Cellular Microbiology (2012) **14**(8), 1193–1205
doi:10.1111/j.1462-5822.2012.01788.x
First published online 12 April 2012

# FK506-binding protein 51 interacts with *Clostridium botulinum* C2 toxin and FK506 inhibits membrane translocation of the toxin in mammalian cells

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## Summary

**The binary *Clostridium botulinum* C2 toxin consists of the binding/translocation component C2IIa and the separate enzyme component C2I. C2IIa delivers C2I into the cytosol of eukaryotic target cells where C2I ADP-ribosylates actin. After receptor-mediated endocytosis of the C2IIa/C2I complex, C2IIa forms pores in membranes of acidified early endosomes and unfolded C2I translocates through the pores into the cytosol. Membrane translocation of C2I is facilitated by the activities of host cell chaperone Hsp90 and the peptidyl-prolyl *cis/trans* isomerase (PPIase) cyclophilin A. Here, we demonstrated that Hsp90 co-precipitates with C2I from lysates of C2 toxin-treated cells and identified the FK506-binding protein (FKBP) 51 as a novel interaction partner of C2I *in vitro* and in intact mammalian cells. Prompted by this finding, we used the specific pharmacological inhibitor FK506 to investigate whether the PPIase activity of FKBPs plays a role during membrane translocation of C2 toxin. Treatment of cells with FK506 protected cultured cells from intoxication with C2 toxin. Moreover, FK506 inhibited the pH-dependent translocation of C2I across membranes into the cytosol but did not interfere with the enzyme activity of C2I or binding of C2 toxin to cells. Furthermore, FK506 treatment delayed intoxication with the related binary actin ADP-ribosylating toxins from *Clostridium perfringens* (iota toxin) and *Clostridium difficile* (CDT) but not with the Rho-glucosylating *Clostridium difficile* toxin A (TcdA). In conclusion, our results support the hypothesis that clostridial binary actin-ADP-ribosylating toxins share a specific FKBP-dependent translocation mechanism during their uptake into mammalian cells.**

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## Introduction

C2 toxin from *Clostridium* (*C.*) *botulinum* is the prototype of binary clostridial actin-ADP-ribosylating toxins (Ohishi *et al.*, 1980; Aktories *et al.*, 1986), which further comprises *C. perfringens* iota toxin, *C. difficile* binary toxin (CDT) (Popoff and Boquet, 1988) and *C. spiroforme* toxin (Stiles and Wilkens, 1986). These toxins directly attack the cytoskeleton of eukaryotic cells by ADP-ribosylating G-actin (Aktories *et al.*, 1986; Vandekerckhove *et al.*, 1988). This results in depolymerization of actin filaments and cell rounding (Wiegers *et al.*, 1991), and eventually cell death (Heine *et al.*, 2008). The toxins are composed of two individual proteins. A binding/translocation component binds to the surface of target cells, forms a complex with the separate enzyme component and mediates the transport of the latter into the cytosol (for review see Barth and Stiles, 2008).

We and others have investigated the cellular uptake of C2 toxin in detail during recent years and found that the binding/translocation component C2II (~ 80 or 100 kDa, depending on the strain) requires limited poteolysis to form ring-shaped heptamers (C2IIa, ~ 420 kDa), which represent the biologically active species (Ohishi *et al.*, 1980; Barth *et al.*, 2000). C2IIa binds to a carbohydrate receptor on target cells (Blöcker *et al.*, 2000; Eckhardt *et al.*, 2000), assembles with the enzyme component C2I (~ 49 kDa) and the C2IIa/C2I complex is internalized by receptor-mediated endocytosis (Pust *et al.*, 2010). Finally,

C2I is released from acidified early endosomes into the cytosol (Barth *et al*., 2000) and most likely, the pH-gradient between the endosomal lumen and the cytosol is the driving force for membrane translocation of C2I (Barth *et al*., 2000). The acidic conditions in the endosomes trigger a conformational change of the C2IIa heptamers, which results in insertion into the endosomal membrane and formation of a translocation pore with an inner diameter of about 1–2 nm (Barth *et al*., 2000; Schleberger *et al*., 2006). C2IIa pores are essential for translocation of C2I across endosomal membranes (Blöcker *et al*., 2003a,b) and C2I translocates in a (partially) unfolded conformation through the pores into the cytosol (Haug *et al*., 2003a). Membrane translocation of C2I is facilitated by the chaperone heat shock protein (Hsp) 90 (Haug *et al*., 2003b) and the folding helper enzyme cyclophilin A (CyPA) (Kaiser *et al*., 2009). CyPs belong to the family of peptidyl-prolyl *cis/trans* isomerases (PPIases), which assists refolding of proteins to their native structures by accelerating the *cis/trans* isomerization of prolyl peptide bonds (Fischer *et al*., 1984; 1989; Lang *et al*., 1987; Schmid, 1993; Schmid *et al*., 1993; Wang and Heitman, 2005). We have demonstrated that CyPA interacts with C2I *in vitro* and in intact cells (Kaiser *et al*., 2009) and more recently we showed that Hsp90 directly binds to C2I *in vitro* (Kaiser *et al*., 2011).

Here, by using pull-down protocols, we demonstrated that C2I interacts with Hsp90 in intact cells and thereby identified the FK506-binding protein (FKBP) 51 as a novel C2I-binding protein. FKBP51 belongs to the FKBP subfamily of PPIases, which are specifically inhibited by FK506 (for review see Galat, 2003). We used FK506 to explore whether the activity of FKBPs plays a role during cellular uptake of C2 toxin. Indeed, treatment of cells with FK506 protected cultured cells from intoxication with C2 toxin. Prompted by this observation, we investigated the underlying mechanism in more detail and tested the effect of FK506 on intoxication of cells with the related binary iota and CDT toxins.

## Results

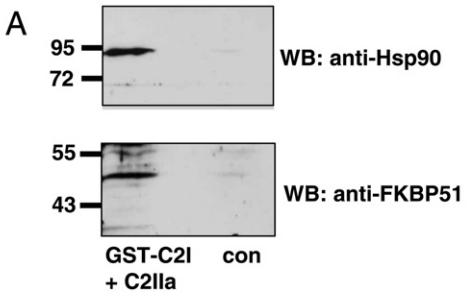
### *C2I interacts with Hsp90 and FKBP51 in intact cells and* in vitro

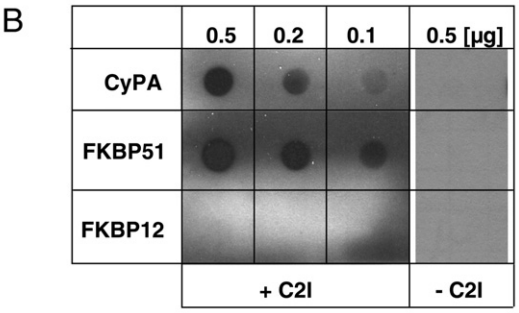
Due to the fact that Hsp90 binds to C2I *in vitro* (Kaiser *et al*., 2011), we performed pull-down experiments to investigate whether C2I interacts with Hsp90 in C2 toxin-treated cells. To this end, HeLa cells were incubated for 2 h with C2IIa and GST-C2I or left untreated for control. Subsequently, cells were lysed, the cytoplasmic fraction was obtained by centrifugation and equal amounts of protein were incubated with glutathion-sepharose beads to pull-down GST-C2I and associated proteins. The precipitates were separated by SDS-PAGE and co-precipitation of Hsp90 was tested in an immunoblot analysis with a specific antibody. As shown in Fig. 1A, Hsp90 was detected in precipitates from cells which were treated with C2IIa plus GST-C2I prior to lysis. Moreover, we found FKBP51 in the same precipitates when we probed the membrane with specific antibody against FKBP51 (Fig. 1A). We confirmed by Western blot analysis that this antibody recognized FKBP51 but not FKBP52 (not shown). In contrast, there were only negligible signals of Hsp90 or FKBP51 in the precipitates from untreated cells, indicating a specific interaction between GST-C2I and these proteins. From these results, it could not be concluded whether GST-C2I directly interacts with Hsp90 and/or FKBP51 in the cells or whether these factors are components of a multi-protein complex that interacts with the translocated C2I. To exclude any effect of the GST-tag on the interaction of C2I with Hsp90 or FKBP51, we repeated the experiment with biotin-C2I instead of GST-C2I and obtained comparable results (not shown).

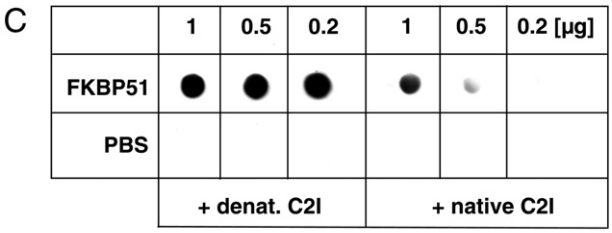
Next, we tested *in vitro* whether C2I directly binds to purified FKBP51. To this end, recombinant FKBP51 and FKBP12 were spotted onto a nitrocellulose membrane and subsequently incubated with C2I in solution. The bound C2I was then detected with a specific antibody against C2I (Fig. 1B). We included CyPA as a positive control, because we demonstrated a direct binding to C2I under such conditions earlier (Kaiser *et al*., 2009). As expected, C2I bound to CyPA and also to FKBP51. There were no signals in the absence of C2I, clearly demonstrating that the binding of C2I to the immobilized PPIases was specific. Interestingly, C2I did not bind to FKBP12 under these conditions, indicating a selective interaction of C2I with some FKBPs but not with others. When this experiment was performed with denatured C2I, a stronger binding to immobilized FKBP51 was observed compared to native C2I (Fig. 1C), suggesting that FKBP51 mainly interacts with the unfolded C2I. Taken together, we identified FKBP51 as a novel interaction partner of C2I in mammalian cells and *in vitro* analysis indicated a direct interaction between both proteins.

### *The pharmacological inhibitor FK506 protects HeLa and Vero cells from intoxication with C2 toxin*

Prompted by this finding, we tested whether the active site of FKBPs is involved in the mode of action of C2 toxin. Although the FK506 affinity of FKBP51 is about 20-fold lower when compared to FKBP12, the inhibition constant is still in the nanomolar range (Weiwad *et al*., 2006). To this end, HeLa cells were treated for 30 min with FK506 to inhibit the PPIase activity of FKBPs in the cells. For control, cells were left untreated. Then, the cells were challenged with C2 toxin and the toxin-mediated cell



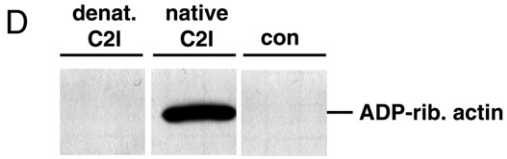




**Fig. 1.** Hsp90 and FKBP51 interact with C2I.
A. Co-precipitation of Hsp90 and FKBP51 from HeLa lysates with GST-C2I as bait. HeLa cells were incubated for 2 h at 37°C with C2IIa (800 ng ml$^{-1}$) plus GST-C2I (400 ng ml$^{-1}$), or without C2 proteins for control (con). The cells were lysed and the cytoplasmic fraction obtained by centrifugation. For pull-down of biotin-C2I, the cytoplasmic fractions were incubated with glutathion-sepharose beads over night at 4°C. The precipitated proteins were washed, separated by SDS-PAGE and blotted onto nitrocellulose. The membrane was cut and Hsp90 as well as FKBP51 were detected with specific antibodies.
B. Dot blot analysis of the interaction between C2I and different PPIases. The purified proteins CyPA, FKBP51 and FKBP12 (0.5, 0.2 and 0.1 µg of each protein) were vacuum aspirated onto a nitrocellulose membrane using a dot-blot system. The membrane was blocked and subsequently incubated with C2I (200 ng ml$^{-1}$). For control, PBS was used for overlay instead of C2I. After washing, the membrane was incubated with a specific antibody against C2I and secondary peroxidase-coupled anti-rabbit antibody to detect the bound C2I by enhanced chemiluminescence reaction.
C. Binding of denatured and native C2I to immobilized FKBP51. Biotin-C2I was incubated with 6 M guanidine hydrochloride, 2 mM DTT, 30 mM TrisHCl for 1 h at 25°C to unfold the protein. Subsequently, denatured biotin-C2I and native biotin-C2I were used for overlay assay and the bound C2I proteins were detected with streptavidin-peroxidase and enhanced chemiluminescence reaction.
D. The denaturing of C2I was confirmed by the loss of C2I ADP-ribosyltransferase activity *in vitro*. Cell lysate (40 µg of protein) was incubated for 10 min at 37°C in the presence of biotin-labelled $NAD^+$ (10 µM) with either denatured C2I, native C2I (each 1 ng ml$^{-1}$) or without C2I for control. Samples were subjected to SDS-PAGE, blotted and ADP-ribosylated actin was detected. The biotin-labelled (i.e. ADP-ribosylated) actin is shown (ADP-rib. actin = ADP-ribosylated actin).

rounding was monitored 2 h after toxin treatment. As shown in Fig. 2A, most of the cells were round after incubation with C2 toxin but only few cells were round when the cells were pretreated with FK506 prior to application of the toxin. FK506 at the applied concentrations had no effect on cell morphology. The quantitative analysis of the toxin-induced cell rounding confirmed the significant protective effect of FK506 towards intoxication of cells with C2 toxin (Fig. 2A). In parallel, we analysed the ADP-ribosylation status of actin from these cells. Cells were lysed and equal amounts of lysate protein were incubated together with fresh C2I and biotin-labelled NAD as co-substrate *in vitro*. In this assay, actin from untreated cells serves as substrate for C2I-catalysed ADP-ribosylation *in vitro* resulting in biotin-labelled actin, as shown in Fig. 2B. In contrast, actin from C2 toxin-treated cells is no substrate for C2I *in vitro* anymore because it was already ADP-ribosylated in the intact cells during incubation with the toxin (Fig. 2B). However, when cells were pretreated with FK506 prior to application of C2 toxin, the actin from these cells was strongly ADP-ribosylated *in vitro* (Fig. 2B), indicating that less actin was ADP-ribosylated by C2 toxin in the cytosol of intact cells. The results imply that the PPIase activity of FKBPs is involved in the mode of action of C2 toxin. The protective effect of FK506 towards C2 toxin was observed for another cell line, too (Fig. 2C). HeLa as well as Vero cells were treated with FK506 prior to application of C2 toxin and the toxin-induced cell rounding was monitored after different incubation periods. The results show overall comparable results for both cell lines. However, treatment of cells with FK506 resulted in a significant delay of cell rounding rather than in a complete inhibition.

### *Treatment of cells with FK506 interferes with the uptake of C2I into the cytosol*

Although the results demonstrate that FK506 treatment protects cells from intoxication with C2 toxin, it remained unclear whether FK506 inhibits the enzyme activity of C2I in the cytosol or whether FK506 interferes with the uptake of C2I into the cytosol. Therefore, we tested

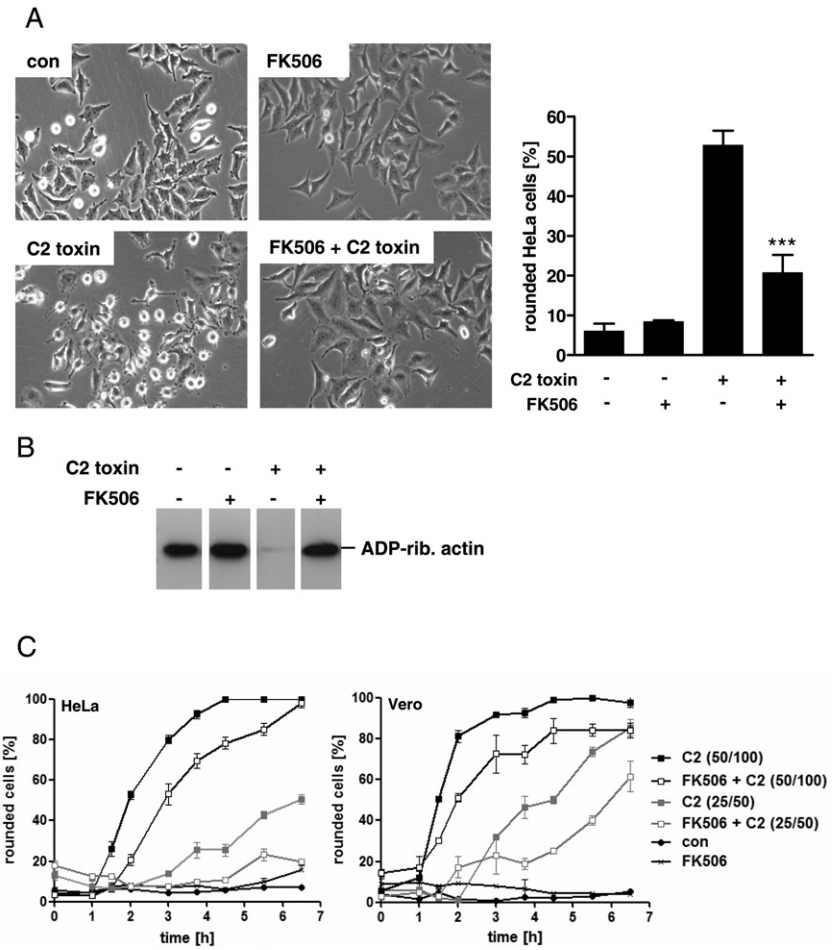



**Fig. 2.** FK506 protects HeLa and Vero cells from intoxication with *C. botulinum* C2 toxin.
A. HeLa cells were pre-incubated with 20 μM FK506 or left untreated (con). After 30 min C2 toxin (50 ng ml$^{-1}$ C2I plus 100 ng ml$^{-1}$ C2IIa) was added and pictures were taken after 2 h. For quantitative analysis, the percentages of rounded cells were calculated from the pictures. Values are given as mean ± SD ($n = 3$). Significance was tested by using Student's *t*-test (***$P < 0.0007$).
B. FK506 prevents ADP-ribosylation of actin by C2I in intact HeLa cells. Cells were treated as described in A. After 2 h of toxin treatment, cells were lysed and the ADP-ribosylation status of actin from these cells was analysed by incubating with C2I and biotin-labelled $NAD^+$. The biotin-labelled (i.e. ADP-ribosylated) actin is shown (ADP-rib. actin = ADP-ribosylated actin).
C. Time-course of intoxication of HeLa and Vero cells with C2 toxin in the absence and presence of FK506. Cells were treated for 30 min with 20 μM FK506 or left untreated. Subsequently, two different concentrations of C2 toxin were applied to each cell line. Black: 50 ng ml$^{-1}$ C2I + 100 ng ml$^{-1}$ C2IIa; Grey: 25 ng ml$^{-1}$ C2I + 50 ng ml$^{-1}$ C2IIa. After the indicated time points pictures were taken to determine the percentage of rounded cells. Values are given as mean ± SD ($n = 3$).

whether FK506 inhibits the C2I-catalysed ADP-ribosylation of actin *in vitro* and found no effect of this inhibitor on the enzyme activity of C2I (Fig. 3A). Next, we investigated whether FK506 interferes with the uptake of C2I into the cytosol. Vero cells were pretreated for 30 min with FK506 and subsequently with C2IIa plus biotin-C2I. For control, cells were treated with C2IIa plus biotin-C2I in the absence of FK506 or left untreated. For further control, cells were pretreated with bafilomycin A1 prior to addition of C2IIa plus biotin-C2I. Bafilomycin A1 prevents acidification of endosomal vesicles and thereby inhibits translocation of C2I from endosomal vesicles into the cytosol. To detect the amount of C2I that was delivered into the cytosol, cells were treated with digitonin to extract the cytosolic fraction which then was analysed for C2I by Western blotting (Fig. 3B). C2I was detectable in C2 toxin-treated cells but not in control cells and, as expected, less C2I was detected in the cytosolic fraction when cells were pretreated with bafilomycin A1 prior to incubation with C2 toxin. Pretreatment of cells with FK506 resulted in a decreased amount of C2I in the cytosol compared to cells, which have been treated with C2 toxin in the absence of the inhibitor. Comparable protein loading was confirmed by immunoblot analysis with an antibody against Hsp90. To exclude that the detected C2I derived from early endosomal vesicles as contaminants in the cytosolic fraction, we tested the cytosolic fraction as well as the extracted cells for the presence of rab5, a marker protein for early endosomes. While rab5 was present in the extracted cells, it was not detectable in the cytosolic fraction, indicating that there was no contamination of C2I-containing early endosomes in this preparation.

In conclusion, this result indicates that after treatment of cells with FK506 a minor amount of C2I reached the cytosol of C2 toxin-treated cells, suggesting that the PPIase activity of FKBPs plays a role for efficient transport of C2I into the host cell cytosol. Prompted by this finding, we investigated which step of toxin uptake was affected by FK506 treatment. First, we excluded by flow cytometry analysis of cell bound C2 toxin that treatment of cells with FK506 interferes with receptor binding of C2 toxin (Fig. 3C). This result was confirmed by Western blot analysis (data not shown). However, pretreatment with FK506 did not protect Vero cells from intoxication with *C. difficile* TcdA, a glucosyltransferase that modifies Rho

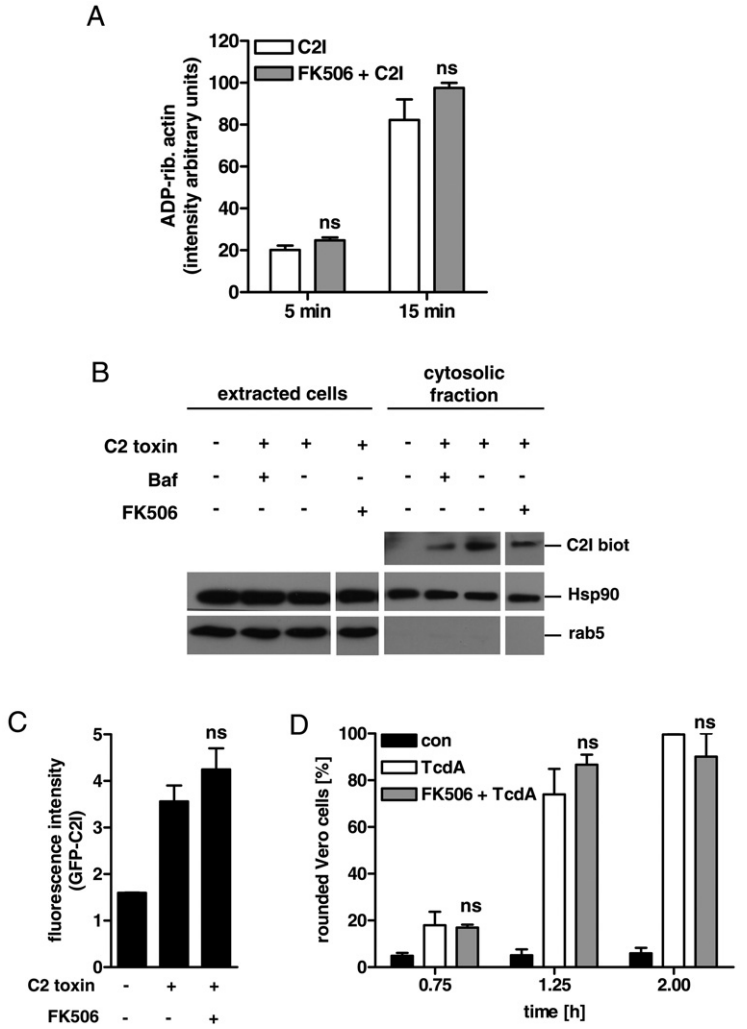



**Fig. 3.** Effect of FK506 on the enzyme activity of C2I, uptake of C2I into the cytosol and binding of C2 toxin to the cell surface.
A. FK506 has no effect on the ADP-ribosyltransferase activity of C2I *in vitro*. Forty micrograms of lysate protein was incubated for 30 min at 37°C with FK506 (20 μM) or left untreated for control. Subsequently C2I (1 ng ml$^{-1}$) and biotin-labelled NAD$^+$ (10 μM) were added and the samples were incubated at 37°C for 5 and 15 min. Samples were subjected to SDS-PAGE, blotted and ADP-ribosylated actin was detected. Intensity of the biotin-labelled actin was determined by densitometry and is given as mean ± SD ($n = 3$). Significance was tested for each time point between cytosol that was not treated with inhibitor and cytosol that was treated with FK506 by using Student's *t*-test (ns, not significant).
B. FK506 interferes with uptake of C2I into the cytosol. Cells were incubated in serum free medium for 30 min at 37°C with either Baf A1 (100 nM), FK506 (20 μM) or without inhibitor for control. Subsequently, cells were incubated with C2 toxin (50 ng ml$^{-1}$ biotin-C2I plus 100 ng ml$^{-1}$ C2IIa) for 1.5 h. The cells were washed and incubated with digitonin buffer (20 μg ml$^{-1}$ digitonin in PBS) for 5 min at 25°C and for further 30 min on ice to extract the cytosolic proteins from the cells. The cytosolic fractions were subsequently subjected to SDS-PAGE and blotted onto nitrocellulose. Biotin-C2I from these fractions was detected with streptavidin-peroxidase and the ECL system. The cytosolic protein Hsp90 and the early endosomal marker protein rab5 confirmed successful separation of the cytosolic fraction from the remaining extracted cells by this protocol (note: there is no rab 5 signal in the cytoslic fractions). Hsp90 detection confirmed equal amounts of protein in the cytosolic fractions.
C. FK506 has no effect on binding of C2 toxin to the cell surface. HeLa cells in suspension ($0.5 \times 10^6$ cells in 300 μl serum-free medium) were pretreated for 30 min at 37°C with 20 μM FK506 or without FK506 for control. Then, cells were incubated for 5 min at 4°C with 100 ng ml$^{-1}$ GFP-C2I plus 200 ng ml$^{-1}$ C2IIa to enable binding of the toxin to the cell surface. For control, cells were left untreated. The cells were washed with ice-cold PBS to remove unbound toxin and the cell-bound fluorescent C2I was analysed by flow cytometry. Values are given as mean ± SD ($n = 3$). Significance was tested by using Student's *t*-test (ns, not significant).
D. FK506 has no effect on intoxication of Vero cells with *C. difficile* TcdA. Vero cells were pre-incubated for 30 min with FK506 (20 μM) or left untreated for control (con). TcdA (50 ng ml$^{-1}$) was added and cells were incubated at 37°C. Pictures were taken after the indicated time points and the percentages of rounded cells calculated. Values are given as mean ± SD ($n = 3$). Significance was tested by using Student's *t*-test (ns, not significant).

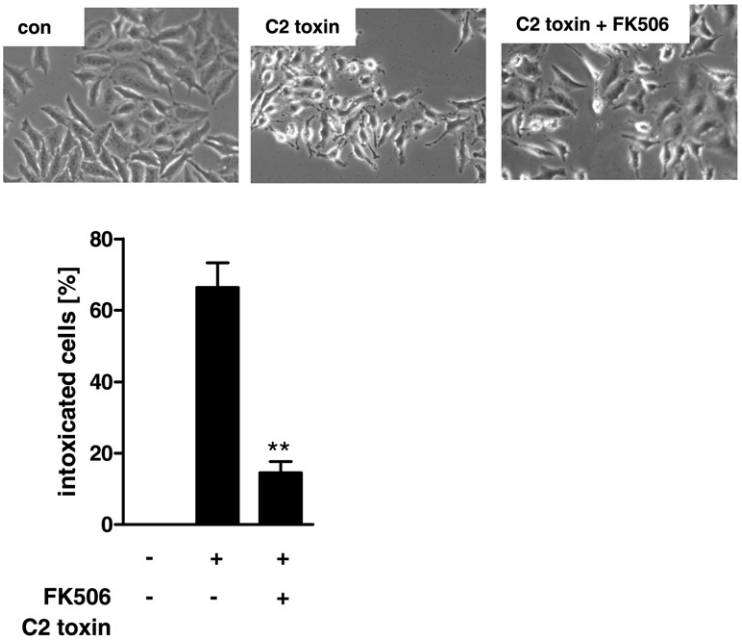



**Fig. 4.** FK506 inhibits the pH-dependent membrane translocation of C2 toxin. Effect of FK506 on the pH-dependent translocation of C2I across cytoplasmic membranes of intact HeLa cells. All cells were incubated for 30 min with 100 nM Baf A1 to block the normal uptake of C2 toxin. One portion of cells was in addition incubated with 20 μM FK506 to inhibit the PPIase activity of FKBPs, the other portion was incubated without FK506. Subsequently, cells were incubated for 30 min at 4°C in serum-free medium with C2 toxin (50 ng $ml^{-1}$ C2I plus 100 ng $ml^{-1}$ C2IIa; the toxin components have been pre-incubated together on ice for 30 min) to enable binding of the toxin to the cells. For control, cells were incubated without toxin. Then the medium was removed and acidic warm medium (pH 4.0, 37°C) was added for 10 min at 37°C to trigger toxin translocation across the cytoplasmic membrane. During this step Baf A1 was present. The cells were then incubated for 1 h at 37°C in neutral medium containing Baf A1. Pictures were taken and the percentages of rounded cells were calculated from the pictures. Values are given as mean ± SD ($n = 3$). Significance was tested by using Student's *t*-test (**$P < 0.005$).

GTPases and thereby induces cell rounding (Just *et al*., 1995) (Fig. 3D). As TcdA is also internalized by receptor-mediated endocytosis and delivers its enzyme domain from acidified endosomes into the cytosol, this finding implies that FK506 does not interfere with receptor-mediated endocytosis or acidification of endosomes in general and thus indicates a toxin-selective function of FKBPs.

### *Treatment of cells with FK506 inhibits membrane translocation of C2 toxin*

Next, we focused on the role of FKBPs during membrane translocation of C2 toxin and tested at first whether pre-treatment of cells with FK506 affected the pH-dependent translocation of C2 toxin across the cytoplasmic membrane of intact cells. All cells were pretreated with bafilomycin A1 to block the normal uptake of C2 toxin via acidified endosomal vesicles. Then, a portion of the cells was pretreated with FK506 and another portion left untreated for control. All cells were incubated at 4°C with C2 toxin to enable toxin binding. Subsequently, cells were exposed to warm, acidic medium to trigger the formation of C2IIa pores in the cytoplasmic membrane and the translocation of C2I through the pores across the membrane into the cytosol. This assay mimics acidified endosomal vesicles and is well established to investigate membrane translocation of bacterial protein toxins including C2 toxin. After 60 min of incubation at 37°C in neutral medium the percentage of intoxicated cells was determined (Fig. 4). While significant cell rounding was observed in the absence of FK506, only marginal cell rounding was observed when cells were pretreated with this inhibitor, indicating that membrane translocation of C2I was inhibited in those cells. The result suggests that the PPIase activity of FKBPs is required for membrane translocation of C2I.

To obtain more information about the role of FKBPs during translocation of C2I, we tested whether FK506 also interferes with the C2IIa-mediated uptake of C2IN into the cytosol. C2IN is the N-terminal domain of C2I (amino acid residues 1–225) which lacks enzyme activity but translocates from endosomes into the cytosol via C2IIa pores as C2I. FK506-treated HeLa cells were incubated with C2II plus C2IN and the cytosolic fractions from these cells were obtained by digitonin extraction. In parallel, C2I was used instead of C2IN. C2IN as well as C2I were detected in the cytosolic fractions by immunoblot analysis with the same antibody against C2IN. While FK506 treatment inhibited the uptake of C2I into

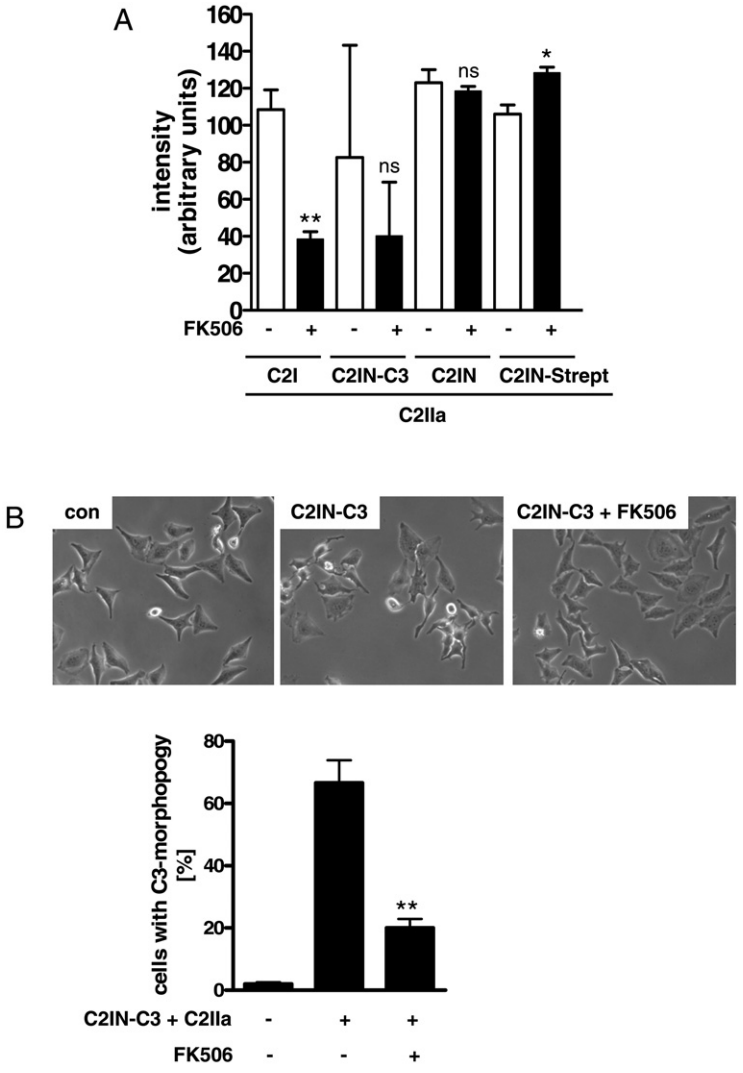



**Fig. 5.** Effect of FK506 on the C2IIa-mediated uptake of C2IN and C2IN fusion proteins into the cytosol of HeLa cells. Cells were incubated in serum free medium for 30 min at 37°C with FK506 (20 μM) or without FK506 for control. Subsequently, cells were incubated with either C2IIa (2 μg ml$^{-1}$) plus C2I (1 μg ml$^{-1}$), C2IIa (2 μg ml$^{-1}$) plus C2IN (1 μg ml$^{-1}$), C2IIa (2 μg ml$^{-1}$) plus C2IN–C3lim (1 μg ml$^{-1}$) or C2IIa (2 μg ml$^{-1}$) plus C2IN–streptavidin. Subsequently the cells were washed and incubated with digitonin buffer (20 μg ml$^{-1}$ digitonin in PBS) for 5 min at 25°C and then for further 30 min on ice. The cytosolic fractions were subjected to SDS-PAGE and blotted onto nitrocellulose. C2I, C2IN, C2IN–C3 and C2IN–strepatavidin were detected by with an antibody against C2IN in a Western blot analysis. Intensity of the signals was determined by densitometry and is given as mean ± SD ($n = 3$). Significance was tested for each individual protein between cytosol that was not treated with FK506 and cytosol that was treated with FK506 by using Student's *t*-test (*$P < 0.05$; **$P < 0.005$; ns, not significant).
B. FK506 protects HeLa cells from intoxication with C2IN–C3lim fusion toxin. HeLa cells were pre-incubated for 30 min with 20 μM FK506 or left untreated (con). C2IN–C3lim (100 ng ml$^{-1}$) plus C2IIa (200 ng ml$^{-1}$) was added, cells were further incubated at 37°C and pictures were taken after 4 h. For quantitative analysis, the percentages of cells with 'C3-morphology' were calculated from the pictures. Values are given as mean ± SD ($n = 3$). Significance was tested between cells treated with C2IN–C3lim plus C2IIa without and with FK506 by using Student's *t*-test (**$P < 0.005$).

the cytosol, it had no effect on the uptake of C2IN (Fig. 5A). This implies that FKBPs facilitate uptake of C2I but not of C2IN, suggesting that FKBPs play a role for membrane translocation of the catalytic domain in the C-terminal portion of C2I, but not for its N-terminal domain. Moreover, FK506 pretreatment of cells had no effect on the C2IIa-mediated uptake of the recombinant C2IN–streptavidin, but it reduced the C2IIa-mediated uptake of the C2IN–C3lim fusion toxin (Fig. 5A), which contains the Rho-modifying ADP-ribosyltransferase from *C. limosum*. In line with this result, FK506 treatment protected cells from intoxication with C2IN–C3lim plus C2IIa (Fig. 5B). Taken together, these results are a hint that FKBPs are specifically involved in the uptake of ADP-ribosylating toxins and fusion toxins, but not of C2IN fusion proteins which contain other cargo proteins. This suggests that FKBPs might selectively facilitate translocation and/or refolding of ADP-ribosyltransferase domains, as it seems to be the case for cyclophilins, too (Dmochewitz *et al.*, 2011).

Taken all together the results imply that the PPIase activities of FKBPs and CyPA facilitate the same step during cellular uptake of C2 toxin, i.e. membrane translocation of the enzyme component C2I. Therefore, we addressed the question whether FK506 and CsA exhibit a synergistic inhibitory effect. As shown in Fig. 6, the protective effect was more efficient when both inhibitors were applied in combination, compared to the single substances, suggesting that the PPIase activities of both FKBPs and CyPs act in a concerted manner during cellular uptake of C2 toxin. We excluded an effect of the inhibitor combination on the enzyme activity of C2I and on the binding of C2 toxin to the cells (not shown).

Finally, we demonstrated that pretreatment with FK506 protected Vero cells from intoxication with the related binary iota toxin from *C. perfringens* and CDT from

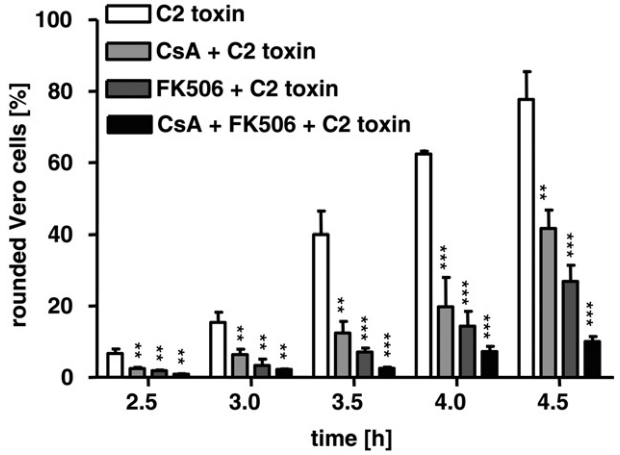



**Fig. 6.** FK506 and CsA inhibit intoxication of cells with C2 toxin in a synergistic manner. Vero cells were pre-incubated with either FK506 (20 μM), or CsA (10 μM), or the combination of FK506 plus CsA (20 μM + 10 μM). Control cells were left untreated (con). C2 toxin (50 ng ml$^{-1}$ C2I plus 100 ng ml$^{-1}$ C2IIa) was added after 30 min and cells were incubated at 37°C. Pictures were taken after the indicated time points and the percentages of rounded cells were calculated from the pictures. Values are given as mean ± SD ($n = 3$). Significance was tested by using Student's *t*-test (**$P = 0.005$; ***$P = 0.0005$).

*C. difficile* (Fig. 7) implying a common FKBP-dependent uptake mechanism for clostridial binary actin ADP-ribosylating toxins.

## Discussion

The present study is a continuation of our investigations that host cell chaperones and PPIases facilitate the intracellular membrane translocation of the enzyme components of clostridial binary actin-ADP-ribosylating toxins into the cytosol of mammalian cells. Here, we observed that treatment of cells with FK506, a specific pharmacological inhibitor of the PPIase activity of FKBPs, protected cultured cells from intoxication with the binary actin ADP-ribosylating C2, iota and CDT toxins. The results imply that the PPIase activity of FKBPs plays a role for the mode of action of these toxins. We used C2 toxin, the prototype of this toxin family, to investigate this inhibitory effect in more detail. FK506 inhibited the pH-dependent membrane translocation of the enzyme component C2I, strongly suggesting that the PPIase activity of FKBPs is involved in the translocation of C2I from the lumen of acidified endosomes into the cytosol of host cells. Interestingly, CyPA, another PPIase, as well as the chaperone Hsp90, facilitate the same step (Haug *et al*., 2003b; Kaiser *et al*., 2009). Specific pharmacological inhibition of CyP or Hsp90 activities protected cells from intoxication with C2, iota and CDT toxins (Haug *et al*., 2004; Kaiser *et al*., 2011) and here, we observed that FK506 protected cells from intoxication with these toxins, too. These results indicate that the PPIase activity of FKBPs plays a role for the mode of action of these toxins as well and suggest that FKBPs are involved in membrane translocation of the enzyme components. Moreover, in combination with our earlier findings they imply a common Hsp90/CyP/FKBP-dependent translocation mechanism of binary actin ADP-ribosylating toxins in mammalian cells. However, it is not clear so far how Hsp90 and the PPIases interact with C2I and whether they facilitate its membrane translocation in a concerted manner, for example as components of a multichaperone translocation complex.

Here we demonstrated for the first time that C2I interacts with a FKBP. FKBP51 was co-precipitated with C2I from lysates of C2 toxin-treated cells. Furthermore, *in vitro* analysis revealed a direct binding between C2I and purified FKBP51. This finding is interesting because FKBP51 is one of the three large immunophilins besides FKBP52 and CyP-40, which were identified as components of Hsp90-containing multichaperone complexes (Pirkl and Buchner, 2001). Such complexes interact with glucocorticoid receptors (GR) and modulate the affinity of GR for steroid hormones in mammalian cells (Pratt and Toft, 1997). Interestingly, one function of FKBP51 in these complexes is to regulate the translocation of GR from the cytoplasm to the nucleus (Stechschulte and Sanchez, 2011). Other Hsp90–multichaperone complexes were described which interact with the enzyme domain of diphtheria toxin and facilitate its membrane translocation into the cytosol of mammalian cells (Ratts *et al*., 2003), but so far it is not known whether these complexes also contain FKBPs.

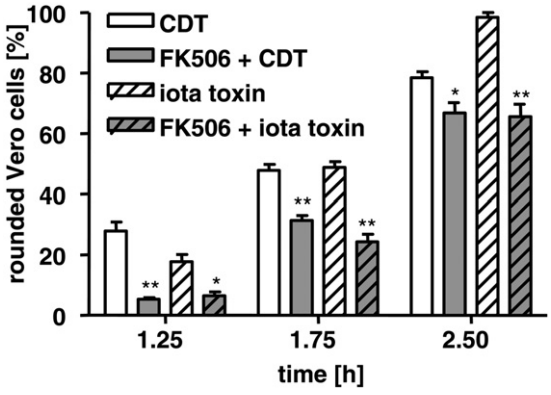



**Fig. 7.** FK506 protects Vero cells from intoxication with *C. perfringens* iota toxin *and C. difficile* binary toxin CDT. Vero cells were pre-incubated for 30 min with 20 μM FK506 or left untreated for control (con) then challenged with either iota toxin (50 ng ml$^{-1}$ Ia plus 100 ng ml$^{-1}$ Ib) or CDT (75 ng ml$^{-1}$ CDTa plus 150 ng ml$^{-1}$ CDTb). Pictures of the cells were taken after the indicated time points and the percentages of rounded cells were calculated from the pictures. Values are given as mean ± SD ($n = 3$). Significance was tested between cells treated without and with FK506 for each toxin by using Student's *t*-test (*$P < 0.05$; **$P < 0.005$).

In front of this view, the findings that both FKBP51 and Hsp90 were co-precipitated with C2I from lysates of C2 toxin-treated cells are interesting. Although we can not conclude from the results that co-precipitated Hsp90 and FKBP51 interact with the same C2I molecule during the same time in the cell, this finding is a first hint that FKBP51 could be a component of an Hsp90-containing multichaperone complex which associates with and facilitates membrane translocation of C2I.

Notably, the FKBP family of PPIases encompasses 16 members in human. They show one or more PPIase domains complemented with other functional polypeptide segments. These segments or domains have been found N-terminally and C-terminally to the catalytic domains. The latter bind to and are inhibited by FK506 for the most isoenzymes. Knowledge on the FK506 binding of the isoenzymes is exclusive based on *in vitro* measurements with purified enzymes and inhibitory potencies are characterized by $IC_{50}$ values in the nanomolar range (Weiwad *et al*., 2006). At the typical FK506 concentration of 20 μM used in our experiments almost complete inhibition of all isoenzymes can be hypothesized. In the cell, however, FK506 has to compete with the isoenzyme–substrate complexes for the active site. Under these conditions, FK506 affinities might be distributed over a wider range of $IC_{50}$ values. Interestingly, the prototypic cytosolic PPIase FKBP12, which is most efficiently inhibited by FK506 among the FKBPs tested (Weiwad *et al*., 2006), did not interact with C2I *in vitro*, a hint that FKBP12 might not be involved in translocation of C2I. However, we have got first evidence that *in vitro* C2I interacts with FKBP52 and CyP-40, too, which are further cochaperones in the GR-associated Hsp90–multichaperone complex (Pratt and Toft, 1997; Pirkl and Buchner, 2001). FKBP51 and FKBP52 share about 60% sequence identity and a four-domain structure (Chambraud *et al*., 1993). The PPIase activity is located in the N-terminal domain and the interaction with Hsp90 occurs C-terminally from this region (Chambraud *et al*., 1993). FKBP12 differs from FKBP51/52 but there is amino acid homology to FKBP51/52 in the N-terminal domains (Callebaut *et al*., 1992). This might be a possible explanation why C2I did not bind to FKBP12 and is a hint that C2I might interact with the C-terminal domains of FKBP51/52 *in vitro.* The active site of FKBP51 was found to be implicated as a positive regulator in androgen receptor signalling in prostate cancer cell lines because FK506 can block androgen-induced cell proliferation (Periyasamy *et al*., 2007; Ni *et al*., 2010). Notably, antagonistic effects were detected investigating the coexpression of FKBP51 and FKBP52 in yeast in receptor-mediated signalling. The presence of FKBP51 eliminated the enhancement of the hormone-dependent GR transcriptional activity by FKBP52 (Mamane *et al*., 2000) and the abundance of FKBP51 is positively correlated with resistance to steroid therapy in lung diseases therapies (Woodruff *et al*., 2007; Holownia *et al*., 2009). Obviously, we cannot yet definitely conclude that FKBP51 is the (only) relevant FKBP which is involved in membrane translocation of C2I.

Currently, we investigate the interaction of C2I with FKBP52 and CyP-40 *in vitro* and in intact cells to test whether these PPIases may also play a role during membrane translocation of C2I. In this regard, the finding that the combination of CsA and FK506 protected cells from intoxication with C2 toxin more efficiently than the single inhibitors might be interesting because it suggests a synergistic mode of action between the PPIase activities of CyP(s) and FKBP(s) during toxin uptake. We already reported that CyPA facilitates translocation of C2I (Kaiser *et al*., 2009), but CsA is not a selective inhibitor for particular CyPs (Handschumacher *et al*., 1984). Hence further CyPs such as CyP-40 might play a role in toxin translocation, too.

We observed that FK506 inhibited the uptake of various ADP-ribosylating toxins but not of toxins which harbour other enzyme activities, such as the large Rho-glucosylating toxins from *C. difficile*. The same holds true for CsA and the pharmacological inhibitors of Hsp90 (Haug *et al*., 2003b; Kaiser *et al*., 2009). Therefore, we speculated that the interaction with Hsp90/CyPA during intracellular membrane translocation of the enzyme moieties might be a selective mechanism for bacterial ADP-ribosylating toxins (Dmochewitz *et al*., 2011). We have demonstrated that an exchange of the protease domain to an ADP-ribosyltransferase domain in the enzyme component of binary anthrax toxins determines the interaction with Hsp90/CyPA (Dmochewitz *et al*., 2011). Therefore, we concluded that the enzyme domain might be of greater impact for the interaction with the host cell factors than the translocation pore or other domains within the enzyme components. The results presented in the present paper for the clostridial actin ADP-ribosylating toxins and the recombinant C2IN–C3lim fusion toxin as well as the C2IN fragment (Barth *et al*., 1998; 2002) suggest that the interaction with FKBPs might also be specific for ADP-ribosyltransferases.

Finally, the results might have a potential medical impact. Many clinical relevant toxins belong to the family of ADP-ribosyltransferases and one major problem is the increasing resistance against most antibiotics by toxin-producing strains of pathogenic bacteria in particular in hospitals. Therefore, the development and use of non-immunosuppressive FK506 derivatives could lead to novel therapeutic approaches to prevent the adverse effects of bacterial ADP-ribosylating toxins for example in combination with non-immunosuppressive CsA derivatives and antibiotics (Barth, 2011). Furthermore, the development of isoenzyme-specific inhibitors would be

especially helpful in assessing the full repertoire of FKBPs responsible for the regulation of the transport of bacterial ADP-ribosylating toxins.

## Experimental procedures

### *Materials*

Cell culture medium (MEM) and fetal calf serum were from Invitrogen (Karlsruhe, Germany). Cell culture materials were from TPP (Trasadingen, Switzerland). Complete® protease inhibitor and Streptavidin-peroxidase were from Roche (Mannheim, Germany). The protein molecular weight markers Page Ruler prestained Protein ladder® and Page Ruler stained Protein ladder® were from Fermentas (St. Leon-Rot, Germany). Biotinylated $NAD^+$ was from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany). Baf A1 was from Calbiochem (Bad Soden, Germany), CsA from Fluka (Munich, Germany) and FK506 from Sigma (Steinheim, Germany). The enhanced chemiluminescence (ECL) system was from Millipore (Schwalbach, Germany). The EZ-Link sulfo-NHS-Biotin was purchased from Pierce (Rockford, USA). Human FKBP12, FKBP51 and FKBP52 were expressed using pET28a (Novagen, Darmstadt, Germany) in *Escherichia coli* Rosetta cells and purified according to published procedures (Edlich *et al*., 2006). The recombinant C2I and C2IIa proteins were prepared and activated as described earlier (Barth *et al*., 1998; 2000). C2IN and C2IN–C3lim were produced and purified as described earlier (Barth *et al*., 1998). C2IN–streptavidin was prepared as described earlier (Fahrer *et al*., 2010). Ia and Ib were purified as described earlier (Perelle *et al*., 1996). Recombinant CDTa and CDTb (from *C. difficile* strain 196) were produced and purified as His-tagged proteins in the *Bacillus megaterium* expression system as described earlier (Papatheodorou *et al*., 2010).

### *Cell culture and cytotoxicity assays*

HeLa and Vero (African green monkey kidney) cells were cultivated in MEM containing 10% heat-inactivated fetal calf serum, 1.5 g $l^{-1}$ sodium bicarbonate, 1 mM sodium-pyruvate, 2 mM L glutamine and 0.1 mM non-essential amino acids. Cells were trypsinized and reseeded for at most 20 times. For cytotoxicity experiments, cells were seeded in culture dishes and incubated in serum-free medium with the respective toxin. For inhibition of the PPIase activity of CyPs or FKBPs, cells were incubated for 30 min with 10 μM CsA or 20 μM FK506 respectively. Subsequently, the respective toxin was added and cells were further incubated at 37°C with toxin plus inhibitor. After the given incubation periods, the cells were visualized by using a Zeiss Axiovert 40CFl microscope (Oberkochen, Germany) with a Jenoptik progress C10 CCD camera (Jena, Germany). The cytopathic effects caused by the toxins were analysed in terms of morphological changes.

### *Detection of cell bound C2 toxin by flow cytometry*

HeLa cells in suspension ($5 \times 10^6$ cells in 300 μl serum-free medium) were incubated for 30 min at 37°C together with 20 μM FK506. Subsequently, the cells were incubated for 5 min at 4°C with 200 ng $ml^{-1}$ C2IIa plus 100 ng $ml^{-1}$ GFP-C2I to enable binding of the toxin to the cell surface. For control, cells were left untreated. The cells were washed with ice-cold PBS to remove unbound toxin and the cell-bound fluorescent C2I was analysed by flow cytometry with FACSCalibur (BD Biosciences, Heidelberg, Germany).

### *Preparation of cell extracts, SDS-PAGE and immunoblot analysis*

Following incubation with the toxins, cells were washed twice with ice-cold PBS and lysed in 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$ and complete® protease inhibitor. Following sonification and centrifugation (20 800 *g*, 7 min, 4°C), the supernatant was stored at –20°C. For immunoblot analysis, equal amounts of lysate protein were subjected to SDS-PAGE according to the method of Laemmli (Laemmli, 1970). The proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany) and subsequently blocked for 45 min with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T) before detection of cell bound biotin-C2I. Therefore, the samples were probed with streptavidin-peroxidase. Subsequently, the membrane was washed three times with PBS-T and proteins were visualized in a chemiluminescence reaction. For the detection of actin, the samples were probed with a mouse monoclonal anti-β-actin antibody (clone AC-15; Sigma-Aldrich, Seelze, Germany). After washing with PBS-T, the membrane was incubated for 1 h with an anti-mouse antibody coupled to horseradish-peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany). The proteins were finally visualized using an ECL system according to the manufacturer's instructions. For detection of Hsp90 and rab5, antibodies from Santa Cruz (Heidelberg, Germany) and BD (Heidelberg, Germany) were taken respectively. The same protocol as described above was used for these antibodies. Anti-CyPA antibody was from Ambion (Foster City, USA). Detection of CyPA was accomplished with a peroxidase-coupled anti-rabbit antibody. Anti-Hsp90 and anti-FKBP51 antibodies were from Santa Cruz (Heidelberg, Germany).

### *Dot blot analysis of the interaction between C2I and immobilized PPIases*

Different amounts of purified CyPA, FKBP51 and FKBP12 proteins were vacuum-aspirated onto a nitrocellulose membrane using a dot blot system (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. The membrane was blocked for 1 h with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T) and subsequently incubated with C2I (200 ng $ml^{-1}$). Then the membrane was washed three times with PBS-T (5 min each) and the bound C2I was detected with a specific antibody against C2I and a peroxidase-coupled anti-rabbit antibody by using the ECL system. To test the binding of unfolded C2I to immobilized FKBP51, biotin-C2I was incubated with 6 M guanidine hydrochloride, 2 mM DTT, 30 mM TrisHCl for 1 h at 25°C before it was tested in the dot blot assay as described before. The bound biotin-C2I was detected with streptavidin-peroxidase and the ECL system as described before. To confirm that this procedure results in denatured C2I, ADP-ribosylation of actin by was performed as described below.

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

HeLa cells were grown in 10 cm dishes and incubated in serum-free medium for 2 h at 37°C with C2IIa (800 ng ml$^{-1}$) plus biotin-C2I (400 ng ml$^{-1}$) or for control without toxin. The cells were lysed with an 18G needle-syringe and the cytoplasmic fraction obtained by centrifugation. The cytoplasmic fraction was incubated with a 60 µl bed volume (1:1 in PBS) of streptavidin-sepharose beads (Pierce, Rockford, USA) overnight at 4°C. Alternatively, GST-C2I was used instead of biotin-C2I and glutathione-sepharose beads were used instead of streptavidin-sepharose beads for pull-down of GST-C2I. The precipitated proteins were washed, heated for 5 min at 95°C in Laemmli sample buffer and subjected to SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and the membrane was divided and probed with specific antibodies against Hsp90 (mouse, 1:1.000 in PBS-T), CyPA (rabbit, 1:5.000 in PBS-T) and FKBP51 (rabbit, 1:5.000 in PBS-T). After incubation with the secondary anti-mouse or anti-rabbit antibodies (1:2.500 in PBS-T), the membranes were washed and co-precipitated proteins visualized using the ECL system.

### Sequential ADP-ribosylation of actin in lysates from toxin-treated cells

For ADP-ribosylation of actin in a cell-free system, 15 µg of whole-cell lysate protein were incubated for 30 min at 37°C in a buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, complete® protease inhibitor, together with biotin-labelled $NAD^+$ (10 µM) and 300 ng of C2I protein. The reaction was stopped with 5× SDS-sample buffer (625 mM Tris/HCl pH 6.8, 20% SDS, 8.5% glycerol, 0.2% bromphenol blue, 100 mM DTT) and heating of the samples for 5 min at 95°C. Subsequently the samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The biotin-labelled ADP-ribosylated actin was detected with peroxidase-coupled streptavidin and a subsequent chemiluminescence reaction. Intensity of the biotin-labelled actin was determined by densitometry using the Adobe Photoshop 7.0 software.

### ADP-ribosylation of actin by C2I in a cell free system

HeLa cell lysate (40 µg of protein) was incubated for 5 and 15 min at 37°C together with 1 ng ml$^{-1}$ of C2I, 10 µM biotin-labelled $NAD^+$ and 20 µM FK506. Samples were subjected to SDS-PAGE, blotted onto a nitrocellulose membrane and the ADP-ribosylated actin was detected with streptavidin-peroxidase. Intensity of the biotin-labelled actin was determined by densitometry using the Adobe Photoshop 7.0 software.

### Toxin-translocation assay with intact cells

The pH-dependent translocation of C2I through C2IIa pores across endosomal membranes was experimentally mimicked on the surface of intact cells as described earlier (Barth *et al.*, 2000). In brief, HeLa cells were exposed to an acidic pulse (pH 4.0) after binding of 50 ng ml$^{-1}$ C2I plus 100 ng ml$^{-1}$ C2IIa to the cell surface. Cell rounding was monitored and documented by photography. To test the influence of FK506 on membrane translocation in this assay, cells were incubated with the inhibitors for 30 min at 37°C prior to toxin binding.

### Detection of C2I in the cytosolic fractions of cells

The cytosolic fraction from cultured cells was obtained by treatment of cells with digitonin as described recently (Kaiser *et al.*, 2009) according to the method described by Wiedlocha and co-workers (Wiedlocha *et al.*, 2005). Vero cells, grown in 12-well plates, were pre-incubated at 37°C in serum-free medium with Baf A1 (100 nM) or with FK506 (20 µM) for 30 min. Control cells were incubated without any inhibitor. Subsequently, C2IIa (100 ng ml$^{-1}$) plus biotin-labelled C2I (50 ng ml$^{-1}$) were applied to cells, and cells were incubated for an additional 1.5 h with toxin and the inhibitors. For a control, cells were incubated without C2 toxin. Cells were washes twice with ice-cold PBS to remove unbound C2I. Then cells were incubated for 5 min at 25°C in the presence of digitonin (20 µg ml$^{-1}$ in PBS) to permeabilize the cell membrane and subsequently for 30 min at 4°C to allow extraction of the cytosolic proteins, including biotin-labelled C2I. The extracted supernatant was collected, equal amounts of protein were subjected to SDS-PAGE and biotin-labelled C2I was detected with streptavidin-peroxidase using the ECL system. The cytosolic fraction was confirmed by immunoblot analysis with antibodies raised against the cytosolic marker protein, Hsp90. The absence of early endosomes in this fraction was confirmed in an immunoblot with an antibody raised against rab5, a marker protein for early endosomes.

### Reproducibility of the experiments and statistics

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

## Acknowledgements

This work was financially supported by the Deutsche Forschungsgemeinschaft (DFG, grant BA 2087/2-1) and the Medical Faculty of the University of Ulm (M.D. Program Experimental Medicine, Natalie Böhm). Katharina Ernst is a member of the International Graduate School in Molecular Medicine Ulm. We thank Ulrike Binder for excellent technical assistance.

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