Cellular uptake of *Clostridium difficile* TcdA and truncated TcdA lacking the receptor binding domain

Ralf Gerhard, Eileen Frenzel, Sebastian Goy and Alexandra Olling

Institut für Toxikologie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

The combined repetitive oligopeptides (CROPs) of *Clostridium difficile* toxins A (TcdA) and B (TcdB) induce clathrin-mediated endocytosis of the toxins. Inconsistently, CROP-truncated TcdA

-1874 is also capable of entering host cells and displaying full cytotoxic properties although with less potency. Pre-incubation of cells with isolated CROPs, however, reconstitutes the reduced uptake of TcdA

-1874 to the level of the full-length toxin. We believe that TcdA exhibits an additional binding motif beyond the C-terminally located CROP domain, which might interact with cellular receptor structures that are associated with alternative internalization pathways. This study therefore evaluated endocytosis routes of CROP-dependent cellular uptake for TcdA and CROP-independent cellular uptake for TcdA

-1874. Clathrin knockdown or inhibition with chlorpromazine affected subsequent internalization of TcdA and TcdA

-1874, although only to some extent, arguing for alternative, clathrin-independent endocytosis routes. Inhibition of dynamin, a GTPase essentially involved in clathrin-mediated endocytosis as well as in various clathrin-independent uptake mechanisms, affected uptake of TcdA to the same extent as clathrin inhibition. In contrast, uptake of TcdA

-1874 was almost completely eliminated in dynamin-inhibited cells. Thus, clathrin-independent uptake of TcdA

-1874 presumably depends on dynamin. These findings demonstrate that the toxins are endocytosed via complex pathways involving clathrin and dynamin, putatively enabling them to adapt to mechanisms of various cell types. With regard to the emergence of *C. difficile* strains producing C-terminally truncated toxins, this study emphasizes the relevance of elucidating toxin uptake as a prerequisite for the development of toxin intervention strategies.

INTRODUCTION

Large clostridial glucosyltransferases [or large clostridial toxins (LCTs)] are single-chain proteins with molecular masses of 250–300 kDa. These huge protein toxins act intracellularly and gain access to their substrate proteins via receptor-mediated endocytosis and translocation of at least the glucosyltransferase domain into the cytosolic compartment of host cells. To accomplish entry into the host cells, the LCTs are composed of several domains specified for each step in this complex process: receptor binding domain, pH-sensitive translocation domain, hydrophobe membrane insertion region, autoproteolytic cysteine protease domain and the glucosyltransferase domain (Jank & Aktories, 2008; Pruitt & Lacy, 2012). A prerequisite for receptor-mediated endocytosis is specific binding of the toxins to receptor structures that are exposed at the cell surface. This ensures inclusion of bound toxins into newly formed endosomes. Although specific binding structures for most clostridial glucosyltransferases have not been identified yet, a core carbohydrate structure [\(\alpha\)-Gal-(1,3)-\(\beta\)-Gal-(1,4)-\(\beta\)-GlcNAc] was found to be important for binding of *Clostridium difficile* toxin A (TcdA). It is generally accepted that all LCTs interact with the cell surface via their combined repetitive oligopeptide (CROP), which has been shown to bind to the carbohydrate structure (Greco et al., 2006), and is therefore defined as the receptor binding domain. The importance of CROPs for the cytotoxic effects of LCTs becomes evident by the fact that antibodies directed against this structure possess high neutralizing capacities (Hussack et al., 2012). Thus, CROPs are considered the essential domain responsible for binding to the cell surface and triggering endocytosis. Some observations have been published that raise doubts about only a single receptor structure on host cells (Keel & Songer, 2007) or CROPs being the exclusive receptor binding domain (Frisch et al., 2003) of TcdA. The description of *Clostridium perfringens* large glucosyltransferase TpeL, which lacks a CROP domain (Arimoto et al., 2007) and proofs that CROPs are not essential for binding and uptake of TcdA (Olling et al., 2011), gives a reason for...
reinvestigation of the endocytosis of TcdA to better understand its cytotoxicity. This is especially important as C. difficile strains exist that show base-pair deletions or mutations within the gene region of TcdA coding for the CROP domain. The strains are classified as toxinotypes II, VI, VII, VIII or XVI, depending on the kind of gene alteration (Rupnik, 2008). These toxinotypes are considered TcdA-negative strains, as it is believed that the genes code for non-functioning TcdA. Based on the assumption that TcdA is capable of using alternative endocytosis routes, the present study evaluated and compared the cellular uptake of TcdA and TcdA1–1874 as representatives of CROP-dependent and CROP-independent endocytosis, respectively. The data reveal that internalization of full-length TcdA is not only restricted to clathrin-mediated endocytosis but is more complex by using redundant pathways.

**METHODS**

**Antibodies and reagents.** The following antibodies were used: anti-Rac1 mAb recognizing non-glucosylated Rac1 (clone 102; BD PharMingen) and total Rac1 (clone 23A8; Upstate, Millipore), β-actin antibody (clone AC15; Sigma), horseradish peroxidase-conjugated secondary mouse antibody (Rockland) and antibody targeting clathrin heavy chain (clone 23; BD Biosciences). Bafilomycin A1 was from Sigma and chlorpromazine, an inhibitor of clathrin coat assembly, was from Calbiochem and the dynamin inhibitor dynasore was from Sigma. The *Bacillus megaterium* expression system was from MoBiTec, the jetPrime transfection system from Polyplus Transfection and clathrin heavy chain siRNA oligo I from Dharmaco. All chemicals were of the highest purity available.

**Expression of recombinant toxins.** C. difficile toxins (strain VPI 10463, GenBank accession no. X51797) were expressed recombinantly in the *B. megaterium* expression system as His-tagged fusion proteins. Expression and purification were performed following a standard protocol, as described previously (Burger et al., 2003). The CROP deletion mutant TcdA1–1874 was generated using a specific endonuclease recognition site (nt 5620) in TcdA, which is located within the first repetitive sequence, and cloning the resulting toxin fragment into the modified *B. megaterium* expression vector pHIS1522.

**Cell culture and cytotoxicity assay.** The human colon crypt cell line HT29 and the human colon carcinoma cell line Caco-2 were cultivated under standard conditions in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 and DMEM, respectively. Culture media were supplemented with 10% fetal bovine serum (FBS), 100 μM penicillin, 100 μg streptomycin ml⁻¹ as well as, exclusively for Caco-2 cells, 1% non-essential amino acids (Hidalgo et al., 1989). Chinese hamster ovary (CHO) C6 cells were cultivated in DMEM/Ham’s F-12 supplemented with 5% FBS, 1 mM sodium pyruvate, 100 μM penicillin and 100 μg streptomycin ml⁻¹ (Huet et al., 1987).

For cytotoxicity assays, cells were seeded in 24-well chambers and grown for 24 h to subconfluence. Toxins were diluted in the respective 4 °C cold medium and the indicated concentrations were added in appropriate volumes to the respective cells. Cells were incubated with toxins for 30 min on ice followed by a temperature shift to 37 °C to induce endocytosis and subsequent further incubation for the indicated time intervals. Toxin-induced cell rounding was monitored by light microscopy and cytopathic effect (CPE) was quantified as the percentage of round cells per total cells. The cell lysates of toxin-treated cells were subjected to SDS-PAGE and Western blot analysis to determine the status of Rac1 glucosylation as a direct marker for the intracellular action of toxins. A glucosylation-sensitive Rac1 antibody was used to specifically detect non-glucosylated Rac1 (clone 102). The level of non-glucosylated Rac1 was normalized to the respective amount of β-actin as a loading control.

**Inhibitor assays.** HT29 or CHO C6 cells were incubated for 30 min on ice with 1 nM TcdA or TcdA1–1874, respectively, with or without previous incubation with 60 nM isolated TcdA CROPs for 30 min on ice. Each incubation step with toxins or toxin fragments was followed by PBS washing. In order to induce and monitor toxin uptake, the incubation temperature was shifted to 37 °C for indicated time intervals.

A blockade of endosomal acidification was accomplished using bafilomycin A1, an inhibitor of the vesicular ATPase. Bafilomycin A1 (100 nM) was applied to HT29 or CHO C6 cells either previously or simultaneously with the toxins or in temporal relation to toxin treatment, as indicated. Intracellular Rac1 glucosylation was determined as a marker for the cellular uptake of toxins. For inhibitor experiments, HT29 or Caco-2 cells were pre-incubated for 45 min at 37 °C with 60 μM chlorpromazine or 80 μM dynasore, respectively, dissolved in serum-free medium. Following washing, the cells were equilibrated on ice and treated with the indicated concentrations of toxins for 30 min. In order to induce endocytosis, toxin-treated cells were subsequently incubated for the indicated time intervals at 37 °C before the cells were harvested in Laemmli sample buffer and light microscopy was performed.

**Small interfering (siRNA) transfection.** HT29 cells were transfected either with siRNA targeting the clathrin heavy chain or with scrambled siRNA a as negative control. Clathrin heavy chain I siRNA was designed according to Hinrichsen & Schultz (1988) and purchased from Dharmacon. Transfection was performed using jetPrime transfection reagent following the manufacturer’s instructions. In brief, cells were incubated with a mixture of siRNA, jetPrime reagent and buffer in DMEM/Ham’s F-12 medium supplemented with 10% FBS and antibiotics, as described above. After 5 h at 37 °C, the cells were washed and further cultivated under standard conditions. Silencing efficacy was analysed after 48 h by Western blot analysis with specific antibody targeting the clathrin heavy chain.

**Western blotting.** Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membrane. After blocking with 5% (w/v) non-fat dried milk in TBST (50 mM Tris/HCl (pH 7.2), 150 mM NaCl, 0.05% (v/v) Tween 20), the membrane was incubated overnight with the primary antibody at 4 °C. Following washing with TBST, the membrane was incubated for 45 min at room temperature with horseradish peroxidase-conjugated secondary antibody in TBST. Detection was performed by means of chemiluminescence.

**Trans epithelial electrical resistance (TER) measurement.** Caco-2 cells were seeded onto 12-well filter inserts (Transwell, pore size 0.4 μm; Becton Dickinson) to measure the TER. Monolayers were cultivated up to an initial resistance of >150 Ω cm⁻². The TER was determined using an epithelial Voltohmmeter (EVOM; World Precision Instruments) equipped with an Endom 24 chamber. To investigate toxin-induced deregulation of the intestinal barrier function, cells were apically or basolaterally treated either concentration-dependently with TcdA and TcdA1–1874 for 4 h or time dependently with 3 nM of the respective toxin at 37 °C. For inhibitor assays, Caco-2 cells were pre-incubated with 60 μM chlorpromazine for 45 min at 37 °C followed by washing and toxin application. TER values are given as means in percentage of the initial value (n = 2). For Western blot analyses, membrane filters were excised from the insert using a scalpel and incubated at 95 °C in Laemmli buffer to generate cell lysates.
RESULTS

Cell-surface-bound CROPs enhance uptake of TcdA<sup>1–1874</sup>

As shown previously, CROPs are not essential for toxin uptake as the recombinantly expressed CROP deletion mutants TcdA<sup>1–1874</sup> and TcdB<sup>1–1852</sup>, respectively, are able to enter cells and display full cytotoxic properties. However, in most of the cell lines tested to date, full-length toxins are up to tenfold more potent than the truncated forms.

Based on these observations, we wondered whether TcdA<sup>1–1874</sup> and the C-terminally located CROP domain might act synergistically during the internalization process. We therefore examined the uptake of the CROP deletion mutant TcdA<sup>1–1874</sup> into HT29 colon cells in terms of its dependence on cell-surface bound CROPs. As revealed by toxin-induced cell rounding (Fig. 1a, left upper panel) and the decrease in the level of non-glucosylated Rac1 compared with constant actin (Fig. 1a, left lower panel), full-length TcdA efficiently entered the cells independently of cell-surface-bound CROPs. In contrast, TcdA<sup>1–1874</sup> glucosylated only half the level of Rac1 compared with the full-length toxin (Fig. 1a, right panels) reflecting the previously observed discrepancy in potency towards HT29 cells (Olling et al., 2011). Interestingly, pre-incubation with the isolated TcdA CROP domain reconstituted uptake efficiency of truncated TcdA<sup>1–1874</sup> into HT29 cells up to the level of the full-length form (Fig. 1b). This phenomenon was corroborated by a kinetic approach. Inhibition of endosomal acidification by bafilomycin A1 was used to prevent toxin uptake at different time points in relation to toxin treatment (Fig. 1c). Simultaneous bafilomycin treatment completely protected the cells from TcdA and TcdA<sup>1–1874</sup>. However, bafilomycin applied even 5 min after TcdA could not prevent the toxin from being endocytosed (filled squares). Almost 50 % of intracellular Rac1 was glucosylated only after 2 h of incubation, which confirmed the very fast internalization process of full-length TcdA. In contrast, uptake of the CROP deletion mutant TcdA<sup>1–1874</sup> was completely blocked at least for 2 h, even if the inhibitor was applied 30 min later than the toxin (filled circles). Thus, CROP-independent endocytosis, for which truncated TcdA<sup>1–1874</sup> is representative, occurs considerably more slowly than CROP-dependent endocytosis. Consistently, cellular uptake of TcdA<sup>1–1874</sup> into those cells extracellularly decorated with the CROPs was almost as effective as that of full-length TcdA, the representative of CROP-dependent internalization (open circles). A direct role of CROPs in the observed efficacy enhancing process was validated by using CHO C6 cells, which do not bind the CROPs

Clathrin is pivotally involved in endocytosis of TcdA and TcdA<sup>1–1874</sup>

As shown previously, endocytosis of clostridial glucosylating toxins involves the formation of clathrin-coated pits (Papatheodorou et al., 2010). We therefore examined and compared the necessity of clathrin for CROP-dependent and CROP-independent uptake processes. Levels of clathrin were effectively reduced in HT29 colon cells by specific siRNA transfection targeting the clathrin heavy chain (Fig. 2a, middle panel). The subsequent concentration-dependent cytotoxic approach revealed that toxin-induced Rac1 glucosylation was not completely abolished as a consequence of clathrin knockdown. Clathrin reduction of about more than 90 % merely attenuated Rac1 glucosylation mediated by TcdA compared with scrambled transfected cells (Fig. 2b, left panel). This phenomenon was similar in cells treated with equipotent concentrations of the CROP deletion mutant TcdA<sup>1–1874</sup>. TcdA<sup>1–1874</sup>-mediated effects were considerably reduced by clathrin knockdown, especially at low toxin concentrations, although they were fully restored at higher concentrations (Fig. 2b, right panel). These data were verified by pharmacological inhibition of clathrin coat assembly (Fig. 2c). Pre-incubation of HT29 cells with chlorpromazine only delayed subsequent TcdA-induced CPE. Whereas a level of 50 % Rac1 glucosylation was achieved approximately after 45 min of toxin treatment in control cells, clathrin inhibition shifted the time point of half-maximum Rac1 modification to 90 min (Fig. 2c, left panel). In concordance with TcdA treatment and the transfection experiments, clathrin inhibition also decelerated the cellular uptake of the CROP deletion mutant TcdA<sup>1–1874</sup> by a factor of two (Fig. 2c, right panel). Hence, clathrin is comparably involved in HT29 cell internalization of full-length TcdA, as well as of its truncated form lacking the CROPs.

We previously characterized the cellular uptake of TcdA and TcdA<sup>1–1874</sup> into polarized Caco-2 cells by monitoring the toxin-induced reduction in TER (Olling et al., 2011). Interestingly, we found that Caco-2 cells were hardly susceptible towards apically applied TcdA<sup>1–1874</sup> whereas basolateral treatment caused a pronounced CPE. In contrast, susceptibility towards full-length TcdA was almost identical and was independent of the side of application. As the differentiated membranes differ in their repertoire of proteins and structures presumably involved

Statistical analysis. Two-tailed t-tests were performed using GraphPad Prism 5.02 to evaluate statistical significance. Significance was set at a P value of <0.05. Data are presented as means ± SEM.
Fig. 1. Cell-surface-bound CROPs enhance cellular uptake of truncated TcdA1–1874. (a) Morphological changes and intracellular level of non-glucosylated and total Rac1 of HT29 cells after treatment with 1 nM full-length (left panel) or CROP-truncated TcdA1–1874 (right panel) in dependence of cell-surface-bound CROPs. (b) Densitometrical evaluation of Western blot analyses with glucosylation-sensitive Rac1 antibody. Level of toxin-induced Rac1 glucosylation of HT29 cells treated with TcdA or TcdA1–1874 after pre-incubation with TcdA CROPs (shaded bars) or without (filled bars). Values are given as means (%) ± SEM (n=4). Significance is indicated by an asterisk (P<0.001). (c) Kinetics of endocytosis of TcdA (■), TcdA1–1874 (●) and TcdA1–1874 + CROPs (○) in HT29 cells determined by addition of bafilomycin A1 at different time points after toxin treatment. Shown are mean levels of Rac1 glucosylation (%). (d) Western blot analyses of CHO C6 cells treated with TcdA or TcdA1–1874, respectively, in dependence of cell-surface-bound TcdA CROPs. (e) Densitometric evaluation of (d). Values are given as means (%) ± SEM (n=4). NS, Not significant.
in endocytotic processes, we wondered whether the discrepancy in apical and basolateral uptake of CROP-truncated TcdA1–1874 might be due to a diverse clathrin involvement. We therefore examined the toxin-mediated decline in TER in the dependence of clathrin inhibition by chlorpromazine. As expected, the concentration-dependent TER reduction by full-length TcdA was effectively neutralized by chlorpromazine up to 1.5 nM of apical or

**Fig. 2.** Clathrin inhibition delays cellular entry of full-length and CROP-truncated toxin A. (a) HT29 cells were treated with a concentration series of full-length TcdA (left panel) or TcdA1–1874 (right panel) for 6 h to determine specific clathrin knockdown. Western blot analyses of lysates showing non-glucosylated Rac1 (upper panel), clathrin heavy chain (middle panel) and β-actin (lower panel). Clathrin levels were reduced to less than 10%. (b) Densitometric evaluation of Western blots from (a) given the level of glucosylated Rac1 in scrambled (filled bars) and siClathrin (shaded bars) transfected cells. Values are given as means (%) ± SEM (n=3). Significance is indicated by an asterisk (P=0.0026). ns, Not significant. (c) Level of glucosylated Rac1 of HT29 cells treated time-dependently with equipotent concentrations of TcdA (left panel) or TcdA1–1874 (right panel) with regard to chlorpromazine (Cp) application. Values are given as means (%) on the basis of Western blot analyses. Inhibition of clathrin coat assembly by chlorpromazine delayed cellular uptake of TcdA and TcdA1–1874 by a factor of two.
Fig. 3. Role of clathrin in apical and basolateral toxin uptake into polarized cells. Clathrin-mediated endocytosis in polarized cells was investigated by determining the inhibition of clathrin by chlorpromazine. (a, c) Determination of toxin-induced reduction in TER of Caco-2 cells treated apically (●) or basolaterally (▼) with a concentration series of TcdA or TcdA1–1874. (b, d) Time-dependent monitoring of the toxin-mediated decrease in TER following the application of 3 nM TcdA or TcdA1–1874. Filled symbols represent controls (ctr) (■) or toxin-treated cells (●, ▼) in the absence of chlorpromazine (Cp); open symbols (□, ○, △, ▼) indicate the presence of chlorpromazine. Data points are normalized to initial values and presented as means (%) (n=2). (e) Western blot analysis of Caco-2 cell lysates from filter inserts of an identical experiment to (a) showing non-glucosylated Rac1 and β-actin.
basolateral toxin treatment (Fig. 3a). Time-dependent application of 3 nM full-length TcdA with regard to dependence on chlorpromazine verified the involvement of clathrin coat assembly during endocytosis (Fig. 3b). In fact, the same was true for cellular uptake of the CROP deletion mutant TcdA\textsuperscript{1–1874} into Caco-2 cells, irrespective of whether endocytosis occurred from the apical or basolateral membrane (Fig. 3c, d). As inhibitor treatment alone apparently increased transepithelial resistance (Fig. 3b, compare the controls), we additionally determined the level of non-glucosylated Rac1 of cells already used for TER measurements to exclude inhibitor-mediated artefacts affecting electrical resistance (Fig. 3e). As expected, chlorpromazine also reduced toxin-induced Rac1 glucosylation, supporting the TER data. Hence, clathrin is a key player in CROP-dependent as well as in CROP-independent cellular uptake of \textit{C. difficile} TcdA. However, additional clathrin-independent routes must exist enabling the toxins to enter host cells in an alternative way.

**Clathrin-independent uptake of TcdA\textsuperscript{1–1874} involves dynamin**

Dynamin is a GTPase mediating scission of newly formed clathrin-coated vesicles, which can be blocked by cell-permeable dynasore (Macia \textit{et al.}, 2006). Thus, inhibition of this enzyme by dynasore should delay TcdA internalization to the same extent as chlorpromazine, the clathrin inhibitor. This was confirmed by monitoring intracellular Rac1 glucosylation as quantification of toxin uptake (Fig. 4). Pre-incubation of HT29 cells with dynasore shifted the time point of TcdA-induced half-maximal effect from approximately 35 to 70 min (Fig. 4b, compare TcdA and TcdA + Dyn). Hence, dynamin blockade, as well as clathrin inhibition, prolonged cellular uptake of full-length TcdA by a factor of two. Interestingly, this was not the case of cells treated with the CROP deletion mutant TcdA\textsuperscript{1–1874}. Whereas uptake rates of full-length and truncated TcdA coincided after blocking clathrin-dependent routes, the dynamin inhibition of the cellular entry of TcdA\textsuperscript{1–1874} was notably stronger (Fig. 4a, lower panels; Fig. 4b). While Rac1 was almost completely glucosylated even in dynasore-treated cells after 90 min of TcdA application, no modification was monitored in those cells incubated with the CROP-truncated TcdA\textsuperscript{1–1874}. This observation indicated that the clathrin-independent pathway presumably additionally used by the toxins is dynamin independent in the case of the full-length toxin, but involves dynamin with regard to CROP-independent internalization by TcdA\textsuperscript{1–1874}.

**DISCUSSION**

It is acknowledged that \textit{C. difficile} TcdA and TcdB enter host cells after binding of their C-terminally located receptor binding domain consisting of CROPs to a so far unknown receptor (Dingle \textit{et al.}, 2008; von Eichel-Streiber \textit{et al.}, 1992). We recently showed that the assumed receptor binding domain is not necessarily involved in toxin uptake. Mutants TcdA\textsuperscript{1–1874} and TcdB\textsuperscript{1–1852}, which lack the CROP domain, still exhibited full cytotoxic properties, although to a lesser extent. Thus, the CROP domain apparently only modulates the internalization efficacy of the toxins. Interestingly, CROP-truncated TcdA\textsuperscript{1–1874} did not compete with binding of full-length TcdA, suggesting at least one additional binding motif beyond the CROPs that interacts with alternative cellular receptor structures (Olling \textit{et al.}, 2011). This notion is in accordance with an earlier competition study (Frisch \textit{et al.}, 2003). It is suggested that the same is true for TcdB. TcdB\textsuperscript{1–1851} merely lacking the CROPs was cytotoxic towards Vero cells. In contrast, TcdB\textsuperscript{1–1500}, a mutant additionally truncated by 350 aa of

![Fig. 4. Role of dynamin in CROP-dependent and CROP-independent cellular uptake of TcdA. (a) Representative Western blots showing the level of non-glucosylated and total Rac1 in HT29 cells treated time-dependently with equipotent concentrations of TcdA (upper panel) or TcdA\textsuperscript{1–1874} (lower panel) with regard to dynamics inhibition by dynasore. β-Actin was used as a loading control. (b) Densitometric evaluation of Western blots presented in (a). Filled symbols represent cells treated with TcdA (■) or TcdA\textsuperscript{1–1874} (○) without dynasore pre-incubation and open symbols (□, ○) those after dynamics inhibition. Values are given as means (%±SEM (n=3)). Significance is indicated by asterisks (P values between 0.0002 and 0.032).](image)
the intermediary region, was not capable of toxin uptake, although it still possessed a pore-forming capacity (Genisyurek et al., 2011).

Interestingly, pre-incubation of HT29 cells with the isolated TcdA CROP domain enhanced the potency of subsequent applied TcdA<sub>1–1874</sub> up to the level of the full-length toxin, as examined by Rac1 glucosylation. CROP-induced stimulation of toxin uptake, however, was restricted to TcdA. Pre-incubation with TcdA CROPs did not alter endocytosis of CROP-truncated TcdB<sub>1–1852</sub> (data not shown). It is therefore conceivable that CROP-truncated TcdA<sub>1–1874</sub> attaches to different cellular structures than full-length TcdA, which might be associated with alternative but less efficient endocytotic pathways. This hypothesis was corroborated by a kinetic experimental setup using the vATPase inhibitor bafilomycin A1. The inhibitor was applied to cells at different time points after toxin treatment in order to block endosomal acidification and consequently to inhibit endosomal toxin passage at predefined time points. The addition of bafilomycin A1 at 30 min after the application of CROP-truncated TcdA<sub>1–1874</sub> completely prevented toxin-induced Rac1 glucosylation under the given conditions, which revealed the very slow transfer of mutant TcdA from the outer-membrane leaflet to the endosomal compartment. Cell-surface-bound TcdA CROPs, however, accelerated the internalization process of TcdA<sub>1–1874</sub> almost up to level of the full-length toxin. Full-length TcdA in fact glucosylated approximately 50% of endogenous Rac1, even if bafilomycin A1 was applied at 5 min after the application of toxin. This observation actually reflects the efficient endocytosis route utilized by CROP-mediated uptake and raises the question of which internalization pathways are used during CROP-dependent (TcdA) and CROP-independent (TcdA<sub>1–1874</sub>) endocytosis, respectively. As analysed in detail for C. difficile TdB, cellular uptake of LCTs involves clathrin (Papatheodorou et al., 2010). In coherence with published data for TdB, siRNA targeting the clathrin heavy chain reduced TcdA-mediated intracellular effects. This was also true for CROP-independent internalization by mutant TcdA<sub>1–1874</sub>. However, although clathrin levels were reduced by more than 90%, toxin uptake was merely attenuated but not completely blocked. Long-term reduction in the amount of clathrin by transfection might upregulate artificial endocytotic mechanisms that are usually much more silent or even non-existent. We therefore showed clathrin involvement by an inhibitory approach applying chlorpromazine. In fact, inhibition of clathrin coat assembly delayed the uptake of full-length as well as truncated TcdA by half, supporting the data obtained by siRNA transfection experiments: clathrin is equally involved in CROP-dependent and CROP-independent endocytosis of TcdA into HT29 cells. In agreement with HT29 inhibitory experiments, clathrin is similarly involved in internalization of TcdA and TcdA<sub>1–1874</sub> into polarized Caco-2 cells, regardless of the side of application (Stubbe et al., 2000).

Thus, differences in toxin uptake from the apical or basolateral side of polarized cells depends on the abundance of receptors rather than the endocytotic routes responsible for uptake. However, as inhibition or knock-down of clathrin did not completely prevent toxin uptake, presumably alternative clathrin-independent routes are utilized. Chemical inhibition of dynamin, which is responsible for scission of invaginated clathrin-coated vesicles (Henley et al., 1998), delayed internalization of full-length TcdA to the same extent as observed by chlorpromazine-mediated clathrin inhibition. In addition, simultaneous application of both inhibitors had no synergistic effect (data not shown). This indicated that the alternative clathrin-independent uptake mechanism is probably also independent of dynamin. Interestingly, endocytosis of CROP-truncated TcdA<sub>1–1874</sub> was almost completely blocked for 2 h as a consequence of dynamin inhibition by dynasore. Aiming to characterize this alternative pathway, we additionally investigated the role of caveolae during uptake, as dynamin is used by several internalization pathways beside clathrin-mediated endocytosis, including caveolin-mediated endocytosis and the flotillin pathway (Doherty & McMahon, 2009). Moreover, in contrast to clathrin-dependent endocytosis, caveolaemediated internalization is described as a slow process, which would be in good agreement with the slow kinetics of TcdA<sub>1–1874</sub> uptake (Broeck et al., 2007). However, an efficient caveolin-1 knockdown in HT29 cells did not affect cellular uptake of full-length TcdA or of its CROP deletion mutant TcdA<sub>1–1874</sub>, arguing against the participation of caveolae during toxin endocytosis (data not shown). This is in accordance with a study focusing on TdB (Papatheodorou et al., 2010) and additionally supported by TER analyses revealing the susceptibility of Caco-2 cells towards the toxins despite being deficient in caveolin (Mirre et al., 1996).

It is acknowledged that binding of the C-terminal repeats to the cell surface induces receptor-mediated endocytosis, which involves clathrin-coated vesicles that are pinched off by dynamin to be further processed. The current study, however, demonstrates that the uptake mechanism of TcdA and TdB is much more complex. First, proposed receptor binding domain is not the only one capable of inducing endocytosis. Obviously, an alternative binding motif within the intermediary toxin region must exist, also accomplishing toxin uptake. Different efficacies of CROP-dependent and CROP-independent internalization reflect the flexibility of the toxins in entering any host cell via alternative endocytosis routes.

Aside from the diversity of target cells, epidemiological variations in the toxin sequence itself benefit from this feature. The prevalence of pathogenic C. difficile strains lacking regions of the tcdA or tcdB gene resulting in C-terminally truncated toxins is increasing (van den Berg et al., 2004). Thus, detailed characterization of endocytosis mechanisms utilized by the different toxin forms and identification of new target molecules might be the basis for the development of toxin intervention strategies.
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