**Abstract**

*Clostridium perfringens* iota-toxin consists of two separate proteins identified as a cell binding protein, iota b (Ib), which forms high-molecular-weight complexes on cells generating Na(+)/-K(+) permeable pores through which iota a (Ia), an ADP-ribosyltransferase, presumably enters the cytosol. Identity of the cell receptor and membrane domains involved in Ib binding, oligomer formation, and internalization is currently unknown. In this study, Vero (toxin-sensitive) and MRC-5 (toxin-resistant) cells were incubated with Ib, after which detergent-resistant membrane microdomains (DRMs) were extracted with cold Triton X-100. Western blotting revealed that Ib oligomers localized in DRMs extracted from Vero, but not MRC-5, cells while monomeric Ib was detected in the detergent-soluble fractions of both cell types. The Ib protoxin, previously shown to bind Vero cells but not form oligomers or induce cytotoxicity, was detected only in the soluble fractions. Vero cells pretreated with phosphatidylinositol-specific phospholipase C before addition of Ib indicated that glycosylphosphatidyl inositol-anchored proteins were minimally involved in Ib binding or oligomer formation. While pretreatment of Vero cells with filipin (which sequesters cholesterol) had no effect, methyl-beta-cyclodextrin (which extracts cholesterol) reduced Ib binding and oligomer formation and delayed iota-toxin cytotoxicity. These studies showed that iota-toxin exploits DRMs for oligomer formation to intoxicate cells.

**Subject Terms**

*Clostridium perfringens*, *iota toxin*, microdomains, detergent-resistance, oligomerization
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Detergent-Resistant Membrane Microdomains Facilitate Ib Oligomer Formation and Biological Activity of *Clostridium perfringens* Iota-Toxin

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* Corresponding author. Mailing address: Toxinology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Mo.) in Hanks balanced salt solution (HBSS) lacking Ca2+.

**C. perfringens** iota-toxin consists of two separate proteins identified as a cell binding protein, iota b (Ib), which forms high-molecular-weight complexes on cells generating Na+/K+-permeable pores through which iota a (Ia), an ADP-ribosyltransferase, presumably enters the cytosol. Identity of the cell receptor and membrane domains involved in Ib binding, oligomer formation, and internalization is currently unknown. In this study, Vero (toxin-sensitive) and MRC-5 (toxin-resistant) cells were incubated with Ib, after which detergent-resistant membrane microdomains (DRMs) were extracted with cold Triton X-100. Western blotting revealed that Ib oligomers localized in DRMs extracted from Vero, but not MRC-5, cells while monomeric Ib was detected in the detergent-soluble fraction of both cell types. The Ib protoxin, previously shown to bind Vero cells but not form oligomers or induce cytotoxicity, was detected only in the soluble fractions. Vero cells pretreated with phosphatidylinositol-specific phospholipase C before addition of Ib indicated that glycosylphosphatidylinositol-anchored proteins were minimally involved in Ib binding or oligomer formation. While pretreatment of Vero cells with filipin (which sequesters cholesterol) had no effect, methyl-beta-cyclodextrin (which extracts cholesterol) reduced Ib binding and oligomer formation and delayed iota-toxin cytotoxicity. These studies showed that iota-toxin exploits DRMs for oligomer formation to intoxicate cells.

*Clostridium perfringens* is a ubiquitous, gram-positive bacillus that produces numerous toxins involved in various human and animal diseases (21, 33). Iota-toxin, one of the four major lethal and dermonecrotic proteins produced by *C. perfringens*, shares many interesting attributes with other bacterial binary lethal and dermonecrotic proteins produced by *animal diseases* (21, 33). Iota-toxin, one of the four major toxins synthesized by spore-forming bacilli such as *Clostridium sporioforme* (iota-like toxin), *Clostridium botulinum* (C2 toxin), *Bacillus anthracis* (lethal and edema toxins), and *Bacillus cereus* (vegetative insecticidal proteins) (13, 16, 18, 28). Iota-toxin consists of a 45-kDa ADP-ribosyltransferase, iota a (Ia), and an 81-kDa cell-binding protein, iota b (Ib), that are transcribed as separate proteins (27, 37). Ib is produced by *C. perfringens* as a biologically inactive 100-kDa protoxin (Ibp) capable of binding to cells but unable to form oligomers or interact with Ia (11, 36). In solution, Ib is cleaved by serine-type proteases into a 20-kDa N-terminal peptide and the 81-kDa biologically active Ib. Ib binds to the cell, forming heptamers and Na+/K+-permeable pores that promote Ia internalization into the cytosol (7, 20, 25, 36). Once inside, Ia ADP-ribosylates G-actin at arginine-177 and disrupts the cytoskeleton (40).

In addition to iota-toxin, *C. perfringens* produces other toxins that also form large complexes and pores in cell membranes (23, 29, 31, 34). The enterically-acting protein, epsilon-toxin, forms surface-associated heptamers that have recently been shown to localize in cholesterol-rich, detergent-resistant membrane microdomains (DRMs) (24). DRMs represent specialized membrane entities on the cell that concentrate lipids and proteins into unique domains, thus facilitating internalization of receptors and other molecules (8, 30).

Other bacterial toxins, such as the *C. perfringens* perfringolysin O, *Aeromonas hydrophila* aerolysin, *Clostridium septicum* alpha-toxin, and *B. anthracis* lethal and edema toxins are found in DRMs, forming large membrane-associated complexes that generate ion-permeable pores through the cell membrane (2, 3, 12, 29). Since DRMs are involved in the binding and internalization of many bacterial toxins, the following study investigated the potential role DRMs play in *C. perfringens* iota-toxin cytotoxicity, in particular focusing upon Ib binding and subsequent oligomer formation on cell membranes.

**MATERIALS AND METHODS**

Iota-toxin and antisera. Purified components of *C. perfringens* iota-toxin (Ia, Ib, and Ibp), as well as rabbit anti-Ib serum, were produced as described previously (28). Goat *C. sporioforme* and *C. perfringens* type C antisera were purchased from TechLab, Inc. (Blacksburg, Va.). Mouse monoclonal antibodies against clathrin heavy chain and a-1 Na+/K+-ATPase as well as affinity-purified rabbit antibody against dynamin II were purchased from Affinity BioReagents (Golden, Colo.). Affinity-purified antibodies against flotillin-1 and caveolin-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). *B. anthracis* PA components (the protoxin PA83 and the enzymatically cleaved PA63), as well as rabbit anti-PA serum, were kind gifts from Stephen Little (U.S. Army Medical Research Institute of Infectious Diseases).

Cells and DRM isolation. MRC-5 (human lung), Vero (African green monkey kidney), SW-13 (human adenocarcinoma), and PC12 (rat pheochromocytoma) cells were obtained from the American Type Culture Collection (Manassas, Va.). Cells were detached from culture flasks with 50 mM EDTA (Sigma, St. Louis, Mo.) in Hanks balanced salt solution (HBSS) lacking Ca2+ and Mg2+. As described previously (35), single-cell suspensions (5 × 10^6/ml) were prepared and incubated with 125 ng of Ib or Ibp in 50 μl of HBSS containing 0.2% bovine serum albumin (HBSS-BSA) for 10 min at 37°C, and then unbound Ib or Ibp was removed by three washes with HBSS-BSA.

To isolate DRMs, we used a procedure developed by Aman and Ravichandran.


RESULTS

Ib oligomers are found in DRMs of iota-toxin-susceptible cells. Iota-toxin-susceptible Vero cells were incubated with Ib and solubilized in cold (4°C) Triton X-100, and membrane components were separated by sucrose gradient centrifugation into detergent-resistant and -soluble fractions. Analysis by Western blotting showed that oligomeric Ib preferentially concentrated within the less-dense DRM fractions (Fig. 1A, lanes 2 to 4), while monomeric Ib appeared in the more-dense, soluble fractions (Fig. 1A, lanes 6 to 8). Additionally, previous investigations showed that Ibpb binds to cells but does not form oligomers or facilitate iota-toxin cytotoxicity (35). Therefore, when Vero cells were incubated with Ibpb and then subjected to Triton X-100 solubilization and gradient fractionation, oligomers were not detected but monomeric Ibpb was found in the soluble fractions (Fig. 1B).

Previous investigations noted that, unlike Vero cells, in which Ib binding is detected when analyzed by FACS, Ibpb was not detected on MRC-5 cells which are resistant to iota-toxin cytotoxicity (35). However, further studies by Western blotting have shown that monomeric, not oligomeric, Ibpb can be detected on these cells (36). When MRC-5 cells were analyzed in the present investigation, Ib oligomers were not found in DRMs (Fig. 1C, lanes 2 to 4), although monomeric Ibpb was detected in the soluble fractions (Fig. 1C, lanes 6 to 8).

Since MRC-5 cells are not susceptible to iota-toxin and Ibpb binding is not observed by FACS analysis, Western blotting experiments were performed in order to determine whether the Ib binding detected in MRC-5 membrane preparations was specific. Previous studies showed that the binary C. spiroforme toxin shares common epitopes with iota-toxin, and antisera prepared against C. spiroforme toxin will neutralize the activity of iota-toxin (28). Conversely, since only the type E strains of C. perfringens produce iota-toxin, antisera prepared against one of the other four major toxin types, like type C, will not neutralize iota-toxin cytotoxicity (21). In the present study, preincubation of Ib with C. spiroforme antisera prevented Ib binding, unlike incubation with C. perfringens type C antisera, thus indicating that Ib interactions with MRC-5 cells were specific (data not shown).

In addition to what occurred in experiments with Vero and MRC-5 cells, iota-toxin-susceptible PC12 and SW13 cells generated Ib oligomers that localized in DRMs, while monomeric Ibpb was detected in the soluble fraction (data not shown). Western blot analysis revealed a protein (220 kDa) consistently present in the soluble fractions of cells incubated with Ib (Fig. 1J, 3, and 5), as well as those not incubated with Ib (Fig. 1D), thus indicating that this protein was not Ibpb specific. To a lesser degree than with anti-Ibpb serum, the band was detected by rabbit anti-PA serum, although not with normal rabbit serum (data not shown).

Cellular proteins detected in DRMs of Ib-treated Vero cells. Upon detergent solubilization and subsequent isolation,
specific marker proteins localize in DRMs (caveolin-1 and flotillin-1) while others remain in the soluble fraction (α-1 Na+,K+-ATPase) (2, 24). When DRMs and detergent-soluble fractions prepared from Vero cells incubated with Ib were analyzed by Western blotting for distinct marker proteins, caveolin-1 and flotillin were found in DRMs while α-1 Na+,K+-ATPase was detected in the soluble fraction (Fig. 2). Two other proteins, clathrin and dynamin, often involved in endocytosis, were found in the soluble fraction (2, 39). DRMs prepared from Vero cells not incubated with Ib also contained caveolin-1 and flotillin-1 in DRMs and α-1 Na+,K+-ATPase, clathrin, and dynamin in the soluble fraction (data not shown).

**GPI-anchored proteins do not represent a receptor for Ib.**

Previous investigations have shown that the Ib cell receptor consists of a protein (35). Often associated with DRMs and used by many bacterial toxins as cell receptors, GPI-anchored proteins are easily discerned by treating cells with PI-PLC, an enzyme that specifically cleaves the GPI anchor, thus releasing proteins from membranes (1, 9, 12, 41). In order to determine whether the receptor for Ib was a GPI-anchored protein, the effects of PI-PLC treatment on Ib binding and oligomer formation were investigated. Although Ib binding and oligomer formation were not abolished by pretreatment of cells with the enzyme, there appeared to be a slight shift of oligomeric Ib from the DRMs to monomeric Ib detected in the soluble fraction (Fig. 3A). In both control and enzyme-treated cells, however, Ib oligomers were detected in DRMs while monomeric Ib was located in the soluble fractions. In addition, when Ia and Ib were added to the cells, with or without PI-PLC pretreatment, there was similar sensitivity to iota-toxin, thus demonstrating that PI-PLC treatment did not alter iota-toxin cytotoxicity (data not shown).

When Vero cells were analyzed for Ib binding by FACS, pretreatment of cells with PI-PLC did not alter the number of Ib-positive cells (Fig. 3B). Histograms (Fig. 3B) showed the relative number of cells binding to Ib, as reflected by an increase in fluorescence from negative to positive when the cells were incubated without (panels 1 and 3) or with (panels 2 and 4) Ib. The amount of Ib binding to PI-PLC-treated cells (panels 3 and 4) did not differ from that in the untreated controls (panels 1 and 2).

**Effects of compounds that remove or sequester cholesterol on Ib binding and iota-toxin cytotoxicity.** In order to determine whether cholesterol was required for iota-toxin cytotoxicity, Ia and Ib were added to Vero cells pretreated with filipin (which sequesters cholesterol) or CD (which removes cholesterol). Then, at various times after intoxication, cells were examined microscopically. When Ia plus Ib were added to cells either untreated (control) or pretreated with filipin, they uniformly rounded and detached within 2 h after addition of toxin (data not shown). If cells were pretreated with CD prior to iota-toxin, there was a noticeable delay in cytotoxicity. Exam-
inDRMs and soluble fractions. FACS analysis would differentiate between Ib oligomers or monomers bound to the cell surface (35). GPI-anchored proteins, as with aerolysin, bind to cell binding and subsequent oligomer formation (24, 36). Ib and epsilon protoxins bind to cells but do not form cell-associated oligomers and do not facilitate cytotoxicity (23, 36). Unlike epsilon-toxin, where both the protoxin and proteolytically activated forms associate with DRMs, Ib was detected in only the soluble fractions.

Although Ib resembles the epsilon protoxin, in that both are cleaved prior to cell binding, the cellular association of Ib more closely resembles that of the PA protoxin (PA83), which binds to a non-DRM protein, the anthrax toxin receptor (2). Cell surface activation of PA83 by furin then leads to monomeric PA63, and the cell receptor is seemingly concentrated into DRMs that facilitate rapid formation of oligomers (3, 17, 19, 22). When Vero cells were incubated with PA83 or PA63 and subjected to Triton X-100 solubilization and sucrose gradient fractionation, analysis by Western blotting detected PA83 in the soluble fractions and PA63 primarily in DRMs.

Previous investigations have indicated that the cellular receptor for Ib is a protein because pronase pretreatment of Vero cells prevents Ib binding (35). GPI-anchored proteins, normally involved in cell signal transduction, associate with DRMs and are often used by bacterial toxins as receptors for binding to cells (9, 12, 41). The toxins may bind to specific GPI-anchored proteins or, as with aerolysin, bind to glycan regions conserved in several different GPI-anchored proteins. Involvement of these proteins as toxin receptors is easily determined, since most GPI-anchored proteins are released from DRMs when cells are treated with PI-PLC (1, 9). While PI-PLC treatment of Vero cells did not inhibit Ib binding and oligomer formation, the treatment resulted in a slight reduction of oligomeric Ib detected in DRMs and an increase of monomeric Ib in the soluble fractions. FACS analysis would not detect this shift, because the rabbit Ib antiserum does not differentiate between Ib oligomers or monomers bound to the cell surface (35).

Since PI-PLC treatment of Vero cells did not alter iota-toxin cytotoxicity and did not inhibit oligomer formation in DRMs, the Ib receptor was apparently not a GPI-anchored protein. The slight shift from oligomeric to monomeric Ib, however, is not a consistent finding. Currently, it is not clear whether the Ib receptor is GPI-anchored or not, but future investigations will be needed to determine the role of this receptor in Vero cell toxicity.
indicated that GPI-anchored proteins could be involved, either directly or indirectly, with Ib forming oligomers in DRMs. While specific for cleavage of a GPI anchor, PI-PLC is not specific in terms of the protein moiety, and the enzyme will release these anchors from various proteins that are normally contained within DRMs, the removal of which could influence DRM formation and stability (9). Further investigation is needed to understand the role these proteins may play in Ib binding and oligomer formation.

Removal or sequestration of cholesterol results in dispersal of DRM-associated components so that certain protein toxins can no longer bind to normally targeted cells (24, 26). Although filipin and CD reduce the availability of membrane-associated cholesterol, their mode of action differs and often results in differential effects on receptor binding and signaling processes within the membrane (6, 15, 42). Filipin, a polyene macrolide antibiotic, inserts into the membrane and sequesters cholesterol, thus inducing structural disorder that distorts caveolar domains and inhibits caveolar function. The drug preferentially binds to cholesterol within caveolae and has been used to identify caveola-associated processes (32). Pretreatment of Vero cells with filipin did not inhibit iota-toxin cytotoxicity or alter either Ib binding or oligomer formation, thus indicating no involvement by caveolae (26). These results are supported by the findings of M. Gibert et al. (unpublished data), showing that cells pretreated with filipin or a related compound, nystatin, did not prevent iota-toxin cytotoxicity as measured by actin filament depolymerization. Further analysis also suggested that caveolae are not involved in Ib oligomer formation because caveolin-1, a major component of caveolae, was detected in DRMs isolated from MRC-5 cells that are toxin resistant and do not form oligomers (36). Conversely, Ib oligomers were found in DRMs from toxin-sensitive SW-13 cells after incubation with Ib, but caveolin-1, as initially described by Holwell and coworkers (14), was not detected (data not shown).

While filipin selectively complexes with membrane cholesterol, CD is a small cyclic oligosaccharide that concentrates at the cell surface and extracts cholesterol from the membrane (6, 42). Recent investigations note that the membrane contains both CD-sensitive and -resistant cholesterol pools, primarily located outside DRMs (15, 42). Since monomeric Ib was not detected in DRMs and CD treatment diminished Ib binding, the cell receptor for Ib more than likely associated with CD-sensitive cholesterol pools, and cholesterol removal resulted in membrane release or conformational distortion of the receptor (15). Additionally, cholesterol extraction by CD could also affect accessibility of the Ib cell receptor, leading to decreased binding of Ib, fewer Ib-Ib interactions, and ultimately decreased, slower oligomer formation (3). As suggested by the
delay in time to cell death, however, sufficient oligomers were ultimately formed to facilitate the entry of Ia. Very few molecules of Ia in the cytosol are likely needed to cause cell death, but this aspect of iota-toxin cytotoxicity remains largely unexplored. The CD effect was specific, because both the delay in cytotoxicity and the decrease in oligomer formation did not occur if cholesterol was added to the medium after CD pretreatment (data not shown). The results from cholesterol-reducing agents indicated that while the cell receptor was not found in DRMs, the receptor probably resided in an area associated with cholesterol that, upon Ib binding, helped to facilitate oligomer formation within DRMs.

Oligomer formation dependence upon an acidic microenvironment and a reduction of PA binding in cells that lack functional clathrin-dependent endocytosis led Abrami and colleagues (2) to conclude that the initial entry of PA into acidic endosomes is by a clathrin-dependent process. Cells lacking functional clathrin-dependent endocytosis or pretreatment of Vero cells with chlorpromazin, a drug that blocks endocytosis through clathrin-coated pits, does not alter Ib binding or iota-toxin cytotoxicity (Gibert et al., unpublished). While caveolin-1 and flotillin-1, proteins commonly found in DRMs, were detected in DRMs extracted from Vero cells incubated with or without Ib, clathrin and dynamin were found in the soluble fractions, thus indicating again that Ib did not use a clathrin-dependent route for endocytosis (2). Additional experiments showed that pretreatment of cells with antibodies prepared against these proteins did not prevent or delay iota-toxin cytotoxicity (data not shown), thus providing further evidence that Ib does not use clathrin-coated pits or caveolae as an entry portal.

Finally, evidence that the cell likely plays an active role in oligomerization is also suggested by experiments with paraformaldehyde-fixed Vero cells that bind Ib but do not form Ib oligomers (B. G. Stiles et al., unpublished data). Ib oligomers rapidly form (in less than 1 min) on Vero cells at 37°C, but oligomers and increased ion permeability are not evident at 4°C (36). Likewise, oligomers were observed in DRMs isolated from cells incubated with Ib at 37°C but not 4°C (data not shown). These studies indicate that Ib oligomer formation is not a random event and requires a metabolically active cell.

In summary, the investigations presented here suggest that Ib binds to a specific protein located outside DRMs. Although the Ib receptor does not reside in DRMs, association with DRMs likely facilitates Ib-Ib interactions and stabilizes nascent oligomer formation. These investigations showed that while the binary bacterial toxins share many similarities, there are also marked differences in binding and subsequent oli-

FIG. 4. Phase-contrast microscopy showing delay of iota-toxin cytotoxicity in Vero cells pretreated with or without CD. Ia plus Ib (125 ng of each) were incubated with Vero cells (left side) and CD-treated Vero cells (right side). Photographs were taken immediately (0 h), 2 h, and 6 h after addition of toxin.
gomerization. Further investigations will provide a greater understanding of how iota-toxin and other binary toxins bind to cells, form oligomeric complexes, and facilitate protein transport across cell membranes.

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REFERENCES


FIG. 5. Western blotting detection of monomeric and oligomeric Ib on Vero cells pretreated with compounds that affect membrane cholesterol. After treatment, cells were solubilized in Triton X-100 and fractions were separated by sucrose discontinuous density gradient centrifugation. (A) No treatment; (B) filipin; (C) CD. For panels A to C, lanes 1 to 8 represent fractions from a 5, 30, and 40% discontinuous sucrose density gradient. (D) Western blotting detection in DRMs (fractions 2 and 3) of PA63 on Vero cells pretreated as for panels A to C.
cells is not associated with the level of expression of apoE, sterol carrier protein-2, or caveolin. J. Lipid Res. 40:1440–1452.