor to basolateral effector by vesicular traffic. Though on opposite sides of the membrane after A_1-peptide translocation, the subunits may not fully dissociate during transport through the cell.

Recent studies by Rodighiero et al. provide additional evidence in support of the importance of B-subunit in toxin function [114]. These studies were initiated to define the structural basis for the well known differences in potency of LTI and CT in vitro and in vivo [11]. To do so, chimeric toxins were prepared associating the A-subunits of CT with the B-subunits of LTI and vice versa. The chimeric toxins were analyzed for bioactivity in the T84 cell system. These studies showed that the increased potency of CT relative to LTI did not stem from differences in enzymatic activity of the A-subunits or differences in receptor recognition by the B-subunits. Rather, the differences in potencies could be attributed to the increased ability of the A_2-fragment of CT to maintain holotoxin stability during uptake and transport through the cell [114]. These studies emphasize the importance of the B-subunit in toxin biology and raise questions of when and where in the cell the A_1-peptide and B-subunit separate fully, if ever. In support of this view, available data indicate the structurally related shiga- and shiga-like toxins also depend on B-subunit function for entry into the ER of target cells [69,70,78,79]. Unlike CT and LTI, shiga- and shiga-like toxins do not exhibit K(R)DEL motifs, and the importance of B-subunit dependent glycolipid-based targeting in this system is more readily self-evident.

In summary, the exact events which lead to CT-induced signal transduction after A_1-peptide translocation in both polarized and non-polarized cells remain incompletely defined. At this time, we interpret the available data as evidence in favor of the idea that the A_1-peptide may remain membrane associ-

ated and enzymatically active after translocation. Indeed, the fact that the A_1-peptide interacts with ARF-family proteins in vitro [17] suggests to us that the A_1-peptide may be involved in directing its own vesicular transport after membrane translocation in vivo. Abundant evidence indicates that ARF-family GTP-binding proteins function in membrane transport to induce assembly of coat proteins for vesicular traffic between ER and Golgi, within Golgi cisternae, at exit from *trans*-Golgi, and between endosomes or synaptic vesicles [17,115–120]. Thus, we speculate that the A_1-peptide may ensure its own movement back out the secretory pathway by entry into anterograde transport pathways and interaction with ARF. A related hypothesis would be that ADP-ribosylated-Gs rather than the A_1-peptide moves from ER or Golgi cisternae in anterograde directed vesicles to activate adenylyl cyclase on the plasma membrane. Such a mechanism of signal transduction would also depend on ARF-mediated membrane traffic and, if Gs traveled together with CT B-subunit, would fit the experimental evidence [72,109]. On the other hand, we acknowledge the available data which support the alternative possibility that the A_1-peptide may separate from the membrane after translocation to diffuse freely in the cytosol. This view, until proven otherwise, will always remain attractive for its simplicity in mechanism of action.

9. Molecular mechanisms of targeting and transport

Some of the most exciting recent work in toxin biology has addressed the molecular mechanisms which account for targeting CT and LTI into the cell. Two recent studies show that this is not a stochastic process. Rather, CT must associate with specific lipid-defined plasma membrane domains to elicit an effect [6,121].

Two laboratory groups have proposed that targeting CT and LTI into non-polarized [5] and polarized cells [109] may plausibly depend on structural features of the toxin's endogenous receptor, ganglioside GM1. Such a dependence on glycolipid structure and function is also true for shiga toxin and shiga-like toxins (which bind Gb3, [122]), *E. coli* heat labile type II toxins (which bind GD1a or GD1b, [23]), and likely tetanus toxin (which binds GD1b, [123,124]). Glycosphingolipids, such as ganglioside GM1, are located exclusively in the outer (exo-cytoplasmic) leaflet of cell membranes (reviewed in [125–128]). They contribute to the structure and function of membrane domains in the plasma membrane and throughout the central vacuolar network of eukaryotic cells, except perhaps ER where they may be present in extremely low concentrations. Transport between distinct intracellular membrane compartments occurs only by vesicular traffic. Thus, ganglioside GM1, which has been shown to recycle between the cell surface and *trans*-Golgi network in murine NCTC 2071 and rat glioma C6 cells [129], represents an ideal target for toxins entering the endocytic and retrograde secretory pathway.

The idea that ganglioside structure may affect CT and LTI function was first tested experimentally by Pacuszka et al. [4,5] (see [21,22] for recent reviews). To show specificity of structure and function, these investigators replaced the ceramide moiety on GM1 with aliphatic amines, cholesterol, or phospholipids and assessed these neo-ganglioside' receptors for potency in mediating CT-induced activation of adenylyl cyclase in rat glioma C6 cells. GM1 neo-gangliosides constructed by attaching the GM1 oligosaccharide to cholesterol or aliphatic amines of greater than 14 carbon chain length were more potent than GM1 in mediating CT action. GM1 oligosaccharide attached to phospholipids or to the protein transferrin were less potent. Thus, receptor structure affects function.

How ganglioside structure may affect toxin trafficking has been explained by two recent studies. In many cell types, a large fraction of GM1 clusters in caveolae [130–132]. Caveolae and related membrane structures display light density and resistance to detergent extraction by virtue of their lipid and possibly protein composition [133–136]. These specialized membrane domains exist on both intracellular [137] and plasma membranes [138]. Caveolae are thought to mediate key cellular functions, which include ligand induced signal transduction, protein and lipid sorting, endocytosis, and (in vascular endothelium) transcytosis [125,126,131,139–146]. Two laboratory groups, including our own, have now shown that caveolae-like membrane domains mediate toxin-induced signal transduction in human intestinal

Caco-2 and T84 cell lines [6,121]. Orlandi and Fishman used the cholesterol-binding agent filipin to disrupt the structure of caveolae in Caco-2 cells and this correlated with inhibition of CT-induced activation of adenylyl cyclase and endocytosis [121]. Wolf et al. utilized the related *E. coli* heat-labile type II enterotoxin LTIIb to show that ganglioside structure was critical for toxin action, and this effect depended on association of toxin with caveolae-like membrane domains [6].

The two toxins used in our studies, CT and LTIIb, distinguish between gangliosides GM1 and GD1a at the cell surface by virtue of their dissimilar receptor binding B-subunits [10,147]. The enzymatically active A-subunits, however, are homologous [148,149] (Fig. 1). While both CT and LTIIb bound specifically to human intestinal T84 cells, only CT elicited a cAMP-dependent Cl^- secretory response. LTIIb, however, was shown to be functional and more potent than CT in eliciting a cAMP-dependent response from mouse Y1 adrenal cells. In T84 cells, CT fractionated with caveolae-like detergent insoluble membranes, but LTIIb did not. In Y1 cells, both toxins associated with caveolae-like membrane domains. Thus, toxin function correlated with binding to gangliosides within caveolae-like plasma membrane domains suggesting the two may be related. To confirm these results, chimeric toxins associating the CT A- with LTIIb B-subunits (and vice versa) were prepared. Analysis of these chimeric toxins showed that toxin-induced signal transduction and association with caveolae depended critically on binding to gangliosides with specific structure, a function of the toxin's B-subunit. Thus, the mechanism(s) by which ganglioside GM1 functions in signal transduction likely depends on coupling CT with caveolae or caveolae-related membrane domains. These data fit nicely with earlier morphologic studies showing that CT enters hepatocytes, fibroblasts, A431, and endothelial cells via smooth, non-clathrin-coated membrane invaginations characteristic of caveolae [77,131,150–152].

Exactly how specific gangliosides may function as discrete sorting motifs for partitioning CT and LTI into caveolae-like membrane subdomains, however, remains undefined. The structures of GM1 and GD1a differ most significantly in their respective carbohydrate head groups, and even these are closely related [153]. Nonetheless, detergent insoluble membrane microdomains of human intestinal T84 cells clearly distinguish between these similar glycolipids. Thus, one possibility is that carbohydrate head groups may define specificity in this system. If so, these studies imply that epitopes on proteins or lipids resident in the exoplasmic bilayer leaflet provide motifs for sorting of membrane components at the cell surface and possibly within endosomes and other transport vesicles (as previously proposed [136,154]). Alternatively, glycosphingolipids exhibit heterogeneity in structure of their lipid tails [153], and this may also impart specificity to glycolipid function as suggested by Sandvig, Mayor, and Pacuszka and colleagues [5,70,155].

10. Summary and significance

In nature, CT and LTI must enter polarized epithelial cells through the apical membrane. This requires endocytosis of toxin–receptor complexes into the apical endosome, retrograde transport into Golgi cisternae and ER [38,41,50,72,73,75], and finally transport of the translocated toxin (or possibly another signaling molecule) across the cell to its site of action on the basolateral membrane. In the human intestine, this process leads to intestinal salt and water secretion and the massive diarrhea seen in cholera. Targeting into this pathway depends critically on the biology and structure of the toxin's cell surface receptor, ganglioside GM1. Binding or clustering GM1 sorts CT and LTI into caveolae or caveolae-like membrane domains [4–6,121]. Thus, the cellular basis of CT and LT action depends on molecular mechanisms which move specific membranes and membrane components throughout the cell. Membrane dynamics are fundamental to the structure and function of eukaryotic cells themselves, and in particular to the polarized epithelial cells which line the gastrointestinal, renal and pulmonary systems of animals in nearly all kingdoms and phyla. Epithelial cells lining the lumen of these organs depend on vesicular transport to establish and maintain cell polarity and function. It should be no surprise that CT and LTI, which must interact with polarized epithelial cells in nature, have evolved to co-opt endogenous mechanisms of membrane transport for their own advantage.

The importance of toxin biology is underscored by the fact that toxigenic secretory diarrheas account for nearly 20% of the identifiable diarrheal disease worldwide [156,157]. In children, less than 5 years old, diarrhea remains the leading cause of death (5 million deaths/year) [157] and a significant factor in malnutrition [158]. Several other deadly enterotoxins, such as shiga- and shiga-like toxins, are related structurally to CT and LTI and also require entry into the cell by glycolipid-dependent vesicular transport. Further definition of the cellular mechanisms which account for the bioactivities of CT and related toxins may lead ultimately to new approaches of high impact for the prevention and treatment of these common diarrheal diseases.

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