Large clostridial toxins glucosylate some small G proteins on a threonine residue, thereby preventing their interactions with effector molecules and regulators. We show that the glucosyltransferase domain of lethal toxin from Clostridium sordellii (LTcyt; amino acids 1–546), which is released into the cytosol during cell infection, binds preferentially to liposomes containing phosphatidylinerine as compared with other anionic lipids. The binding of LTcyt to phosphatidylinerine increases by two orders of magnitude the rate of glucosylation of liposome-bound geranyl-geranylated Rac-GDP. Limited proteolysis and deletion studies show that the binding site for phosphatidylinerine lies within the first 18 N-terminal residues of LTcyt. Deletion of these residues abolishes the effect of phosphatidylinerine on the activity of LTcyt on liposome-bound geranyl-geranylated Rac-GDP and prevents the morphological effects induced by LTcyt microinjection into various cells, but it does not affect the intrinsic activity of LTcyt on non-geranyl-geranylated Rac-GDP in solution. We conclude that the avidity of LTcyt for phosphatidylinerine facilitates its targeting to the cytosolic leaflet of cell membranes and, notably, the plasma membrane, where this anionic lipid is abundant and where several targets of lethal toxin reside.

Numerous bacterial toxins affect the GDP/GTP cycle of G proteins. Large clostridial toxins, which include toxins A and B from C. difficile, lethal toxin (LT)1 from Clostridium sordellii, and alpha toxin from Clostridium novyi add a glucosyl group on various small G proteins (1–3). The targets of C. difficile toxins A and B are members of the Rho/Rac/Cdc42 family, a small G protein subfamily that plays a critical role in the control of cell morphology. The spectrum of LT partially overlaps that of C. difficile toxins. LT glucosylates Rac but also members of the Ras subfamily (Ras, Rap1, and Rap2) (2). In all cases the toxin acts as a glucosyltransferase, catalyzing the transfer of a glucosyl group from a nucleotide sugar (usually UDP-glucose) to a conserved threonine residue. This threonine (Thr-35 in Rac) participates in the coordination of the nucleotide, belongs to one of the two switch regions that undergo a conformational change upon GDP/GTP exchange and GTP hydrolysis, and is involved in effector binding. Threonine glucosylation prevents the interaction of the small G protein with effector molecules and interferes with the catalysis of GDP/GTP exchange and GTP hydrolysis by guanine nucleotide exchange factors and GTPase-activating proteins (4–6).

Large clostridial toxins are composed of three domains that act sequentially during cell infection (Fig. 1A) (7). The 500-amino acid C-terminal domain is involved in the specific recognition of cell surface receptors. After endocytosis, a central hydrophobic domain penetrates through the endosome membrane in a pH-dependent manner and drives the translocation of the N-terminal domain from the luminal side to the cytosolic side (8–10). The N-terminal domain (amino acids 1–546 in LT and toxin B), which is responsible for the glucosyltransferase activity, is then liberated by proteolytic cleavage in the cytosol and glucosylates intracellular targets (11, 12).

Most in vitro studies of the glucosyltransferase activity of large clostridial toxins have been carried out in solution using non-modified, recombinant small G proteins as substrates (1, 2, 4–6, 11). In cells, however, small G proteins are generally lipid-modified. The members of the Ras family are geranylgeranlated at their C terminus and, as such, interact with lipid membranes. Alternatively, the members form a soluble 1:1 complex with GDI, a protein that masks the geranylgeranylated group. RhoA in complex with GDI is not a substrate for glucosylation by toxin B from C. difficile (13). Indeed, GDI interacts not only with the lipid group but also with the switch regions of Rho proteins and thereby hinders threonine glucosylation (14–16). Considering the binary partitioning of Rho proteins between lipid membranes and GDI, it is therefore likely that only membrane-bound Rho proteins should be substrates for large clostridial toxins. This hypothesis was tested here using geranyl-geranylated Rac bound to liposomes of defined lipid composition. We show that because of a specific phos-
phatidylycerine-binding site located in the first 18 residues, the cystolic fragment of lethal toxin glucosylates liposome-bound prenylated Rac-GDP very efficiently when the lipid bilayer displays a composition approaching that of the inner leaflet of the plasma membrane. Moreover, deletion of this N-terminal site abolishes the cytotoxic effects of LT observed in vivo.

**EXPERIMENTAL PROCEDURES**

**Lipids and Reagents**—Lipids were purchased (Avanti Polar Lipids). Glucosylceramide, cholesterol, and synthetic lipids were from Avanti Polar Lipids. Egg phosphatidylycerine (PG) and phosphatic acid (PA) were from Sigma. [14C]UDP-glucose was from Amersham Biosciences.

**Protein Expression and Purification**—Full-length LT was produced and purified from strain IP 82 as described (17). Fragments corresponding to amino acids 1–546 of LT (15), 1852–2366 of LT, and 1–546 of C. difficile toxin B were cloned in a pET28 vector (Merck-Novagen). This vector adds a hexahistidine tag and a thrombin cleavage site at the N terminus. The constructs were expressed in E. coli and purified by nickel chromatography. In typical glucosylation and liposome binding experiments we observed that removal of the hexahistidine tag with thrombin did not modify the properties of LT<sub>cyt</sub>. Therefore, for most experiments the hexahistidine tag was not removed.

The complex between GDI (with an N-terminal FLAG) and geranyl-geranylated Rac-GDP (with an N-terminal His<sub>6</sub> tag) was expressed in E. coli as a glutathione S-transferase fusion and purified using glutathione-agarose beads followed by glutathione S-transferase cleavage.

**Liposome Preparation**—Stock solutions of lipids in chloroform were mixed in a 20 ml pear-shaped glass container. The fluorescent lipid was removed using a rotary evaporator. The lipid film was resuspended in 20 mM Hepes (pH 7.5) and 100 mM KCl for 15 min at 25 °C. The liposomes were resuspended at a final lipid concentration of 10 mM in the same buffer and stored under argon at room temperature before use.

**Liposome Binding Experiments**—Protein and liposomes were mixed at a final concentration of 1 μM and 3 mM, respectively, in buffer A (20 mM Hepes, pH 7.5, 100 mM KCl, and 1 mM dithiothreitol) supplemented with 1 mM MgCl<sub>2</sub> and 1 mM MnCl<sub>2</sub> (total volume 50 μl). After 5 min at 30 °C, the sample was centrifuged at 400,000 × g for 15 min at 25 °C. The liposomes were resuspended at a final lipid concentration of 10 mM in the same buffer and stored under argon at room temperature before use.

**GDI Removal Protocol**—The complex between GDI and Rac-GDP (1 μM) was incubated for 40 min at 30 °C with liposomes (6 mM lipids) and 20 μM GTP in buffer A supplemented with 1 mM MgCl<sub>2</sub> and 2 mM EDTA to give a Mg<sup>2+</sup>-free of 1 μM. The sample was centrifuged at 400,000 × g for 20 min at 4 °C. The pellet was washed with cold buffer A (Mg<sup>2+</sup>-free = 1 μM), centrifuged again for 5 min at 4 °C, and resuspended in buffer A supplemented with 1 mM MgCl<sub>2</sub> and no EDTA (Mg<sup>2+</sup>-free = 1 mM). The Rac-loaded liposomes were then incubated at 30 °C for 30 min to promote GTP hydrolysis on Rac and stored on ice before use. Aliquots (20 μl) from the initial lipid/protein suspension, the supernatants, and the final liposome suspension were withdrawn and analyzed by fluorometry and SDS-PAGE to determine liposome and protein recovery.

**Rac Glucosylation**—Glucosylation reactions were performed at 30 °C. [14C]UDP-glucose (10 μM) was used as a glucose donor. Liposome-bound Rac-GDP, which was obtained by the GDI removal protocol, was diluted 2-fold in buffer A supplemented with 1 mM MgCl<sub>2</sub> and 1 mM MnCl<sub>2</sub> to give a final lipid concentration of 3 mM and a final protein concentration of 0.25 μM (as estimated by SDS-PAGE). Glucosylation was initiated by the addition of LT. At the indicated times, 20-μl aliquots were withdrawn, diluted in 2 ml of ice-cold buffer (20 mM Hepes (pH 7.5), 100 mM KCl, and 10 mM MgCl<sub>2</sub>) and filtered on 25-mm BA55 nitrocellulose filters (Schleicher & Schüll). After two additional washes with 2 ml of ice-cold buffer, the filters were dried and counted.

**Limited Proteolysis**—An LT cystolic fragment (200 μg/ml) was incubated at 30 °C with trypsin (0.5 μg/ml) in buffer A supplemented with 1 mM MgCl<sub>2</sub> and 1 mM MnCl<sub>2</sub>. At the indicated times, the reaction was stopped by the addition of 0.2 mM phenylmethylsulfonyl fluoride and analyzed by SDS-PAGE using Sypro Orange staining.

**Cell Microinjection**—NIH-3T3 cells were plated at low confluency on 15-mm glass coverslips for 48 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin G (100 units/ml), and streptomycin (100 μg/ml) (Invitrogen). Cytosolic microinjection (Eppendorf 5171 microinjector) was performed with the indicated toxin (50 μg/ml) and with dextran-TRITC (1 mg/ml) in phosphate-buffered saline. After a 3-h incubation at 37 °C the cells were fixed with a solution of 3.7% paraformaldehyde, and the polymerized actin was stained with phallolidin-fluorescein isothiocyanate (Molecular Probes, Leiden, Netherlands). The glass coverslips were mounted in Mowiol (Calbiochem and VWR International) and observed by fluorescence microscopy using a confocal laser-scanning microscope (Leica).

**RESULTS**

In pilot experiments we tested the susceptibility of liposome-bound geranyl-geranylated Rac-GDP to glucosylation by full-length LT using liposomes made of a crude mixture of soybean lipids (azolectin). We observed that the rate of glucosylation largely exceeded the rate observed in solution using E. coli-expressed, non-geranyl-geranylated Rac-GDP, the substrate that is classically used in vitro experiments. This finding suggested that the lipid bilayer provided a favorable environment for the glucosylation reaction. This hypothesis was also supported by experiments using membranes from cells overexpressing Ras, which showed that detergents such as Triton X-100, at a concentration that did not modify the glucosylation of soluble substrates, strongly inhibited the glucosylation of Ras by LT (data not shown).

These preliminary observations prompted us to examine the lipid binding properties of LT and notably that of the N-terminal domain, because this domain is released into the cytosol by proteolysis and glucosylates cellular substrates such as Rac or Ras.

**Lipid Binding Properties of the Various Domains of LT**—To assess the lipid binding properties of LT, we performed sedimentation experiments using sucrose-loaded liposomes of defined composition. Liposome-bound and unbound proteins were separated by centrifugation and quantified by fluorometry after SDS-PAGE using Sypro Orange staining. The liposome composition was based on a background of neutral lipids consisting of PC (30–60%), PE (20%), and cholesterol (20%). When indicated, anionic lipids such as PS, PG, PA, or PI were included at the expense of PC.

Fig. 1 compares the lipid binding properties of full-length LT with that of its N-terminal cytosolic domain (amino acids 1–546) or its C-terminal “receptor binding” domain (amino acids 1852–2366). These two latter forms were expressed in E. coli with an N-terminal hexahistidine tag. Full-length LT bound completely to all liposomes tested, including neutral PC/PE/cholesterol liposomes (Fig. 1B). In contrast, the C-terminal domain showed no binding to liposomes, either neutral or anionic (Fig. 1C). Interestingly, the N-terminal cytosolic domain (hereafter abbreviated as LT<sub>cyt</sub>) bound weakly (15%) to neutral PC/PE/cholesterol liposomes but showed better binding to liposomes containing anionic lipids (Fig. 1D). Of all the anionic lipids tested, PS, the major anionic lipid of eukaryotic cell membranes, was the most potent. At 30 mol % PS, approximately 80% of LT<sub>cyt</sub> was recovered in the liposome pellet (Fig. 1D). PG, which is abundant in bacterial membranes, and PIP<sub>2</sub>, which is present at low amounts in cell membranes, induced a moderate binding of LT<sub>cyt</sub>. Other anionic lipids, including PA and PI, were less potent (Fig. 1D). Similar lipid binding properties
were observed after removal of the N-terminal hexahistidine tag (data not shown). Taken together, these experiments show that LT displays complex lipid binding properties that are related to its composite domain organization. The central hydrophobic “translocation” domain probably accounts for the strong avidity of full-length LT for all liposomes (Fig. 1B). More intriguing was the binding of the cytosolic domain to PS-containing liposomes (Fig. 1D) insofar as PS is abundant in the cytosolic leaflet of cell membranes and notably the plasma membrane, where small G proteins such as Ras are found.

**Phosphatidylserine Facilitates Glucosylation of Liposome-bound Rac by LT**—Next, we wished to test the effect of PS on the glucosylation of liposome-bound Rac-GDP by LT. For this task, it was first necessary to assess the minimal lipid composition required to prepare liposome-bound Rac.

Geranyl-geranylated Rac-GDP was purified as a 1:1 soluble complex with GDI. To prepare liposome-bound Rac-GDP, we used a GDI removal protocol that takes advantage of the transient displacement of Rac from GDI and its binding to liposome during a single round of GTP binding and hydrolysis (19). The rationale of this protocol is that the replacement of GDP by GTP weakens the interaction of Rac with GDI and thus facilitates the association of Rac with liposomes. Liposome-bound Rac-GTP can then be separated from soluble GDI by centrifugation and subsequently incubated with a millimolar concentration of magnesium to promote the conversion to the GDP-bound form because of the GTPase activity of Rac (19). Fig. 2A shows that under conditions favoring GDP to GTP exchange (low MgCl₂ concentration), Rac accumulates strongly and with similar efficiency to all liposomes containing 30 mol % anionic lipids but not to neutral PC/PE/cholesterol liposomes. This finding suggested that nonspecific electrostatic interactions with anionic lipids contribute to the translocation of geranylgeranylated Rac from GDI to lipid membranes during GDP to GTP exchange. It is likely that these interactions involve a cluster of six basic residues adjacent to the C-terminal geranylgeranylated cysteine of Rac. The juxtaposition of basic residues with a lipid modification helps to anchor many proteins to lipid membranes, including prenylated small G proteins (20–25).
Having established that liposome-bound Rac-GDP could be prepared on any anionic liposome, we conducted glucosylation reactions. We observed that the nature of the anionic lipid present in the liposomes had a dramatic effect on the rate of Rac-GDP glucosylation by full-length LT (Fig. 2B) as well as by LTcyt (Fig. 2C). In both cases, glucosylation proceeded slowly with PA as the sole source of anionic lipid, five times faster with PG, and 60 times faster with PS. Thus, the activity of LTcyt toward liposome-bound Rac-GDP correlated with its lipid binding properties (compare Figs. 2C and 1D). Moreover, despite the fact that full-length LT bound avidly to all liposomes tested (Fig. 1B), its activity also depended on the presence of PS (Fig. 2B), suggesting that PS favors the correct positioning of the cytosolic and glucosyltransferase N-terminal domain toward membrane-bound Rac-GDP.

To further assess the specificity of LTcyt for PS, we conducted glucosylation reactions using liposomes containing increasing amounts of synthetic PS. Because the yield of liposome-bound Rac-GDP obtained by the GDI removal protocol depended on the presence of anionic lipids, PS was added at the expense of another anionic lipid (e.g., PI or PG) to keep constant the total amount of all anionic lipids at 30 mol %. Fig. 3A shows that the rate of Rac glucosylation increased 10-fold when PI was gradually replaced by dioleoyl-PS (DOPS), dipalmitoyl-PS (DPPS), dioleoyl-PG (DOPG), or dipalmitoyl-PG (DPPG) (30 mol %).

**FIG. 2.** Phosphatidylserine facilitates the glucosylation of liposome-bound Rac-GDP by LT. A, preparation of liposome-bound Rac. The complex between Rac-GDP and GDI (1 μM) was incubated for 40 min at 30 °C with GTP (20 μM) and with or without PC/PE/cholesterol liposomes supplemented with 30 mol % PA, PG, or PS as indicated. The concentration of free Mg²⁺ was 1 μM. After incubation, the sample was centrifuged, and the pellet (P) and the supernatant (S) were analyzed by SDS-PAGE and Sypro Orange staining. N.A., no addition of liposomes. B and C, glucosylation experiments. Rac-GTP bound to anionic liposomes was prepared as described for panel A and converted to the GDP-bound state by a further incubation at 1 mM free Mg²⁺ to promote GTP hydrolysis. Glucosylation was initiated by the addition of 1 mM full-length LT (panel B) or 0.25 mM LTcyt (panel C) in the presence of [¹⁴C]-UDP-glucose (10 μM). Protein-bound radioactivity was determined on 20 μl aliquots (∼5 pmol of Rac per aliquot). Continuous traces represent best single exponential fits.
suggest that lipid packing influences the accessibility of polar head groups to LTcyt.

A Specific Binding Site for Phosphatidylserine in the N Terminus of Lethal Toxin—To search for the presence of subdomains in LTcyt, which are potentially involved in PS binding, we conducted limited proteolysis experiments. LTcyt was relatively resistant to digestion by trypsin, chymotrypsin, and subtilisin (Fig. 4 and data not shown). In all cases, the N-terminal hexahistidine tag used for purification was immediately cleaved (as revealed by Western blot). After 30 min, fragments that displayed an apparent molecular mass of at least 90% of the initial construct were observed. This suggests that LTcyt folds as a single domain probably flanked by somehow flexible short ends. Interestingly, centrifugation experiments revealed that the fragment generated by trypsin treatment that had the lowest molecular mass (apparent molecular mass/54 kDa) bound less avidly to PS-containing liposomes as compared with the other fragments (Fig. 4). N-terminal sequencing revealed that this fragment starts from the 19th amino acid of LT. Therefore, we constructed an N-terminal truncated version of LTcyt lacking the first 18 amino acids ([18]LTcyt) and compared its lipid binding properties with that of LTcyt.

Fig. 5A shows that, in contrast to LTcyt, [18]LTcyt showed no PS-dependent binding to liposomes. Importantly, LTcyt and [18]LTcyt showed the same glycosyltransferase activity on non geranyl-geranylated Rac-GDP in solution, suggesting that residues 1–18 in LT play no role in the glycosyltransferase mechanism per se (Fig. 5B). It should be noted that these experiments were performed with an ~100-fold higher concentration of LTcyt and [18]LTcyt as compared with experiments on liposome-bound Rac (see Figs. 2C and 5C), thus illustrating the catalytic advantage provided by the lipid membrane environment.

Next, we assessed the activity of [18]LTcyt on liposome-bound Rac-GDP (Fig. 5C). In marked contrast with what was observed for LTcyt, the rate of glucosylation was slow and was not influenced by the nature of the anionic lipid present in the liposomes (compare Figs. 2C and 5C). We concluded from these experiments that the first 18 residues of LTcyt participate in the formation of a PS-specific binding site that helps to anchor the cytosolic domain of LT to lipid membranes, thereby facilitating the glucosylation of membrane-bound Rac.

Amino Acids 1–18 Are Required for the Cytotoxicity of LTcyt—To determine the importance of the N terminus of LT for its cytotoxic effect in vivo, we microinjected LTcyt or [18]LTcyt FIG. 5. Deletion of the first 18 amino acids in LTcyt abolishes its sensitivity to phosphatidylserine. A, binding of LTcyt or the [18] truncated form ([18]LTcyt) to PC/PE/cholesterol liposomes (3 mM lipids) containing increasing amounts of PS at the expense of PC. S, supernatant; P, pellet. B, time course of glucosylation of non-prenylated Rac (0.5 μM) in solution in the presence of 50 nM LTcyt (black circles) or 50 nM [18]LTcyt (white circles). C, glucosylation of geranyl-geranylated Rac (0.25 μM) by [18]LTcyt (0.5 μM) on PC/PE/cholesterol liposomes supplemented with 30% PS (white circles), 30% PG (white triangles), or 30% PA (crosses). For comparison, an experiment with 0.5 nM LTcyt on PS-containing liposomes is shown (black circles).
positive for dextran-TRITC. 

...the cells microinjected with ...ical actin cytoskeleton. The cells lost their typical flat assembly of actin stress fibers and the formation of a thick cor-

...four large Clostridial toxins, toxin B from C. difficile was visualized by phalloidin-fluorescein isothiocyanate. The data for NIH-3T3 cells are shown in Fig. 6, and similar results were observed for the other cell lines. In agreement with previous findings (2), we observed that LTcyt triggered the disassembly of actin stress fibers and the formation of a thick cortical actin cytoskeleton. The cells lost their typical flat morphology and became round and smaller (Fig. 6A). In contrast, the cells microinjected with [Δ18]LTcyt were intact and indistinguishable from non-injected cells (Fig. 6B). Thus, although not involved in the glucosyl-transferase activity per se, the N terminus is essential for the cytotoxic activity of LTcyt.

...from C. difficile is not specific for PS—Among the four large Clostridial toxins, toxin B from C. difficile is the closest homolog to LT (3). Yet, as illustrated in Fig. 7A, the two toxins show substantial differences in their first 18 residues. Both toxins are rich in hydrophobic and basic residues, but toxin B contains a glutamic residue (Glu-10), whereas LT contains no acidic residue. In addition, there are some non-conservative substitutions between polar, charged, and hydrophobic residues (Ala → Lys, Val → Ala, and Tyr → Asn; see Fig. 7A). Fig. 7B shows that the cytotoxic domain of toxin B was less sensitive to the anionic composition of liposomes than LT. The time course of Rac glucosylation by the cytotoxic domain of toxin B was the same whether PS or PG was included in the liposomes, suggesting that some amino acid differences in the N terminus of LT and toxin B contribute to the striking PS dependence of LT. In addition, the cytotoxic domain of toxin B showed only modest levels of binding to PS-containing liposomes (<30%; data not shown).

...shown to favor the catalysis of GDP/GTP exchange by guanine nucleotide exchange factors and GTP hydrolysis by GTPase-activating proteins (19, 26, 27). Here we show that the cytotoxic domain of a bacterial toxin also takes advantage of the lipid environment to inactivate Rac. The catalytic domain of LT, which is released into the cytosol after cell infection, glucosylates the small G protein Rac-GDP much more efficiently in a minimal lipid environment than in solution. With an optimal lipid composition, the “kinetics gain” provided by the membrane environment results in at least a 100-fold increase in the rate of Rac glucosylation by LTcyt. Indeed, whereas non-preny-

...domain of the trans-

...membrane SNARE proteins, act more efficiently in the presence of negatively charged lipids (28). The tarantula venom toxin VTX1 partitions into the membrane through a hydrophobic surface and thereby inhibits efficiently voltage-dependent ion channels despite a weak protein-protein interaction (30).

...shows a marked preference for PS as compared with other anionic lipids, including PG, PA, and phosphoinositides (Figs. 1D, 2C, and 3). PS is the most abundant anionic lipid in eukaryotic cells and is enriched in the cytosolic leaflet of cellular membranes, notably at the plasma membrane (31). The specificity of LTcyt for PS thus provides a straightforward mechanism for targeting the glucosyltransferase domain to prenylated and membrane-associated small G proteins. In addition, the low abundance of PS in the outer leaflet of the...
plasma membrane should prevent the glucosyltransferase domain from interfering with key membrane binding events that occur early during LT infection, including the recognition of cell surface receptors by the C-terminal domain and the translocation of the central hydrophobic domain through the endosomal membrane.

Removal of the first 18 N-terminal residues of LT<sub>cyt</sub> was sufficient to abolish the effect of PS on LT<sub>cyt</sub> binding to liposomes (Fig. 5A) and on the LT-catalyzed glucosylation of liposome-bound Rac (Fig. 5C). However, the N-terminal truncation had no effect on LT<sub>cyt</sub> activity on soluble recombinant Rac (Fig. 5B), suggesting that the lipid binding motif of LT<sub>cyt</sub> is not structurally coupled to the catalytic and Rac-binding domains, which have been localized, respectively, in the central and the C-terminal region of LT<sub>cyt</sub> (3, 32, 33). The N terminus of LT<sub>cyt</sub> contains four basic residues, eight hydrophobic residues, and no acidic residues (Fig. 7A) and is therefore adapted to interact with negatively charged lipid membranes. Because this region is highly sensitive to proteolysis (Fig. 4A), we suggest that it forms a flexible chain that adsorbs at the surface of negatively charged membranes. Experimental and theoretical studies on proteins and model peptides have detailed the various combinations of electrostatic and hydrophobic forces that allow membrane adsorption of basic/hydrophobic clusters (21, 22). When electrostatic interactions prevail, the basic cluster remains at some distance from the lipid polar head because of the energy cost of desolvating charged groups. The interaction is thus nonspecific, and anionic lipids with the same net charge such as PS and PG are interchangeable in promoting membrane adsorption. However, when hydrophobic residues act in concert with basic residues, they lead to a deeper penetration of the protein region within the lipid polar head region and thus may impose some specificity toward given lipid head groups. The fact that LT<sub>cyt</sub> interacts preferentially with liposomes having anionic lipids with unsaturated acyl chains also argues for a deep membrane adsorption (Fig. 3B). Interestingly the cytosolic domain of C. difficile toxin B did not show a marked preference for PS (Fig. 7). Although highly homologous, lethal toxin and toxin B show a few substitutions in their N-terminal sequence between charged, polar, and hydrophobic residues (Fig. 7A). Mutagenesis and structural studies should help to further characterize the PS-binding site of LT.

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