Membrane translocation of binary actin-ADP-ribosylating toxins from *Clostridium difficile* and *Clostridium perfringens* is facilitated by Cyclophilin A and Hsp90†

Eva Kaiser*, Claudia Kroll*, Katharina Ernst*, Carsten Schwan§, Michel Popoff#, Gunter Fischer†, Johannes Buchner§, Klaus Aktories§, and Holger Barth*1

*Institute of Pharmacology and Toxicology, University of Ulm Medical Center, Ulm, Germany; †Department of Anaerobic Bacteria, Pasteur Institute, Paris, France; ‡Max Planck Research Unit for Enzymology of Protein Folding Halle, Halle, Germany; ¶Munich Center for Integrated Protein Science and Department Chemistry, Technische Universität München, Garching, Germany; §Institute of Experimental and Clinical Pharmacology and Toxicology, University of Freiburg, Freiburg, Germany

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†Correspondent footnote:

Dr. Holger Barth

Institute of Pharmacology and Toxicology, University of Ulm Medical Center

Albert-Einstein-Allee 11, D-89081 Ulm, Germany

Tel.: 49-731-50065503, Fax: 49-731-50065502

E-mail: holger.barth@uni-ulm.de
Some hypervirulent strains of *Clostridium difficile* produce the binary actin-ADP-ribosylating toxin CDT in addition to the Rho-glucosylating toxins A and B. It has been suggested that the presence of CDT increases the severity of the *C. difficile*-associated diseases including pseudomembranous colitis. CDT contains a binding and translocation component CDTb, which mediates the transport of the separate enzyme component CDTa into the cytosol of target cells, where CDTa modifies actin. Here, we have investigated the cellular uptake mechanism of CDT and found that bafilomycin A1 protects cultured epithelial cells from intoxication with CDT, implying that CDTa translocates from acidified endosomal vesicles into the cytosol. Consistently, CDTa translocates across the cytoplasmic membranes into the cytosol when cell-bound CDT is exposed to acidic medium. Radicicol and cyclosporin A, inhibitors of the heat shock protein Hsp90 and cyclophilins, respectively, protected cells from intoxication with CDT but not with toxins A and B. Moreover, both inhibitors blocked the pH-dependent membrane translocation of CDTa, strongly suggesting that Hsp90 and cyclophilin are crucial for this process. In contrast, the inhibitors did not interfere with ADP-ribosyltransferase activity, receptor-binding and endocytosis of the toxin. We obtained comparable results for the closely related iota toxin from *Clostridium perfringens*. Moreover, CDTa as well as Ia, the enzyme component of iota toxin specifically bound to immobilized Hsp90 and cyclophilin A in vitro. In combination with our recently obtained knowledge on the C2 toxin from *C. botulinum*, the results imply a common Hsp90/cyclophilin A-dependent translocation mechanism for the family of binary actin-ADP-ribosylating toxins.
INTRODUCTION

*Clostridium difficile* (C. difficile) infection causes human diseases ranging from mild diarrhea to severe and potentially life-threatening pseudomembranous colitis. The *C. difficile*-associated diseases occur in patients treated with broad-spectrum antibiotics. Under these conditions the disturbed gut flora allows germination of *C. difficile* spores and colonization of the gut by this pathogen. *C. difficile* produces two exotoxins, toxin A (308 kDa) and toxin B (270 kDa), which are the causative agents of pseudomembranous colitis. The toxin-catalyzed glucosylation of Rho, Rac and Cdc42 results in the inhibition of GTPase-mediated cell-signalling, destruction of the actin cytoskeleton, and cell rounding that is the reason for loss of integrity of the intestinal wall (for review see (20,21)).

During the last 10 years, hypervirulent *C. difficile* strains were identified, which produce in addition to toxins A and B a third exotoxin, the binary *C. difficile* transferase (CDT). Up to 35% of the strains produce CDT and it has been suggested that the presence of CDT correlates with severity of the *C. difficile*-associated diseases (12,14,25,27,52). CDT belongs to the family of binary actin-ADP-ribosylating toxins and consists of two non-linked proteins: the binding/translocation component CDTb and the separate enzyme component CDTa, which harbors the ADP-ribosyltransferase activity. Like the other members of this toxin family, CDT exerts its toxic effects on mammalian cells by mono-ADP-ribosylation of G-actin at arginine-177 (1) thereby inhibiting actin polymerization (54). This results in cell-rounding. Recently, it was reported that CDT interferes with the organization of microtubule structures, too, resulting in formation of long microtubule-based protrusions around the cell body. Importantly, *C. difficile* can bind to these microtubule-based protrusions resulting in increased adherence and colonization of bacteria in infection models (46).
The family of binary actin-ADP-ribosylating toxins comprises the C2 toxin from *C. botulinum* and the iota-like toxins. The latter includes iota toxin, which is produced by *C. perfringens* type E strains and causes sporadic diarrheic outbreaks in farm animals (47,50,51), as well as CDT from *C. difficile* (37) and *C. spiroforme* transferase (CST) (36). Iota, CDT and CST are distinguished from the C2 toxin (30) because they are closer related than to C2 toxin regarding sequence homology, functional and immunological aspects (34) and the different modification of individual actin isoforms (31,43).

The sophisticated mechanism by which the binding/translocation component mediates the transport of the enzyme components into the cytosol of mammalian target cells was discovered for C2 toxin and iota toxin (3,5). The binding/translocation component of iota toxin, Ib (98 kDa) becomes proteolytically activated and then binds to an unknown protein receptor and forms heptamers, which act as a docking platform for the enzyme component Ia (47 kDa) (5,16,49). After receptor-mediated endocytosis of the Ib/Ia complex, Ib mediates the translocation of Ia from acidified endosomal vesicles into the cytosol (29,48). Under acidic conditions Ib heptamers convert into a pore-conformation and form pores in endosomal membranes (5), which serve as translocation channels for the enzyme component. A widely comparable uptake mechanism was reported for C2 toxin. For both toxins, pore formation by the binding/translocation components is an essential prerequisite for translocation of the enzyme components C2I and Ia, respectively into the cytosol (6,23). It was shown earlier that C2I unfolds to translocate cross endosomal membranes (19). Most likely, the enzyme components translocate through the pore lumen driven by the pH gradient between the endosomal lumen and cytosol (23). Although both toxins share an overall comparable uptake mechanism via acidified endosomes, translocation of the enzyme components from endosomal vesicles to the cytosol are different. While Ia appears to escape from endocytotic carrier vesicles, which are in a state between early and late endosomes (13), C2I
translocates from early endosomes to the cytosol (3), suggesting that Ia requires more acidic conditions to cross membranes. Moreover, translocation of Ia seems to require a membrane potential gradient in addition to the pH gradient (13). We have reported earlier that the membrane translocation of C2 as well as iota toxin is facilitated by the chaperone heat shock protein 90 (Hsp90) (17,18) and more recently, that cyclophilin A (CypA), a peptidyl-prolyl cis/trans isomerase (PPlase) (22) is crucial for translocation of C2 toxin. PPlases catalyze cis-trans isomerization of proline-peptide bonds, often a rate-limiting step during protein refolding (2,9,44,45). So far it is not clear whether PPlases are also involved in membrane translocation of iota toxin.

In contrast to C2 toxin and iota toxin, the cellular uptake mechanism of CDT is not known. Therefore, we have investigated the uptake of CDT into cultured African green monkey kidney epithelial cells (Vero cells) and in particular studied the membrane translocation of the toxin. We focused on the role of the host cell factors Hsp90 and cyclophilin A in membrane translocation of CDT in comparison to iota toxin. The specific pharmacological inhibition of Hsp90 by radicicol (Rad), as well as the inhibition of cyclophilin by cyclosporin A (CsA), protected Vero cells from intoxication by CDT and iota toxin and inhibited the pH-dependent membrane translocation of both toxins.
MATERIAL AND METHODS

Materials - Cell culture medium (MEM) and fetal calf serum were purchased from Invitrogen (Karlsruhe, Germany) and cell culture materials were from TPP (Trasadingen, Switzerland). Complete® protease inhibitor and streptavidin-peroxidase were from Roche (Mannheim, Germany). The protein molecular weight markers Page Ruler prestained Protein ladder® and Page Ruler stained Protein ladder® were obtained from Fermentas (St. Leon-Rot, Germany). Biotinylated NAD⁺ was supplied by R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany). Bafilomycin A1 (BafA1) was obtained from Calbiochem (Bad Soden, Germany), CsA from Fluka (Munich, Germany) and Rad from Sigma Aldrich (Munich, Germany). The enhanced chemiluminescence (ECL) system was purchased from Millipore (Schwalbach, Germany). Alexa568-maleimide was from Invitrogen (Karlsruhe, Germany).

Protein expression, purification and biotinylation – Ia and Ib were purified as described earlier (33). Recombinant CDTa and CDTb (from C. difficile strain 196) were produced and purified as His-tagged proteins in the B. megaterium expression system as it was described by others for the large clostridial glycosylating toxins (32,55). Labelling of CDTa with Alexa568-maleimide was performed according to the manufacturer’s protocol (Invitrogen, Karlsruhe, Germany). The purified proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie blue and the protein concentration was determined via densitometry by using Photoshop 7.0 software (Adobe Systems Inc.). CypA was purified as described earlier (8) and human Hsp90 was purified as described (41). The biotinylation of C2I, Ia and CDTa was performed with sulfo-NHS-biotin (Pierce, Rockford, Illinois, USA) according to the manufacturer’s instructions.
Cell culture and cytotoxicity assays - African green monkey kidney (Vero) cells and the human intestinal Caco-2 cells were cultivated at 37 °C and 5% CO₂ in MEM containing 10% heat-inactivated fetal calf serum, 1.5 g/L sodium bicarbonate, 1 mM sodium-pyruvate, 2 mM L glutamine, 0.1 mM non-essential amino acids and 10 mg/mL Penicillin/Streptomycin. Cells were trypsinized and reseeded for at most 15-20 times. For cytotoxicity experiments, cells were seeded in culture dishes and incubated in serum-free medium with CDT or iota toxin. To inhibit the PPlase activity of Cyps or the activity of Hsp90, the cells were incubated for 30 min with the indicated concentrations of CsA or Rad, respectively. Subsequently, toxin was added and cells were further incubated at 37 °C with toxin plus inhibitor. After the given incubation periods, the cells were visualized by using a Zeiss Axiovert 40CFI microscope (Oberkochen, Germany) with a Jenoptik progress C10 CCD camera (Carl Zeiss GmbH, Jena, Germany). The cytopathic effects caused by the toxins were analyzed in terms of morphological changes.

Fluorescence microscopy to detect internalized CDTa - Caco-2 cells were preincubated with 10 µM CsA, 10 µM Rad for 30 min at 37 °C. Subsequently cells were cooled to 4°C and 1 µg/mL CDTa labelled with Alexa568 plus 2 µg/mL CDTb was added. Cells were incubated at 4°C for 30 min to allow toxin binding. Cells were transferred to 37°C for 20 min to induce endocytosis and fixed. Actin was stained by FITC-phalloidin. Fixed samples were analyzed with an inverted Axiovert 200M microscope (Carl Zeiss GmbH, Jena, Germany) equipped with plan-apochromat objectives, driven by Metamorph imaging software (Universal Imaging, Downingtown, PA). Confocal images were collected with a Yokogawa CSU-X1 spinning disc confocal head (Tokyo, Japan) with an emission filter wheel, a Coolsnap HQ II digital (Roper Scientific, Tucson, AZ) camera and 488 nm, 561 nm laser lines.
Preparation of cell extracts, SDS-PAGE and immunoblot analysis - Following incubation with the toxin, cells were washed twice with ice-cold PBS and lysed in 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 5 mM MgCl₂ and complete protease inhibitor. Following lysis of the cells and centrifugation (20,800 × g, 7 min, 4 °C), the supernatant was stored at −20 °C. For immunoblot analysis, equal amounts of lysate protein were subjected to SDS-PAGE according to the method of Laemmli (24). Subsequently, the proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany). The membrane was blocked for 30 min with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T). For the detection of actin, the samples were probed with a mouse monoclonal anti-β-actin antibody (clone AC-15; Sigma-Aldrich, Seelze, Germany). After washing with PBS-T, the membrane was incubated for 1 h with an anti-mouse antibody coupled to horseradish-peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany). The membrane was washed and the proteins visualized using an enhanced chemiluminescence (ECL) system according to the manufacturer’s instructions.

Sequential ADP-ribosylation of actin in lysates from toxin-treated cells - For ADP-ribosylation of actin in a cell-free system, 20 µg of whole-cell lysate protein were incubated for 30 min at 37 °C in a buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 5 mM MgCl₂, complete protease inhibitor, together with biotin-labelled NAD⁺ (10 µM) and 300 ng of C2I protein. The reaction was stopped with 5 x SDS-sample buffer (625 mM Tris/HCl pH 6.8, 20% SDS, 8.5% glycerol, 0.2% bromphenol blue, 100 mM DTT) and heating of the samples for 5 min at 95 °C. The samples were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and the biotin-labelled ADP-ribosylated actin was detected with peroxidase-coupled streptavidin and a subsequent chemiluminescence reaction.
ADP-ribosylation of actin by CDTa in a cell free system - Vero cell lysate (50 µg of protein) was incubated for 2, 5 and 15 min at 37 °C together with 50 ng/mL of CDTa, 10 µM biotin-labelled NAD⁺, and 10 µM CsA. Samples were subjected to SDS-PAGE, blotted onto a nitrocellulose membrane and the ADP-ribosylated actin was detected with streptavidin-peroxidase. Intensity of the biotin-labelled actin was determined by densitometry using the Adobe Photoshop 7.0 software.

Toxin-translocation assay with intact cells - The pH-dependent translocation of CDTa and Ia through their corresponding pores across endosomal membranes was experimentally mimicked on the surface of intact cells as described for iota toxin earlier (5). In brief, Vero cells were exposed to an acidic pulse (pH 4.0) after binding of either CD Tb/CDTa or Ib/Ia to the cell surface. Under acidic conditions CDTa and Ia translocate across the cytoplasmic membrane into the cytosol. Cell rounding was monitored and documented by photography.

Dot Blot analysis of the interaction between immobilized CypA and Hsp90 with CDTa, Ia and C2I – Different amounts of CypA and Hsp90 were vacuum aspirated onto a nitrocellulose membrane using a dot-blot system (Bio-Rad, Munich, Germany) according to the manufacturer’s instructions. Subsequently the membrane was blocked for 1 h with PBS-T containing 5 % non fat dry milk and incubated with either biotin-labelled C2I, Ia or CDTa (200 ng/mL) for 1 h. The membrane was washed three times with PBS-T and the bound biotinylated proteins were detected with streptavidin-peroxidase using the ECL system.

Reproducibility of the experiments and statistics - All experiments were performed independently at least 2 times. Results from representative experiments are shown in the figures. In each
individual immunoblot panel shown in the figures, the protein bands were originally detected on the same membrane and only cut out and recombined for presentation in the figures. Values ($n \geq 3$) are calculated as mean ± standard deviation (S.D.) using the Prism4 Software (GraphPad Software, Inc.).
RESULTS

CDT translocates from acidified endosomal vesicles into the host cell cytosol – The binary actin-ADP-ribosylating C2 and iota toxins deliver their A-components from endosomal vesicles into the cytosol. Importantly, acidification of the endosomal lumen is an essential prerequisite for this process. Therefore, we used BafA1, which inhibits the vesicular ATPase and thereby prevents acidification of the endosomes to test whether CDT translocates from acidified endosomes to the cytosol. Pre-treatment of Vero cells with BafA1 protected Vero cells from intoxication with CDT very efficiently. While there was a time-dependent increase in the amount of round cells after application of CDT, the toxin-induced cell-rounding was inhibited in the presence of 100 nM BafA1 (Fig. 1A). This concentration of BafA1 exhibited its protective effect even when the cells were incubated with CDT for 24 h in the presence of BafA1 but had no effect on the morphology of cells (not shown). Moreover, 0.1% final concentration of DMSO as solvent control had no inhibitory effect on the CDT-induced cell rounding (not shown). This result suggests that the A-component CDTa translocates from acidified endosomal vesicle to the cytosol.

We performed an alternative assay to verify that the membrane translocation of CDTa essentially requires acidic conditions. Therefore, we experimentally mimicked the acidic conditions of the endosomal lumen on the surface of intact cells. Vero cells were pre-treated with BafA1 to block the “normal” uptake of CDTa into the cytosol and then the cells were incubated at 4 °C with CDTb plus CDTa to enable toxin binding to the cell surface receptors. Subsequently, the pH of the culture medium was adjusted to pH 4.5 (for control pH 7.5) and cells were incubated at 37 °C to trigger membrane translocation of CDTa. Cell rounding was monitored to detect that CDTa was delivered into the cytosol and modified actin. Cell rounding was observed only when CDT-treated cells were exposed to low pH (Fig. 1B), indicating that a pH-gradient is essential for translocation of cell-bound CDTa across the cytoplasmic membrane into the cytosol.
Taken together, the results imply that CDTa translocates from acidified endosomal vesicles to the host cell cytosol which is in agreement with other members of this toxin family (3,5).

Pharmacological inhibition of host cell cyclophilin and Hsp90 protects Vero cells from intoxication with CDT – To investigate whether the cellular uptake of CDT depends on the host cell cyclophilin and/or Hsp90, we first tested whether pharmacological inhibition of these factors by CsA and Rad, respectively, has any effect on intoxication of cells with CDT. Pre-treatment of Vero cells with each inhibitor alone and with the combination of CsA and Rad protected Vero cells from CDT-induced cell-rounding within 4 h after toxin application (Fig. 2A). The inhibitors alone had no effects on cell morphology under these conditions (not shown). A quantitative analysis revealed that CsA and Rad caused a significant and time-dependent delay in intoxication rather than a complete inhibition (Fig. 2B). The combination of CsA and Rad had a slightly stronger protective effect compared to the individual inhibitors however this effect was not statistically significant (Fig. 2B). Because higher concentrations of CsA induced some morphological effects on Vero cells, the concentration of 10 µM was used in this study.

The morphology-based analysis of the protective effect was confirmed by analyzing the ADP-ribosylation status of actin from cells. Cells were treated with CDT in the absence and presence of the inhibitors. After 1.5 h, cells were lysed and lysates were incubated with fresh C2I and biotin-NAD$^+$ as a co-substrate to enable ADP-ribosylation of actin in vitro and thereby its biotin-labelling. The biotin-labelled, i.e. ADP-ribosylated actin was detected in a Western blot analysis with streptavidin-peroxidase (Fig. 2C, upper panel) and the intensity of the bands was quantified (Fig. 2C, the bars correspond to the bands of ADP-ribosylated actin in the upper panel). In this assay, actin from control cells gives a strong signal, because it was not ADP-ribosylated in the intact cells before lysis. In contrast, actin from CDT-treated cells gives a weaker signal, because
most of the actin was already modified in the intact cells by the toxin and is, therefore, no more
substrate in vitro. Most importantly, cells, incubated with CDT in the presence of CsA, Rad or
the combination of both inhibitors, gave a stronger signal of biotin-labelled actin compared to
cells, which have been treated with CDT alone. This result indicates that less actin was modified
by the toxin in intact cells when CsA or Rad was present. An anti-β-actin immunoblot of the
identical lysates confirmed comparable protein loading (Fig. 2C).

Taken together, the results clearly indicate that there was less CDTa activity in the cytosol of
cells in the presence of CsA or Rad, strongly suggesting that cyclophilin and Hsp90 are crucial
for intoxication of cells with CDT. However, from this result it is not clear whether the inhibitors
interfere with the enzyme activity of CDTa and/or the uptake of CDTa into the host cell cytosol.

Therefore, we first excluded that CsA and Rad inhibit the CDTa-catalyzed ADP-ribosylation of
actin in vitro (data not shown). This finding implies that the inhibitors interfere with the uptake of
CDTa into the cytosol and, therefore, we investigated which step during toxin uptake into the
cytosol is affected. First, we investigated whether the inhibitors interfere with binding of
CDTa/CDTb to the cell surface and the subsequent internalization of the toxin into endosomal
vesicles. Caco-2 cells, which have been pre-treated with either CsA or Rad, were incubated for
30 min at 4°C with CDTb and Alexa568-labelled CDTa to allow binding and for 20 min at 37°C
for internalization of the toxin complex. The internalized CDTa-Al568 protein was visualized by
fluorescence microscopy. As shown in Fig. 3, there was a comparable amount of CDTa
detectable in the cells, independent whether the cells have been treated with inhibitors. Thus,
neither CsA nor Rad inhibited binding of the toxin to the receptor or internalization by receptor-
mediated endocytosis. CsA and Rad did not inhibit uptake of the C. difficile toxins A and B into
Vero cells under comparable experimental conditions (data not shown). These toxins are
internalized via receptor-mediated endocytosis and translocate from acidified endosomal vesicles
into the cytosol, likewise, where they modify Rho proteins leading to cell rounding. In our experiments, the combination of CsA and Rad did not cause a significant delay in toxin A-induced rounding-up of Vero cells (data not shown), indicating endocytosis in the presence of these inhibitors.

CsA and Rad inhibit the pH-dependent membrane translocation of CDTa – Having excluded that CsA and Rad interfere with the early steps of toxin uptake we focused on the membrane translocation of CDTa. To test an effect of the inhibitors on this process, we performed a well-established assay, which mimics endosomal conditions on the surface of intact cells. In brief, Vero cells were pre-treated with BafA1 to block the “normal” uptake of CDT. Then, cells were incubated at 4 °C with CDTb plus CDTa and, thereafter, cells were exposed to warm acidified medium (37 °C, pH 4.5) as described before to trigger translocation of cell-bound CDTa across the cytoplasmic membrane into the cytosol. During this step, CsA, Rad or the combination of both inhibitors was present in the culture medium. The successful translocation of CDTa into the cytosol was determined by the amount of round cells (Fig. 4). In the presence of CsA or Rad there was a significant decrease in the amount of round cells after 1, 1.5 and 2 h, indicating that both CsA and Rad inhibit membrane translocation of CDTa. The combination of CsA and Rad, however, exhibited a synergistic inhibitory effect which caused a prolonged delay in intoxication of cells compared to the single substances. In conclusion, these results imply that cyclophilin as well as Hsp90 are crucial for the pH-dependent membrane translocation of CDTa and suggest that both factors might act in a synergistic manner during this process.

CsA and Rad inhibit membrane translocation of the C. perfringens iota toxin and thereby protect cells from intoxication – We have observed earlier that pharmacological inhibition of Hsp90
protected Vero cells from intoxication with iota toxin; however, the underlying molecular mechanism was not investigated so far. Prompted by the results obtained for CDT, we finally tested whether Hsp90 is crucial for membrane translocation of the enzyme component Ia and whether cyclophilin is also involved in this process. To determine whether cyclophilins are involved in the uptake of iota toxin, Vero cells were incubated with iota toxin in the presence or absence of CsA. Toxin-induced cell rounding was analyzed after 4 h (Fig. 5A). Most of the toxin-treated cells were round while the presence of CsA prevented cell rounding. The observed iota toxin-induced cell rounding correlated with the ADP-ribosylation status of actin in these cells (not shown). CsA inhibited the iota toxin-induced cell rounding in a time- and concentration-dependent manner (Fig. 5B) and as observed before for CDT, the combination of CsA and Rad showed a synergistic protective effect compared to the single inhibitors (Fig. 5C). Most important, CsA as well as Rad inhibited the pH-dependent translocation of cell-bound iota toxin across the cytoplasmic membrane into the cytosol. This becomes evident in a significantly decreased amount of round cells in the presence of the inhibitors (Fig. 5D). In conclusion, the data imply that Hsp90 as well as cyclophilin are crucial for membrane translocation of iota toxin which is consistent to the results obtained for the closely related binary toxin CDT. Moreover, in this aspect the iota-like toxins behave comparable to the binary actin-ADP-ribosylating C2 toxin from *C. botulinum*.

The enzyme components of CDT and iota toxin directly interact with Hsp90 and CypA in vitro – From these results we were not able to conclude which particular cyclophilin is involved in uptake of CDT and iota toxins. We hypothesized, however, that cyclophilin A might interact with the enzyme components of both toxins. This hypothesis is plausible because cyclophilin A is the prominent cyclophilin in the cytosol of mammalian cells and the major molecular target of CsA.
Moreover, we have reported earlier that it interacts with C2I, the enzyme component of the C2 toxin. Therefore, we have finally investigated whether purified cyclophilin A and Hsp90 proteins interact with CDTa and Ia in a dot blot analysis *in vitro* and we included C2I as a positive control (Fig. 6). Starting with 1 µg of protein, decreasing amounts of Hsp90 and CypA proteins were spotted onto a nitrocellulose membrane and the membranes (for control PBS, indicated as con) were incubated in an overlay assay with either biotin-labelled CDTa, Ia or C2I protein in solution (200 ng/ml final concentration). After extensive washing, the membrane-bound enzyme components of the toxins were detected. Most importantly, CDTa, Ia and C2I bound to Hsp90 as well as CypA and this binding was specific because there was no toxin bound to the membrane in the absence of Hsp90 or CypA. Moreover, there was no signal when the immobilized Hsp90 and CypA proteins were mock-incubated with PBS instead of toxin or incubated with the non-binding lethal factor from *Bacillus anthracis*, as demonstrated recently (7).

In conclusion, this result indicates that the enzyme components of the binary CDT and iota toxins directly interact with Hsp90 and CypA *in vitro*. This is in line with our recently obtained results for the C2 toxin, implying that this interaction with Hsp90 and CypA might be a common feature of the family members of binary actin-ADP-ribosylating toxins.
In the present paper, we have performed a series of experiments to analyze the cellular uptake of the binary actin-ADP-ribosylating toxin CDT from *C. difficile*, in particular the membrane translocation of its enzyme component CDTa. We demonstrate that CDTa translocates from acidified endosomal vesicles into the cytosol and that the translocation depends on a pH-gradient across the membrane. This is in agreement to earlier findings on the translocation of the binary iota and C2 toxins (3,5). Recently, we reported that membrane translocation of some binary toxins but not of others are facilitated by host cell chaperones and PPIases (7,17,18,22). Therefore, we tested here whether CDT and the closely related iota toxin the iota-like toxins require such factors for translocation. Indeed, membrane translocation of the enzyme components CDTa and Ia was blocked by the pharmacological inhibitors Rad and CsA, implying that Hsp90 as well as cyclophilin facilitate this step. Consequently, both inhibitors protected cultured cells from intoxication by CDT and iota toxins and the relative effects of CsA and Rad on CDT and iota actions were overall comparable. The inhibitory effect on the intoxication of Vero cells with iota toxin was significantly stronger when CsA and Rad were combined compared to CsA or Rad alone while this synergistic inhibitory effect was less pronounced and not statistically significant for the intoxication of Vero cells with CDT. Importantly, we ruled out that the inhibitors did influence the enzyme activities of CDTa and Ia or other steps in toxin internalization, such as binding of the toxin complex to the cell surface or endocytosis. Thus, we conclude that the inhibitors exclusively interfere with toxin translocation and thereby inhibit uptake of CDTa and Ia into the cytosol. To investigate membrane translocation of CDTa and Ia, we mimicked the endosomal conditions on the surface of intact Vero cells. Only when cells were exposed to an acidic pulse, membrane translocation of the cell-bound toxin was triggered. CsA and Rad, however, blocked pH-driven translocation of CDTa and Ia. This assay was originally established
to investigate pH-dependent membrane translocation of diphtheria toxin (42) and successfully
used for a variety of toxins, which translocate from acidified endosomes into the cytosol
(10,15,28), including the binary actin-ADP-ribosylating C2 and iota toxins (3,5,6). Interestingly,
the inhibitory effect of CsA and Rad on the CDT-induced cell rounding appeared less efficient
when CDTa was introduced into the cytosol by acidic shift in comparison to the “normal” uptake
of the toxin via receptor-mediated endocytosis and subsequent translocation from acidified
endosomes. One possible explanation for this observation might be the synchronous translocation
of a comparatively large amount of CDTa across the cytoplasmic membrane under these artificial
conditions while less CDTa might translocate into the cytosol when the toxin is taken up via
acidified endosomes. In agreement with this hypothesis, cell rounding was faster when CDT was
introduced into cells by acidic pulse compared to “normal” uptake. On the other hand, there
might be differences regarding the recruitment of chaperones/PPIases which are crucial for
translocation of CDT to the endosomal membrane compared to the cytoplasmic membrane. From
our results obtained by this method, we conclude that Hsp90 and cyclophilins facilitate
translocation of CDTa and Ia across the membranes of acidified endosomes during uptake of the
toxin into mammalian cells. Moreover, this is most likely the explanation for an earlier finding
that Rad prevents intoxication of cells with iota toxin although it did not inhibit ADP-
ribosyltransferase activity of Ia (17).

The results corroborate our recent finding that cyclophilins and Hsp90 facilitate membrane
translocation of C2I, the enzyme component of the binary actin-ADP-ribosylating C2 toxin (22).
Immunoprecipitation experiments revealed that CypA, the most abundant cyclophilin in the
cytosol of mammalian cells and the major target for CsA, interacts with C2I (22). In the present
study we found that CDTa as well as Ia bound to immobilized Hsp90 and CypA proteins in vitro,
a hint that CypA might be the relevant cyclophilin that interacts with CDT and iota toxins during
cellular uptake, too. As observed for CDT and iota toxin in the present study, the inhibitors Rad or CsA, respectively, prevented membrane translocation of C2I. Thus, in the presence of Rad or CsA less, if any, C2I reached the cytosol and therefore cells were protected from intoxication by C2 toxin (22). The finding that membrane translocation of CDTa, Ia, and C2I is facilitated by Hsp90 and cyclophilins is interesting, because differences have been reported between C2 and iota toxins during uptake of their enzyme components into the target cell cytosol. First, C2I translocates from early endosomes into the cytosol while Ia is released at a later stage in vesicle transport between early and late endosomes, implying that translocation of Ia is triggered by more acidic conditions (13). Second, translocation of Ia but not of C2I requires a membrane potential gradient in addition to the pH-gradient (13). Finally, there are different regions within the Ia and C2I proteins that respectively mediate their interaction with Ib and C2IIa, as well as their membrane translocation (4,26).

The observation that membrane translocation of C2I but also of CDTa and Ia is facilitated by the same host cell factors is a strong hint for a common role of Hsp90 and cyclophilins during translocation of binary actin-ADP-ribosylating toxins. Interestingly, the intoxication of cells with the binary lethal toxin from *Bacillus anthracis* was not influenced by Rad and CsA (7,18,57), although lethal toxin shares significant sequence and structural homology and an overall common cellular uptake mechanism with binary actin-ADP-ribosylating toxins (for review see (38,56)).

Just like Ib and C2IIa, the activated binding-/translocation component, protective antigen (PA63), forms heptameric pores in membranes of acidified endosomes, which facilitate pH-dependent membrane translocation of the enzyme component lethal factor (56). However, when the enzyme domain of lethal factor (LF), a protease, was replaced by the enzyme domain of diphtheria toxin (DTA), an ADP-ribosyltransferase, the PA63-dependent uptake of the LFn-DTA fusion toxin was inhibited by Rad and CsA (7). Moreover, we demonstrated that the inhibitors blocked the pH-
dependent membrane translocation of this fusion toxin across endosomal membranes, as found for the binary actin-ADP-ribosylating toxins (7). This unexpected finding strongly suggests that the interaction with Hsp90 and cyclophilin during membrane translocation might be specific for bacterial ADP-ribosyltransferases. This hypothesis is confirmed by earlier reports that Hsp90 is crucial for the membrane translocation of the enzyme moieties of diphtheria toxin (40) and cholera toxin (53).

The findings are in agreement with an earlier report by Ratts and co-workers that translocation of diphtheria toxin from early acidified endosomes is facilitated by a multi-protein translocation complex containing Hsp90 and thioredoxin reductase (40). The composition of such complexes and contribution of individual PPIases might differ depending on the type of toxin. However, PPIases such as Cyp-40, FKBP51 and FKBP52 have been identified as functional co-chaperones in Hsp90-containing protein complexes (35,39). Therefore, we can not exclude at present that other cyclophilins besides CypA are involved in translocation of CDTa and/or Ia because CsA inhibits the PPIase activity of most human cyclophilins. Moreover, it will be important to investigate whether further PPIases besides the cyclophilins, for instance FK506 binding proteins (11), are also involved in translocation of the binary actin-ADP-ribosylating toxins.

In conclusion, our study provides new information on the interaction of binary clostridial toxins with target cell cyclophilins and Hsp90. However, further investigation is required to unravel the precise molecular mechanisms how these host cell factors facilitate membrane translocation of the toxins in mammalian cells.
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REFERENCES


demonstration of channel formation by the activated binding component Ib and channel block by the enzyme component Ia. J.Biol.Chem. 277:6143-6152.


46. Schwan, C., B. Stecher, T. Tzivelekidis, M. van Ham, M. Rohde, W. D. Hardt, J. Wehland, and K. Aktories. 2009. Clostridium difficile toxin CDT induces formation of
570  microtubule-based protrusions and increases adherence of bacteria. PLoS.Pathog. 5:e1000626.
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Fig. 1. Effect of BafA1 on intoxication of Vero cells with CDT. A. Vero cells were pre-incubated with 100 nM BafA1 or left untreated (con). After 30 min CDT (40 ng/mL CDTa plus 80 ng/mL CDTb) was added and pictures were taken after 6 h. Quantitative analysis of rounded Vero cells after the given time points of toxin treatment is shown. Values are given as mean ± S. D. (n = 3). Significance versus CDT-treated cells was tested by using the student’s t-test (*** = p < 0.0001).

B. Acidic pH triggers membrane translocation of CDTa. Vero cells were pre-incubated with 100 nM BafA1 for 30 min at 37 °C and then at 4°C with CDT (100 ng/mL CDTa plus 200 ng/mL CDTb; for control without toxin). Subsequently the medium was adjusted to pH 4.5 with HCl and cells were incubated at 37 °C in this acidic medium (for control in neutral medium). Pictures were taken after 1.5 h. The percentage of round cells was determined from these pictures. Values are given as mean ± S. D. (n = 3).

Fig. 2. CsA and Rad inhibit the intoxication of Vero cells by CDT. A. Vero cells were preincubated with 10 µM of CsA, 10 µM of Rad or a combination of both for 30 min at 37 °C. Subsequently toxin was added (40 ng/mL CDTa plus 80 ng/mL CDTb). The shown pictures were taken after 4 h. B. Time course of Vero cell intoxication with CDT in the presence of CsA and Rad. Vero cells were pre-incubated with 10 µM of CsA, 10 µM of Rad or the combination of both inhibitors. Afterwards, CDT (40 ng/mL CDTa plus 80 ng/mL CDTb) was applied. At the indicated time points the percentage of rounded cells was determined. Values are given as mean ± S. D. (n = 3). Significance was tested for each time point versus cells treated with CDT alone (if not indicated otherwise by lines) by using the student’s t-test (* = p < 0.05, ** = p < 0.001, *** = p < 0.001, NS = not significant). C. Effect of CsA and Rad on the ADP-ribosylation status of actin in CDT-treated Vero cells. After 1.5 h of incubation with CDT (40 ng/mL CDTa plus 80
ng/mL CDTb) cells were lysed and the ADP-ribosylation status of actin from these cells was analyzed by incubating with C2I and biotin-labelled NAD$^+$. The biotin-labelled (i.e. ADP-ribosylated) actin is shown in the upper panel (ADP-rib actin = ADP-ribosylated actin). Equal protein loading was confirmed by an anti-β-actin antibody (lower panel). The intensity of the ADP-ribosylated actin bands was measured by using photoshop software and is given below. The bars correspond to the bands of ADP-ribosylated actin in the upper panel.

**Fig. 3. Influence of CsA and Rad on binding and endocytosis of CDT into Caco-2 cells.**

Caco-2 cells were preincubated with 10 µM CsA, 10 µM Rad for 30 min at 37 °C. Subsequently cells were cooled to 4°C and toxin was added (1 µg/mL CDTa labelled with Alexa568 plus 2 µg/mL CDTb). Control consists of cells in media alone. Cells were incubated at 4°C for 30 min to allow toxin binding. Cells were transferred to 37°C for 20 min to induce endocytosis and fixed. Actin was stained by FITC-phalloidin. Pictures were acquired with a confocal microscope.

**Fig. 4. CsA and Rad inhibit the pH-dependent membrane translocation of CDTa.** Vero cells were incubated with 100 nM BafA1 in combination with either 10 µM CsA, 10 µM Rad or the combination of both inhibitors at 37 °C. Subsequently CDT was added (80 ng/mL CDTa plus 160 ng/mL CDTb; for control without toxin) and cells were incubated on ice for 30 min. The medium was adjusted to pH 4.5 and the cells were incubated at 37 °C for 2 h. After 1, 1.5 and 2 h pictures were taken and the percentage of round cells was determined from these pictures. Values are given as mean ± S. D. (n = 3). Significance versus CDT-treated cells was tested (if not indicated otherwise by lines) by using the student’s t-test (*** p < 0.0005, ** = p < 0.005, * = p < 0.05, NS = not significant).
Fig. 5. CsA and Rad protect Vero cells from intoxication with the actin-ADP-ribosylating iota toxin from *C. perfringens*. A. Vero cells were pre-incubated with 10 µM CsA or left untreated (con). After 30 min iota toxin (20 ng/mL Ia plus 40 ng/mL Ib) was added and pictures were taken after 4 h. B. Quantitative analysis of rounded Vero cells after 4 h of toxin treatment is shown. Values are given as mean ± S. D. (n = 3). Significance versus iota-treated cells was tested by using the student’s t-test (* = < 0.05, ** = p < 0.005, *** = p < 0.0005). C. Vero cells were pre-incubated with 10 µM CsA, 10 µM Rad, the combination of both or left untreated. After 30 min iota toxin (20 ng/mL Ia + 40 ng/mL Ib) was added and pictures were taken at the indicated time points. The percentage of round cells was determined from the pictures. Values are given as mean ± S. D. (n = 3). Significance was tested versus cells treated with iota toxin alone (if not indicated otherwise by lines) by using the student’s t-test (** = p < 0.0001, *** = p < 0.005, NS = not significant). D. CsA and Rad inhibit the pH-dependent membrane translocation of Ia. Vero cells were incubated with 100 nM BafA1 in combination with either 10 µM CsA or 10 µM Rad at 37 °C. Subsequently cells were exposed to acidic medium (pH 4.0, 37 °C) containing iota toxin (50 ng/mL Ia plus 100 ng/mL Ib; for control without toxin) and incubated for 15 min at 37 °C, still in the presence of BafA1. The medium was removed, neutral medium was added and after further 30 min at 37 °C pictures of the cells were taken. The percentage of round cells was determined from the pictures. Values are given as mean ± S. D. (n = 3). Significance versus iota-treated cells was tested by using the student’s t-test (** = p < 0.005).

Fig. 6. CDTa, Ia and C2I directly interact with Hsp90 and CypA *in vitro*. The proteins Hsp90 and CypA (1, 0.5 and 0.25 µg of each protein) were vacuum aspirated onto a nitrocellulose membrane using a dot-blot system. For control, PBS was aspirated instead of protein. The membrane was blocked and subsequently incubated with 200 ng/mL of the biotinylated
proteins CDTa, Ia, and C2I. For control, PBS was used for overlay. After washing, the membrane was incubated with streptavidin-peroxidase to detect the bound biotinylated proteins by enhanced chemiluminescence reaction.