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***Clostridium difficile* toxin A binds colonocyte Src causing dephosphorylation of focal adhesion kinase and paxillin**

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Abstract

C. difficile toxin A impairs tight junction function of colonocytes by glucosylation of Rho family proteins causing actin filament disaggregation and cell rounding. We investigated the effect of toxin A on focal contact formation by assessing its action on focal adhesion kinase (FAK) and the adapter protein paxillin. Exposure of NCM460 human colonocytes to toxin A for 1 hour resulted in complete dephosphorylation of FAK and paxillin, while protein tyrosine phosphatase activity was reduced. Blockage of toxin A-associated glucosyltransferase activity by co-incubation with UDP 2'3' dialdehyde did not reduce toxin A-induced FAK and paxillin dephosphorylation. GST-pull down and *in vitro* kinase activity experiments demonstrated toxin A binding directly to the catalytic domain of Src with suppression of its kinase activity. Direct binding of toxin A to Src, independent of any effect on protein tyrosine phosphatase or Rho glucosylation, inhibits Src kinase activity followed by FAK/paxillin inactivation. These mechanisms may contribute to toxin A-inhibition of colonocyte focal adhesion that occurs in human colonic epithelium exposed to toxin A.

Introduction

Clostridium difficile, an anaerobic pathogen responsible for antibiotic-associated colitis, exerts its pathogenic effects via release of toxins A and B (1-6), high molecular weight cytotoxic proteins, into the colonic lumen. After receptor binding and internalization, toxin A triggers disaggregation of actin microfilaments and cell rounding, causes apoptosis and stimulates proinflammatory responses in cultured epithelial cells and in experimental animal models. The primary molecular mechanism by which these toxins mediate actin disaggregation and cell rounding is glucosylation of Rho, Rac and cdc42 at threonine 37 leading to inactivation of these small GTP binding proteins (7). Actin disaggregation following Rho protein inactivation leads to tight junction impairment (7), barrier dysfunction and eventual disruption of the colonic epithelium.

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In addition to disruption of actin filaments in cultured cells, toxin A also causes detachment of epithelial cells in native human colon. Riegler *et al* reported that toxin A caused exfoliation of superficial but not crypt epithelial cells in human colonic mucosal explants in Ussing chambers (7). Ottlinger *et al* reported that toxin A disrupted the normal spatial distribution of the focal adhesion plaque molecules, vinculin and talin (8), suggesting that disruption of focal adhesions following tight junction breaks may be responsible for toxin A-induced epithelial cell detachment. However, the molecular mechanisms mediating rapid disruption of focal contact formation in colonocytes exposed to toxin A remains unclear.

Adhesion of epithelial cells to the underlying extracellular matrix occurs by focal contact formation (9). Focal adhesions link the matrix and the cell interior, and mediate critical signaling networks (10,11) which regulate barrier function and epithelial permeability (12). Integrin-mediated focal contact formation requires activation of the tyrosine kinases Src and FAK (13). The levels of tyrosine phosphorylation of FAK, paxillin and Src correlate with the assembly of focal adhesion complexes (14). FAK and paxillin are phosphorylated by Src, a critical regulator of their activities (15).

In view of the potential importance of focal contact formation on barrier function induced by toxin A, we studied its effect on the major focal adhesion molecules, Src, FAK and paxillin. We found that exposure of human colonocytes to toxin A resulted in dephosphorylation of FAK and paxillin that was independent of the known effect of the toxin on inactivation of Rho. We observed direct binding of toxin A to the catalytic domain of Src, leading to reduced Src autophosphorylation and Src activity. These results provide a Rho-independent mechanism to explain the disruption of focal contact formation in colonocytes exposed to toxin A.

Material and methods

C. difficile toxin A and biotin labeling reaction

Toxin A was purified from culture supernatants of *C. difficile* strain VPI 10463 (American Type Culture Collection, Rockville, Maryland, USA) as previously described (16). Toxin A was biotinylated using a commercially available kit following the manufacturer's instructions (Sulfo-NHS-LC Biotinylation Kit; Pierce, Rockford, IL). Briefly, one mg of toxin A was added to a Sulfo-NHS-LC-Biotin solution and incubated on ice for 2 h. Unbound biotin reagent was removed by a Streptavidin column. The purity of native toxin A and biotinylated toxin A was assessed by gel electrophoresis, confirming the expected molecular mass of 307 kDa.

Reagents

The polyclonal antibody for FAK was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against paxillin, phospho-paxillin (Tyr-118), Src and phospho-Src (Tyr-416) and phospho-Src (Tyr-527) were from Cell Signaling Technology (Beverly, MA). The UDP-2'3' dialdehyde, KCl, Bafilomycin A1 and rhodamine-phalloidin were from Sigma-Aldrich (St, Louis, MO). Src inhibitor (SU6656), JAK inhibitor (AG490) and PKC inhibitor (GF109203X) were from Calbiochem (San Diego, CA). Recombinant Src fragment proteins, GST-UD (UD), GST-UD+SH3 (SH3), GST-UD+SH3+SH2 (SH2) and full size GST-Src (fSrc) were from Lab Vision Corporation (Fremont, CA). The GST-Src catalytic domain (KD) was from MRL Corporation (Woburn, MA). Human NCM460 colonocytes and M3D culture medium were obtained from INCELL Corporation (San Antonio, TX).

Immunoblot analysis

Human colonocytes were washed with cold PBS, then lysed in 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 on SDS-polyacrylamide gels. Antigen-antibody complexes were detected with LumiGlo reagent (New England Bio labs Inc.).

Protein tyrosine phosphatase (PTP) assay

Colonocyte extracts were prepared in a low detergent lysis buffer (0.25% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl). Protein tyrosine phosphatase activity from cell extracts was determined by measuring free PO_4 generated from the phosphopeptide RRA(pT)VA (Promega, Madison, WI). A standard curve was prepared using free phosphate.

In vitro kinase assay

Colonocytes were incubated with toxin A for 30 min and Src was recovered by immunoprecipitation with a Src antibody. The peptide KVEKIGEGTYGVVYK was used as a phosphorylation substrate for immunoprecipitated Src. Immunoprecipitated Src, substrate peptide (150 μM), and diluted [^{32}P] ATP (3000 Ci/mmol; NEN Life Science Products) were mixed in a kinase assay buffer. After incubation for 30 min at 30°C, the phosphorylated substrate was separated from residual free [^{32}P] ATP using a P81 phosphocellulose paper and ^{32}P incorporated into the substrate was assayed by liquid scintillation counting.

Autophosphorylation of GST-Src catalytic domain

The recombinant catalytic domain of c-Src protein diluted in kinase buffer was mixed with either control buffer or toxin A and then allowed to incubate at 37°C for 30 min. The amount of phosphorylated catalytic domain of Src was measured following the manufacturer's instructions (Cyclex c-Src kinase assay kit, MRL Corporation, Woburn, MA).

Binding of toxin A to Src

NCM460 cells (5×10^7) 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 cell lysates were obtained by centrifugation. Native toxin A (5 or 10 μg) was added to each lysate and incubated for 4 h for protein binding. Immunoprecipitation was performed with Src antibody or toxin A antibody for 16 h and immune complexes were recovered with protein G-Sepharose beads. Isolated protein lysates were then subjected to SDS-PAGE.

GST-full down assay

Biotinylated toxin A (1 μg) was incubated with GST fusion-Src fragment proteins (1 μg) in pull-down buffer (20 mM HEPES/KOH, pH 7.6, 100 mM KCl, 0.5 mM EDTA, 0.05% NP-40, 1 mM dithiothreitol, 5 mM MgCl_2 , 0.02% BSA) at 4°C for 16 h. Immune complexes were recovered with protein G-Sepharose beads and were analyzed by immunoblotting with a GST antibody.

Inactivation of toxin A

C. difficile toxin A was treated with UDP-2'3' dialdehyde (1 mmol/L) dissolved in modification buffer (20 mmol/L Tris-HCl [pH 7.2], 150 mmol/L NaCl) at 37 °C for 3 hours. The reaction mixture was applied to a 100-kilodalton cutoff membrane (Microcon 100; Amicon, Beverly, MA) to remove the remainder of the UDP-2'3' dialdehyde, followed by extensive washing with PBS.

Glucosylation of Src or cdc42

Glucosylation of Src or cdc42 by toxin A were performed in a buffer containing 30 μM UDP-[^{14}C] glucose (50 nCi), 3 mM MgCl_2 , 0.3 mM GDP, 150 mM KCl, 50 mM triethanolamine HCl, pH 7.5 and 10 $\mu\text{g/ml}$ toxin A plus either recombinant cdc42 protein (50 $\mu\text{g/ml}$) or Src

protein (50 µg/ml) µl at 37 °C for 45 min (17). Next, Laemmli sample buffer was added and proteins were separated on 12.5% SDS-PAGE. Gels were dried and exposed to x-ray film.

Immunocytochemistry

CHO cells plated on fibronectin (Gibco Life Technologies) were exposed to toxin A for 1 h. Cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and then permeabilized with 0.2% Triton X-100 in PBS for 5 min. These samples were pre-incubated with 3% BSA in PBS for 1h followed by incubation with mouse monoclonal antibody to FAK overnight at 4 °C. Cells were washed extensively with PBS and then incubated with goat anti-mouse antibody coupled with FITC with 1 unit/ml of rhodamine-phalloidin (Molecular probes, Eugene, OR) for 30 min at room temperature in order to visualize actin filaments. Cells were examined on a confocal microscope (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

Results are presented as mean values $\pm$ SEM. Data was analyzed using the SIGMA-STAT professional statistics software program (Jandel Scientific Software, San Rafael, CA). Analyses of variance with protected *t* test were used for intergroup comparisons.

Results

Toxin A induces tyrosine dephosphorylation of FAK and paxillin

Activities of FAK and paxillin, essential components for focal contact formation, are regulated by tyrosine phosphorylation (15,18). Since toxin A causes disruption of cell-matrix interactions in various cell types, we measured phosphorylation of FAK and paxillin in NCM 460 human colonocytes exposed to toxin A. Constitutive phosphorylation of paxillin and FAK disappeared after 1 h of toxin A exposure (Figure 1A and B). Toxin A-induced FAK and paxillin dephosphorylation was not reversible even after 48 h of culture (data not shown). Toxin A-induced dephosphorylation of FAK and paxillin was also observed in colonic adenocarcinoma T84 cells and CHO cells (Fig. 1C). We next examined the effect of toxin A on focal adhesion complexes using FAK staining. In NCM460 colonocytes in which actin stress fibers were stained with rhodamine-phalloidin, toxin A caused complete loss of sub-cortical actin filaments and cell rounding (Fig. 1D). The typical localization of FAK in focal adhesion plaques at the sub-cortical ends of actin filaments was also completely abolished in cells exposed to toxin A, indicating loss or disruption of focal adhesion complexes.

Dephosphorylation of FAK/paxillin and glucosyltransferase activity of toxin A

Because actin stress fibers anchored at focal adhesions are disrupted by toxin A by a mechanism involving Rho glucosylation (8,19), we assessed whether glucosyltransferase activity of toxin A is required for FAK and paxillin dephosphorylation. As reported previously (20), UDP-2'3' dialdehyde inhibited glucosyltransferase activity of toxin A (Fig. 2A), but it did not affect toxin A-induced tyrosine dephosphorylation of FAK or paxillin (Fig. 2B, lanes 2 vs 3). To exclude a direct effect of UDP 2'3'-dialdehyde on colonocytes, cells were exposed to toxin A inactivated by incubation with UDP 2'3'-dialdehyde followed by filtration with a 100-kilodalton cut off membrane. Inactivated toxin A also increased tyrosine dephosphorylation of FAK and paxillin (Fig. 2, lanes 5 vs 6), suggesting that toxin A-induced tyrosine dephosphorylation of FAK and paxillin in colonocytes is largely independent of glucosylation of Rho proteins.

Effect of toxin A internalization and receptor binding on paxillin dephosphorylation

Toxin A-receptor binding and cell entry precede Rho protein glucosylation, and by themselves contribute to receptor activation of p38 MAPK and p53 which are independent of Rho glucosylation (16). We next assessed whether receptor binding or toxin A internalization are

also involved in the signaling mechanism of FAK and paxillin dephosphorylation. To assess this, we measured the times for receptor binding and cellular uptake of toxin A into colonocytes. Colonocytes were exposed to toxin A for intervals of 0 – 10 min and fixed with 3% paraformaldehyde. Cells were sequentially stained with a goat-anti-toxin A antibody and a corresponding secondary antibody conjugated with FITC, and toxin A receptor binding was analyzed by FACS. As shown in Figure 3A, binding of toxin A to the colonocyte membrane was highest at 3 min, consistent with our previous results in CHO cells (21). No measurable toxin A binding was observed at 10 min, indicating rapid toxin A internalization into colonocytes (Fig. 3B). We previously reported that toxin A stimulated rapid over production of reactive oxygen species (ROS) in colonocytes (22), and that activation of p38 MAPK-dependent signaling pathways in toxin A-exposed colonocytes was mediated by rapid ROS production (23). Therefore, we assessed whether suppression of ROS generation by *N*-acetyl-L-cysteine (NAC) or p38 MAPK inhibition by SB203580 (SB) influenced tyrosine dephosphorylation of paxillin in response to toxin A. Neither of these inhibitors had any effect on toxin A-induced paxillin dephosphorylation (Fig. 3C), suggesting that toxin A-induced disruption of focal adhesion molecules is not related to ROS generation and p38 MAPK activation.

Cellular uptake of toxin A (24,25) requires acidification of endosomal vesicles, a process that can be blocked by the vesicular H⁺-ATPase inhibitor bafilomycin A1 (24). Henriques *et al* also reported that 200 mM of KCl, a lysosomotropic blocker, prevents toxin A-induced cytotoxicity (26). Colonocytes pretreated with 200 mM KCl or 1 μ M bafilomycin A 30 min prior to toxin A exposure to inhibit endocytosis exhibited no toxin A-mediated paxillin dephosphorylation (Fig. 3D), indicating that paxillin dephosphorylation requires toxin A internalization.

Toxin A dephosphorylation of paxillin is not associated with protein tyrosine phosphatases

Tyrosine phosphorylation levels of proteins are determined by the dynamic balance between tyrosine kinases and tyrosine phosphatases. Toxin A reduced activity of whole cell protein tyrosine phosphatases with a maximum at 30 min and a return to baseline at 1 h (Fig. 4A). To further clarify the effect, colonocytes were treated with the tyrosine phosphatase inhibitor sodium orthovanadate (SO, 1 to 5 μ M) (27) and the serine/threonine phosphatase inhibitor okadaic acid (OA, 10 μ M) (28) for 1 h prior to toxin A exposure. Neither of these inhibitors reversed toxin A-induced tyrosine dephosphorylation of paxillin (Fig. 4B, lanes 2-4).

Src and toxin A-induced dephosphorylation of paxillin

To explore the potential role of Src in toxin A-induced dephosphorylation of FAK and paxillin, colonocytes were treated with the Src inhibitor SU6656 (4 μ M) (29), the JAK inhibitor AG490 (50 μ M) (23), a serine/threonine kinase PKC inhibitor GF109203X (10 nM) (30) or toxin A for 1 h and tyrosine dephosphorylation of paxillin was determined by Western blot analysis. As shown in Figure 5A, inhibition of Src or exposure to toxin A completely reduced basal phosphorylation of paxillin, while none of the other tyrosine kinase inhibitor had any effect. This suggests that the observed dephosphorylation of paxillin by toxin A could be mediated by toxin A inhibition of Src. Src phosphorylates FAK/paxillin regulating focal contact formation in several cell types (15). Src activity is differentially regulated by tyrosine phosphorylation at two sites: phosphorylation of Tyr-416 on the catalytic domain of Src increases enzyme activity, whereas phosphorylation of Tyr-527 in the carboxyl-terminal tail is inhibitory (31). Moreover, phosphorylation of Tyr-416 initiates a conformational change that enhances Src binding to its substrates (32). To assess an effect of toxin A on Src phosphorylation, we exposed colonocytes to either medium or toxin A for 10 min, followed by incubation with EGF, a physiological Src activator (33). EGF strongly increased colonocyte Src phosphorylation at Tyr-416, which was completely blocked in cells pre-exposed to toxin

A (Fig. 5B). Constitutive Src phosphorylation at Tyr-416 was also inhibited by toxin A (lanes 1 vs 5). No significant changes in Src phosphorylation at Tyr-527 were detectable. EGF also induced active phosphorylation of ERK1/2 at Thr-202/Tyr-204 in colonocytes, but this was not inhibited by toxin A (Fig. 5B).

Toxin A interacts with the catalytic domain of Src

We next examined the possibility that toxin A binds to Src in human colonocytes. Five or 10 μ g of toxin A was incubated with colonocyte extracts for 4 h, and toxin A-cell lysate complexes were immunoprecipitated with a Src antibody. Proteins were separated by SDS-PAGE, then subjected to Western blot analysis using goat anti-toxin A antibody. As shown in Figure 6A, toxin A bound to intracellular Src in cell lysates in a dose-dependent manner. To better confirm the binding of toxin A to intracellular Src, toxin A was added to cytoplasmic extracts of colonocytes for 4 h, and toxin A-cell lysate complexes were immunoprecipitated with a toxin A antibody. As shown in Fig 6 B probing the immunoprecipitate with src antibody revealed src bound to toxin A (mol mass 308 kDa) near the origin at the top of the gel, where the toxin-src complex would be expected to migrate. We next assessed whether specific Src domains shown in Figure 6C interacted with toxin A. Biotinylated toxin A (1 μ g) was incubated with recombinant GST-Src fragments for 4 h, immunoprecipitated with streptavidin, separated by SDS-PAGE, and then probed with a GST antibody against Src. Toxin A bound to a GST-fusion recombinant Src protein *in vitro* (Figure 6D left panel, lane 5). The right panel of Figure 6D indicates expected size of Src fragments. Using Src fragment analysis, we observed that only the catalytic Src domain (KD, lane 4, left panel) bound the toxin A. Since toxin A bound to the catalytic Src domain and inhibited 416-tyrosine phosphorylation on the catalytic domain (Fig. 5B), we measured autophosphorylation activity using recombinant catalytic domain as described in Material and Methods. Recombinant catalytic domain of Src (226-536 a.a) was incubated with toxin A in kinase buffer for 30 min and the intensity of autophosphorylation of the recombinant catalytic domain of Src was measured by anti-phosphotyrosine antibody and ELISA analysis. Compared to control, addition of toxin A inhibited the autophosphorylation of the catalytic domain in a dose dependent manner (Fig. 6E), confirming the results shown in Figure 5B. In addition, kinase activity of Src in colonocytes was inhibited by toxin A exposure (Fig. 6F). Taken together, binding of toxin A to the catalytic domain of Src inhibits autophosphorylation of Src that in turn reduces its binding to its substrates FAK and paxillin. Since toxin A's catalytic activity in cells involved glucosylation of Rho proteins (17), we assessed whether toxin A is capable of glucosylating Src. Our results indicate that toxin A did not catalyze the incorporation of [^{14} C] glucose from UOP-[^{14} C] glucose into recombinant Src protein, in contrast to its expected glucosylation of cdc42, a Rho family member (Fig. 6G).

Overexpression of Src restores toxin A-induced paxillin dephosphorylation

Since binding of toxin A to Src and subsequent inhibition of its autophosphorylation are strongly associated with FAK/paxillin dephosphorylation, we overexpressed colonocyte Src to determine if this would prevent toxin A-induced dephosphorylation of paxillin. Colonocytes were infected with adenovirus expressing the human c-Src gene (Ad-Src) or LacZ (Ad-LacZ), as a negative control gene and then tyrosine dephosphorylation of paxillin after toxin A exposure was measured. Western blot analysis showed that overexpression of c-Src in colonocytes partially reversed toxin A-induced tyrosine dephosphorylation of paxillin in a dose-dependent manner (Fig. 7).

Discussion

Cell detachment is strongly dependent on the disruption of focal contact formation (18). Toxin A disrupts the distribution of the focal adhesion plaque proteins vinculin and talin (8) and also causes dose-dependent detachment of epithelial cells in human colonic mucosal sheets (7). In

the setting of intestinal inflammation following toxin A exposure, massive cell detachment as well as loss of tight junctions would result in more severe barrier disruption and increased intestinal permeability and inflammation. We report here that FAK and paxillin, both important components of focal contact formation, were dephosphorylated in human colonocytes exposed to toxin A, and demonstrate that this is mediated by a direct interaction of toxin A with the catalytic domain of Src. This observation provides a novel cytotoxic mechanism for this toxin, which is distinct from its well documented ability to inactivate Rho proteins.

The striking effects of toxin A on cytoskeletal actin (7,8) are ascribed to glucosylation of Thr 37 on Rho proteins (17,34), rendering these molecules functionally inactive, and leading to disaggregation of actin microfilaments and cell rounding. The critical involvement of Rho in focal adhesion formation in different cell types is well established. For example, microinjection of a dominant active RhoA (RhoAV14) induces the formation of focal adhesions (35). Here we show that FAK/paxillin dephosphorylation by toxin A was not related to Rho protein inactivation, as blockage of toxin glucosyltransferase activity did not inhibit FAK/paxillin dephosphorylation (Fig. 2). Consistent with our observations, focal adhesion components such as Src, FAK, and vinculin are not altered in primary fibroblasts isolated from Rac1 deficient mice (36), and Cdc42-deficient cells show no apparent defects in actin stress fiber formation and FAK phosphorylation compared to wild type cells (37).

In addition to the pathophysiologic importance of enzymatic modification of the Rho family proteins by toxin A, receptor binding and cell entry are also crucial steps for intoxication (38). For example, translocation of *C. difficile* toxin A from early endosomal compartments into the cytosol requires toxin A cleavage (39) as well as acidification of the endosomal compartment (24,25). Reineke *et al* demonstrated that cleavage of toxin A is strongly required for cellular uptake and release of the amino terminal region of toxin A into the cytosol (40). We observed that blockade of toxin A endocytosis inhibited toxin A-induced FAK/paxillin dephosphorylation (Fig. 3C), indicating that toxin A internalization precedes and is required for dephosphorylation of FAK/paxillin in colonocytes.

FAK and paxillin, both Src substrates, are involved in integrin-mediated signaling pathways through focal contact formation (41). In addition, Tyr-416 and Tyr-527 phosphorylation represent essential modifications for kinase activity of Src (31,32,42). Phosphorylation of Tyr-416 initiates a conformational change on Src, relieving a steric barrier for substrates (32). Our results indicate that toxin A inhibits Src kinase activity at Tyr-416 by binding to the catalytic domain of Src (Fig. 7E). Src binding proteins may either increase or decrease the kinase activity of Src. For example, the regulatory component of caveolin binds Src and suppresses its kinase activity (43). RACK1 also binds Src and suppresses its kinase activity (44). To our knowledge, this represents the first example of a bacterial enterotoxin suppressing Src kinase via a direct protein-protein interaction.

A large body of recent evidence supports the view that bacterial protein toxins are multifunctional and pleiomorphic, causing damage to host target cells via multiple pathways. For example, *Helicobacter pylori* vacuolating cytotoxin (VacA) causes cellular damage by several pathways including 1) cell vacuolation via formation of anion-selective channels in endosomal membranes 2) reduction of mitochondrial membrane permeability and apoptosis 3) activation of mitogen activated protein (MAP) kinases p38 and ERK and 4) rapid changes in intracellular calcium concentration (45). The latter two VacA-associated effects appear to be independent of ion channel formation, and probably result from toxin-receptor ligation.

In summary, FAK and paxillin were dephosphorylated in colonocytes exposed to toxin A. These responses are independent of Rho glucosylation and associated with endocytosis-dependent toxin A internalization and direct binding of toxin A to the catalytic domain of Src,

resulting in inhibition of Src activity. These observations document a dual attack by the toxin on tight junction permeability, a critical pathophysiologic target in the colon of patients afflicted with *C. difficile* colitis and diarrhea.

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Abbreviations

FAK	focal adhesion kinase
TER	transepithelial resistance
FACS	fluorescence-activated cell sorting
UDP 2'3'-dialdehyde	uridine diphosphate 2'3' dialdehyde
PTP	protein tyrosine phosphatase
IP	immunoprecipitation
GST	glutathion S transferase

Figure 1. Dephosphorylation of FAK and paxillin in colonocytes exposed to toxin A

A. NCM460 colonocytes were incubated with toxin A (3 nM) for the indicated time points. Cell lysates were resolved on 10% polyacrylamide gel and probed with antibodies against paxillin, phospho-paxillin and β -actin. **B.** Colonocytes exposed to toxin A for the indicated time points were lysed and then immunoprecipitated with FAK antibody. Cell lysates were resolved on 10% polyacrylamide gel and probed with either an anti-phospho tyrosine antibody (4G10) or a FAK antibody. **C.** T84 cells and CHO cells were incubated with toxin A for the indicated times. Cell lysates were resolved on 10% polyacrylamide gel and probed with antibodies against the indicated proteins. All results are representative of three separate experiments. **D.** NCM460 colonocytes exposed to toxin A for 1 h were fixed in 4% paraformaldehyde in PBS. The cells were incubated with a FAK antibody, followed by secondary incubation with FITC-conjugated antibody and rhodamine-phalloidin. Cells were examined on a confocal microscope. All results are representative of three separate experiments.

Figure 2. Toxin A-induced dephosphorylation of FAK/paxillin in colonocytes does not involve glucosyltransferase activity of toxin A
A. Glucosylation of Rho by toxin A. **B.** NCM460 cells were pretreated with UDP-2'3' dialdehyde for 1 h and then exposed to toxin A (lanes 1 - 4). Colonocytes were exposed to UDP 2'3'-dialdehyde-inactivated toxin A (inactive) for 1 h (lanes 5 and 6). Quantitative densitometry of lanes 1, 3 and 6 from three separate experiments is provided in the bar graph.

Figure 3. Involvement of Toxin A internalization on toxin A-induced paxillin dephosphorylation
A. NCM460 colonocytes were fixed in paraformaldehyde after toxin A and sequentially incubated with a goat-anti-toxin A antibody and a secondary antibody conjugated with FITC. Binding of toxin A to its receptor was measured by FACS analysis. **B.** Toxin A binding to colonocyte membranes (*, $P < 0.001$ vs medium-treated cells). **C.** Colonocytes were pretreated with the p38 MAPK inhibitor, SB203580 (10 μ M) or the hydrogen peroxide scavenger NAC (10 mM) for 30 minutes and then incubated with either medium or toxin A for 1 h. **D.** Colonocytes were pretreated with the endocytosis inhibitors, KCl (200 mM) or Bafilomycin A (1 μ M) for 30 minutes and then incubated with toxin A for 1 h.

Figure 4. Paxillin dephosphorylation by toxin A is independent of protein tyrosine phosphatase
A. NCM460 cells were incubated with toxin A (3 nM) for the indicated time points. The activity of protein tyrosine phosphatase in total cell extracts was measured as described in *Materials and Methods* (*, $P < 0.005$ vs medium-treated cells). **B.** Colonocytes were pretreated with sodium orthovanadate or okadaic acid (10 μM) for 30 minutes and then exposed to toxin A for 1 h. Results are representative of three separate experiments.

Figure 5. The role of Src on toxin A-induced paxillin dephosphorylation

A. NCM460 cells were treated with DMSO (control), or the tyrosine kinase inhibitors, Src (SU6656, 4 μ M), JAK (AG490, 50 μ M), or the PKC inhibitor (GF109203X, 10 nM), or toxin A for 1 h. **B.** NCM460 cells were pre-incubated with either medium or toxin A for 10 minutes prior to exposure of EGF (10 ng/ml) for the indicated time points (min). Quantitative densitometry is provided in the bar graph. Results are representative of three separate experiments.

Figure 6. Toxin A binds and inhibits to the catalytic domain of Src

A. Toxin A (1 or 5 μg) was added to colonocyte extracts and immune complexes were recovered with protein G-Sepharose beads. Equal amounts of Src protein in each cell lysate used as a loading control (input). Results are representative of three separate experiments. **B.** Reverse immunoprecipitation was performed with a goat-anti-toxin A antibody (1 μg) and the blot was probed with the Src antibody. **C.** Schematic presentation of recombinant Src fragments. **D.** Identification of the Src domains involved in the interaction with toxin A. GST-fusion Src fragment proteins (1 μg) and biotinylated toxin A (1 μg) were coupled to the glutathion-Sepharose beads in pull-down buffer at 4°C for 16 h and the beads were then recovered by addition of streptavidin against biotinylated-toxin A. Supernatants were then subjected to polyacrylamide gel and probed with a GST antibody. **E.** Various concentrations of toxin A were mixed with a recombinant catalytic domain of Src in kinase buffer for 30 minutes. Autophosphorylation of Src catalytic domain was measured by an anti-phosphotyrosine antibody and ELISA analysis as described in *Materials and Methods*. Bars represent mean ± SEM of three separate experiments (*, $P < 0.0005$ vs buffer control). **F.** NCM460 cells were incubated with either medium or toxin A for 1 h and kinase activity of intracellular Src was measured (*, $P < 0.005$ vs medium-treated cells). **G.** Toxin A does not glucosylate Src.

Figure 7. Overexpression of Src inhibits toxin A-induced paxillin dephosphorylation
 NCM460 cells were infected with adenovirus expressing LacZ (Ad- LacZ) or c-Src (Ad-Src) for 36 h and then exposed to toxin A for 1 h. MOI (multiplicity of infection; 1,000 particles/cell). Cell lysates were subjected to polyacrylamide gel and probed with antibodies against phospho-paxillin (Tyr-118) and β -actin. Western blot analysis shows successful c-Src gene overexpression (upper panel). Results are representative of three separate experiments.