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## Functional significance of active site residues in the enzymatic component of the *Clostridium difficile* binary toxin

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### ABSTRACT

*Clostridium difficile* binary toxin (CDT) is an ADP-ribosyltransferase which is linked to enhanced pathogenesis of *C. difficile* strains. CDT has dual function: domain *a* (CDTa) catalyses the ADP-ribosylation of actin (enzymatic component), whereas domain *b* (CDTb) transports CDTa into the cytosol (transport component). Understanding the molecular mechanism of CDT is necessary to assess its role in *C. difficile* infection. Identifying amino acids that are essential to CDTa function may aid drug inhibitor design to control the severity of *C. difficile* infections. Here we report mutations of key catalytic residues within CDTa and their effect on CDT cytotoxicity. Rather than an all-or-nothing response, activity of CDTa mutants vary with the type of amino acid substitution; S345A retains cytotoxicity whereas S345Y was sufficient to render CDT non-cytotoxic. Thus CDTa cytotoxicity levels are directly linked to ADP-ribosyltransferase activity.

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### 1. Introduction

*Clostridium difficile* is a gram positive, anaerobic bacterium that is the leading cause of antibiotic-associated pseudomembranous colitis worldwide. It produces two large potent exotoxins TcdA and TcdB that are the causative agents of infection and some strains of *C. difficile* produce an ADP-ribosyltransferase binary toxin (CDT), which is made up of two individually produced components, CDTa and CDTb [1]. CDTa is the enzymatically active component (48 kDa), whereas CDTb is the transport component (74 kDa) (Fig. 1A), assisting CDTa's entry into target cells [2–4]. It has been shown that CDT is toxic to African green monkey kidney epithelial cells (Vero cells) [5] but its precise role in pathogenesis is unclear [6]. Using time-lapse and immunofluorescence microscopy, Schwan et al., have shown that CDT forms dynamic microtubule protrusions on the surface of human colon carcinoma cells (Caco-

2) concomitantly with ADP-ribosylation of actin and depolymerisation of microfilaments [7]. It is thought that the binary toxin increases adherence of bacteria to the intestinal epithelial cells through these cell surface extensions. Schwan et al. also reported that the protrusions form a dense meshwork in which the bacteria were caught, contributing to the colonisation of *C. difficile*. Similar results were also demonstrated for the homologues *C. botulinum* toxin C2 (C2) and *C. perfringens* Iota toxin (Ia) [2,7,8]. In addition to protrusion formation, cellular microtubule structures were also altered to increase bundling of microtubules.

The N-terminus of CDTa is responsible for interaction with CDTb, whereas the C-terminus harbours the enzymatic activity [6,9]. Structural evidence from the complex of actin with the enzymatic component of Ia shows that Arg-177 of actin is the ribosylation site [10]. Based on homology and biochemical evidence, we can predict that CDTa would also irreversibly ADP-ribosylate monomeric G-actin at the Arg-177 residue. This ADP-ribosylation blocks polymerisation of G-actin to F-actin and subsequently disrupts the F-actin:G-actin equilibrium [11,12].

Previously we have reported the crystal structure of CDTa at three different pH values, 4.0, 8.5 and 9.0 and in complex with NADPH and NAD at pH 9.0 [13]. Both the structure of CDTa combined with the mechanism of ADP-ribosylation of Ia (which shares 84% sequence identity with CDTa), have been used to propose a detailed mechanism of ADP-ribosylation [11–13]. It was postulated that CDTa transfers the ADP ribose group of NAD/NADPH to monomeric G-actin at Arg-177, blocking polymerisation of actin

Abbreviations: CDT, *C. difficile* binary toxin; CDTa, *C. difficile* toxin domain a; rCDTa, recombinant wild-type *C. difficile* toxin domain a; CDTb, *C. difficile* toxin domain b residues 1–876; CDTb', residues 42–876 of CDTb (no signal peptide sequence); CDTb'', residues 209–876 of CDTb (no activation domain); TcdA, *C. difficile* exotoxin A; TcdB, *C. difficile* exotoxin B; ARTT, ADP-ribosyl turn-turn; Ia, *C. perfringens* Iota binary toxin; C2, *C. botulinum* toxin C2.

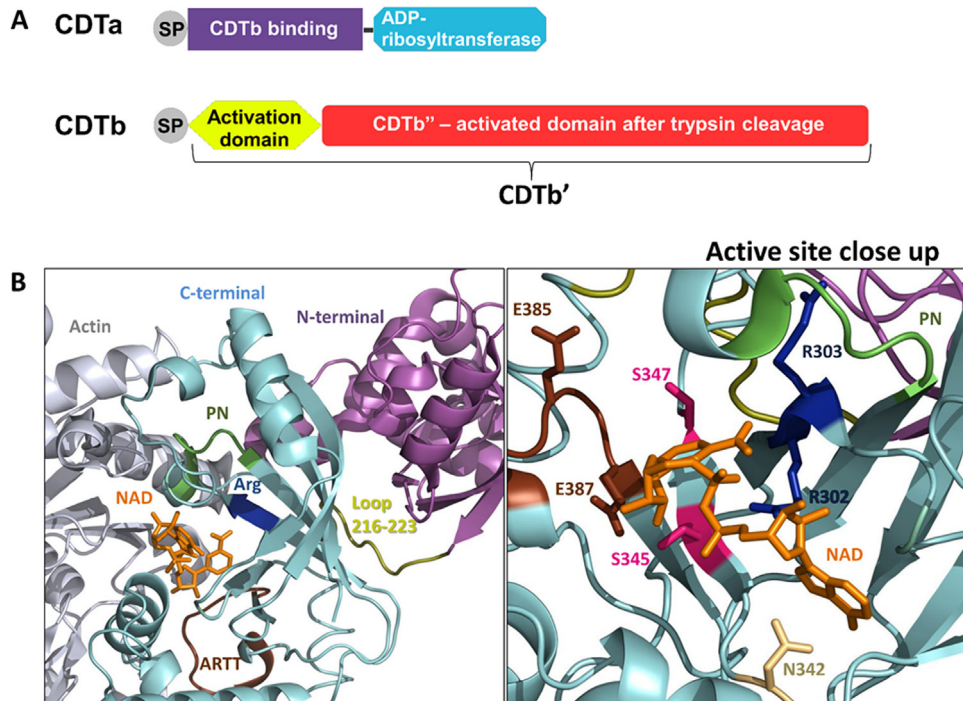
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**Fig. 1.** (A) Schematic representation of the domain organisation of CDTa and CDTb of the *C. difficile* binary toxin. The N-terminal signal peptide is displayed in grey for both components. CDTa N-terminal domain (CDTb binding, purple) and the C-terminal domain (ADP-ribosyltransferase activity, cyan) are shown. The CDTb component has an activation domain (yellow) that must be cleaved to give the activated domain (red). (B) Left: Key features in the CDTa active site. The N-terminal domain (residues 1–125), the C-terminal domain (residues 224–240), and the loop region connecting both domains (residues 216–223) are displayed in purple, cyan, and yellow respectively. Actin is shown in grey. Also shown are PN-loop (green), ARRT-loop (brown). Right: Close-up of catalytic residues. Residues of the ARRT-loop (E385, E387), Arg-motif (blue, R302, R303), NAD (orange), STS-motif (pink, S345, S347) are shown. Images were created using PyMOL (Version 1.5.0.4 Schrödinger, LLC) using previously described CDTa structure [13].

and therefore leading to the collapse of the cell cytoskeleton [3].

The CDTa N-terminal domain (residues 1–215; numbering refers to mature toxin without signal peptide) is proposed to interact with CDTb whereas the C-terminal domain (residues 224–420) is thought to interact with actin (Fig. 1B). These domains are linked by a short loop (seven residues) [13]. Both NAD and NADPH bind to the catalytic cleft of CDTa via the interacting residues Arg-302, Arg-303, Gln-307, Asn-342 and Ser-345 (Fig. 1B) [13].

Fig. 1B also displays key catalytic residues in addition to a number of other important features of the ADP-ribosylating family of toxins, including the PN-loop and Arg-motif. The EXE-motif which is thought to be the key motif involved in the ADP-ribosylation mechanism is located on the highly flexible ADP-ribosyl turn-turn (ARTT)-loop which is important for substrate binding. In CDTa, the EXE-motif is composed of residues Glu-385 and Glu-387, which are thought to be involved in stabilising the substrate–enzyme complex as confirmed with the corresponding residues Glu-378 and Glu-380 in Ia [10]. The unusual arrangement in this structure is that these residues are not in direct contact with NAD or NADPH, which might suggest that the EXE-motif in CDTa is not necessary for ligand binding and stabilisation of this complex. However, this could be due to the location of both Glu-385 and Glu-387 on the flexible ARTT-loop [3]. The final feature shown in Fig. 1B is the STS-motif, which includes the residues Ser-345 and Ser-347. The Ser-345 residue forms a strong hydrogen bond with Glu-387, and also directly with NAD and NADPH, therefore suggesting a role for the STS-motif in ligand binding and catalysis [3,13].

An  $SN_1$  mechanism has been proposed for ADP-ribosylation of monomeric G-actin. The mechanism was based on the mutagenesis studies of Ia and the crystallographic structure of actin in complex with Ia [10]. The  $SN_1$  reaction proceeds via two consecutive intermediates: an oxocarbenium ion intermediate and a

cationic intermediate [3]. This mechanism is thought to be consistent amongst the ADPRT family of toxins [14]. As Ia is the closest homologue to CDT, a similar  $SN_1$  reaction has been proposed with the EXE-motif playing a key role [13].

Here we report a detailed mutagenesis and biochemical study on CDTa active site residues to determine their role in CDTa activity and thereby testing the hypothesis of the  $SN_1$  mechanism. This study also provides experimental evidence on the importance of the EXE-motif in the catalytic activity of CDTa.

## 2. Materials and methods

### 2.1. Cloning and expression

CDTa mutants were generated by site-directed mutagenesis using pMAL p2X-CDTa plasmid as template. Oligonucleotides (primers) for mutagenesis of active site residues are summarised in Table 1. Plasmids containing *cdtA* inserts were transformed into *E. coli* Top 10 cells and mutations confirmed by DNA sequencing.

CDTa mutants were expressed in *E. coli* BL21 (DE3)-CodonPlus cells. It was noted that the expression yield of both recombinant wild-type CDTa (rCDTa) and the mutants was low. To improve protein expression yield, pGEX-6p1 was used as expression vector: *cdtA* DNA inserts were released from pMAL-p2X plasmids by restriction digest (BamH1 and Sal1) and ligated into the pGEX-6p1 vector. *cdtA* DNA inserts were checked by sequencing.

### 2.2. Expression of CDTa constructs

CDTa constructs were grown in LB medium containing the appropriate antibiotic at 37 °C until  $OD_{600}=0.8$ –1.0. Cultures were induced at 20 °C with 1 mM IPTG for 4 h. Cells were harvested at

**Table 1**  
Oligonucleotides designed for site directed mutagenesis of CDTa active site residues.

Mutation	Primers
<b>S345A</b>	5'-CCG AAC TTT ATT GGC ACC AGC-3' 5'-GCT GGT GCC AAT AAA GTT CGG-3'
<b>S345F</b>	5'-CCG AAC TTT ATT TTC ACC AGC-3' 5'-GCT GGT GAA AAT AAA GTT CGG-3'
<b>S345R</b>	5'-CCG AAC TTT ATT CGT ACC AGC-3' 5'-GCT GGT ACG AAT AAA GTT CGG-3'
<b>S345Y</b>	5'-CCG AAC TTT ATT TAC ACC AGC-3' 5'-GCT GGT GTA AAT AAA GTT CGG-3'
<b>E385Q</b>	5'-GCT ATG CGG GCC AAT ATG AAG TG-3' 5'-CAC TTC ATA TTG GCC CGC ATA GC-3'
<b>E387Q</b>	5'-GGG CGA ATA TCA AGT GCT GCT G-3' 5'-CAG CAG CAC TTG ATA TTC GCC G-3'
<b>E387R</b>	5'-GC GAA TAT GAT GTG CTG CTG-3' 5'-CAG CAG CAC ACG ATA TTC GC-3'

4 °C and stored at –80 °C until further use.

### 2.3. Purification of CDTa constructs (rCDTa and mutants)

Cell pellets were re-suspended in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA; 2 ml/g of pellet). Cells were passed through a homogeniser twice at a pressure of 20kpsi at 4 °C. The cell lysate was centrifuged at 25,000 rpm for 35 min at 4 °C and the resultant supernatant was filtered through both a Minisart<sup>®</sup> GF-pre-filter (Sartorius) and 0.45 µm milllex-HA filter (Millipore). The filtered supernatant was loaded onto a 5 ml GSTrap-FF column (GE Healthcare) at 0.5 ml/min in lysis buffer. GST-tagged CDTa constructs were eluted at 0.5 ml/min in 20 mM reduced glutathione in lysis buffer. The GST-tag was removed during overnight dialysis at 4 °C against 50 mM Tris-HCl pH 8.0 and 20 mM NaCl, by adding PreScission<sup>™</sup> protease (1U per 100 µg of protein, GE Healthcare) to the purified protein. Dialyzed protein was centrifuged at 4000g to remove any precipitated protein and re-loaded onto the GSTrap-FF column as above. Purified CDTa constructs were collected in flow through, concentrated to 5.0 mg/ml (Amicon Ultra-15, Millipore), flash frozen in liquid nitrogen and stored at –80 °C until further use.

### 2.4. Vero cell cytotoxicity assays

The cytotoxicity of CDTa mutants was assessed using a previously established protocol [5] with some modifications. Vero cells have previously shown cell death in the presence of both CDTa and CDTb' (CDTb lacking the N-terminal signal peptide, residues 41–876, Fig. 1A), as well as TcdA and TcdB. Vero cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum (FCS) and 2 mM glutamine at 37 °C in the presence of 5% CO<sub>2</sub>. Cells were routinely trypsinized and used to coat 96-well plates in a volume of 200 µl (10<sup>4</sup> cells/well) of complete DMEM (supplemented with 10% FCS and 2 mM glutamine) and were incubated for 24 h to allow formation of a confluent monolayer. Prior to assays, the medium was carefully removed and the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS) and the cells were incubated for 1 h in 100 µl DMEM (supplemented with 10% FCS and 2 mM glutamine).

To test the cytotoxic effect of CDT on Vero cells, cells were incubated with various components (100 µl reaction volume) for 24 h at 37 °C, 5% CO<sub>2</sub>. TcdA and TcdB were purified from *C. difficile* as previously described [15,16] and used as a positive control for cell death. Cells were incubated with 50 ng/ml TcdA and 0.5 ng/ml

TcdB to ensure the Vero cells were responsive to the standard cytotoxicity assay. To establish baseline activity of CDTa-CDTb' complex and compare it with CDTa mutants, cells were incubated with 250 ng of each CDTa and trypsin activated CDTb'. Trypsin activated CDTb' was used rather than CDTb' (Fig. 1A) as this shows greater cytotoxicity when combined with CDTa [5]. To exclude cell death caused by any of the assay components, cells were incubated with either serum free DMEM, CDTa buffer, CDTb' buffer, 250 ng CDTa or 250 ng trypsin activated CDTb' (negative controls). The cells were examined after 24 h for evidence of cytotoxic effect.

### 2.5. Western blot analysis

To assess whether the mutations affect CDTa binding to NAD or transfer of ADP-ribose to actin, CDTa was incubated with biotinylated NAD (biotin-NAD) and actin and reactions were analysed by Western blotting for the presence of biotin-NAD. The NAD used here is biotinylated at the ADP-ribose end and is accessible to an enzyme-conjugated streptavidin antibody after ADP-ribosylation. CDTa constructs were incubated with NAD-biotin at equal molar ratio. Prior to adding actin to the CDTa-biotin-NAD mix in a 1:1 M ratio, actin was incubated with latrunculin A at equal molar concentration to maintain actin in its monomeric form. Negative controls included reactions without biotinylated-NAD or rCDTa to confirm that neither biotinylated-NAD nor rCDTa alone would be detected in the blot (not shown). Reactions were incubated at room temperature with gentle rocking for 1 h and stopped by addition of SDS-PAGE sample loading dye (no heating). Samples were resolved on a 10% Tris-glycine SDS-PAGE (200 V for 1 h) and transferred to nitrocellulose membrane using standard techniques for 1 h at 50 V. The membrane was blocked overnight in 1% bovine serum albumin (BSA) in phosphate buffered saline pH 7.4 containing 0.05% Tween-20 (PBST). The membrane was then washed 2 × 5 min in PBST, after which the membrane was incubated with streptavidin horseradish peroxidase (strep-HRP) 1:1000 in PBST for 1 h. The membrane was washed 6 × 5 min before adding the developing solution: 50 mM Tris-HCl pH 7.5, 0.03% H<sub>2</sub>O<sub>2</sub> and 0.1% 3, 3'-Diaminobenzidine (DAB). Developing solution was removed immediately after bands appeared on the membrane to prevent overexposure.

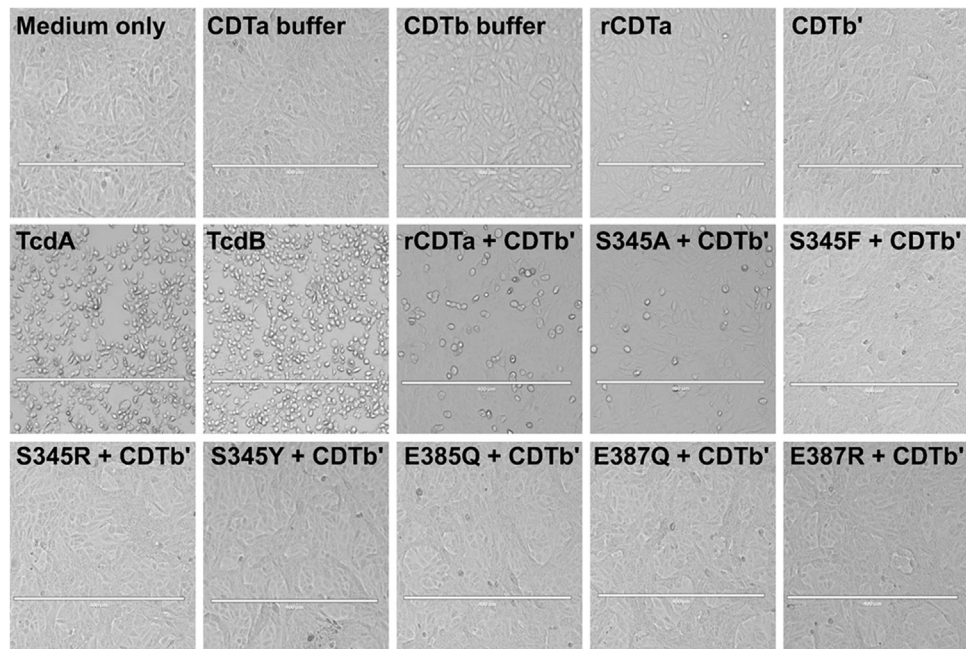
## 3. Results

### 3.1. CDTa mutations reduce or abolish cytotoxicity

To assess the cytotoxicity of the CDTa mutants, Vero cell assays were performed. The negative controls (Fig. 2, top row) confirm that protein buffers, rCDTa and trypsin activated CDTb' on their own are non-cytotoxic to Vero cells. TcdA and TcdB, which are known to have a clear cytotoxic impact on Vero cells, were included as positive controls to ensure that the Vero cells were responsive to the standard cytotoxicity assay. As expected TcdA or TcdB induce cell rounding leading to cell death (Fig. 2).

rCDTa-CDTb' (Fig. 2) also induces cell death, but not to the same extent as TcdA and TcdB. These results are consistent with those previously reported [1].

CDTa mutation S345A shows some cytotoxicity but the number of rounded cells is significantly less than in the case of rCDTa (Fig. 2). CDTa mutants S345F, S345R, S345Y, E385Q, E387Q and E387R show no sign of Vero cell death (cell rounding) i.e. they abolish CDTa-CDTb' toxicity. This suggests that these mutations prevent ADP-ribosylation of G-actin.



**Fig. 2.** Vero cell cytotoxicity assay. Amount of rCDTa, mutants and CDTb' present in cell assays was 250 ng. Reaction volume was 100  $\mu$ l. TcdA and TcdB were added to a final concentration of 50 ng/ml and 0.5 ng/ml respectively. Size bars, 400  $\mu$ m.

### 3.2. ADP-ribose transfer is essential for CDTa cytotoxicity

*In vitro* cell assays suggested that impaired ADP-ribosylation may be the major reason preventing cytotoxicity of CDTa mutants. To determine if cytotoxicity depends only on biotin-NAD transfer to actin or also on CDTa binding of biotin-NAD, CDTa constructs were incubated with biotin-NAD and actin. The reactions were resolved on SDS-PAGE (Fig. 3A) and subjected to Western blot analysis following the presence of biotin-NAD by strep-HRP antibody (Fig. 3B). As a control rCDTa was incubated with biotin-NAD in the absence of actin (-actin sample) to assess binding of biotin-NAD to CDTa. As positive control (+ve control) rCDTa was incubated with biotin-NAD and actin to confirm both, biotin-NAD binding by rCDTa as well as transfer of the ADP-ribose group to actin by rCDTa. Western blot analysis confirms that rCDTa strongly binds to biotin-NAD and transfers biotin-NAD to actin (Fig. 3B). In comparison, in the absence of actin, rCDTa does not bind biotin-NAD well.

CDTa mutant S345A binds and transfers biotin-NAD to the same extent as rCDTa. These results correlate with the cytotoxicity assays in which S345A was the only mutation that induced cell death. S345F and S345R bind biotin-NAD but transfer to actin is negligible (very faint band). S345R binds more biotin-NAD than S345F but the level of biotin-NAD transfer appears similar between these mutants. Both mutants were non-cytotoxic in Vero cell assays. Mutants S345Y, E385Q, E387Q and E387R are unable to transfer biotin-NAD to actin. Low level of biotin-NAD binding to E385Q, E387Q and E387R can be detected, whereas S345Y does not bind biotin-NAD. Based on the physical difference between serine and tyrosine, it is conceivable that the mutation caused a significant reduction in binding and cytotoxic efficiency.

## 4. Discussion

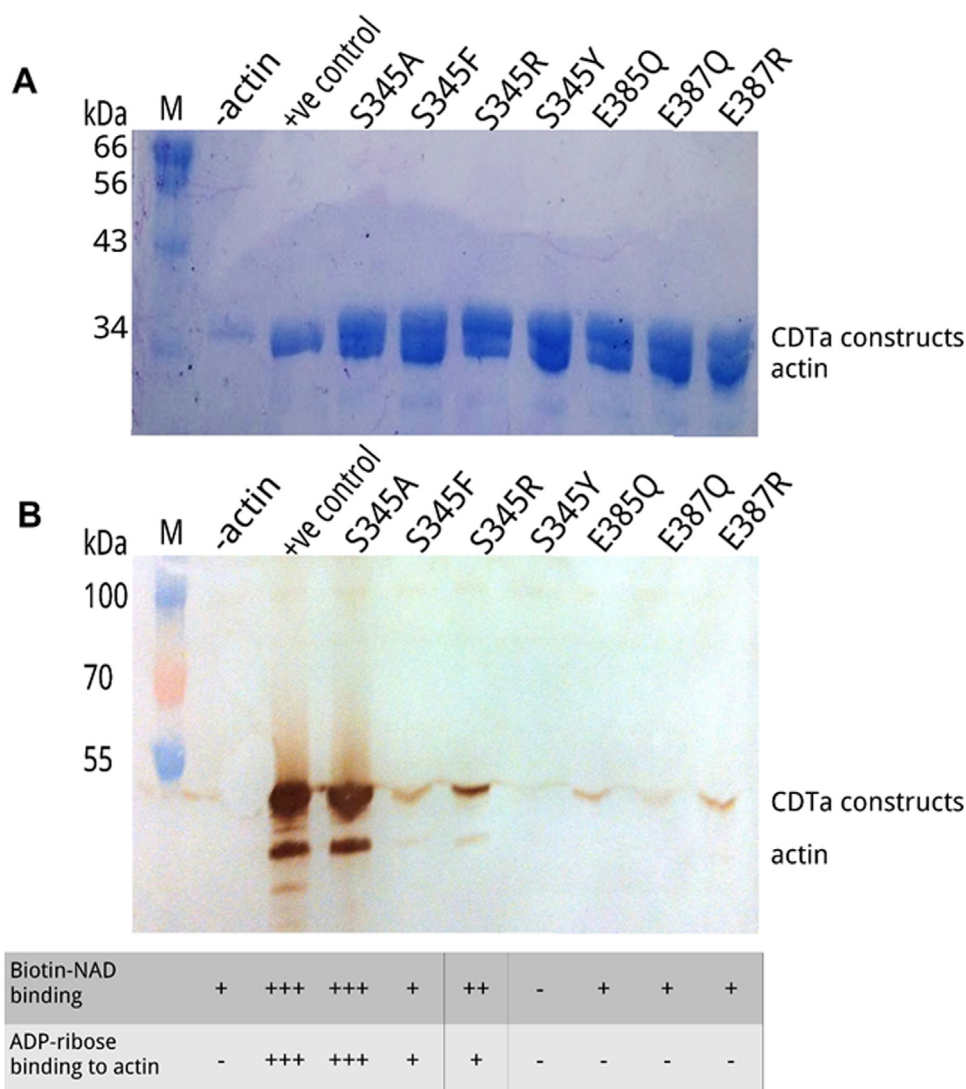
The selected residues Glu-385 and Glu-387 of the EXE-motif of rCDTa were mutated, as this motif is known to play a strong role in ligand binding and catalysis [3]. They directly correspond to residues Glu-378 and Glu-380 which were shown to play key roles in the

cytotoxicity of Ia [17]. In addition Ser-345 was also selected for mutagenesis studies due to its key role in binding Glu-387 and NAD.

CDT ADP-ribosylates monomeric actin, which leads to cytoskeletal collapse and eventually to cell death. Cytotoxicity cell assays confirmed that rCDTa-CDTb' induces cell death and Western blot analysis confirmed that rCDTa ADP-ribosylates G-actin. In the absence of actin, rCDTa binds lower levels of biotin-NAD as expected. SDS-PAGE reveals that the -actin sample does not correlate with the other samples although reactions were handled in the same manner. This is likely a result of gel loading. Consequently the lower amount of protein present is reflected by the almost undetectable biotin-NAD signal in Western analysis. Notably, in the absence of actin, biotin-NAD is still hydrolysed and released from the rCDTa active site, which given its molecular weight (244.31) is likely to run off SDS-PAGE before Western transfer. In this case, the signal will be even lower and it would be impossible to compare samples. To overcome inaccuracies caused by uncontrolled biotin-NAD hydrolysis reactions contained actin. In the presence of actin, the biotin-NAD signal can be monitored either whilst bound to CDTa constructs or actin. Western blot analysis of CDTa mutants which had diminished or no cytotoxic effect, confirmed that majority of mutants had lost ADP-ribosyl transferase activity but not the ability to bind biotin-NAD. In cytotoxicity assays S345A is the only mutant inducing significant cell death, *albeit* less than rCDTa-CDTb', there is still evidence that this mutation has not inhibited the activity of CDTa but may have slightly reduced its efficacy. In Western blot analysis S345A activity matches that of rCDTa.

The inability of CDTa mutants to transfer biotin-NAD to actin correlates with the proteins being unable to induce cell death *in vitro*. Mutants E385Q, E387Q and E387R are unable to transfer biotin-NAD but retain some ability to bind biotin-NAD.

Both the Western blot analysis and the Vero cell assay have confirmed that S345A is the only mutation that retains cytotoxicity. Ser-345 together with Thr-346 and Ser-347, forms the STS motif. The S345A mutation retains the overall arrangement of NAD in the active site and salt bridges between Arg-302, Arg-359 and NAD are unaffected (Fig. 4A). As expected the hydrogen bond in

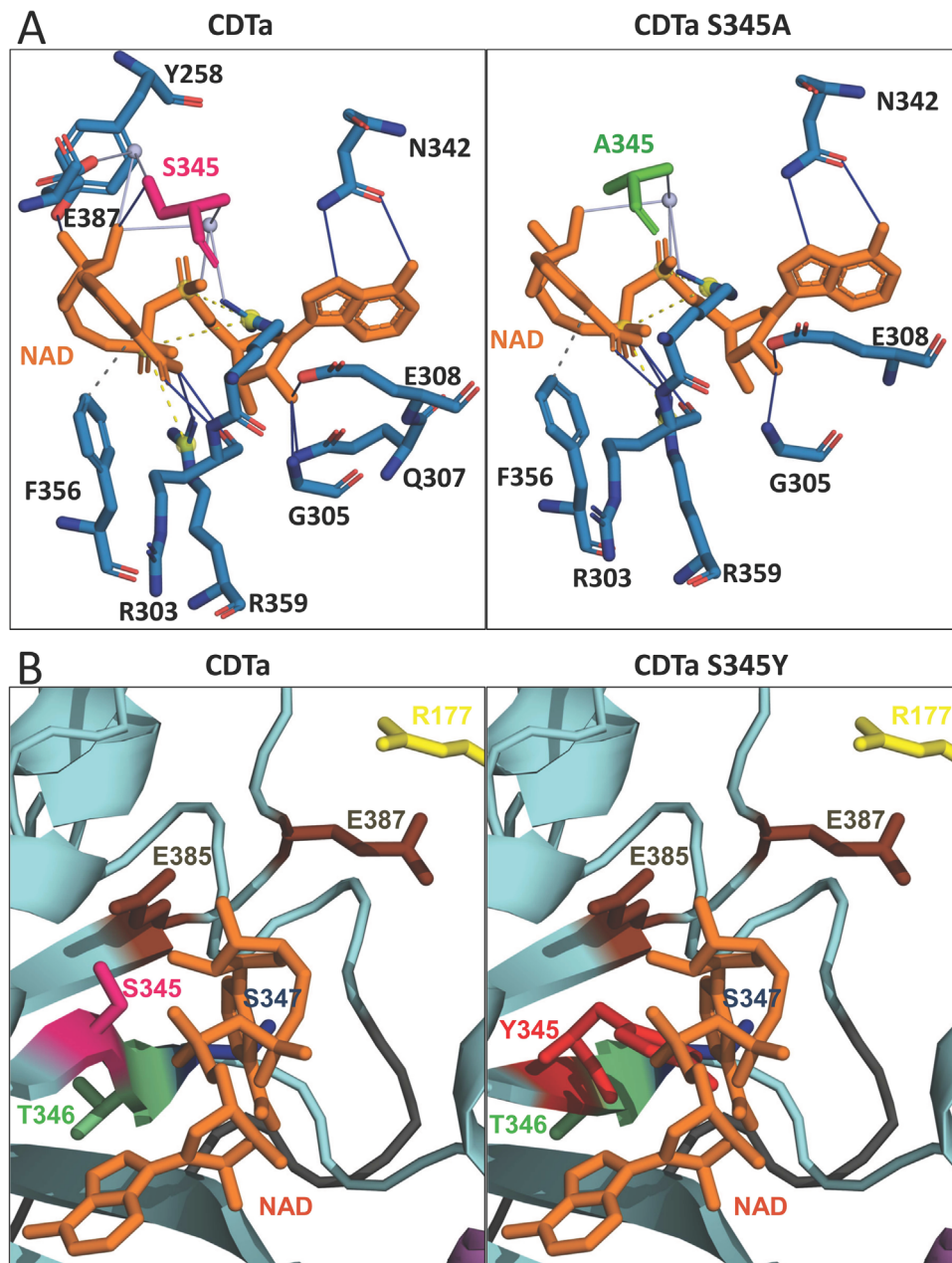


**Fig. 3.** Enzyme activity of rCDTa and CDTa mutants. 10% Tris-glycine SDS-PAGE of biotin-NAD binding and transfer assays (A) which was used for subsequent Western blot detection of biotin-NAD via strep-HRP antibody (B). Reaction in lane -actin contained rCDTa + biotin-NAD. All other reactions contained biotin-NAD, actin and the protein construct stated. The Western blot was developed with DAB displaying strep-HRP bound to biotin-NAD where present on the membrane. The lanes correspond with gel picture A. The table summarises the activity of CDTa constructs to bind and transfer NAD-biotin to actin where +++ is maximum activity (rCDTa control) and - is lack of activity.

rCDTa between the Ser-345 hydroxyl group and one of the hydroxyl groups of the nicotinamide carrying ribose, as well as the hydrogen bond with the catalytic Glu-387 are lost (Fig. 4A). Yet, cytotoxicity assays confirm that the loss of these interactions only reduces activity of the S345A mutant. This is in agreement with previous mutagenesis studies on corresponding residues of the STS motif in Ia [18] and C2 [19]. By mutating a serine residue to alanine, the -OH group is removed, whereas mutations to phenylalanine, arginine or tyrosine are much more significant in terms of size and functional group properties. This would suggest that the functional hydroxyl group of serine does not play an important role in the active site. Mutation of Ser-345 to alanine only marginally reduced the cytotoxicity whereas mutants S345F, S345R and S345Y are non-cytotoxic. Thus increase in size of the Ser-345 residue has an adverse effect on cytotoxicity which could be due to a number of reasons. It could prevent the residue from interacting with and stabilising Glu-387, or it could block the transfer of ADP-ribose to actin. We identified a single mutation, S345Y, which was able to abolish both biotin-NAD binding and transfer whereas S345F and S345R only affect transfer. Hence, targeting CDTa ability

to transfer NAD to actin is sufficient to inhibit cytotoxicity. The enlarging effect of the residue does not seem to affect NAD binding as demonstrated by the Western blot analysis, with the exception of S345Y which is likely due to steric hindrance in the active site, reducing the NAD binding capacity. Notably, phenylalanine which is similar in structure and physical properties to tyrosine does not block NAD binding. A model of the rCDTa active site illustrates how S345Y blocks the space usually occupied by NAD in the wild type structure (Fig. 4B).

The EXE-motif mutations also show interesting results. Previously it has been suggested that the hydrolysis of NAD and ADP-ribosylation of actin take place via an  $S_N1$  reaction, with Glu-387 playing a key role in oxocarbenium ion formation by interaction with the 2'OH ribose group of NAD. By creating E387Q, the hydroxyl group (-OH) of Glu-387 is substituted with an amine group (-NH<sub>2</sub>), which appears to completely abolish ADP-ribosylation of actin. This suggests that the proposed  $S_N1$  reaction involving Glu-387 has some validity. In addition E385Q also completely eliminates ADP-ribosylation of actin, which indicates that the -OH functional group plays a crucial role in the mechanism. The theory is that Glu-385 brings Arg-177 from actin into



**Fig 4.** (A) Interaction of rCDTa and CDTa S345A with NAD. Hydrogen bonding, water bridges, salt bridges, and hydrophobic interactions are shown in blue, grey (dotted), and grey (dotted) lines respectively. Ser-345 is shown in pink and Ala-345 in green. CDTa S345A was generated using Swiss Model [20–23]. Protein ligand profiles were generated using PLIP [24]. (B) Comparison of the active sites of rCDTa and CDTa S345Y. The protein structure for rCDTa has been reported previously [13], PDB accession 2WN7. rCDTa C-terminal region is shown in cyan. NAD cofactor is shown in orange. Residue Arg-177 (ribosylation site) from the modelled Actin is shown in yellow as reference. The active site of S345Y was modelled using Swiss-Model [20–23].

the active site via the –OH functional group of Glu-385. Again, the present mutagenesis study indicates this theory may also be correct. Having already established that substitution of the functional group of Glu-387 to –NH<sub>2</sub> abolishes actin ADP-ribosylation, it confirms the view that increasing the size of this residue and substituting the functional group (E387R mutation) also hinders ADP-ribosylation.

In summary, the purpose of this mutagenesis study was to monitor the effect on cytotoxicity, and to determine whether or not these residues are vital individually for the activity of CDTa. Combination of both the Vero cell assays and the Western blot analysis has proven that altering three key residues has a significant impact on CDTa activity. This study is also supporting the proposed SN<sub>1</sub> mechanism of CDTa. If the activity of CDTa can be inhibited, then ADP-ribosylation of actin can also be prevented, which in turn will protect

from cell rounding and cell death. The study shows that mutating CDTa key residues affects activity but that the amino acid substitution determines the extent by which CDTa activity is affected.

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#### Appendix A. Transparency document

Transparency document associated with this article can be

found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.08.011>.

## References

- [1] M.R. Popoff, E.J. Rubin, D.M. Gill, P. Boquet, Actin-specific ADP-ribosyltransferase produced by a *Clostridium difficile* strain, *Infect. Immun.* 56 (1988) 2299–2306.
- [2] H. Barth, Uptake of binary actin ADP-ribosylating toxins, *Rev. Physiol. Biochem. Pharmacol.* 152 (2004) 165–182.
- [3] A.H. Davies, A.K. Roberts, C.C. Shone, K.R. Acharya, Super toxins from a super bug: structure and function of *Clostridium difficile* toxins, *Biochem. J.* 436 (2011) 517–526.
- [4] S. Perelle, M. Gibert, P. Bourlioux, G. Corthier, M.R. Popoff, Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196, *Infect. Immun.* 65 (1997) 1402–1407.
- [5] A. Sundriyal, A.K. Roberts, R. Ling, J. McGlashan, C.C. Shone, K.R. Acharya, Expression, purification and cell cytotoxicity of actin-modifying binary toxin from *Clostridium difficile*, *Protein Expr. Purif.* 74 (2010) 42–48.
- [6] D.N. Gerding, S. Johnson, M. Rupnik, K. Aktories, *Clostridium difficile* binary toxin CDT: mechanism, epidemiology, and potential clinical importance, *Gut Microbes* 5 (2013) 15–27.
- [7] C. Schwan, B. Stecher, T. Tzivelekidis, M. Van Ham, M. Rohde, W.D. Hardt, et al., *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria, *PLoS Pathog.* 5 (2009) e1000626.
- [8] H. Barth, K. Aktories, M.R. Popoff, B.G. Stiles, Binary bacterial toxins: biochemistry, biology, and applications of common *Clostridium* and *Bacillus* proteins, *Microbiol. Mol. Biol. Rev.* 68 (2004) 373–402.
- [9] I. Gülke, G. Pfeifer, J. Liese, M. Fritz, F. Hofmann, K. Aktories, et al., Characterization of the enzymatic component of the ADP-ribosyltransferase toxin CDTa from *Clostridium difficile*, *Infect. Immun.* 69 (2001) 6004–6011.
- [10] H. Tsuge, M. Nagahama, M. Oda, S. Iwamoto, H. Utsunomiya, V.E. Marquez, et al., Structural basis of actin recognition and arginine ADP-ribosylation by *Clostridium perfringens* iota-toxin, *Proc. Natl. Acad. Sci. USA* 105 (2008) 7399–7404.
- [11] J. Vandekerckhove, B. Schering, M. Bärman, K. Aktories, *Clostridium perfringens* iota toxin ADP-ribosylates skeletal muscle actin in Arg-177, *FEBS Lett.* 225 (1987) 48–52.
- [12] S. Mauss, C. Chaponnier, I. Just, K. Aktories, G. Gabbiani, ADP-ribosylation of actin Isoforms by *Clostridium botulinum* C2 Toxin and *Clostridium perfringens* Iota Toxin, *Eur. J. Biochem.* 194 (1990) 237–241.
- [13] A. Sundriyal, A.K. Roberts, C.C. Shone, K.R. Acharya, Structural basis for substrate recognition in the enzymatic component of ADP-ribosyltransferase toxin CDTa from *Clostridium difficile*, *J. Biol. Chem.* 284 (2009) 28713–28719.
- [14] K.P. Holbourn, C.C. Shone, K.R. Acharya, A family of killer toxins: exploring the mechanism of ADP-ribosylating toxins, *FEBS J.* 273 (2006) 4579–4593.
- [15] A.K. Roberts, C.C. Shone, Modification of surface histidine residues abolishes the cytotoxic activity of *Clostridium difficile* toxin A, *Toxicon* 39 (2001) 325–333.
- [16] A. Roberts, J. McGlashan, A.A. Ibrahim, R. Ling, H. Denton, S. Green, et al., Development and evaluation of an ovine antibody-based platform for treatment of *Clostridium difficile* infection, *Infect. Immun.* 80 (2012) 875–882.
- [17] S. Perelle, M. Domenighini, M.R. Popoff, Evidence that Arg-295, Glu-378, and Glu-380 are active-site residues of the ADP-ribosyltransferase activity of Iota toxin, *FEBS Lett.* 395 (1996) 191–194.
- [18] M. Nagahama, Y. Sakaguchi, K. Kobayashi, S. Ochi, J. Sakurai, Characterization of the enzymatic component of *Clostridium perfringens* Iota-toxin, *J. Bacteriol.* 182 (2000) 2096–2103.
- [19] H. Barth, J.C. Preiss, F. Hofmann, K. Aktories, Characterization of the catalytic site of the botulinum C2 toxin by site-directed mutagenesis characterization of the catalytic site of the ADP-ribosyltransferase *Clostridium botulinum* C2 toxin by site-directed mutagenesis, *J. Biol. Chem.* 273 (1998) 29506–29511.
- [20] K. Arnold, L. Bordoli, J. Kopp, T. Schwede, The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling, *Bioinformatics* 22 (2006) 195–201.
- [21] M. Biasini, S. Bienert, A. Waterhouse, K. Arnold, G. Studer, T. Schmidt, et al., SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information, *Nucleic Acids Res.* 42 (2014) 252–258.
- [22] N. Guex, M.C. Peitsch, T. Schwede, Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a historical perspective, *Electrophoresis* 30 (2009) 162–173.
- [23] F. Kiefer, K. Arnold, M. Künzli, L. Bordoli, T. Schwede, The SWISS-MODEL repository and associated resources, *Nucleic Acids Res.* 37 (2009) 387–392.
- [24] S. Salentin, S. Schreiber, V.J. Haupt, M.F. Adasme, M. Schroeder, PLIP: fully automated protein-ligand interaction profiler, *Nucleic Acids Res.* 43 (2015) W443–W447.