

Cyclophilin-Facilitated Membrane Translocation as Pharmacological Target to Prevent Intoxication of Mammalian Cells by Binary Clostridial Actin ADP-Ribosylated Toxins

Katharina Ernst¹, Simon Langer¹, Eva Kaiser¹, Christian Osseforth², Jens Michaelis², Michel R. Popoff³, Carsten Schwan⁴, Klaus Aktories⁴, Viktoria Kahlert⁵, Miroslav Malesevic⁶, Cordelia Schiene-Fischer⁶ and Holger Barth¹

¹ - Institute of Pharmacology and Toxicology, University of Ulm Medical Center, 89081 Ulm, Germany

² - Institute of Biophysics, University of Ulm, 89081 Ulm, Germany

³ - Department of Anaerobic Bacteria, Pasteur Institute, 75724 Paris, France

⁴ - Institute of Experimental and Clinical Pharmacology and Toxicology, University of Freiburg, 79104 Freiburg, Germany

⁵ - Max Planck Research Unit for Enzymology of Protein Folding Halle, 06120 Halle (Saale), Germany

⁶ - Institute for Biochemistry and Biotechnology, Martin Luther University Halle-Wittenberg, 06120 Halle (Saale), Germany

Correspondence to Cordelia Schiene-Fischer and Holger Barth: Department of Enzymology, Institute for Biochemistry and Biotechnology, Martin Luther University Halle-Wittenberg, Weinbergweg 22, D-06120 Halle (Saale), Germany; H. Barth, Institute of Pharmacology and Toxicology, University of Ulm Medical Center, Albert-Einstein-Allee 11, D-89081 Ulm, Germany. cordelia.schiene-fischer@biochemtech.uni-halle.de; holger.barth@uni-ulm.de.

<http://dx.doi.org/10.1016/j.jmb.2014.07.013>

Edited by D. Mokranjac

Abstract

Clostridium botulinum C2 toxin, *Clostridium perfringens* iota toxin and *Clostridium difficile* CDT belong to the family of binary actin ADP-ribosylating toxins and are composed of a binding/translocation component and a separate enzyme component. The enzyme components ADP-ribosylate G-actin in the cytosol of target cells resulting in depolymerization of F-actin, cell rounding and cell death. The binding/translocation components bind to their cell receptors and form complexes with the respective enzyme components. After receptor-mediated endocytosis, the binding/translocation components form pores in membranes of acidified endosomes and the enzyme components translocate through these pores into the cytosol. This step is facilitated by the host cell chaperone heat shock protein 90 and peptidyl-prolyl *cis/trans* isomerases including cyclophilin A. Here, we demonstrate that a large isoform of cyclophilin A, the multi-domain enzyme cyclophilin 40 (Cyp40), binds to the enzyme components C2I, Ia and CDTa *in vitro*. Isothermal titration calorimetry revealed a direct binding to C2I with a calculated affinity of 101 nM and to Ia with an affinity of 1.01 μ M. Closer investigation for the prototypic C2I revealed that binding to Cyp40 did not depend on its ADP-ribosyltransferase activity but was stronger for unfolded C2I. The interaction of C2I with Cyp40 was also demonstrated in lysates from C2-treated cells by pull-down. Treatment of cells with a non-immunosuppressive cyclosporine A derivative, which still binds to and inhibits the peptidyl-prolyl *cis/trans* isomerase activity of cyclophilins, protected cells from intoxication with C2, iota and CDT toxins, offering an attractive approach for development of novel therapeutic strategies against binary actin ADP-ribosylating toxins.

© 2014 Elsevier Ltd. All rights reserved.

Introduction

Various clostridia produce potent exotoxins, which mono-ADP-ribosylate G-actin at arginine 177 in the cytosol of mammalian target cells (for review, see Ref. [1]). This toxin-catalyzed modification turns

G-actin into a capping molecule that prevents further polymerization of actin filaments [2–5] and results in the breakdown of the actin cytoskeleton. Finally, the mode of action of these toxins results in cell rounding [6–11] and cell death [12,13] associated with severe enteric diseases in humans and animals [14–18].

Binary actin ADP-ribosylating toxins are the C2 toxin from *Clostridium botulinum*, the prototype of this toxin family [11], and the iota-like toxins that comprise the iota toxin from *Clostridium perfringens* type E strains [19,20] as the prototype of this group, CDT from hypervirulent strains of *Clostridium difficile* [21–24] and the *Clostridium spiroforme* transferase [25]. The iota-like toxins are closer related among each other than to C2 toxin regarding their degree of sequence homology [26], the interchangeability of their components and the modification of actin isoforms that serve as a substrate [27] (for review, see Ref. [1]). Moreover, all iota-like toxins bind to the lipolysis-stimulated receptor on particular target cells [28,29] while C2 toxin binds to a carbohydrate receptor that is present on all mammalian cell types tested so far [30].

However, C2 toxin and the iota-like toxins share an overall comparable unique structure and mode of action. They are composed of two non-linked proteins, an enzyme component (A-component) and a separate binding and translocation component (B-component). Both components must assemble on the surface of target cells where the B-component binds to its cellular receptor, forms a complex with the A-component and facilitates the receptor-mediated endocytosis and subsequently the transport of the A-component from acidified endosomal vesicles into the cytosol (for review, see Ref. [1]). The cellular uptake of C2 and iota toxins was discovered by us and by others during recent years. C2 toxin consists of the enzyme component C2I (~49 kDa) [31,32] and the binding/translocation component C2II (~80 or 100 kDa, depending on the strain [33,34]). Proteolytically activated C2II forms ring-shaped heptamers (C2IIa) that bind to asparagine-linked carbohydrate structures on mammalian cells [30,33,35–37] and form complexes with C2I. After their receptor-mediated endocytosis, the C2IIa/C2I complexes reach early endosomal vesicles where the C2IIa heptamers change their conformation, insert as pores into endosomal membranes and serve as channels for translocation of unfolded C2I from the endosomal lumen into the cytosol [33,38–42]. The cellular uptake and intracellular membrane transport of iota toxin is widely comparable [43]. After receptor-mediated endocytosis of the Ib/Ia complexes, the proteolytically activated binding/translocation component Ib also forms heptameric transmembrane pores, which mediate translocation of the enzyme component Ia (47 kDa) across endosomal membranes into the cytosol [43–47]. However, there are some differences during cellular uptake of C2 toxin and iota toxin since Ia likely translocates from endosomes, which are in a state between early and late endosomes [48], while C2I translocates from early endosomes [33]. Most likely, translocation of Ia requires more acidic conditions and a membrane potential gradient in addition to the pH gradient [48].

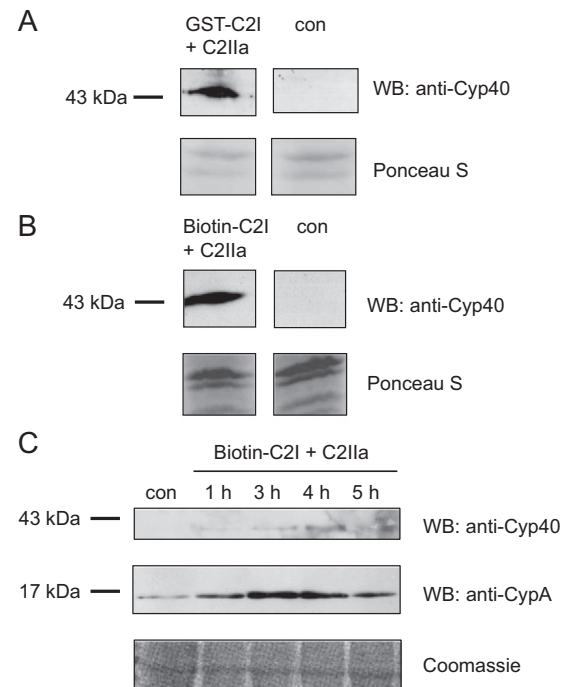


Fig. 1. Co-precipitation of C2I with Cyp40 from lysates of C2 toxin-treated HeLa cells. Cells were incubated for 2 h at 37 °C with C2IIa (800 ng/ml) plus either GST-C2I (400 ng/ml) (A) or biotin-C2I (400 ng/ml) (B) or without any protein for control (con). Cells were lysed and the cytoplasmic fraction was obtained by centrifugation. For pull-down of GST-C2I or biotin-C2I, the cytoplasmic fractions were incubated with glutathione-Sepharose beads (a) or streptavidin-agarose beads (b), respectively, overnight at 4 °C. The beads were washed and precipitated proteins separated by SDS-PAGE and analyzed by Western blotting with a specific antibody against Cyp40. Comparable protein loading was confirmed by Ponceau S staining of the blot membranes. (C) Cells were incubated at 37 °C with C2IIa (600 ng/ml) plus biotin-C2I (300 ng/ml) for different periods of time as indicated. The experiment was performed as described above. Precipitates were analyzed by Western blotting with specific antibodies against Cyp40 and CypA and comparable amounts of protein in the input controls were confirmed by SDS-PAGE and subsequent Coomassie staining.

Despite these differences, membrane translocation of C2 toxin and iota toxin is facilitated by the same chaperones including heat shock protein 90 (Hsp90) [49,50] and the peptidyl-prolyl *cis/trans* isomerases (PPIases) cyclophilin A (CypA) [51,52] and FK506 binding protein 51 (FKBP51) [53]. Moreover, these host cell factors are also involved in membrane translocation of *C. difficile* CDT and we have got evidence that the Hsp90/PPIase-mediated membrane transport might be a common feature of bacterial ADP-ribosylating toxins [53]. PPIases are protein folding helper enzymes that catalyze the *cis/trans* isomerization of proline-peptide

bonds, which often represents a rate-limiting step during protein refolding even after membrane transport of proteins [54–58].

Pharmacological inhibition of the PPlase activity of Cyps in cultured cells by cyclosporine A (CsA) [59,60] protected cells from intoxication with C2, iota and CDT toxins because the intracellular membrane transport of the enzyme components of these toxins was inhibited [51,52], indicating that Cyps are crucial for this step. Prompted by this observation, we demonstrated earlier that CypA interacts with C2I *in vitro* and in intact cells [51] and inhibition of CypA prevented translocation of C2I from endosomal vesicles to the cytosol *in vitro* [51]. However, since CsA inhibits the PPlase activity of most Cyp isoenzymes, further Cyps besides CypA might also interact with C2I in mammalian cells. Here, we investigated the interaction of C2, iota and CDT toxins with cyclophilin 40 (Cyp40), a large isoform of CypA that is part of a multi-chaperone complex facilitating intracellular membrane transport of the steroid hormone receptor and containing Hsp90 and FKBP51 [61,62], which bind to the enzyme components of these toxins [49–53].

We report that Cyp40 binds to C2I *in vitro* and in intact cells and characterize this interaction by dot blot and isothermal titration calorimetry (ITC) analysis in more detail. Cyp40 also interacts with the enzyme components of iota toxin and CDT, suggesting a common principle for this toxin family. Since Cyps are crucial for uptake of binary actin ADP-ribosylating toxins into the cytosol of mammalian cells, we tested whether a non-immunosuppressive CsA derivative protects cells from intoxication, which might be an

attractive pharmacological option against the members of this toxin family.

Results

Cyp40 co-precipitates with C2I in lysates from C2 toxin-treated cells

HeLa cells were incubated with GST (glutathione S-transferase)-C2I plus C2IIa or left untreated for control. Subsequently, cells were lysed and lysates incubated with glutathione-Sepharose beads to pull-down GST-C2I and associated proteins. Co-precipitated Cyp40 was analyzed by Western blotting with a specific antibody. As shown in Fig. 1A, Cyp40 was detected in precipitates from HeLa cells treated with GST-C2I plus C2IIa prior to lysis but not in the precipitates from untreated cells, indicating that Cyp40 interacts with GST-C2I. Moreover, Hsp90 and FKBP51 were detected in the same experiment as host cell proteins that co-precipitate with C2I from C2-treated cells, as demonstrated earlier [53]. To ensure that the GST-tag had no effect on this interaction, we repeated the experiment with biotin-C2I instead of GST-C2I. As shown in Fig. 1B, a comparable result was obtained, clearly indicating that Cyp40 specifically interacted with C2I in intact cells. The specific binding of the co-precipitated host cell proteins to C2I was confirmed by probing the membranes with an antibody against Rho-GDI α , an abundant cytosolic protein that does not interact with C2I. Rho-GDI α was detected in the HeLa lysate that was used for the co-precipitation but did not

Fig. 2. Interaction of the enzyme components of C2, CDT and iota toxins with Cyp40 *in vitro*. (A) C2I, CDTa and Ia directly interact with Cyp40 *in vitro*. Purified recombinant Cyp40 (starting concentration, 1 μ g; serial dilution) was spotted onto a nitrocellulose membrane via vacuum aspiration using the dot blot system. For negative control, PBS and purified recombinant C3bot, a Rho-modifying ADP-ribosyltransferase of *C. botulinum* (starting concentration, 1 μ g; serial dilution), were spotted. The membrane was blocked and incubated for 1 h with biotin-labeled C2I, CDTa or Ia (200 ng/ml) or for control with PBST alone. After extensive washing, we detected the bound proteins via streptavidin-peroxidase using the ECL system. (B) Enzymatically inactive C2IE387/389Q interacts with Cyp40 *in vitro*. Purified Cyp40 and, for negative control, the proteins FKBP12 and C3bot, as well as PBS, were spotted onto a nitrocellulose membrane (starting concentration, 0.5 μ g; serial dilution). Wild-type C2I and the enzymatically inactive C2IE387/389Q (each 200 ng/ml) were used for overlay. Bound C2I and C2IE387/389Q proteins were detected by an antibody against C2I and peroxidase-coupled secondary antibody using the ECL system. (C) Binding of denatured versus native C2I, CDTa and Ia to Cyp40. For unfolding, the biotin-labeled enzyme components C2I, CDTa and Ia were incubated with 6 M guanidine hydrochloride, 2 mM DTT and 30 mM Tris-HCl for 1 h or with PBS for control. Subsequently, a nitrocellulose membrane with spotted Cyp40 (starting concentration, 1 μ g; serial dilution) and FKBP12, C3bot and PBS for negative control were probed for 1 h with denatured or native C2I, CDTa and Ia proteins (each 200 ng/ml). The bound enzyme components were detected via streptavidin-peroxidase using the ECL system. (D) Interaction of Cyp40 with the binding/translocation component C2IIa and the C2I/C2IIa complex. Purified recombinant proteins were spotted onto a nitrocellulose membrane as described in (a). The membrane was blocked and incubated with either the binding/translocation component C2IIa or the enzyme component C2I alone or with pre-incubated C2I plus C2IIa (as described before [61]). For negative control, PBST was used. After extensive washing, we detected the bound proteins by an antibody against C2I or C2II and a peroxidase-coupled secondary antibody using the ECL system.

co-precipitate with C2I (data not shown). Furthermore, the co-precipitation of Cyp40 with biotin-C2I from cell lysates was analyzed by Western blotting after incubation of cells with biotin-C2I plus C2IIa for different periods of time. As shown in Fig. 1C, the amount of detected co-precipitated Cyp40 increased over time with a peak at 4 h in this experiment, followed by a decrease in the amount of co-precipitated Cyp40. A resembling pattern of interaction was observed when the co-precipitated CypA was detected, for which a specific interaction with C2I has been demonstrated

earlier [51]. Noteworthy, the overall amounts of lysate protein used for the co-precipitation step were comparable between control and toxin-treated samples, as confirmed by Coomassie staining of the lysate proteins after SDS-PAGE (Fig. 1C, lower panel).

C2I, CDTa and Ia directly interact with Cyp40 *in vitro*

From the pull-down experiments, it could not be concluded whether C2I directly interacts with Cyp40

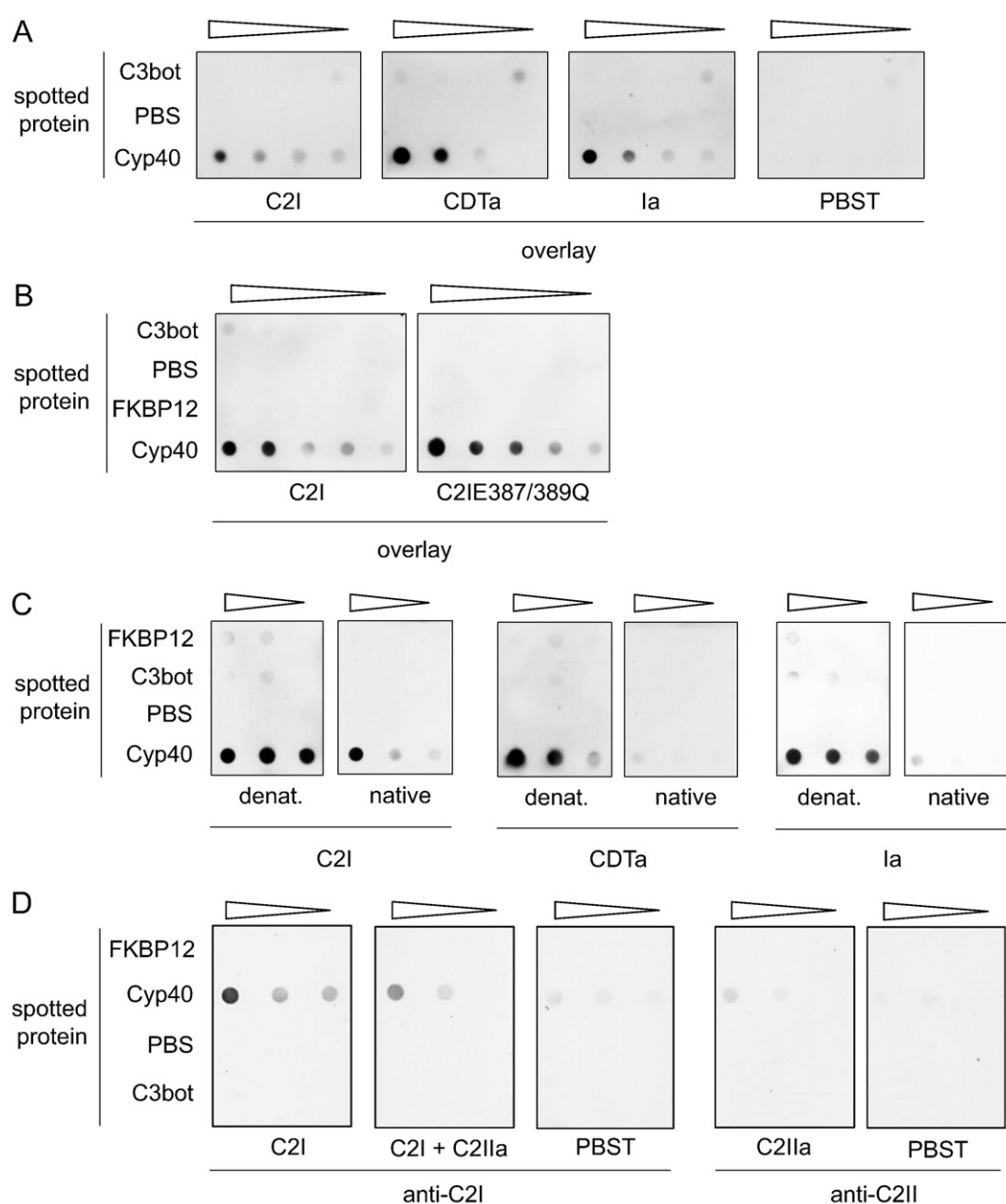


Fig. 2 (legend on previous page)

or whether other proteins might serve as an adaptor for this interaction. Therefore, dot blot analysis was performed to test whether C2I directly binds to Cyp40 *in vitro*. Purified recombinant Cyp40 was spotted onto a nitrocellulose membrane followed by an overlay with biotin-labeled C2I. The result shown in Fig. 2A revealed a direct interaction between Cyp40 and C2I. This interaction was specific since biotin-C2I did not bind to the non-relevant C3bot protein or to the membrane and there was no signal when the overlay buffer was applied instead of biotin-C2I (Fig. 2A). Comparable results were obtained when CDTa and Ia were analyzed instead of C2I, clearly indicating that Cyp40 directly interacts with the enzyme components of C2 toxin and of the iota-like toxins *in vitro*. Prompted by this finding, we next investigated whether this interaction depends on the enzyme activity of the toxins and analyzed the enzymatically inactive C2IE387/389Q [32] in parallel with C2I. The signal of bound protein was comparable for the mutant and the wild-type C2I, indicating that the loss of enzyme activity does not interfere with binding of C2I to Cyp40 (Fig. 2B).

However, pre-treatment of C2I, CDTa and Ia by guanidine hydrochloride dramatically enhanced the binding of these proteins to Cyp40 *in vitro* (Fig. 2C), strongly suggesting that the interaction with Cyp40 preferentially occurs in the unfolded conformation of the enzyme components. The difference in binding affinity of unfolded CDTa and Ia was so strong that detection of both signals, unfolded and native, during the same exposure time was difficult. Respective binding of the native CDTa and Ia was confirmed by longer exposure times (data not shown) and was comparable to binding shown in Fig. 2A. The denaturing effect of guanidine hydrochloride treatment on the enzyme components of the toxins was confirmed by *in vitro* ADP-ribosylation of actin (data not shown). In contrast to C2I, C2IIa did not bind to Cyp40 *in vitro* (Fig. 2D). However, C2I was still able to bind to Cyp40 when pre-incubated with C2IIa under conditions that allow C2I/C2IIa complex formation [63] (Fig. 2D).

Quantitative analysis of the interaction between Cyp40 and C2I or Ia by ITC

ITC was used to determine the thermodynamic parameters of the association reaction between Cyp40 and C2I or Ia, respectively. Measurements were performed at 20 °C and pH 7.8. The ITC measurements of the reaction between Cyp40 and CDTa were hindered by severe aggregation during the process of titrating highly concentrated Cyp40 into CDTa. The titration of 13 µM C2I with 65 µM Cyp40 (Fig. 3A) revealed a binding enthalpy ΔH_{ITC} of -4.0 ± 0.25 kcal/mol, a $T\Delta S_{ITC}$ of 5.4 kcal/mol and a ΔG_{ITC} of -9.4 kcal/mol resulting in a

dissociation constant K_D of 101 nM. Similarly, the titration of 13 µM Ia with 65 µM Cyp40 (Fig. 3B) revealed a binding enthalpy ΔH_{ITC} of -3.9 ± 0.53 kcal/mol, a $T\Delta S_{ITC}$ of 4.1 kcal/mol and a ΔG_{ITC} of 8.0 kcal/mol resulting in a dissociation constant K_D of 1.01 µM.

The negative ΔH and the positive $T\Delta S$ showed that the association of Cyp40 with C2I or Ia was both enthalpically and entropically driven. The enthalpic contribution suggests that the protein complex is stabilized by hydrogen bonds or van der Waals interactions [64]. The positive entropic contribution hints to a burial of solvent-accessible surface area on binding since the release of ordered water molecules to bulk solvent often contributes to a favorable entropic contribution to the interaction [64]. The 10-fold reduced affinity of Ia and Cyp40 mostly results from the less favorable binding entropy compared to C2I and Cyp40.

Interestingly, the binding stoichiometry in the titration experiment was about one Cyp40 per two enzyme component molecules in the case of both C2I ($N = 0.42$) and Ia ($N = 0.36$). As a negative control, Cyp40 at the same concentration was titrated into buffer alone. As shown in Fig. 3C, no heat change was detectable.

Human Cyp40 is inhibited by CsA and the non-immunosuppressive CsA derivative VK112

CsA as the generic CypA inhibitor with a K_i value of 2.9 nM [65] suppresses the PP1ase activity of human Cyp40 with a K_i value of 0.320 ± 0.076 µM. The non-immunosuppressive CsA derivative VK112 retains potency in inhibition of human Cyp40 with a K_i value of 2.34 ± 0.34 µM. The reduction about 7-fold of the affinity of VK112 toward Cyp40 compared to CsA is similar to the 6-fold reduced affinity found for the same inhibitor pair for CypA [66].

The intoxication of Vero cells with C2 toxin, iota toxin and CDT is inhibited by the non-immunosuppressive CsA derivative VK112

In a first experiment, we performed bright-field and super-resolution stimulated emission depletion (STED) microscopy to investigate the change in intracellular distribution of C2I under the treatment of CsA. The data (data not shown) suggest an entrapment of fluorescently labeled C2I in endosomal compartments when treated with bafilomycin A1 (BafA1), an established inhibitor of C2 toxin uptake, or CsA, but not when the cells were treated with C2 toxin in the absence of inhibitors. In line with these findings, cell rounding as a measure of uptake of C2I into the host cell cytosol was significantly elevated in C2 toxin-treated cells as opposed to cells protected by BafA1 or CsA. Prompted by these findings, we

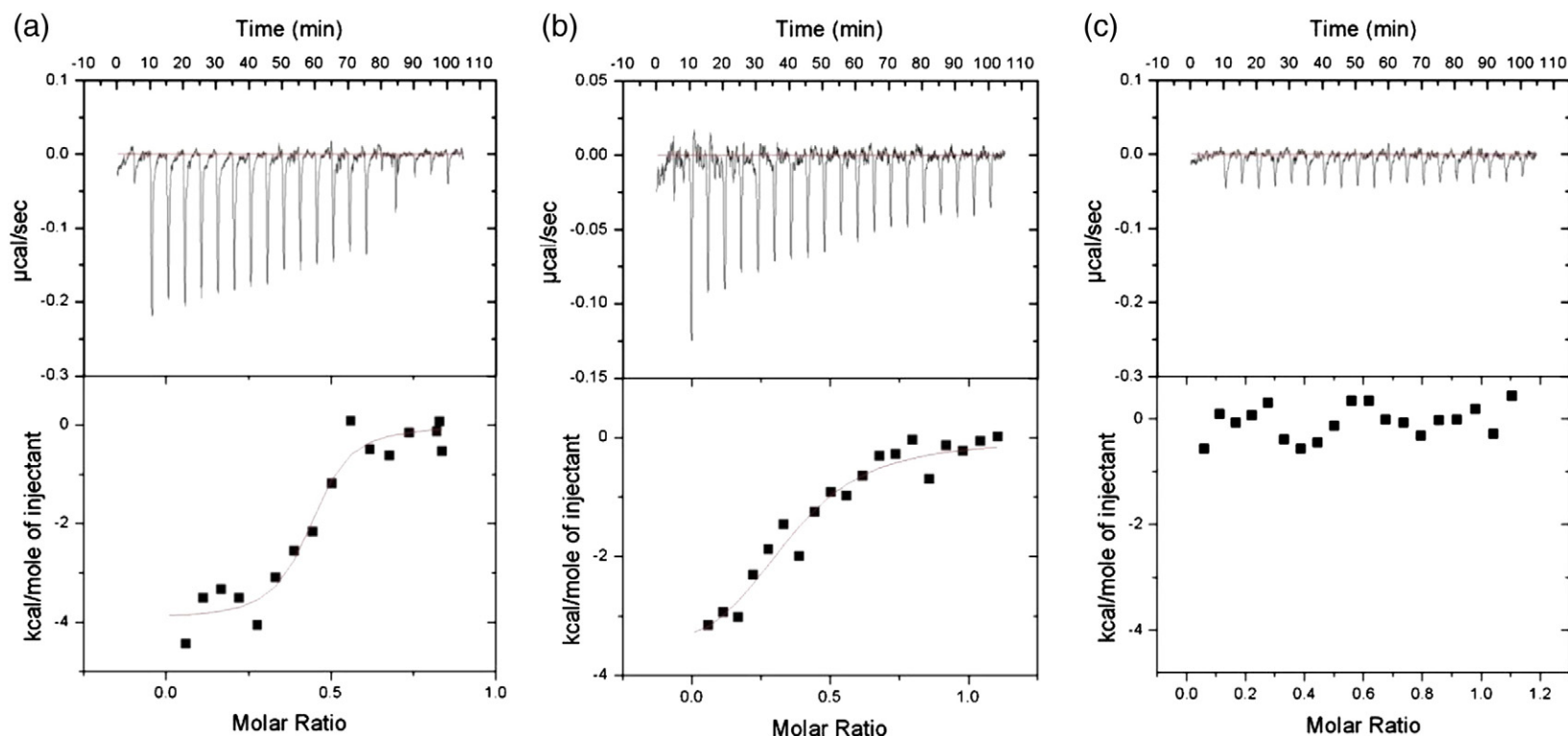


Fig. 3. Isothermal titration of C2I or Ia with Cyp40. Upper panel, raw titration data; lower panel, integrated enthalpies. (A) A solution of 13 μ M C2I (initial concentration) in 35 mM Hepes buffer (pH 7.8) was titrated at 20 $^{\circ}$ C with 65 μ M Cyp40 and fitted to a single-site binding model, resulting in a K_D of 101 nM, with stoichiometry $N = 0.42$, $\Delta H_{ITC} = -4.0 \pm 0.25$ kcal/mol and $T\Delta S_{ITC} = 5.4$ kcal/mol. (B) A solution of 13 μ M Ia (initial concentration) in 35 mM Hepes buffer (pH 7.8) was titrated at 20 $^{\circ}$ C with 65 μ M Cyp40 and fitted to a single-site binding model, resulting in a K_D of 1.01 μ M, with stoichiometry $N = 0.36$, $\Delta H_{ITC} = -3.9 \pm 0.53$ kcal/mol and $T\Delta S_{ITC} = 4.1$ kcal/mol. (C) ITC control experiment was carried out by titrating Cyp40 at the same concentration into buffer only (35 mM Hepes, pH 7.8). No heat change was detectable.

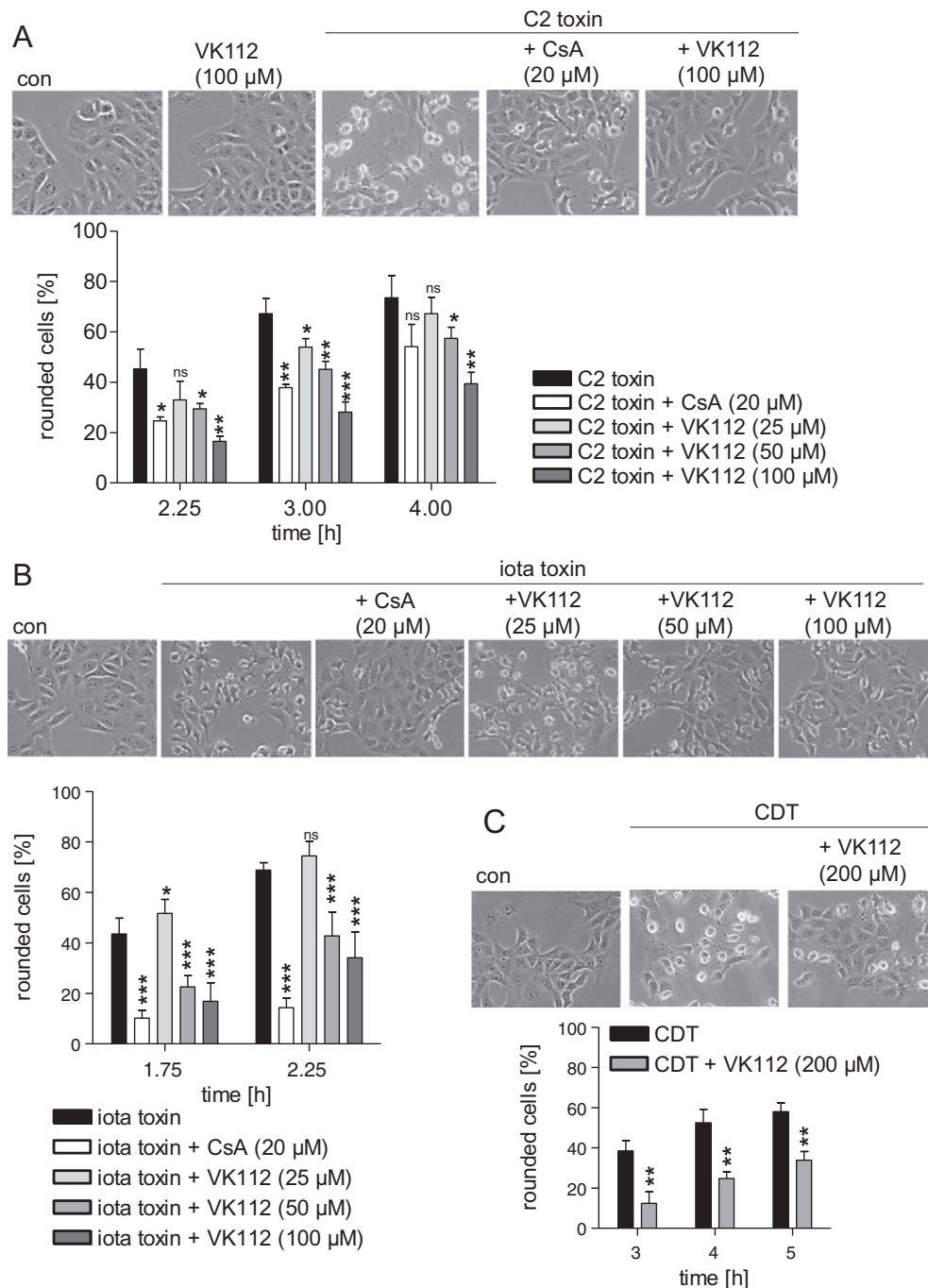


Fig. 4. The non-immunosuppressive CsA derivative VK112 delays intoxication with C2 toxin, iota toxin and CDT. (A) Effect of VK112 on intoxication of cells with C2 toxin. Vero cells were pre-incubated for 30 min at 37 °C with increasing concentrations of VK112 and for control with 20 μ M CsA. For further control, cells were left untreated (con). Subsequently, C2 toxin (75 ng/ml C2I plus 150 ng/ml C2IIa) was added and cells were further incubated at 37 °C. Morphological changes were monitored at the indicated time points. The pictures shown were taken after 2.25 h. For quantitative analysis, percentage of rounded cells was determined from the pictures taken at the indicated time points. Values are given as mean $\pm$ SD ($n = 3$). Significance was tested by using the Student's t test and values refer to samples treated with toxin only (ns = not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). (B) Effect of VK112 on intoxication of cells with iota toxin. Vero cells were treated with iota toxin (40 ng/ml Ia plus 80 ng/ml Ib) in the presence or absence of CsA and VK112 exactly as described for C2 toxin in (A). The pictures shown were taken after 3 h. (C) Effect of VK112 on intoxication of cells with CDT. Vero cells were incubated with CDT (30 ng/ml CDTa plus 30 ng/ml CDTb) in the presence or absence of VK112 exactly as described for C2 toxin in (A). The pictures shown were taken after 5 h of toxin incubation.

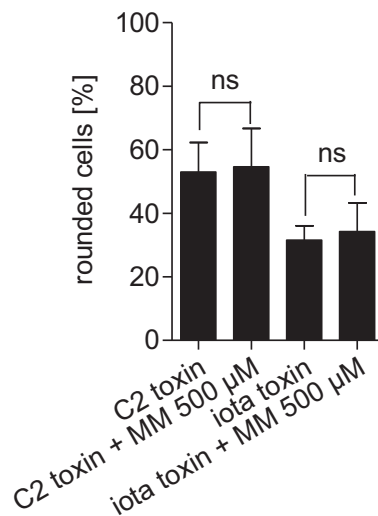


Fig. 5. Effect of the non-cell-permeable CsA derivative MM284 on intoxication of cells with C2 and iota toxin. Vero cells were pre-incubated for 30 min at 37 °C with MM284 or left untreated. Subsequently, C2 toxin (50 ng/ml C2I plus 100 ng/ml C2IIa) or iota toxin (30 ng/ml Ia plus 60 ng/ml Ib) were added and cells were further incubated at 37 °C for 5 h. Morphological changes were monitored. For quantitative analysis, percentage of rounded cells was determined from the pictures taken. Values are given as mean $\pm$ SD ($n = 3$). Significance was tested by using the Student's t test (ns = not significant).

tested whether the non-immunosuppressive CsA derivative VK112 also protects cells from intoxication with C2 toxin and iota toxin. To this end, Vero cells pre-treated with increasing concentrations of VK112 or with CsA for control were challenged with either C2 toxin or iota toxin and cell rounding was analyzed (Fig. 4). Although less efficient compared to CsA, pre-treatment of cells with VK112 significantly delayed the toxin-induced cell rounding in a time-dependent and concentration-dependent manner, indicating that VK112 also interferes with mode of action of the toxins. Moreover, VK112 protected Vero cells from intoxication with CDT as well, even though a 2-fold higher concentration was required to achieve a significant delay of intoxication compared to C2 toxin and iota toxin (Fig. 4C). Treatment of cells with VK112 alone had no effect on cell morphology (Fig. 4A). Noteworthy, pre-treatment of cells with the non-cell-permeable CsA derivative MM284 had no inhibiting effect on intoxication with C2 or iota toxin (Fig. 5). Furthermore, pre-treatment with VK112 inhibited the pH-driven translocation of cell-bound C2 toxin across the cytoplasmic membrane of intact cells (Fig. 6), implicating that VK112 inhibits the translocation of C2I from the lumen of acidic endosomes into the

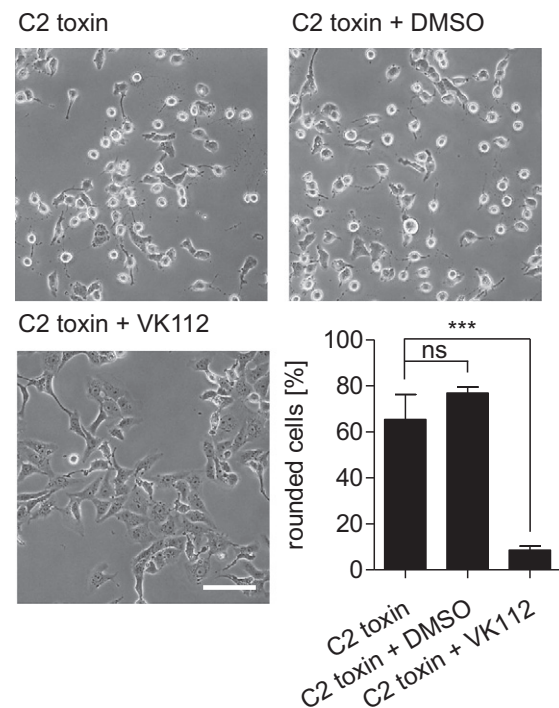


Fig. 6. The non-immunosuppressive CsA derivative VK112 inhibits the pH-dependent translocation of cell-bound C2 toxin across the cytoplasmic membrane. Vero cells were pre-incubated for 30 min at 37 °C with 100 nM BafA1 and 200 μ M VK112. For control, cells were treated with DMSO, the solvent of VK112, or left untreated. Subsequently, cells were cooled to 4 °C and C2 toxin (150 ng/ml C2I plus 300 ng/ml C2IIa) was added. After 15 min at 4 °C, the medium was adjusted to pH 4.6 and the cells were incubated at 37 °C for 10 min. Afterward, the acidic medium was removed and cells were further incubated with FCS- and BafA1-containing medium at 37 °C. Toxin-induced morphological changes were monitored. The pictures shown were taken after 5.5 h (the scale bar represents 100 μ m). For quantitative analysis, percentage of rounded cells was determined from the pictures taken at the indicated time points. Values are given as mean $\pm$ SD ($n = 3$). Significance was tested by using the Student's t test (ns = not significant, *** $p < 0.001$).

host cell cytosol and thus interferes with exactly the same step during toxin uptake into cells as CsA. This is a well-established approach in the field of toxin research to mimic the situation in the lumen of acidified endosomal vesicles directly on the surface of living cells, which was used to characterize the molecular mechanisms underlying membrane translocation of various bacterial protein toxins [67] including C2, iota and CDT toxins *in vitro* [33,43,52]. In summary, prompted by our earlier findings that the PPIase activity of Cyps facilitates membrane translocation of

clostridial binary actin ADP-ribosylating toxins, Cyp40 was identified as a new binding partner for the enzyme components of C2, iota and CDT toxins and the protective effects of a non-immunosuppressive CsA derivative against these toxins were established in a cell model.

Discussion

The results described in this article are an extension of our ongoing research on the role of host cell chaperones/PPLases for membrane translocation of bacterial protein toxins. In summary, we discovered that, besides Hsp90, the PPLase activity of Cyps and FKBP facilitates the membrane translocation of clostridial actin ADP-ribosylating toxins C2, iota and CDT from acidic endosomes into the cytosol, and we demonstrated that pharmacological inhibition of Hsp90, Cyps or FKBP prevents toxin translocation and thereby intoxication of cultured cells with C2 toxin, iota toxin and CDT. In contrast, the membrane translocation of bacterial toxins, which deliver their enzyme moieties also from acidic endosomes into the cytosol but are not ADP-ribosyltransferases, does not require Hsp90 and the PPLases. In conclusion, the results implicate a common Hsp90/Cyp/FKBP-dependent membrane translocation mechanism, which is selective for ADP-ribosylating toxins.

In vitro, purified recombinant Hsp90, CypA and FKBP51 directly bound to the enzyme components of the binary actin ADP-ribosylating toxins, and for the prototype of this family, C2 toxin, the interaction between the enzyme component C2I and these factors was demonstrated in by pull-down experiments. Recently, by using the actin ADP-ribosylating *Photobacterium luminescens* toxin PTC3, we could show for the first time that, besides Hsp90, CypA and FKBP51, Cyp40 also interacts with the isolated ADP-ribosyltransferase domain of this toxin in lysates from intact toxin-treated cells [68]. This was an interesting finding since the PPLase activity of Cyp40 is inhibited by CsA and Cyp40 is part of a multi-chaperone complex that contains Hsp90 and FKBP51 [61,62], both binding partners of the enzyme components of the binary actin ADP-ribosylating toxins, C2I, Ia and CDTa. In cells, this multi-chaperone complex mediates the translocation of the steroid hormone receptor across the nuclear membrane. Moreover, another Hsp90-containing translocation factor complex facilitates the translocation of diphtheria toxin (DT), another ADP-ribosylating bacterial toxin, from acidified endosomal vesicles into the cytosol *in vitro*, as discovered earlier by Ratts *et al.* [69]. We found previously that the membrane translocation of DTA, the enzyme domain of DT, also requires the PPLase activity of Cyps and showed the direct

interaction between DTA and recombinant CypA *in vitro*. However, it is not known so far whether PPLases are part of the cytosolic translocation complex that mediates the membrane transport of the toxin.

In human cells, there are 18 Cyp isoenzymes, most of which strongly bind the immunosuppressive drug CsA that inhibits their PPLase activity below the micromolar concentration range. Furthermore, when complexed to the most abundant host cell PPLase, CypA, CsA potently suppresses the catalytic activity of the protein phosphatase calcineurin by gain of function [70]. Thus, pharmacological dissection of each Cyp isoenzyme by CsA in the toxin translocation pathway is hampered by a lack of inhibitor specificity. In addition, evidence from *in vitro* and *in vivo* studies indicate that several Cyps have been shown in the extracellular space to control distinct signaling functions [71]. Using specifically designed Cyp inhibitors such as the CsA derivatives MM284 and VK122, we could address the role of isoenzymes and Cyp organismic subfractions in toxin translocation.

First, we have investigated whether C2I and the enzyme components of the iota-like toxins interact with Cyp40. We demonstrated by dot blot analysis that C2I, Ia and CDTa directly bind to Cyp40 *in vitro*. In contrast, the binding/translocation component of C2 toxin, C2IIa, did not interact with Cyp40 *in vitro*. We characterized this interaction in more detail for C2 toxin, which represents the prototype of this toxin family. The ADP-ribosyltransferase activity of C2I is not essential for the binding to Cyp40 *in vitro*, which is in line with our earlier result obtained for DTA and CypA [72]. However, treatment of C2I with guanidine hydrochloride decreased its enzyme activity indicating the unfolding of C2I [53] and obviously enhanced the binding of C2I to Cyp40 *in vitro*. Comparable results were obtained for Ia and CDTa. Although the folding state of the toxin components on the dot blot membrane might deviate from the fully unfolded protein in the denaturant solution, partially folded chain segments might dominate the protein population on the membrane. This finding strongly suggests that Cyp40 shows preferred interaction with the enzyme components of the binary actin ADP-ribosylating toxins in their unfolded conformation, which is plausible since they unfold, at least partially, during translocation across endosomal membranes through the transmembrane pores formed by the separate binding/translocation components of these toxins. Although we cannot explain the binding stoichiometry of about one Cyp40 per two molecules of either C2I or Ia so far, the results suggest that this interaction might be common for the interaction between Cyp40 and the enzyme components of the binary actin ADP-ribosylating toxins. However, we were not able to perform this

experiment with CDTa because this protein precipitated when added to Cyp40 in the concentrations, which are necessary for ITC.

The results from the pull-down experiments performed in this and earlier studies [51,53] clearly indicate that C2I specifically interacts with Cyp40, CypA, Hsp90 and FKBP51 in living cells. Here, we could show for the first time that this interaction of C2I with Cyp40 and CypA is increasing over time and drops after a peak. A plausible explanation for this result might be that, after completion of the translocation of C2I from the endosomal vesicles to the cytosol, no interaction with the PPLases occurs, which supports the hypothesis that the PPLases facilitate membrane translocation of C2I. However, from the pull-down results, it cannot be concluded whether the interaction between C2I and Cyp40 in intact cells is direct or whether Cyp40 is part of a protein complex, such as an Hsp90/multi-chaperone complex, which binds to C2I. Therefore, the logical next steps will be the closer investigation of the interaction between the enzyme components of these toxins with Hsp90 and the PPLases during their membrane translocation in living cells and to test whether Cyp40 interacts with the enzyme moieties of other clinically relevant ADP-ribosyltransferases including DT.

Cyp40 encompasses a single Cyp-like catalytic domain complemented by an extended C-terminal stretch incorporating three units of the tetratricopeptide repeat (TPR) and a calmodulin binding site. Cyp40 protein interactions in steroid receptor hetero-complexes are described to be mediated by the TPR domains [73]. Since a partially folded TPR repeat is trapped in a particular crystal form of Cyp40, the conformational flexibility of TPR might easily adapt unfolded conformations in protein-protein recognition [74].

The non-immunosuppressive CsA derivative VK112, which inhibits CyPs without targeting calcineurin, protects Vero cells from the intoxication by C2 toxin concentration dependently. This indicates the importance of the Cyp PPLase site for the intoxication and the minor role of the cyclosporine-insensitive TPR motifs in the toxin pathway. Additionally, VK112-mediated inhibition of cell intoxication by C2 toxin ruled out gain-of-function inhibition of calcineurin as the reason for inhibited intoxication of cells. The fact that VK112 is slightly less effective than CsA in reducing intoxication at similar concentrations might result from the lower affinity to CyPs compared to the parent compound CsA [66]. Indeed, VK112 exhibited a sevenfold decrease in inhibitory potency compared to CsA in an *in vitro* Cyp40 activity assay. To exclude participation of extracellular CyPs, which are known to contribute to cell-cell communication, in the membrane translocation process of toxins, we made use of the cell-impermeable CsA derivative

MM284. Notably, this compound strongly inhibits the extracellular fractions of CypA and CypB [75], as well as Cyp40. Its inability to inhibit intoxication of cells with C2 toxin, the prototype of the binary actin ADP-ribosylating toxins, and iota toxin, the prototype of the subgroup of iota-like toxins, indicates that the extracellular pathway is not involved in the Cyp-mediated membrane translocation of the members of this toxin family.

The finding that CyPs are crucial for the uptake of the medically relevant group of bacterial ADP-ribosylating exotoxins including CDT [76], which is associated with severe forms of pseudomembranous colitis associated with *C. difficile* [77–79], should also be of clinical interest. CyPs might serve as novel drug targets to prevent the uptake of the toxins into their human and/or animal target cells, which protects cells from intoxication. Since the toxin-induced cell damage represents the reason for the clinical symptoms of the toxin-associated diseases caused by the pathogenic bacteria, pharmacological inhibitors of PPLases such as CsA could be considered for treatment and/or prevention of such diseases. This might be, in particular, attractive for diseases caused by toxin-producing bacteria that became multi-resistant against our common antibiotics. However, CsA is an immunosuppressive drug and might not be indicated during bacterial infections, but non-immunosuppressive CsA derivatives such as VK112 used in the cell-based experiments of this study might be an alternative and should be tested in animal models.

Materials and Methods

Materials

MEM (cell culture medium) and fetal calf serum (FCS) were acquired from Invitrogen (Karlsruhe, Germany). Materials for cell culture were purchased from TPP (Trasadingen, Switzerland). Complete® protease inhibitor and streptavidin-peroxidase were obtained from Roche (Mannheim, Germany). Protein molecular weight marker Page Ruler prestained protein ladder was acquired from Thermo Scientific (Bonn, Germany). Biotin-labeled NAD⁺ was purchased from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany). BafA1 was obtained from Calbiochem (Bad Soden, Germany) and CsA was obtained from Fluka (Munich, Germany). Enhanced chemiluminescence (ECL) system was acquired from Millipore (Schwalbach, Germany). The streptavidin-Sepharose beads were from Pierce (Bonn, Germany).

Protein expression and purification

Human FKBP12 was expressed using pET28a (Novagen, Darmstadt, Germany) in *Escherichia coli* Rosetta cells and purified according to published

procedures [80]. To obtain the expression plasmid of human (His)₆-Cyp40, we PCR-amplified the gene of the protein using gene-specific primers from an ORF encoding human PPID (imaGenes, Berlin) and cloned it into pET28a. Purification of the His-tag fusion protein was performed using affinity chromatography on Ni-NTA resin followed by size-exclusion chromatography in 10 mM Hepes (pH 7.8), 150 mM KCl and 1.5 mM MgCl₂. The purified protein was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry confirming its correct molecular weight.

The recombinant C2I, C2IE387/389Q and C2IIa proteins were purified and activated as described before [32,33]. Recombinant C3bot was purified as described earlier [81]. Ia and Ib were prepared as described earlier [82]. Recombinant CDTa and CDTb (from *C. difficile* strain 196) were produced and purified as a His-tagged proteins in the *Bacillus megaterium* expression system as described earlier [83].

For biotin labeling, we incubated C2I, Ia and CDTa with EZ-Link sulfo-NHS-Biotin (Pierce) for 2 h at 4 °C. For removal of remaining free sulfo-NHS-Biotin, Micro Bio-Spin™ Chromatography Columns from Bio-Rad (Munich, Germany) were employed. For fluorescence labeling, we incubated C2I with a 3-fold molar excess of Atto-590-NH-S-Ester (Atto-Tec, Siegen, Germany) for 30 min at room temperature. For removal of remaining free dye illustra, MicroSpin G-25 Columns from GE Healthcare (Munich, Germany) were used according to manufacturer's instructions. For both labeling methods, it was confirmed that the biological activity of the labeled enzyme component was comparable to unlabeled enzyme component by cytotoxicity assays (see below).

Synthesis of the CsA derivatives MM284 and VK112

Synthesis of the CsA derivatives MM284 [75] and VK112 [66] was performed as previously described.

PPIase inhibition experiments

PPIase activity assays were performed using the protease-coupled assay as described by Fischer *et al.* [84]. Measurements were carried out in 35 mM Hepes buffer (pH 7.8) at 10 °C using a Hewlett Packard 8452 diode array UV/Vis spectrophotometer (Böblingen, Germany). For isomer-specific proteolytic cleavage, chymotrypsin was used at final concentrations of 0.83 mg/ml (400 U/mg; Merck, Darmstadt, Germany). The stock solution of the substrate Suc-Ala-Ala-Pro-Phe-pNA was prepared in DMSO to a concentration of 10 mg/ml. The assay was started by adding 3 µl of the peptide to the reaction mixture. The concentration of Cyp40 was 50 nM. Measurements were performed in the presence of increasing inhibitor concentrations up to 10 µM in triplicate.

Cell culture and cytotoxicity assays

Vero (African green monkey kidney) and HeLa cells were cultivated in MEM containing 10% heat-inactivated FCS, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate,

2 mM L-glutamine and 0.1 mM non-essential amino acids at 37 °C and 5% CO₂. Cells were detached using trypsin and reseeded no more than 20 times. For cytotoxicity assays, cells were seeded in culture dishes and incubated in MEM plus FCS with the respective toxin. To inhibit the PPIase activity of Cyps, we pre-incubated the cells with indicated concentrations of CsA or the non-immunosuppressive CsA derivative VK112 for 30 min and these inhibitors were present in the medium during the further incubation of the cells with the toxin. Cells were visualized after the indicated incubation times by using a Zeiss Axiovert 40CFI microscope (Oberkochen, Germany) with a Jenoptik progress C10 CCD camera (Jena, Germany). The toxin-induced changes in cell morphology (i.e., cell rounding) were analyzed and percentage of round cells was determined from the images as described earlier [51].

Toxin translocation assay with intact cells

The pH-dependent translocation of C2I through C2IIa pores across the endosomal membrane was experimentally mimicked at the cytoplasmic membrane of intact Vero cells as described earlier [33]. In brief, Vero cells were pre-incubated with BafA1 and cooled to 4 °C, and C2 toxin was added. After a binding period of 15 min, an acidic pulse was given (pH 4.6). Under the acidic conditions, C2IIa forms pores into the cytoplasmic membrane and C2I translocates directly into the cytosol. Cell rounding was monitored and visualized by photography.

Imaging

Bright-field images were recorded using a 10× objective as described for cytotoxicity assays. Fluorescence images were acquired with a custom-built STED microscope described elsewhere [85]. In short, the microscope can operate in confocal and STED mode. While the confocal mode will yield standard diffraction-limited images, the STED mode greatly improves lateral resolution to ~35 nm. The system uses a super-continuum laser source to provide all spectral bands necessary for excitation and depletion (SC-450-PP-HE; Fianium Ltd., UK). The sample is scanned by a piezo stage (733.2DD; Physik Instrumente, Karlsruhe, Germany) and fluorescence is recorded by an avalanche photodiode. Instrument control and acquisition software was custom written in LabVIEW (National Instruments, USA).

Interaction analysis between C2I and immobilized Cyp40 using the dot blot system

Serial dilutions of the purified proteins Cyp40, FKBP12 and C3bot were vacuum aspirated onto a nitrocellulose membrane using the Bio-Rad dot blot system according to the manufacturer's manual. A Ponceau S staining was performed and then the membrane was blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST). Subsequently, the membrane was cut and probed with the respective overlay proteins (200 ng/ml) for 1 h. After extensive washing, we detected the bound proteins by streptavidin-peroxidase

(biotin-labeled overlay proteins) or a specific antibody against C2I or C2II and a peroxidase-coupled anti-rabbit antibody from Santa Cruz (Heidelberg, Germany) by using the ECL system. To investigate the binding of unfolded C2I, CDTa and Ia to immobilized Cyp40, we incubated the biotin-labeled enzyme components with 6 M guanidine hydrochloride, 2 mM DTT, 30 mM Tris-HCl or PBS for control for 1 h before they were tested in the dot blot assay as described before. To confirm that this procedure results in denatured C2I, CDTa and Ia, we tested *in vitro* ADP-ribosylation of actin as described below.

Isothermal titration calorimetry

ITC experiments were performed using a VP-ITC (MicroCal, Munich, Germany). Prior to the experiment, all buffers were filtered through filter membranes with a pore size of 0.2 μ m (Whatman, Dassel, Germany) and degassed. Protein solutions were dialyzed against the assay buffer (35 mM Hepes, pH 7.8). In a typical experiment, 300 μ l of a 65- μ M solution of Cyp40 was titrated in 15- μ l steps to a 13- μ M enzyme component solution (C2I or Ia) at 20 °C. The instruments' stirring speed was set to 310 RPM and the feedback gain mode was set to "high". Since the signal from the first injection can usually not be used for data analysis, only 2 μ l was titrated in this step and the data point was omitted. Measured data were analyzed using the "Origin" software (MicroCal).

Pull-down experiments with GST-C2I and biotin-C2I

The pull-down experiments were performed as described earlier [51,53]. In brief, HeLa cells were grown in 10-cm dishes and incubated in serum-free MEM at 37 °C for 2 h with biotin-C2I plus C2IIa or for control without toxin. Subsequently, cells were lysed using an 18G needle syringe, and the cytoplasmic fraction was obtained by centrifugation and subsequently incubated with a 60- μ l-volume bed (1:1 in PBS) of streptavidin-Sepharose beads overnight at 4 °C. As an alternative approach, GST-C2I was applied instead of biotin-C2I and glutathione-Sepharose beads were used in place of streptavidin-Sepharose beads for pull-down of GST-C2I. The precipitated proteins were washed and subjected to SDS-PAGE according to the methods of Laemmli [86], and the proteins were transferred onto a nitrocellulose membrane that was probed with a specific antibody against Cyp40 from Thermo Fisher (Bonn, Germany), CypA from Ambion (Kaufungen, Germany) or Rho-GDIa from Santa Cruz Biotechnology (Heidelberg, Germany). After incubation with a secondary peroxidase-coupled anti-rabbit antibody (Santa Cruz Biotechnology), the co-precipitated proteins were visualized using the ECL system.

Reproducibility of the experiments and statistics

All experiments were performed independently at least two times. Results from representative experiments are shown in the figures. In each individual Western blot panel shown in the figures, the protein bands were originally detected on the same membrane and cut out and

recombined for presentation only in the figures. Values ($n \geq 3$) are calculated as mean $\pm$ standard deviation (SD) using the Prism4 Software (GraphPad Software).

Acknowledgements

This work was financially supported by the Deutsche Forschungsgemeinschaft (grant BA 2087/2-2 to H.B.). K.E. is a fellow of the International Graduate School in Molecular Medicine Ulm. We thank Ulrike Binder, Barbara Korge and Suzanne Ross for excellent technical assistance.

Received 30 April 2014;

Received in revised form 3 July 2014;

Accepted 13 July 2014

Available online 21 July 2014

Keywords:

bacterial protein toxin;
cellular uptake;
membrane transport;
cyclophilin 40;
PPLase

Abbreviations used:

PPLase, peptidyl-prolyl cis/trans isomerase; Hsp90, heat shock protein 90; CypA, cyclophilin A; Cyp40, cyclophilin 40; FKBP51, FK506 binding protein 51; CsA, cyclosporine A; ITC, isothermal titration calorimetry; STED, stimulated emission depletion; BafA1, bafilomycin A1; DT, diphtheria toxin; TPR, tetratricopeptide repeat; ECL, enhanced chemiluminescence; FCS, fetal calf serum; PBS, phosphate-buffered saline.

References

- [1] Barth H, Aktories K, Popoff MR, Stiles BG. Binary bacterial toxins: biochemistry, biology, and applications of common *Clostridium* and *Bacillus* proteins. *Microbiol Mol Biol Rev* 2004;8:373–402.
- [2] Aktories K, Bärnmann M, Ohishi I, Tsuyama S, Jakobs KH, Habermann E. *Botulinum* C2 toxin ADP-ribosylates actin. *Nature* 1986;322:390–2.
- [3] Ohishi I, Tsuyama S. ADP-ribosylation of nonmuscle actin with component I of C2 toxin. *Biochem Biophys Res Commun* 1986;136:802–6.
- [4] Vandekerckhove J, Schering B, Bärnmann M, Aktories K. *Clostridium perfringens* iota toxin ADP-ribosylates skeletal muscle actin in Arg-177. *FEBS Lett* 1987; 225:48–52.
- [5] Vandekerckhove J, Schering B, Bärnmann M, Aktories K. *Botulinum* C2 toxin ADP-ribosylates cytoplasmic beta/gamma-actin in arginine 177. *J Biol Chem* 1988;263: 696–700.
- [6] Aktories K, Wegner A. ADP-ribosylation of actin by clostridial toxins. *J Cell Biol* 1989;109:1385–7.

- [7] Wegner A, Aktories K. ADP-ribosylated actin caps the barbed ends of actin filaments. *J Biol Chem* 1988;263:13739–42.
- [8] Weigt C, Just I, Wegner A, Aktories K. Nonmuscle actin ADP-ribosylated by *botulinum* C2 toxin caps actin filaments. *FEBS Lett* 1989;246:181–4.
- [9] Wieggers W, Just I, Müller H, Hellwig A, Traub P, Aktories K. Alteration of the cytoskeleton of mammalian cells cultured *in vitro* by *Clostridium botulinum* C2 toxin and C3 ADP-ribosyltransferase. *Eur J Cell Biol* 1991;54:237–45.
- [10] Miyake M, Ohishi I. Response of tissue-cultured cynomolgus monkey kidney cells to *botulinum* C2 toxin. *Microb Pathog* 1987;3:279–86.
- [11] Ohishi I, Iwasaki M, Sakaguchi G. Purification and characterization of two components of *botulinum* C2 toxin. *Infect Immun* 1980;30:668–73.
- [12] Heine K, Pust S, Enzenmüller S, Barth H. ADP-ribosylation of actin by the *Clostridium botulinum* C2 toxin in mammalian cells results in delayed caspase-dependent apoptotic cell death. *Infect Immun* 2008;76:4600–8.
- [13] Hilger H, Pust S, von Figura G, Kaiser E, Stiles BG, Popoff MR, et al. The long-lived nature of *Clostridium perfringens* iota toxin in mammalian cells induces delayed apoptosis. *Infect Immun* 2009;77:5593–601.
- [14] Ohishi I. Response of mouse intestinal loop to *botulinum* C2 toxin: enterotoxic activity induced by cooperation of non-linked protein components. *Infect Immun* 1983;40:691–5.
- [15] Ohishi I, Odagiri Y. Histopathological effect of *botulinum* C2 toxin on mouse intestines. *Infect Immun* 1984;43:54–8.
- [16] Songer JG. Clostridial enteric diseases of domestic animals. *Clin Microbiol Rev* 1996;9:216–34.
- [17] Geric B, Rupnik M, Gerding DN, Grabnar M, Johnson S. Distribution of *Clostridium difficile* variant toxinotypes and strains with binary toxin genes among clinical isolates in an American hospital. *J Med Microbiol* 2004;53:887–94.
- [18] McDonald LC, Killgore GE, Thompson A, Owens RC, Kazakova SV, Sambol SP, et al. An epidemic, toxin gene-variant strain of *Clostridium difficile*. *N Engl J Med* 2005;353:2433–41.
- [19] Stiles BG, Wilkins TD. Purification and characterization of *Clostridium perfringens* iota toxin: dependence on two nonlinked proteins for biological activity. *Infect Immun* 1986;54:683–8.
- [20] Stiles BG, Wilkins TD. *Clostridium perfringens* iota toxin: synergism between two proteins. *Toxicon* 1986;24:767–73.
- [21] Perelle S, Gibert M, Bourlioux P, Corthier G, Popoff MR. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. *Infect Immun* 1997;65:1402–7.
- [22] Popoff MR, Rubin EJ, Gill DM, Boquet P. Actin-specific ADP-ribosyltransferase produced by a *Clostridium difficile* strain. *Infect Immun* 1988;56:2299–306.
- [23] Stubbs S, Rupnik M, Gibert M, Brazier J, Duerden B, Popoff M. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol Lett* 2000;186:307–12.
- [24] Gülke I, Pfeifer G, Liese J, Fritz M, Hofmann F, Aktories K, et al. Characterization of the ADP-ribosyltransferase CDTa from *Clostridium difficile* by site directed mutagenesis. *Infect Immun* 2001;69:6004–11.
- [25] Popoff MR, Boquet P. *Clostridium spiroforme* toxin is a binary toxin which ADP-ribosylates cellular actin. *Biochem Biophys Res Commun* 1988;152:1361–8.
- [26] Perelle S, Scalzo S, Kochi S, Mock M, Popoff MR. Immunological and functional comparison between *Clostridium perfringens* iota toxin, *C. spiroforme* toxin, and anthrax toxins. *FEMS Microbiol Lett* 1997;146:117–21.
- [27] Schering B, Bärman M, Chhatwal GS, Geipel U, Aktories K. ADP-ribosylation of skeletal muscle and non-muscle actin by *Clostridium perfringens* iota toxin. *Eur J Biochem* 1988;171:225–9.
- [28] Papatheodorou P, Carette JE, Bell GW, Schwan C, Guttenberg G, Brummelkamp TR, et al. Lipolysis-stimulated lipoprotein receptor (LSR) is the host receptor for the binary toxin *Clostridium difficile* transferase (CDT). *Proc Natl Acad Sci USA* 2011;108:16422–7.
- [29] Papatheodorou P, Wilczek C, Nölke T, Guttenberg G, Hornuss D, Schwan C, et al. Identification of the cellular receptor of *Clostridium spiroforme* toxin. *Infect Immun* 2012;80:1418–23.
- [30] Eckhardt M, Barth H, Blöcker D, Aktories K. Binding of *Clostridium botulinum* C2 toxin to asparagine-linked complex and hybrid carbohydrates. *J Biol Chem* 2000;275:2328–34.
- [31] Fujii N, Kubota T, Shirakawa S, Kimura K, Ohishi I, Moriishi K, et al. Characterization of component-I gene of *botulinum* C2 toxin and PCR detection of its gene in clostridial species. *Biochem Biophys Res Commun* 1996;220:353–9.
- [32] Barth H, Preiss JC, Hofmann F, Aktories K. Characterization of the catalytic site of the ADP-ribosyltransferase *Clostridium botulinum* C2 toxin by site-directed mutagenesis. *J Biol Chem* 1998;273:29506–11.
- [33] Barth H, Blöcker D, Behlke J, Bergsma-Schutter W, Brisson A, Benz R, et al. Cellular uptake of *Clostridium botulinum* C2 toxin requires oligomerization and acidification. *J Biol Chem* 2000;275:18704–11.
- [34] Sterthoff C, Lang AE, Schwan C, Tauch A, Aktories K. Functional characterization of an extended binding component of the actin-ADP-ribosylating C2 toxin detected in *Clostridium botulinum* strain (C) 2300. *Infect Immun* 2010;78:1468–74.
- [35] Blöcker D, Barth H, Maier E, Benz R, Barbieri JT, Aktories K. The C terminus of component C2II of *Clostridium botulinum* C2 toxin is essential for receptor binding. *Infect Immun* 2000;68:4566–73.
- [36] Stiles BG, Blöcker D, Hale ML, Guethoff MA, Barth H. *Clostridium botulinum* C2 toxin: binding studies with fluorescence-activated cytometry. *Toxicon* 2002;40:1135–40.
- [37] Schleberger C, Hochmann H, Barth H, Aktories K, Schulz GE. Structure and action of the binary C2 toxin from *Clostridium botulinum*. *J Mol Biol* 2006;364:705–15.
- [38] Schmid A, Benz R, Just I, Aktories K. Interaction of *Clostridium botulinum* C2 toxin with lipid bilayer membranes. Formation of cation-selective channels and inhibition of channel function by chloroquine. *J Biol Chem* 1994;269:16706–11.
- [39] Bachmeyer C, Benz R, Barth H, Aktories K, Gilbert M, Popoff MR. Interaction of C2 toxin with lipid bilayer membranes and Vero cells: inhibition of channel function by chloroquine and related compounds *in vitro* and intoxication *in vivo*. *FASEB J* 2001;15:1658–60.
- [40] Blöcker D, Pohlmann K, Haug G, Bachmeyer C, Benz R, Aktories K, et al. *Clostridium botulinum* C2 toxin: low pH-induced pore formation is required for translocation of the enzyme component C2I into the cytosol of host cells. *J Biol Chem* 2003;278:37360–7.
- [41] Blöcker D, Bachmeyer C, Benz R, Aktories K, Barth H. Channel formation by the binding component of *Clostridium botulinum* C2 toxin: glutamate 307 of C2II affects channel

- properties *in vitro* and pH-dependent C2I translocation *in vivo*. *Biochemistry* 2003;42:5368–77.
- [42] Haug G, Wilde C, Leemhuis J, Meyer DK, Aktories K, Barth H. Cellular uptake of *Clostridium botulinum* C2 toxin: membrane translocation of a fusion toxin requires unfolding of its dihydrofolate reductase domain. *Biochemistry* 2003;42:15284–91.
- [43] Blöcker D, Behlke J, Aktories K, Barth H. Cellular uptake of the binary *Clostridium perfringens* iota-toxin. *Infect Immun* 2001;69:2980–7.
- [44] Stiles BG, Hale ML, Marvaud JC, Popoff MR. *Clostridium perfringens* iota toxin: characterization of the cell-associated iota b complex. *Biochem J* 2002;367:801–8.
- [45] Knapp O, Benz R, Gibert M, Marvaud JC, Popoff MR. Interaction of the binding component of *Clostridium perfringens* iota-toxin with lipid bilayer membranes: demonstration of channel formation by the activated binding component Ib and channel block by the enzyme component Ia. *J Biol Chem* 2002;277:6143–52.
- [46] Nagahama M, Nagayasu K, Kobayashi K, Sakurai J. Binding component of *Clostridium perfringens* iota-toxin induces endocytosis in Vero cells. *Infect Immun* 2002;70:1909–14.
- [47] Hale ML, Marvaud JC, Popoff MR, Stiles BG. Detergent-resistant membrane microdomains facilitate Ib oligomer formation and biological activity of *Clostridium perfringens* iota-toxin. *Infect Immun* 2004;72:2186–93.
- [48] Gibert M, Marvaud JC, Pereira Y, Hale ML, Stiles BG, Boquet P, et al. Differential requirement for the translocation of clostridial binary toxins: iota toxin requires a membrane potential gradient. *FEBS Lett* 2007;581:1287–96.
- [49] Haug G, Leemhuis J, Tiemann D, Meyer DK, Aktories K, Barth H. The host cell chaperone Hsp90 is essential for translocation of the binary *Clostridium botulinum* C2 toxin into the cytosol. *J Biol Chem* 2003;278:32266–74.
- [50] Haug G, Aktories K, Barth H. The host cell chaperone Hsp90 is necessary for cytotoxic action of the binary iota-like toxins. *Infect Immun* 2004;72:3066–8.
- [51] Kaiser E, Pust S, Kroll C, Barth H. Cyclophilin A facilitates translocation of the *Clostridium botulinum* C2 toxin across membranes of acidified endosomes into the cytosol of mammalian cells. *Cell Microbiol* 2009;11:780–95.
- [52] Kaiser E, Kroll C, Ernst K, Schwan C, Popoff MR, Fischer G, et al. Membrane translocation of binary actin-ADP-ribosylating toxins from *Clostridium difficile* and *Clostridium perfringens* is facilitated by cyclophilin A and Hsp90. *Infect Immun* 2011;79:3913–21.
- [53] Kaiser E, Böhm N, Ernst K, Langer S, Schwan C, Aktories K, et al. FK506-binding protein 51 interacts with *Clostridium botulinum* C2 toxin and FK506 blocks membrane translocation of the toxin in mammalian cells. *Cell Microbiol* 2012;14:1193–205.
- [54] Bang H, Fischer G. Slow conformational changes in protein folding can be accelerated by enzymes. *Biomed Biochim Acta* 1991;50:137–42.
- [55] Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T, Schmid FX. Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. *Nature* 1989;337:476–8.
- [56] Schmid FX. Prolyl isomerase: enzymatic catalysis of slow protein-folding reactions. *Annu Rev Biophys Biomol Struct* 1993;22:123–42.
- [57] Schmid FX, Mayr LM, Mucke M, Schonbrunner ER. Prolyl isomerases: role in protein folding. *Adv Protein Chem* 1993;44:25–66.
- [58] Galat A. Peptidylprolyl *cis/trans* isomerases (immunophilins): biological diversity—targets—functions. *Curr Top Med Chem* 2003;3:1315–47.
- [59] Handschumacher RE, Harding MW, Rice J, Drugge RJ, Speicher DW. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* 1984;226:544–7.
- [60] Fanghänel J, Fischer G. Thermodynamic characterization of the interaction of human cyclophilin 18 with cyclosporin A. *Biophys Chem* 2003;100:351–66.
- [61] Pratt WB, Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev* 1997;18:306–60.
- [62] Pirkil F, Buchner J. Functional analysis of the Hsp90-associated human peptidyl prolyl *cis/trans* isomerases FKBP51, FKBP52 and Cyp40. *J Mol Biol* 2001;308:795–806.
- [63] Kaiser E, Haug G, Hliscs M, Aktories K, Barth H. Formation of a biologically active toxin complex of the binary *Clostridium botulinum* C2 toxin without cell membrane interaction. *Biochemistry* 2006;45:13361–8.
- [64] Klebe G, Böhm HJ. Energetic and entropic factors determining binding affinity in protein–ligand complexes. *J Recept Signal Transduct Res* 1997;17:459–73.
- [65] Daum S, Schumann M, Mathea S, Aumüller T, Balsley MA, Constant SL, et al. Isoform-specific inhibition of cyclophilins. *Biochemistry* 2009;48:6268–77.
- [66] Prell E, Kahlert V, Rücknagel KP, Malešević M, Fischer G. Fine tuning the inhibition profile of cyclosporine A by derivatization of the MeBmt residue. *ChemBioChem* 2013;14:63–5.
- [67] Sandvig K, Olsnes S. Diphtheria toxin entry into cells is facilitated by low pH. *J Cell Biol* 1980;87:828–32.
- [68] Lang AE, Ernst K, Lee H, Papatheodorou P, Schwan C, Barth H, et al. The chaperone Hsp90 and PPIases of the cyclophilin and FKBP families facilitate membrane translocation of *Photobacterium luminescens* ADP-ribosyltransferases. *Cell Microbiol* 2013;16:490–503.
- [69] Ratts R, Zeng H, Berg EA, Blue C, McComb ME, Costello CE, et al. The cytosolic entry of diphtheria toxin catalytic domain requires a host cell cytosolic translocation factor complex. *J Cell Biol* 2003;160:1139–50.
- [70] Liu J, Farmer JD, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 1991;66:807–15.
- [71] Hoffmann H, Schiene-Fischer C. Functional aspects of extracellular cyclophilins. *Biol Chem* 2014. <http://dx.doi.org/10.1515/hsz-2014-0125>.
- [72] Dmochewitz L, Lillich M, Kaiser E, Jennings LD, Lang AE, Buchner J, et al. Role of CypA and Hsp90 in membrane translocation mediated by anthrax protective antigen. *Cell Microbiol* 2011;13:359–73.
- [73] Ratajczak T, Carrello A. Cyclophilin 40 (CyP-40), mapping of its hsp90 binding domain and evidence that FKBP52 competes with CyP-40 for hsp90 binding. *J Biol Chem* 1996;271:2961–5.
- [74] Taylor P, Dornan J, Carrello A, Minchin RF, Ratajczak T, Walkinshaw MD. Two structures of cyclophilin 40: folding and fidelity in the TPR domains. *Structure* 2001;9:431–8.
- [75] Malešević M, Gutknecht D, Prell E, Klein C, Schumann M, Nowak RA, et al. Anti-inflammatory effects of extracellular cyclosporins are exclusively mediated by CD147. *J Med Chem* 2013;56:7302–11.

- [76] Schwan C, Stecher B, Tzivelekidis T, van Ham M, Rohde M, Hardt WD, et al. *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. *PLoS Pathog* 2009;5:e1000626.
- [77] Heinlen L, Ballard JD. *Clostridium difficile* infection. *Am J Med Sci* 2010;340:247–52.
- [78] Goncalves C, Decre D, Barbut F, Burghoffer B, Petit JC. Prevalence and characterization of a binary toxin (actin-specific ADP-ribosyltransferase) from *Clostridium difficile*. *J Clin Microbiol* 2004;42:1933–9.
- [79] Martin H, Willey B, Low DE, Staempfli HR, McGeer A, Boerlin P, et al. Characterization of *Clostridium difficile* strains isolated from patients in Ontario, Canada, from 2004 to 2006. *J Clin Microbiol* 2008;46:2999–3004.
- [80] Edlich F, Weiwad M, Wildemann D, Jarczowski F, Kilka S, Moutty MC, et al. The specific FKBP38 inhibitor *N*-(*N*, *N*-dimethylcarboxamidomethyl)cycloheximide has potent neuroprotective and neurotrophic properties in brain ischemia. *J Biol Chem* 2006;281:14961–70.
- [81] Fahrer J, Kuban J, Heine K, Rupps G, Kaiser E, Felder E, et al. Selective and specific internalization of clostridial C3 ADP-ribosyltransferases into macrophages and monocytes. *Cell Microbiol* 2010;12:233–47.
- [82] Perelle S, Domenighini M, Popoff MR. Evidence that Arg-295, Glu-378, and Glu-380 are active-site residues of the ADP-ribosyltransferase activity of iota toxin. *FEBS Lett* 1996;395:191–4.
- [83] Papatheodorou P, Zamboglou C, Genisyurek S, Guttenberg G, Aktories K. Clostridial glucosylating toxins enter cells via clathrin-mediated endocytosis. *PLoS One* 2010;5:e10673.
- [84] Fischer G, Bang H, Mech C. Determination of enzymatic catalysis for the *cis-trans*-isomerization of peptide bonds in proline-containing peptides. *Biomed Biochim Acta* 1984;43:1101–11.
- [85] Osseforth C, Moffitt JR, Schermelleh L, Michaelis J. Simultaneous dual-color 3D STED microscopy. *Opt Express* 2014;22:7028–39.
- [86] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.