**Clostridium difficile** Toxin A Inhibits the Kinase Activity of Extracellular Signal-Related Kinases 1 and 2 Through Direct Binding

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**Clostridium difficile** toxin A glucosylates Rho family proteins, resulting in actin filament disaggregation and cell rounding in cultured colonocytes. Given that the cellular toxicity of toxin A is dependent on its receptor binding and subsequent entry into the cell, we herein sought to identify additional colonocyte proteins that might bind to toxin A following its internalization. Our results revealed that toxin A interacted with ERK1 and ERK2 in two human colonocyte cell lines (NCM460 and HT29). A GST-pulldown assay also showed that toxin A can directly bind to ERK1 and ERK2. In NCM460 cells exposed to PMA (an ERK1/2 activator), the phosphorylation of ERK1/2 did not affect the interaction between toxin A and ERK1/2. However, an *in vitro* kinase assay showed that the direct binding of toxin A to ERK1 or ERK2 inhibited their kinase activities. These results suggest a new molecular mechanism for the cellular toxicity seen in cells exposed to toxin A.

**Keywords:** *Clostridium difficile* toxin A, extracellular signal-related kinases-1/2, protein–protein interaction, antiproliferatory effect

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**Clostridium difficile**, an anaerobic pathogen that can cause antibiotics-associated diarrhea and pseudomembranous colitis, releases two exotoxins: toxin A and toxin B. These toxins use separate cell surface receptors [18], and only toxin A has been shown to trigger severe diarrhea and inflammation in animal intestines [14, 16, 20]. Both toxins have glucosyltransferase activities that are capable of inactivating Rho proteins, leading to actin disaggregation and cell rounding [9, 10]. The catalytic domain of toxin A is located in its N-terminal region, whereas the hydrophobic middle region was thought to be involved in the translocation of the toxin to the cytosol [9, 10]. The receptor-binding domain, which is located in the C-terminal region [8, 9], contains a solenoid-like structure that is involved in protein–carbohydrate interactions [7]. After receptor binding, toxin A is rapidly internalized to the cytosol of the target cells [5, 6, 13]; this step is critical for the ability of the toxin to trigger actin filament disaggregation and cell rounding [5, 6]. Accordingly, some recent studies have focused on seeking to identify intracellular proteins that can bind to the internalized toxin A, in an effort to explain the varied cellular toxicities observed in different cell types. However, only a few proteins have been clearly shown to interact with toxin A. For example, the interaction of toxin A with glycoprotein 96 (gp96), a membrane protein and member of the heat shock protein family, was found to mediate cell rounding and apoptosis in human colonocytes [17], and we previously reported that binding of toxin A to the catalytic domain of Src inhibited its kinase activity, which in turn caused cell detachment [13]. These findings suggest that the dysfunction or unusual coupling of one or more toxin A-interacting protein(s) may trigger cellular toxicity following exposure to toxin A.

Na *et al.* [18] reported that *C. difficile* toxin B-mediated induction of IL-8 transcription was markedly blocked by treatment with the ERK1/2 inhibitor, PD98059. Therefore, we assessed in the present study whether toxin A interacts with ERK1/2 in human colonocytes. We used a GST-pulldown assay to confirm the direct binding of toxin A to ERK1 and ERK2 *in vitro*, and performed an *in vitro* kinase assay to show that the interaction with toxin A decreased the kinase activities of ERK1/2. Since ERK1 and ERK2 are essential mediators of cell proliferation and
differentiation in various cell types, our finding that toxin A directly blocks their activities provides new insight into the molecular mechanisms underlying toxin A-induced cellular toxicity.

**Materials and Methods**

*C. difficile* Toxin A and Biotin Labeling Reaction

Toxin A was purified from the culture supernatants of *C. difficile* strain VPI 10463 (American Type Culture Collection, Rockville, MD, USA), as previously described [22]. For the biotinylation of toxin A, we used a Sulfo-NHS-LC biotinylation kit (Pierce, Rockford, IL, USA). Briefly, 1 mg of toxin A was added to the Sulfo-NHS-LC-Biotin solution and incubated on ice for 2 h, and then the unbound biotin reagent was removed with a streptavidin column. The purities of the native and biotinylated toxin A proteins were assessed by gel electrophoresis, which confirmed the expected molecular mass of 307 kDa [13].

**Reagents**

Polyclonal antibodies against ERK1/2 and p38MAPK were obtained from Cell Signaling Technology (Beverly, MA, USA). PMA (phorbol 12-myristate 13-acetate), an ERK1/2 agonist, was purchased from Culbiochem (San Diego, CA, USA). GST-ERK1, GST-ERK2, and recombinant activated ERK1 and ERK2 were obtained from Upstate Biotechnology (Lake Placid, NY, USA). The streptavidin-agarose beads were from Sigma-Aldrich (St. Louis, MO, USA), and the human NCM460 colonocytes and M3D culture medium were obtained from INCELL Corporation (San Antonio, TX, USA).

**Immunoblot Analysis**

Human colonocytes were washed with cold PBS and mixed with lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% Nonidet P-40), and equal amounts of protein were fractionated by SDS-polyacrylamide gel electrophoresis, which confirmed the expected molecular mass of 307 kDa [13].

**In Vitro Kinase Assay**

Colonocytes were incubated with PMA or toxin A plus PMA for 30 min, and ERK1/2 was recovered via immunoprecipitation with an ERK1/2 antibody. Major basic protein (MBP) was used as a phosphorylation substrate for the immunoprecipitated ERK1/2 or recombinant active ERK1 and ERK2 proteins (R&D Systems, Minneapolis, MN, USA). The ERK1/2 proteins, MBP (150 µM), and diluted [γ-32P] ATP (3,000 Ci/mmol; NEN Life Science Products, Inc., Boston, MA, USA) were mixed in a kinase assay buffer. After incubation for 30 min at 30°C, the phosphorylated substrate was separated from residual free [γ-32P] ATP using P81 phosphocellulose paper (Fisher Scientific, Pittsburgh, PA, USA), and the amount of 32P incorporated into the substrate was assayed by liquid scintillation counting.

**Binding of Toxin A to ERK1/2**

Human colonocytes (NCM460 and HT29; 5 × 10⁶ cells/tube) were lysed by sonication at 4°C in 1 ml of lysis buffer (10 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1% Triton X-100), and protein extracts were obtained by centrifugation. Biotinylated toxin A (1 µg) was added to the protein extract, and the sample was incubated for 4 h for protein binding. Immunoprecipitation was performed for 16 h with ERK1/2 antibodies or streptavidin-agarose beads against biotinylated toxin A, and immune complexes were recovered with protein G-Sepharose beads. The isolated proteins were resolved by polyacrylamide gel electrophoresis and probed with antibodies against ERK1/2 or toxin A.

**GST-Pulldown Assay**

GST-ERK1 or GST-ERK2 (1 µg) was incubated with biotinylated toxin A (1 µg) in pulldown buffer (20 mM HEPES/KOH, pH 7.6, 100 mM KCl, 0.5 mM EDTA, 0.05% NP-40, 1 mM dithiothreitol, 5 mM MgCl₂, 0.02% BSA) at 4°C for 16 h. The resulting immune complexes were recovered with streptavidin-agarose beads and analyzed by immunoblotting with an antibody against GST.

**Statistical Analysis**

The results are presented as mean values ± SEM. The data were analyzed using the SIGMA-STAT professional statistics software program (Jandel Scientific Software, San Rafael, CA, USA). Analyses of variance with protected *t*-tests were used for intergroup comparisons.

**Results**

**Toxin A Interacts with ERK1/2**

We previously reported that *C. difficile* toxin A interacted with Src and inhibited its kinase activity, resulting in cell detachment [13]. In this study, we assessed whether other MAPK-family proteins, which are known as mediators of cell proliferation [3], differentiation [1], and migration [4, 15, 21, 23], could interact with toxin A in human colonic epithelial cells. To explore this, 1 µg of biotinylated toxin A was incubated with lysates from non-transformed human colonocytes (NCM460) for 4 h, and the resulting complexes were immunoprecipitated with streptavidin, a high-affinity molecule for biotinylated proteins. The immunoprecipitated proteins were separated and subjected to immunoblot analysis using an antibody against ERK1/2. As shown in Fig. 1A, toxin A bound to intracellular ERK1/2 proteins, and the expected ERK1/2 protein size was confirmed through comparison with the lysate lane (positive control). Similar results were obtained with the HT29 human colon cancer cell line (Fig. 1B). As shown in Fig. 1C, reverse immunoprecipitation confirmed that toxin A bound to ERK1/2 in NCM460 colonocytes (Fig. 1C, lanes 3 and 4). We also assessed whether p38 MAPK, another member of the MAPK family, bound to toxin A, but did not observe any such binding (Fig. 1D).

**Toxin A Binds Directly to Recombinant GST-ERK1 and -ERK2 Proteins**

To further confirm the binding of toxin A to intracellular ERK1 and ERK2, biotinylated toxin A was incubated with recombinant GST-ERK1 or GST-ERK2. As shown in Fig. 2,
toxin A bound to both GST-ERK1 and GST-ERK2 in vitro (Fig. 2, lanes 4 and 5). Compared with ERK2, ERK1 showed a higher binding affinity for toxin A. The recombinant proteins had their correct expected sizes (Fig. 2, lanes 6 and 7), and we did not observe any nonspecific affinity of the GST antibody to the biotinylated toxin A (Fig. 2, lane 3). These results indicate that toxin A directly binds to ERK1 and ERK2 without needing an adaptor protein.

Toxin A Binding Inhibits the Kinase Activities of ERK1 and ERK2

We next assessed whether the binding of toxin A affected the kinase activities of ERK1 and ERK2. To do this, we incubated a recombinant active form of ERK1 with MBP, a substrate of ERK1/2, and monitored ERK1 kinase activity. In the absence of ERK1, the incorporation of $^{32}$P into MBP was weak (Fig. 3A). The addition of ERK1 increased $^{32}$P incorporation 36-fold over the basal line. However, the addition of toxin A significantly inhibited the kinase activity of ERK1 in a dose-dependent manner (Fig. 3A). For example, 0.5 µg of toxin A decreased ERK1 activity by about 60% (Fig. 3A). We also observed dose-dependent inhibition of ERK2 activity by toxin A, but this effect was relatively weak in comparison with that of ERK1 (Fig. 3B). Next, we investigated whether the intact protein structure of toxin A was required for its inhibition of ERK1/2 kinase activity. Interestingly, when toxin A was denatured by boiling and then incubated with either ERK1 or ERK2, we did not observe any inhibition of their kinase activities (Fig. 3C). These findings suggest that the direct binding of structurally intact toxin A to ERK1/2 inhibits their kinase activity.

Toxin A Reduces the Kinase Activities of PMA-Phosphorylated and -Activated ERK1/2

We next assessed whether the activating phosphorylations of threonine 202 (thr-202) and tyrosine 204 (tyr-204) [1]...
had any effect on the interactions between toxin A and ERK1 or ERK2. Human colonocytes (NCM460 cells) were serum-starved for 24 h to exclude effects of serum on ERK1/2 phosphorylation. The cells were then exposed to PMA (an activator of ERK1/2) in the presence or absence of toxin A, and changes in the phosphorylation levels of ERK1/2 were investigated. As shown in Fig. 4A, PMA treatment induced thr-202/tyr-204 phosphorylation of ERK1/2 in a time-dependent manner, and this effect was not inhibited by the presence of toxin A (Fig. 4A). Furthermore, the phosphorylation of ERK1/2 by PMA did not affect the interactions between toxin A and ERK1 or ERK2 (Fig. 4B, lane 2 vs. 3). Next, we assessed whether toxin A inhibited the kinase activities of ERK1/2. In NCM460 cells treated for 1 h with PMA, toxin A, or toxin A plus PMA, ERK1/2 proteins were recovered by immunoprecipitation and their kinase activities were measured (Fig. 4C). We detected significant PMA-dependent increases in ERK1/2 activity (Fig. 4C). However, the presence of toxin A significantly blocked the PMA-induced activation of ERK1/2 kinase activity. These results suggest that phosphorylation (an important protein modification) does not affect the binding of toxin A to ERK1/2, resulting in inhibition of its kinase activity.

**DISCUSSION**

Several papers have shown that the MAPKs (MAP kinases), such as ERK1 and ERK2, are critically involved in *C. difficile* toxin-induced inflammation and cellular toxicity in non-transformed or transformed human colonocytes and monocytes [2, 12, 18, 22]. For example, Warny *et al.* [22] also showed that inhibition of p38MAPK and ERK1/2 with SB203580 and PD98059 significantly reduced IL-8 and IL-1β secretion and necrosis in human monocytes exposed to toxin A. Here, we found that toxin A inhibited the kinase activities of ERK1/2 through direct binding, and that this occurred without modification of phosphorylation.
This is consistent with our previous report that toxin A treatment did not increase the phosphorylation of ERK1/2 at thr-202/tyr-204 in NCM460 cells [12]. The apparent discrepancies in the effects of toxins A and B on ERK1/2 may reflect the use of different cell types in the experiments, or the fact that the two toxins use separate cell surface receptors that trigger distinct signal transduction pathways.

Receptor binding and cell entry are crucial steps for the cellular effects of toxin A [11]. Furthermore, Reineke et al. [19] demonstrated that cleavage of toxin A is required for the release of its amino-terminal region (which contains the catalytic domains) into the cytosol where it can interact with protein substrates. The glucosyltransferase activity of the amino-terminal region of toxin A was found to inactivate Rho family proteins, resulting in actin filament disaggregation and cell rounding [9, 10]. Similarly, we found that the amino-terminal region of toxin A contains a possible ERK1/2 binding site (DLIEFKFPENNLSQL) at amino acids 496 to 510. This putative ERK1/2 binding site may be responsible for the interaction between toxin A in the future, we will mutate and/or delete this possible binding site and examine the binding of toxin A to ERK1/2.

Several papers have shown physical interactions between toxin A and various intracellular proteins. For example, the direct binding of toxin A to the catalytic domain of Src was shown to inhibit its kinase activity, leading to the deactivation of two downstream signaling molecules, FAK and paxillin [13]. Na et al. [17] also showed that the binding of GP96 to toxin A is a key molecular event that promotes actin filament disaggregation and apoptosis. In this study, our GST-pulldown assay revealed that toxin A directly binds to ERK1 and ERK2. Moreover, we observed that protein modification of ERK1/2 (e.g., phosphorylation) did not affect the interaction of ERK1/2 with toxin A. These results suggest that identifying proteins capable of binding to internalized toxin A may provide the groundwork for explaining the various cellular toxicities seen in response to toxin A.

In summary, ERK1 and ERK2, two signal mediators for cell proliferation, differentiation, and migration, were found to be directly bound and inhibited by toxin A. This finding suggests a novel cytotoxic mechanism for toxin A that is distinct from its well-known ability to inactivate Rho family members.
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Abbreviations

ERK1/2, extracellular signal-related kinases-1/2; IP, immunoprecipitation; GST, glutathione S-transferase; PMA, phorbol 12-myristate 13-acetate; MAPK, mitogen-activated protein kinase.

REFERENCES


