EXPERIMENTAL CELL RESEARCH 315 (2009) 3336–3344

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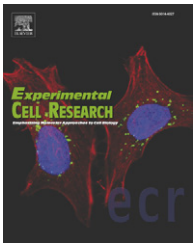

Research Article

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

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ARTICLE INFORMATION

Article Chronology:
Received 20 April 2009
Revised version received 19 May 2009
Accepted 20 May 2009
Available online 27 May 2009

Keywords:
Clostridium difficile
Toxin A
Paxillin
Focal adhesion kinase
Transepithelial resistance
Colitis
Signal transduction
Rho family proteins

ABSTRACT

Clostridium 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 h 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.

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]. Ottlingger et al. reported that

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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, glutathione S transferase

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doi:10.1016/j.yexcr.2009.05.020

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 meditate 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.

Materials 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 dithiotreitol, 5 mM MgCl2, 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.