gp96 Is a Human Colonocyte Plasma Membrane Binding Protein for Clostridium difficile Toxin A

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Clostridium difficile toxin A (TxA), a key mediator of antibiotic-associated colitis, requires binding to a cell surface receptor prior to internalization. Our aim was to identify novel plasma membrane TxA binding proteins on human colonocytes. TxA was coupled with biotin and cross-linked to the surface of HT29 human colonic epithelial cells. The main colonocyte binding protein for TxA was identified as glycoprotein 96 (gp96) by coimmunoprecipitation and mass spectrum analysis. gp96 is a member of the heat shock protein family, which is expressed on human colonocyte apical membranes as well as in the cytoplasm. TxA binding to gp96 was confirmed by fluorescence immunostaining and in vitro coimmunoprecipitation. Following TxA binding, the TxA-gp96 complex was translocated from the cell membrane to the cytoplasm. Pretreatment with gp96 antibody decreased TxA binding to colonocytes and inhibited TxA-induced cell rounding. Small interfering RNA directed against gp96 reduced gp96 expression and cytotoxicity in colonocytes. TxA-induced inflammatory signaling via p38 and apoptosis as measured by activation of BAK (Bcl-2 homologous antagonist/killer) and DNA fragmentation were decreased in gp96-deficient B cells. We conclude that human colonocyte gp96 serves as a plasma membrane binding protein that enhances cellular entry of TxA, participates in cellular signaling events in the inflammatory cascade, and facilitates cytotoxicity.

Clostridium difficile is a gram-positive anaerobic human pathogen that causes pseudomembranous colitis following antibiotic therapy. C. difficile infection is highly prevalent in hospitals and nursing homes where patients frequently receive antibiotics and represents one of the most common hospital infections, causing approximately 3 million cases of diarrhea and colitis per year (20, 33) in the United States.

C. difficile releases two high-molecular-weight exotoxins, toxin A (TxA) and toxin B (TxB). These enzymes share approximately 63% amino acid homology and have identical glucosyltransferase domains that inactivate Rho proteins, leading to actin disassembly and cell rounding in target cells (15, 17, 18, 42). TxA and TxB consist of three major domains. The enzymatic (catalytic) domain containing the critical glucosyltransferase activity is expressed in the first 550 N-terminal amino acids, and the receptor binding domain resides in the C-terminal domain (14, 15). The middle hydrophobic portion is suggested to facilitate toxin translocation into the cytosol. The C-terminal (receptor binding) domain of TxA consists of repeating oligopeptide units with 21, 30, or 50 amino acid residues (7, 30, 42, 43). Recent crystal structure of a 127-amino-acid fragment of the C terminus (toxinotype VI, covering residues 2582 to 2709) revealed a solenoid-like structure.
Antibodies and reagents. Rat monoclonal gp96 antibody was purchased from Neomarkers (Lab Vision Corporation, Fremont, CA). Isotypic control rat immunoglobulin G (IgG) was from Immunotech (Beckman Coulter, Inc., Fuller- ton, CA). Hsp60, Hsp75, Hsp90, and their isotypic control antibodies were purchased from Neomarkers. Antibodies against phosphorylated or total p38 were purchased from Cell Signaling Technology (Beverly, MA). Antibody to Bcl-2 homologous antagonist/killer (BAK) was obtained from Cell Signaling Technology (Danvers, MA), and actin antibody was obtained from Santa Cruz Biocompany (Santa Cruz, CA). Goat anti-TxA was kindly provided by Michel Warny.

FIG. 1. Immunoprecipitation with TxA-biotin. (A) Native and biotinylated TxA (2 μg each) were resolved on SDS-PAGE gels for either Coomassie blue staining or Western blotting (WB) with streptavidin to detect biotinylated TxA. (B) HT29 cells were exposed to either TxA (1 nM) or TxA-biotin (1 nM) for 60 min (60”) at 37°C, and cell rounding was assessed microscopically. (C) HT29 cells were incubated with TxA-biotin (1 nM) at 4°C for 1 h in the presence of BS3. TxA-biotin-associated proteins were immunoprecipitated (IP) against streptavidin. Immunoprecipitates were resolved on SDS-PAGE gels and subjected to Western blotting (WB) with streptavidin-AP. TxA-biotin was loaded as a control.
Coimmunoprecipitation and mass spectrometry. TxA was coupled with biotin according to the manufacturer’s instructions (Pierce Biotechnology, Inc., Rockford, IL). HT29 cells were incubated with TxA-biotin (1 nM) at 4°C for 1 h in the presence of the cross-linker BS3 [bis(sulfosuccinimidyl) suberate] (Pierce Biotechnology, Inc.). We reported previously that at 4°C TxA bound to the cell surface but was not internalized (32). After a 1-h incubation, cells were washed to remove unbound TxA-biotin and lysed in 0.5% NP-40. Cell lysates were immunoprecipitated with streptavidin-coated beads to collect TxA-biotin and TxA-biotin-bound proteins. A complex of TxA-biotin (molecular size of 308 kDa) and protein was observed as a higher-molecular-weight band ($\alpha_11011400$ kDa), which was excised and analyzed by mass spectrometry.

Coimmunoprecipitation assay. NCM460 cells were grown to 100% confluence in T-75 flasks, and TxA (1 nM) was added for 1 h at 4°C. Cells were then lysed and immunoprecipitated (IP) with gp96 antibody or control IgG. Immunoprecipitates were resolved on SDS-PAGE gels and subjected to Western blotting (WB) with gp96 and TxA. Whole-cell lysates were used as a positive control. (C) Native TxA or heat-inactivated TxA (10 µg) was incubated with or without gp96 (10 µg) at either 4 or 37°C. The mixture was immunoprecipitated with gp96 antibody after a 60-min incubation. Immunoprecipitates were then resolved on SDS-PAGE gels and immunoblotted with antibodies to gp96 or TxA.

FIG. 2. gp96 interacts with TxA. (A) Peptide sequences (red) matching gp96 that were identified in the protein interacting with TxA on HT29 cells. (B) NCM460 cells were incubated with TxA (1 nM) for 1 h at 4°C. Cells were then lysed and immunoprecipitated (IP) with gp96 antibody or control IgG. Immunoprecipitates were resolved on SDS-PAGE gels and subjected to Western blotting (WB) with gp96 and TxA. Whole-cell lysates were used as a positive control. (C) Native TxA or heat-inactivated TxA (10 µg) was incubated with or without gp96 (10 µg) at either 4 or 37°C. The mixture was immunoprecipitated with gp96 antibody after a 60-min incubation. Immunoprecipitates were then resolved on SDS-PAGE gels and immunoblotted with antibodies to gp96 or TxA.

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Cell-rounding assay. HT29 or NCM460 cells were seeded on 96-well plates for 16 h and incubated with antibodies to gp96, Hsp60, Hsp75, or Hsp90 or control IgG (40 μg/ml) 5 min before TxA addition. The percentage of rounded cells was counted in 10 separate high-power fields using phase microscopy.

Fluorescence immunostaining. HT29 cells were incubated on four-well slides (Nalge, Rochester, NY) for 16 h, and TxA (1 nM) was added to the cell medium. Cells were then fixed with 4% formaldehyde and doubly immunostained with anti-gp96 monoclonal rat antibody and anti-TxA polyclonal goat antibody, followed by Texas red-conjugated anti-rat and fluorescein isothiocyanate (FITC) green-conjugated secondary antibody. Cells were permeabilized with 0.2% Triton X-100 for 5 min at room temperature after fixation under permeable staining conditions. Expression intensity and location of gp96 and TxA were examined by confocal microscopy.

Immunohistochemical staining. Normal human colonic tissue was obtained from surgical “discards” following removal from patients undergoing elective colectomy for colorectal neoplasm. Immunohistochemical stains were performed on formalin-fixed, paraffin-embedded tissue sections of human colon using monoclonal rat antiserum to human gp96 (Lab Vision Corporation, Fremont, CA) (35) (1:100). Sections were incubated with primary antibodies at room temperature for 60 min, followed by another 60-min incubation with rabbit anti-rat secondary antibodies (Jackson Labs, West Grove, PA). Slides were developed for 10 min at room temperature and counterstained with modified Mayer’s hematoxylin blue. Expression of gp96 was examined using light microscopy.

Apoptosis assay. E4.126 cells deficient in gp96 and control cells derived from the murine pre-B-cell line 70Z/3 (35) were incubated with 10 nM TxA or heat-inactivated TxA (95°C for 1 h) for 60 to 240 min. Cells were lysed, resolved on 15% SDS-PAGE gels, and analyzed by Western blotting for BAK and actin. E4.126 and control cells were incubated with 10 or 50 nM TxA or staurosporine (1 μM), a chemical agent that induces apoptosis at 24 or 48 h. The percentage of cells undergoing apoptosis was determined using the Flurosens Assay kit (Laboratories for Therapeutic Research, New York, NY) and analyzed by Western blotting using antibodies to gp96 and TxA.

Statistical analyses. Data were analyzed using the SIGMA-STAT professional statistics software program (Jandel Scientific Software, San Rafael, CA).
protein on the HT29 cell surface was identified as gp96 (Fig. 2A). To examine whether gp96 also associates with TxA in colonocytes other than HT29, we tested a nontransformed human colonocyte line, NCM460. NCM460 colonocytes were incubated with TxA (1 nM) for 1 h at 4°C, at which toxin binding but not internalization occurs, and lysates were immunoprecipitated with anti-gp96 or control IgG antibodies, fractionated on SDS-PAGE gels, and immunoblotted with antibodies to either TxA or gp96. As shown in Fig. 2B, TxA was identified in immunoprecipitates against gp96 but not in immunoprecipitates against control antibodies, indicating that TxA bound specifically to gp96 in NCM460 cells. To examine if purified gp96 could itself bind to TxA, coinmunoprecipitation was performed using highly purified TxA and gp96. TxA or heat-inactivated TxA (10 μg) was incubated with or without gp96 (10 μg) at 4°C in order to preserve protein structure and at 37°C in order to reflect physiological conditions and immunoprecipitated with gp96 antibody. TxA immunoprecipitated when incubated with gp96, demonstrating that TxA binds to non-membrane-bound gp96 (Fig. 2C). More TxA was detected interacting with gp96 at 4°C than at 37°C, indicating that the native toxin structure was better preserved at 4°C. Heat-inactivated TxA failed to interact with gp96 (Fig. 2C), suggesting that both the functional and structural integrity of TxA is important to its ability to associate with gp96.

**Cellular localization of gp96.** To visualize the cellular localization of gp96 and its interaction with TxA in colonocytes, we performed dual immunofluorescent staining with gp96 and TxA antibodies in HT29 cells at 37°C, a permissive temperature that allows surface binding and entry of TxA into the cells. As shown in Fig. 3A, after exposure to TxA (1 nM) for 5 min, gp96 (red) was colocalized with TxA (green) on the cell plasma membrane (Fig. 3A, yellow in merged images). After 15 min of TxA exposure, gp96 was observed to partially translocate into the cytoplasm, where it was still partially colocalized with TxA. In contrast, expression patterns of several other heat shock proteins, Hsp75, Hsp60, and Hsp90, in HT29 cells examined by immunofluorescence did not reveal cell surface staining (data not shown). At 4°C gp96 and TxA were colocalized on the cell membrane but not internalized (data not shown). gp96 expres-

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**FIG. 5.** gp96 antibody inhibits TxA-induced cell rounding. (A) HT29 cells were incubated with cell medium, TxA (1 nM), gp96 antibody (40 μg/ml) plus TxA (1 nM), or control IgG (40 μg/ml) plus TxA (1 nM). Cell rounding was measured after a 60-min (60') incubation at 37°C and expressed as mean percent cell rounding ± standard error of the mean of three experiments per group. *, P < 0.05 for the percentage of cell rounding in cells exposed to TxA antibody plus gp96 versus cells exposed to TxA plus control IgG. (B) HT29 cells were incubated with antibodies to gp96, Hsp75, Hsp60, Hsp90α, or Hsp90β or with control IgG prior to TxA exposure. Cell rounding and statistical analyses were performed as described for panel A. Ab, antibody.
tion in human colonic mucosa obtained from surgical specimens was evaluated by immunohistochemistry staining and was mainly detected in colonocytes (Fig. 3B) with both membrane and cytoplasmic staining.

**gp96 antibody inhibits TxA binding and cell rounding.** HT29 colonocytes were incubated with either control IgG (40 μg/ml) or gp96 antibody (40 μg/ml) before exposure to TxA (1 nM) at 4°C, and immunofluorescent staining for TxA was used to assess TxA binding to the plasma membrane. As shown in Fig. 4A, TxA binding to the cell surface was markedly decreased in cells preincubated with gp96 antibody. To further study gp96 antibody effects on TxA binding, we incubated HT29 cells with biotinylated TxA at 4°C and compared binding of biotinylated TxA on the cell surface after preincubation with either nonlabeled TxA (100-fold excess), control IgG (40 μg/ml), or gp96 (40 μg/ml) antibody. As shown in Fig. 4B, TxA-biotin binding was concentration dependent with saturation at 100 ng/ml (similar binding curves were also observed in NCM460 cells [data not shown]). Non-labeled or native TxA or gp96 antibodies inhibited the total binding of TxA-biotin to the cell surface by approximately 40%.

We next analyzed the effect of gp96 antibody on TxA-induced cell rounding. HT29 cells were exposed to medium alone (Fig. 5A), TxA alone (1 nM), gp96 antibody (40 μg/ml) plus TxA (1 nM), or control IgG (40 μg/ml) plus TxA (1 nM) for 1 h. As expected, compared to the control, TxA alone caused significant cell rounding, which was significantly blocked by gp96 antibody but not control IgG. gp96 antibody had no effect on TxB-mediated cell rounding under the same conditions (data not shown), consistent with previous observations suggesting that these two toxins bind to separate membrane receptors (27, 29, 41). The effects of antibodies to other heat shock proteins (Hsp60, Hsp75, Hsp90α, and Hsp90β) on TxA-mediated cell rounding were studied to assess the specificity of gp96 antibody. As shown in Fig. 5B, antibodies against Hsp60 or Hsp75 did not prevent rounding, while antibodies to Hsp90α and Hsp90β, which are family members of gp96, demonstrated moderate inhibition.

**Silencer gp96 reduced TxA-mediated cytotoxicity.** Silencer gp96 was used to downregulate the expression of gp96 on human colonocytes. As shown in Fig. 6A, exposure of NCM460 cells to TxA (1 nM) led to slight gp96 overexpression. Introducing silencer gp96 into the cells inhibited TxA-induced gp96 production slightly at 24 and significantly more at 48 h. Cell rounding after TxA exposure was decreased in proportion to the degree of gp96 inhibition (Fig. 6B).

**gp96 is required for TxA-mediated cell signaling.** E4.126, a B-cell line deficient in gp96, was identified by Randow and Seed (35) by screening for genes required for the lymphocyte response to bacterial endotoxins. E4.126 cells are at least 10,000 times less sensitive to LPS than nonmutant cells because Toll-like receptors require gp96 for their translocation from the endoplasmic reticulum to the cell surface. We have reported that p38, a key mediator in inflammatory signaling cascades, is phosphorylated after exposure to TxA in monocytes (44) and colonocytes (22) and serves as a marker for TxA receptor binding, internalization, and activation of signal transduction pathways. We exposed E4.126 and control cells to either LPS (positive control) or TxA. As shown in Fig. 7A, p38 activation by either LPS or TxA was reduced in gp96-deficient E4.126 cells. Because epithelial cell apoptosis occurs after TxA exposure in human colon (36), we examined TxA-induced apoptosis in B cells with intact or deficient gp96 (control and E4.126 cells). BAK is a proapoptotic member of the Bcl-2 family expressed on the outer membrane of mitochondria (9), which is important for conducting apoptotic signals through the mitochondrial pathway (46, 47). Upon apoptotic stimulation, an upstream stimulator induces conformational changes in BAK to form oligomer channels in the mitochondrial membrane for cytochrome c release (6, 45). We previously showed that TxA strongly induces colonocyte BAK expression and regulates mitochondrial cytochrome c release and subsequent activation of caspase-3, leading to apoptosis (21). As shown in Fig. 7B (part a), TxA-induced expression of BAK was higher in control versus E4.126 cells, consistent with the suggested role of GP96 as a binding site for the toxin. We also compared TxA-induced DNA fragmentation (laddering effect) in these two cell lines (Fig. 7B, part b) using staurosporine as a positive control. In E4.126 cells with deficient gp96 expression, TxA caused apoptotic degradation of genomic DNA (12 kDa) only after 48 h of TxA exposure and at a concentration of 50 nM. In contrast, in control cells with intact gp96, genomic DNA degradation was
FIG. 7. gp96 and TxA-mediated cell signaling. (A) Control (wild type) and E4.126 (gp96-deficient B lymphocytes) cells were exposed to either LPS (10 μg/ml) (a) or TxA (10 nM) (b). Cell lysates were resolved on SDS-PAGE gels and subjected to Western blotting (WB) against phosphorylated p38 or gp96. Antibodies against total p38 demonstrated equal protein loading. (B) Control (wild type) and E4.126 (gp96-deficient B lymphocytes) cells were exposed to TxA (10 nM) for different time periods (a) and treated with Staurosporine (10 nM and 50 nM) (b). Cell lysates were resolved on agarose gels and subjected to genomic DNA analysis (c).
evident after 24 h of incubation with the lower concentration of TxA, similar to the effect seen with staurosporine. Heat-inactivated TxA failed to induce DNA degradation in control cells with intact gp96 (Fig. 7 B, part c).

DISCUSSION

We report here that gp96 is a human colonocyte plasma membrane binding protein for C. difficile TxA on human colonocytes, including the malignant cell line HT29 and a nontransformed cell line, NCM460. Moreover, absence of gp96 on a mutant B-cell line, E4.126, renders these cells partially but not completely resistant to p38 signaling and apoptosis. We also observed substantial but incomplete blocking of TxA effects by gp96 antibodies. We suspect that partial blocking or delay of binding of TxA-biotin (Fig. 4) and cell rounding of HT29 cells (Fig. 5) may be related to low affinity of gp96 antibody binding versus high-affinity TxA binding to gp96. We also observed partial inhibition of rounding in NCM460 cells exposed to silencer gp96 (Fig. 6). In this experiment we were unable to completely silence gp96 (Fig. 6A), which may explain the partial inhibition of rounding (Fig. 6B). However, in E4.126 lymphocytes lacking gp96, we observed attentuated p38 signaling to TxA, as well as to LPS, as reported earlier by Randow and Seed (35).

Several interpretations can be generated from our data. One is that gp96 is the major (or only) membrane receptor for TxA and that residual cell rounding in the presence of gp96 blocking antibody (Fig. 5) or in the presence of silencer gp96 (Fig. 6) reflects incomplete activity of these two experimental manipulations, allowing residual expression of gp96 on target cells. Alternatively, some of the target cells might take up TxA by non-receptor-mediated endocytosis. A second hypothesis is that gp96 is a TxA coreceptor whose main function is to present the toxin to a high-affinity coreceptor. A similar situation has been described for the role of cell surface glypican, which mediates low-affinity binding of endostatin to endothelial cells (19). Interestingly, the related Hsp90 interacts with the glucocorticoid receptor to hold it in a conformation conducive to ligand binding (2). In this context it should be noted that heat shock proteins themselves have specific cell surface receptors. For example, internalization of gp96 into immune cells is mediated by its binding to scavenger receptor class A on the cell surface (1). We speculate that internalization of TxA in target cells is a multistep process and that initial binding to plasma membrane gp96 is critical.

Previous studies demonstrated that the C-terminal repetitive units of TxA interact with cell surface Galα1-3Galβ1-4GlcNAc as an initial step in pathogenesis (24). Recent cocrystal structure analysis using a TxA C-terminal fragment and a synthetic carbohydrate containing Galα1-3Galβ1-4GlcNAc revealed that this critical trisaccharide binds to a specific region in the C terminus of TxA that is formed by long peptide repeats and a hairpin turn of the following short peptide repeat (8). Galα1-3Galβ1-4GlcNAc is thought to be essential for the TxA receptor in rodents and other animals but apparently not in humans, who lack the specific α-galactosyltransferase required to form α-galactosyl bonds (23). Whether other glycans are critical for toxin binding in human tissues is unknown. gp96 is composed of 803 amino acids with five potential N-linked glycosylation sites (28), but the glycan moieties of this molecule have not been studied in detail. gp96 was found to have different glycosylation patterns between various tumors, and the presence or absence of specific glycosyl motifs was associated with variations in cancer phenotype (40). Detailed structural characterization of gp96 glycan motifs in colonic epithelial cells may be required to further characterize gp96 interaction with TxA.

gp96 is expressed on plasma membranes of porcine hepatocytes, endothelial cells, and Kupffer cells (5), but its location and physiologic function in the intestine have not been determined. In order for gp96 to function as a toxin receptor for C. difficile, it should be expressed on the apical plasma membrane facing the colonic lumen where TxA is released. Alternatively, gp96 on the basolateral membrane could also bind TxA, but the large size of the toxin (308 kDa) would prevent it from crossing the tight junction barrier in the intact colon epithelium. We observed gp96 on the exterior aspect of HT29 colonocyte plasma membranes (Fig. 3A). After TxA binding, cell surface gp96 was translocated into the cytoplasm, where it remained colocalized with TxA (Fig. 3A), suggesting a role in both plasma membrane binding and internalization. Cell rounding (cytotoxicity) in response to TxA requires its translocation to the cytoplasm where it glucosylates Rho family proteins that regulate actin filament formation (18). gp96 antibody partially blocked TxA binding to the colonocyte cell surface and also inhibited cell rounding (Fig. 4 and 5).

gp96 is recognized as a key mediator of the innate immune response related to its ability to bind pathogenic bacteria or their products. Cabanes et al. reported recently that gp96 is a plasma membrane receptor for Vip, a novel Listeria monocytogenes virulence factor that is required for cell invasion and downstream signaling events (3). gp96 expression was detected at the cell surface of Ca2 (human colonocyte) and L2071 (mouse fibroblast cells) (3) cells. A gp96 homologue, Ecpp, expressed on the surface of human brain microvascular endothelial cells has been shown to interact with the outer membrane protein A of Escherichia coli K1 (34).

gp96 has also been linked to the Toll-like receptor pathway, which recognizes pathogens and activates transcription factors (e.g., NF-κB) that participate in immune responses to pathogens (16, 35). gp96 directly binds Toll-like receptors, deter-
mines their subcellular localization, and reduces sensitivity to LPS secondary to intracellular retention of Toll-like receptors (35). We observed (Fig. 7A) that a gp96-deficient B-cell line also exhibits markedly reduced sensitivity to TxA-induced p38 activation and apoptosis compared to parental cells with normal expression of gp96, supporting a role for gp96 in binding and internalization of this toxin.

In addition to directing innate immune responses, gp96 also has important functions in the adaptive immune response. gp96 binds to a wide range of peptides and delivers them to antigen-presenting cells (25). Recent studies demonstrate that gp96 elicits antigen-presenting cell activation, making it an attractive candidate to facilitate tumor vaccines (37–39). One potential clinical application of our observations here would be to determine if the gp96-TxA complex provides enhanced immunogenicity to a C. difficile vaccine.

It was previously reported that Hsp72, a member of the Hsp70 heat shock family of proteins, has a protective role against C. difficile TxA (26). Colonic epithelial cells transfected with Hsp72 antisense were more sensitive than control cells to Hsp72 heat shock family of proteins, has a protective role in the adaptive immune response. Creating a C-terminal repeat from Hsp90 is essential for the steroid glucocorticoid receptor. J. Biol. Chem. 277:1076–1081.


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