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Role of CypA and Hsp90 in membrane translocation mediated by anthrax protective antigen

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Summary

Bacillus anthracis **lethal toxin consists of the protective antigen (PA) and the metalloprotease lethal factor (LF). During cellular uptake PA forms pores in membranes of endosomes, and unfolded LF translocates through the pores into the cytosol. We have investigated whether host cell chaperones facilitate translocation of LF and the fusion protein LF_NDTA. LF_N mediates uptake of LF_NDTA into the cytosol, where DTA, the catalytic domain of diphtheria toxin, ADP-ribosylates elongation factor-2, allowing for detection of small amounts of translocated LF_NDTA. Cyclosporin A, which inhibits peptidyl-prolyl *cis*/*trans* isomerase activity of cyclophilins, and radicicol, which inhibits Hsp90 activity, prevented uptake of LF_NDTA into the cytosol of CHO-K1 cells and protected cells from intoxication by LF_NDTA/PA. Both inhibitors, as well as an antibody against cyclophilin A blocked the release of active LF_NDTA from endosomal vesicles into the cytosol *in vitro*. In contrast, the inhibitors did not inhibit cellular uptake of LF. *In vitro*, cyclophilin A and Hsp90 bound to LF_NDTA and DTA but not to LF, implying that DTA determines this interaction. In conclusion, cyclophilin A and Hsp90 facilitate translocation of LF_NDTA, but not of LF, across endosomal membranes, and thus they function selectively in promoting translocation of certain proteins, but not of others.**

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Introduction

The anthrax toxin produced by *B. anthracis*, consists of three different protein components: lethal factor (LF), a 90 kDa metalloprotease that cleaves MAP kinase kinases (Duesbery *et al.*, 1998; Vitale *et al.*, 1998; Tonello and Montecucco, 2009); oedema factor (EF), an 89 kDa adenylyl cyclase (Leppla, 1982; 1991); and protective antigen (PA), an 83 kDa binding and translocation component. PA mediates the transport of LF and EF into the cytosol of target cells, where they exhibit their activities [for review see (Young and Collier, 2007; Collier, 2009)].

The first step in toxin uptake is binding of PA to one of the two anthrax toxin receptors on the cell surface, TEM8 (tumour endothelial marker 8, *alias* ANTXR1) and CMG2 (capillary morphogenesis 2, *alias* ANTXR2) (Young and Collier, 2007). Cell-bound PA becomes proteolytically activated and the resulting biologically active fragment, PA_{63}, forms ring-shaped oligomers (heptamers and octamers), which bind LF and EF (Young and Collier, 2007). Subsequently, the receptor-bound toxin complexes are internalized by actin- and clathrin-dependent endocytosis and delivered to acidic endosomes (Friedlander, 1986; Abrami *et al.*, 2003; 2004; 2010; Zornetta *et al.*, 2010). There, the acidic condition triggers conformational changes of the PA_{63} oligomers and their conversion into membrane-spanning pores (Blaustein *et al.*, 1989; Miller *et al.*, 1999), which serve as translocases for LF and EF. The pH gradient across the endosomal membrane has been proposed to be the major driving force for translocation of LF and EF through the pores to the cytosol (Wesche *et al.*, 1998; Krantz *et al.*, 2006). The PA_{63} pore is a mushroom-shaped structure with a membrane-spanning β-barrel stem that is ~10 nm long and ~1.5 nm wide (Benson *et al.*, 1998; Nassi *et al.*, 2002) and thus necessitates unfolding of LF and EF to pass through the narrow pores (Krantz *et al.*, 2004).

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Unfolding of LF and EF is promoted by the acidic pH of the endosomal lumen, which destabilizes the proteins and causes their N-terminal domains LF_N and EF_N, respectively, to become molten globules (Krantz *et al*., 2004). Moreover, the pH gradient across the endosomal membrane is believed to promote directionally biased diffusion (ratcheting) of LF and EF through the pore, according to a Brownian ratchet model of toxin translocation (Krantz *et al*., 2005). In the oligomeric PA_{63} pore, the phenylalanine-427 residues form the so-called Φ-clamp, which has a chaperone-like function and catalyses translocation of unfolded proteins through the pore (Krantz *et al*., 2005).

It was demonstrated recently that delivery of LF_NDTA from the lumen of acidified endosomes to the cytosol requires cytosolic host cell factors (Tamayo *et al*., 2008). The N-terminal domain of LF (LF_N) interacts with PA and mediates internalization and membrane translocation of LF_NDTA. The catalytic domain of diphtheria toxin (DTA) harbours ADP-ribosyltransferase activity and allows sensitive detection of translocated LF_NDTA. Therefore, LF_NDTA is an established tool to study membrane translocation via the PA pore [see (Collier, 2009) for review]. Membrane translocation of LF_NDTA from endosomal vesicles is facilitated by the COPI coatomer complex *in vitro* and components of the COPI coatomer complex interact with the LF_N domain (Tamayo *et al*., 2008).

In the present report, we have used LF_NDTA to address whether host cell chaperones and peptidyl prolyl *cis*/*trans* isomerases (PPIases), in particular cyclophilins (Cyps), are involved in membrane translocation via the PA pore. Cyps accelerate the *cis*/*trans* isomerization of proline–peptide bonds, often a rate-limiting step of protein folding (Fischer *et al*., 1989; Bang and Fischer, 1991; Schmid, 1993; Schmid *et al*., 1993). We have found that cyclosporin A (CsA), which inhibits the PPIase activity of Cyps (Handschumacher *et al*., 1984), and radicicol (Rad), an inhibitor of Hsp90 acitivity [for recent review see (Wandinger *et al*., 2008)], protected CHO-K1 cells from intoxication by LF_NDTA/PA, but not by PA/LF. Moreover, both compounds inhibited the PA-mediated release of active LF_NDTA, but not of LF, from purified endosomal vesicles to the cytosol *in vitro*, and we identified CypA as the essential Cyp involved in this process. *In vitro*, Hsp90 as well as CypA specifically bound to LF_NDTA and DTA but not to LF. In conclusion, our data show that both host cell factors facilitate the PA-mediated translocation/refolding of LF_NDTA but not of LF. The results support the hypothesis that among bacterial toxins the interaction with Hsp90/CypA during intracellular membrane translocation of the catalytic domains might be selective for bacterial ADP-ribosyltransferases.

Results

CsA protects cells from intoxication by LF_NDTA/PA

In this study, we first investigated whether Cyps are involved in cellular uptake of LF_NDTA, which consists of the N-terminal part of LF (LF_N, aa 1-263) and the catalytic domain of diphtheria toxin (DTA). DTA ADP-ribosylates mammalian elongation factor 2 (EF-2) and inhibits protein biosynthesis in intoxicated cells. Thus, cellular uptake of LF_NDTA can be easily followed by monitoring protein synthesis levels. CHO-K1 cells were incubated for 4 h with LF_NDTA + PA in the absence or presence of CsA, an inhibitor of the PPIase activity of Cyps. DTA-mediated inhibition of protein synthesis was then monitored by measuring the incorporation of [^{3}H]-leucine into newly synthesized proteins. To this end, following incubation with LF_NDTA + PA, cells were further incubated for 1 h with [^{3}H]-leucine and the amount of incorporated [^{3}H]-leucine was determined by scintillation counting. As expected, treatment of cells with LF_NDTA + PA strongly inhibited protein synthesis, indicating that EF-2 was inactivated by the toxin (Fig. 1). Addition of CsA increased protein synthesis levels in PA/LF_NDTA treated cells by ~4-fold (Fig. 1), while

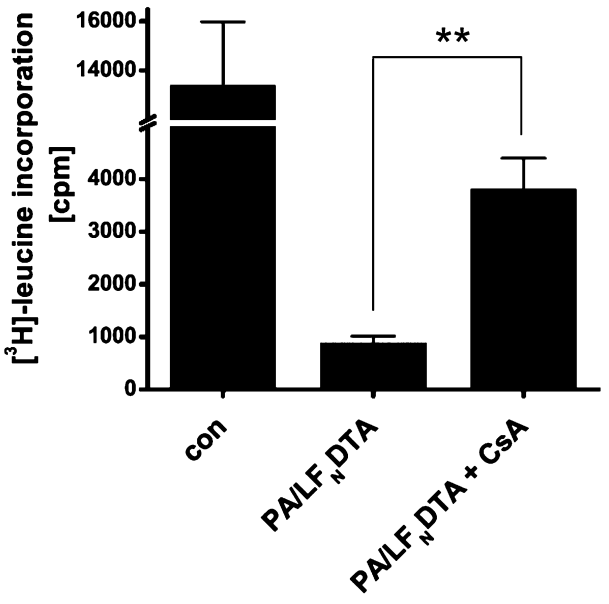


Fig. 1. CsA protects CHO-K1 cells from cytotoxic effects caused by LF_NDTA. CHO-K1 cells were pretreated for 30 min at 37°C in the presence or absence of 1 M CsA. Subsequently, PA_{83} (10 nM) and LF_NDTA (10 nM) were added to the cells. As a control, buffer alone was applied (denoted: con). Cells were incubated for an additional 4 h before adding [^{3}H]-leucine. After the addition of [^{3}H]-leucine, cells were incubated at 37°C for 1 h before harvesting the cells and measuring the amount of incorporated [^{3}H]-leucine using a scintillation counter. The readout is counts per minute (CPM). The values shown are the average of three trials and the error bars represent standard error of the mean. Significance was tested by using the student's *t*-test (**$P < 0.005$).

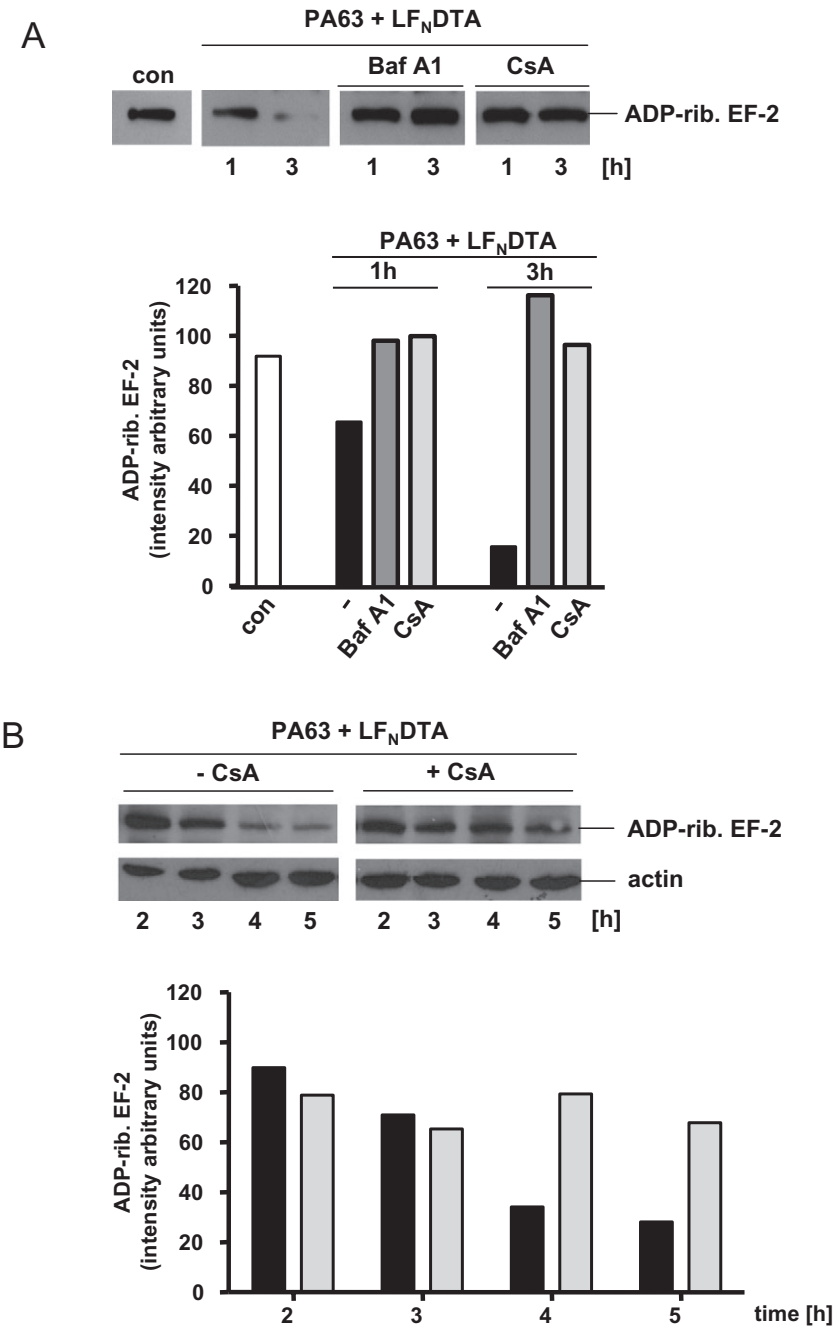


Fig. 2. CsA prevents ADP-ribosylation of EF-2 in CHO-K1 cells treated with PA_{63} plus LF_NDTA.
A. Effects of Baf A1 and CsA on the LF_NDTA-mediated ADP-ribosylation of EF-2 in toxin-treated CHO cells. Cells were pretreated for 30 min with Baf A1 (100 nM) or CsA (10 μM) and PA_{63} (1.6 nM) + LF_NDTA (1.9 nM) were added to the cells. For control, cells were left untreated (con). After 1 and 3 h of incubation, cells were lysed and the ADP-ribosylation status of EF-2 from these cells was analysed by post-ADP-ribosylation. Intensity of the ADP-ribosylated EF-2 was quantified by densitometry.
B. Time course of the ADP-ribosylation of EF-2 by LF_NDTA in CHO-K1 cells in the presence and absence of CsA. CHO-K1 cells were incubated for 30 min at 37°C in the absence or presence of CsA (20 μM) and subsequently PA_{63} (1.2 nM) + LF_NDTA (1.4 nM) were added. Cells were incubated for the indicated periods, lysed and the ADP-ribosylation status of EF-2 from these cells was analysed (upper panel). Equal amounts of protein were confirmed by anti-β-actin-immunoblot (lower panel). Intensity of the ADP-ribosylated EF-2 was quantified by densitometry (black bars: PA + LF_NDTA; grey bars: CsA + PA + LF_NDTA).

treatment with CsA alone (in the absence of toxin) had no effect on protein synthesis (data not shown). These results suggest that CsA alleviates the toxin-mediated block of protein synthesis by preventing the inactivation of EF-2 by LF_NDTA.

Next, we directly investigated the effect of CsA on the ADP-ribosylation status of EF-2 in CHO-K1 cells after treatment with LF_NDTA + PA. Cells were pretreated with or without CsA for 30 min, after which time buffer alone or buffer containing LF_NDTA + PA was added, and the cells were further incubated at 37°C. As a positive control, before the addition of LF_NDTA + PA, cells were treated with bafilomycin A1, which blocks PA-mediated translocation of LF_NDTA by preventing endosomal acidification. After 1 and 3 h, cells were lysed and the ADP-ribosylation status of EF-2 was analysed by post-ADP-ribosylation with biotin-NAD^+ (Fig. 2A, quantification of the signals is shown in the lower panel). In cells treated with LF_NDTA + PA without inhibitor, there was a weak signal of biotin-ADP-ribosylated EF-2 after 3 h, indicating that at this time point most of the EF-2 had been ADP-ribosylated by LF_NDTA in the intact cells. In

contrast, there was strong *in vitro* labelling of EF-2 after both 1 and 3 h when cells were treated with either bafilomycin A1 or CsA, indicating that only a minor portion, if any, of EF-2 was ADP-ribosylated by LF_NDTA in the intact cells when cells were treated with these inhibitors. Prompted by this finding, we investigated the inhibitory effect of CsA in more detail by analysing the effect of CsA on the time-dependent ADP-ribosylation status of EF-2 following treatment of cells with $LF_NDTA + PA$ (Fig. 2B, quantification of the signals is shown in the lower panel). The results demonstrate that CsA inhibits the LF_NDTA-catalysed ADP-ribosylation of EF-2 in the cytosol of CHO-K1 cells treated with $LF_NDTA + PA$. When we used the epithelial Vero cell line instead of CHO-K1 fibrobasts, we obtained comparable results (data not shown), indicating that the observed effects are not restricted to a single cell line but have a rather general impact. Importantly, CsA had no inhibitory effect on the *in vitro* ADP-ribosyltransferase activity of LF_NDTA, as demonstrated by ADP-ribosylation of EF-2 from CHO and Vero lysates (data not shown). Taken together, these findings strongly suggest that CsA prevents uptake of LF_NDTA into the cytosol of mammalian cells.

CsA prevents the uptake of LF_NDTA into the cytosol of toxin-treated cells

In the presence of CsA, less LF_NDTA protein was detected in the cytosolic fractions of LF_NDTA/PA-treated CHO-K1 cells. Cells were pretreated for 30 min with or without CsA, and then biotin-LF_NDTA and PA were added. After 1.5 h of incubation, the cytosolic fractions of these cells were obtained by digitonin extraction as described recently (Kaiser *et al.*, 2009) and analysed for the presence of biotin-LF_NDTA. The successful separation of cytosolic fractions from the remaining extracted cells was confirmed by immunoblot analysis with antibodies against early endosomal antigen 1 (EEA1), a marker protein for early endosomes, and MEK1, a cytosolic marker protein (Fig. 3A). Most importantly, no EEA1 was detectable in the cytosolic fractions, indicating that there was no contamination by early endosomes, which might contain LF_NDTA. Figure 3B shows the amount of biotin-LF_NDTA in the cytosolic fractions in the presence and absence of CsA. Quantification of these signals revealed that there was significantly less LF_NDTA protein in the cytosol when cells were pretreated with CsA (Fig. 3B). These data imply that Cyps play a role in the uptake of LF_NDTA into the cytosol of mammalian target cells.

We investigated at which individual step CsA interferes with cellular uptake of LF_NDTA and found that CsA did not prevent the PA-mediated binding of LF_NDTA to the cell surface, as detected by Western blot analysis of

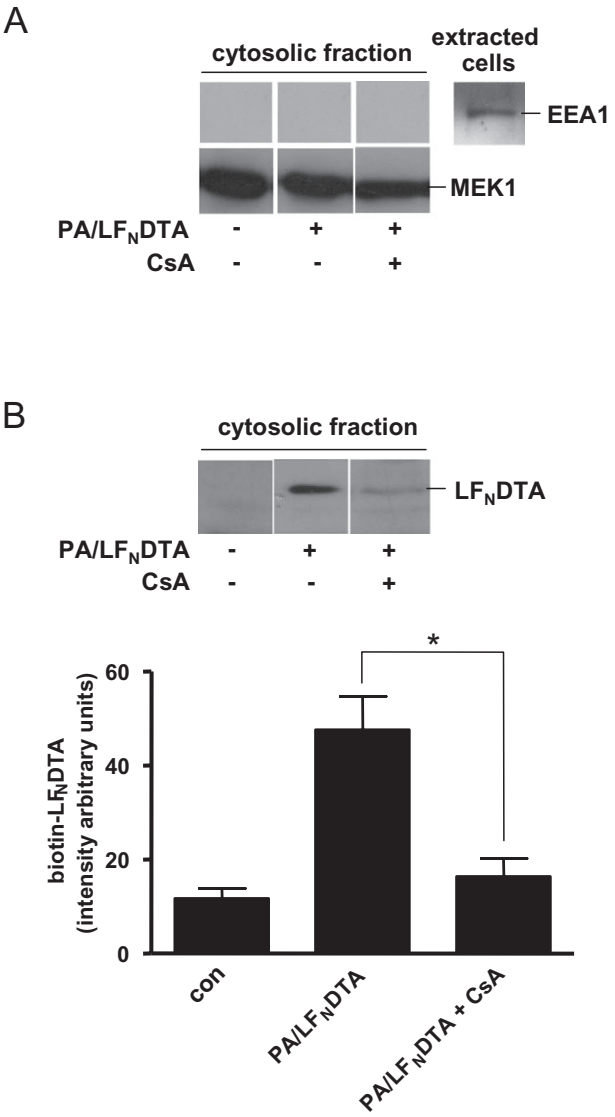


Fig. 3. Less LF_NDTA is detected in the cytosol from CsA-treated CHO-K1 cells. CHO-K1 cells were pre-incubated with 20 μM CsA for 30 min at 37°C or left untreated. Subsequently, cells were incubated with PA_{63} (6.3 nM) + biotin-LF_NDTA (7.4 nM) for 1.5 h. Thereafter, cells were washed and incubated with digitonin buffer (20 μg ml^{-1} digitonin in PBS) for 5 min at 25°C followed by 30 min incubation on ice to extract the cytosol.
A. Characterization of the cytosolic fractions from digitonin extraction by immunoblot analysis. Cytosolic fractions were blotted and probed with antibody against EEA1 to exclude contamination by early endosomes (upper panel). Extracted cells were taken for control. In parallel, cytosols were analysed with an antibody against the cytosolic marker protein MEK1 (lower panel).
B. Detection of biotin-LF_NDTA from the cytosolic fractions with streptavidin-peroxidase. Intensity of the biotin-LF_NDTA signals was quantified by densitometry and is given as mean ± SD ($n = 3$); Significance was tested against the cells without CsA by using the student's *t*-test (*$P < 0.05$).

cell bound LF_NDTA (not shown). Therefore, we focused on the effect of CsA on membrane translocation of the toxin.

CsA has an inhibitory effect on the in vitro *translocation of LF_NDTA from enriched endosomal vesicles*

We tested the effect of CsA on *in vitro* translocation of LF_NDTA from the lumen of enriched endosomes into the cytosol. Endosomes were preloaded with LF_NDTA as described earlier by Tamayo *et al*. (Tamayo *et al*., 2008). Briefly, CHO cells were incubated with LF_NDTA + PA in the presence of bafilomycin A1, cells were lysed, and endosomal vesicles were purified by discontinuous sucrose density gradient ultracentrifugation. The partially purified endosomal vesicles were used for *in vitro* translocation studies. Translocation of LF_NDTA across the membranes of the endosomal vesicles was induced by addition of freshly prepared CHO cytosol and ATP. The assay mixture, which contained also biotin-NAD^+, was incubated for 30 min at 37°C and the ADP-ribosyltransferase activity of translocated LF_NDTA was detected by monitoring the ADP-ribosylation, i.e. biotinylation, of EF-2 in the cytosolic supernatant (Fig. 4A). We confirmed by anti-MEK1 immunoblot analysis that the analysed samples contained comparable amounts of protein (Fig. 4A, lower panel).

To test the influence of CsA on translocation of LF_NDTA from endosomal vesicles, cytosol was pre-incubated for 30 min at 37°C with 20 μM CsA before application to the vesicles. Thereafter, the endosomal vesicles were pelleted by ultracentrifugation. The cytosolic supernatant was subjected to SDS-PAGE, blotted and the biotin-labelled EF-2 was detected and quantified (Fig. 4B). The pretreatment of the cytosol with CsA (20 μM) resulted in significantly less ADP-ribosylation of EF-2. Importantly, equal protein loading was confirmed by MEK1 antibody (see Fig. 4B, lower panel). When higher final concentrations of CsA (60 μM) were used in this assay, only negligible ADP-ribosylation of EF-2 was detected. However, 60 μM of CsA had a moderate inhibitory effect on the enzyme activity of LF_NDTA *in vitro* and therefore we did not use such concentrations of CsA in our experiments. These results indicate that there was less active LF_NDTA in CsA-treated cytosol, implying that Cyps facilitate the transport of active LF_NDTA from LF_NDTA/PA-loaded endosomal vesicles into the cytosolic supernatant *in vitro*. These experiments, together with our results showing that CsA decreases the amount of LF_NDTA found in the cytosol of LF_NDTA/PA-treated cells (Fig. 3), favour a model in which CsA inhibits PA-mediated translocation across the endosomal membrane, rather than a subsequent step, such as refolding.

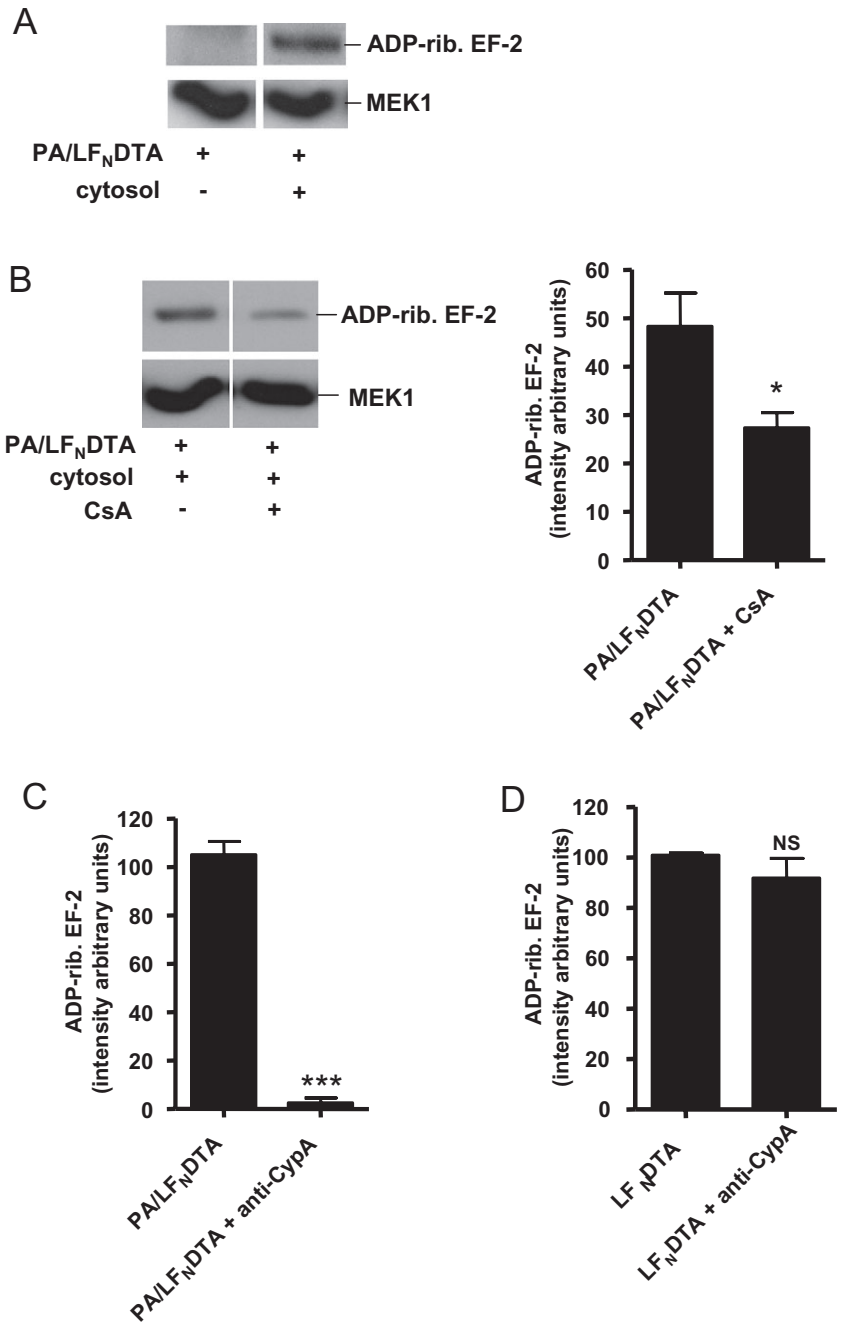
An antibody against CypA inhibits in vitro *translocation of LF_NDTA from enriched endosomal vesicles*

CsA inhibits the PPIase activity of various Cyps. Therefore, to determine the particular host cell Cyp that facilitates membrane translocation of LF_NDTA, we pretreated the cytosol with a specific antibody against CypA, the most prominent cytosolic Cyp and the major target of CsA in mammalian cells, before adding the cytosol to the LF_NDTA-containing endosomal vesicles to induce translocation. As a control, untreated cytosol was used. The *in vitro* translocation assay was performed as described above. Equal protein loading was confirmed by MEK-1 immunoblot analysis (not shown). As shown in Fig. 4C, there was only negligible ADP-ribosylation of EF-2 when LF_NDTA-containing vesicles were incubated with cytosol pretreated with anti-CypA. Importantly, the anti-CypA antibody had no influence on the ADP-ribosyltransferase activity of LF_NDTA as tested in a cell free system *in vitro* (Fig. 4D). In conclusion, these results indicate that CypA is the relevant Cyp that facilitates membrane translocation of LF_NDTA *in vitro*.

Rad has an inhibitory effect on the PA-mediated uptake of active LF_NDTA from endosomal vesicles and protects CHO-K1 cells from intoxication by LF_NDTA/PA

Finally, we investigated whether Hsp90 is also involved in PA-mediated uptake of LF_NDTA. LF_NDTA preloaded endosomal vesicles were incubated with cytosol to induce *in vitro* translocation of LF_NDTA. The cytosol was treated for 30 min with or without Rad, an inhibitor of Hsp90, before addition to the endosomal vesicles. As shown in Fig. 5A and B, less EF-2 was ADP-ribosylated by LF_NDTA when the cytosol was pretreated with Rad. However, Rad had no effect on the ADP-ribosyltransferase activity of LF_NDTA (Fig. 5C). These findings strongly suggest that Hsp90 activity is crucial for the PA-mediated uptake of active LF_NDTA from endosomal vesicles *in vitro*.

Prompted by this finding, we tested whether inhibition of Hsp90 protects CHO-K1 cells from intoxication by LF_NDTA/PA. Cells were pretreated with or without Rad, then LF_NDTA and PA were applied, and the toxin-catalysed inactivation of cytosolic EF-2 was measured by incorporation of [^{14}C]-leucine into the cellular proteins (Fig. 5D). In the absence of Rad there was strongly decreased incorporation of [^{14}C]-leucine, indicating inhibition of protein biosynthesis as a result of LF_NDTA-mediated inhibition of EF-2. In contrast, in Rad-treated cells, there was no significant inhibition of protein biosynthesis by LF_NDTA. Moreover, in the presence of Rad, less biotin-LF_NDTA protein was detected in the digitonin extracted cytosolic fractions of LF_NDTA/PA-treated CHO-K1 cells compared with cells that were not pre-



treated with Rad before toxin application (data not shown). In line with these results, pretreatment of Vero cells with Rad partially inhibited the LF_NDTA-catalysed ADP-ribosylation of EF-2 in the cytosol of cells treated with LF_NDTA + PA (data not shown). Thus, the observed effects are not restricted to a single cell line. In combination with the result from the *in vitro* uptake assay, the data strongly suggest that Rad inhibits the release of active LF_NDTA from endosomes to the cytosol, implying that Hsp90 likely facilitates PA-mediated translocation or a subsequent step (e.g. refolding) of LF_NDTA in mammalian target cells.

LF_NDTA directly interacts with Hsp90 and CypA in vitro

To investigate whether LF_NDTA interacts with CypA or Hsp90 *in vitro*, we analysed binding of biotinylated LF_NDTA and LF proteins to immobilized CypA and Hsp90 in a dot-blot assay. Importantly, LF_NDTA showed specific and concentration dependent binding to both proteins. In

Fig. 4. CsA as well as an antibody against CypA have inhibitory effects on translocation of LF_NDTA from the lumen of endosomal vesicles into the cytosol *in vitro*. PA_{63}/LF_NDTA-loaded endosomal vesicles were purified by sucrose density gradient centrifugation from CHO-K1 cells, previously treated with PA_{83} (10 nM) + LF_NDTA (10 nM) in the presence of Baf A1.
A. Enriched toxin-containing endosomes were subjected to *in vitro* translocation assay by incubating 5 μl of vesicles together with freshly prepared cytosol from CHO-K1 cells (40 μg of protein) in 20 μl translocation buffer containing biotin-NAD^+ (10 μM) for 30 min at 37°C. For control, toxin-containing vesicles were incubated with translocation buffer instead of cytosol. Vesicles were pelleted and the supernatant was then incubated with cytosol containing biotin-NAD^+ as described before. Proteins were separated by SDS-PAGE, blotted and the biotin-labelled, i.e. ADP-ribosylated, EF-2 was detected with streptavidin-peroxidase.
B. To test the influence of CsA on translocation of LF_NDTA from endosomal vesicles, cytosol was pre-incubated for 30 min at 37°C with 20 μM CsA before application to the vesicles. Thereafter, vesicles were pelleted by ultracentrifugation, the cytosolic supernatant was subjected to SDS-PAGE, blotted and the biotin-labelled, i.e. ADP-ribosylated, EF-2 was detected with streptavidin-peroxidase (upper panel). Comparable protein loading was confirmed by anti-MEK1 immunoblot analysis (lower panel). Intensity of the biotin-labelled EF-2 bands was determined by densitometry and is given as mean ± SD ($n = 3$). Significance was tested by using the student's *t*-test ($^*P < 0.05$).
C. An antibody against CypA prevents translocation of LF_NDTA from early endosomes into the cytosol *in vitro*. PA_{63}/LF_NDTA-loaded endosomal vesicles from CHO-K1 cells were incubated for 30 min at 37°C together with freshly prepared CHO-K1 cytosol (40 μg of protein) in 20 μl translocation buffer containing biotin-NAD^+ (10 μM). In parallel, cytosol that was pre-incubated for 30 min at 37°C with 4 μl of anti-CypA was applied to endosomal vesicles in this assay. Thereafter, the endosomes were pelleted by ultracentrifugation, the cytosolic supernatant was subjected to SDS-PAGE, blotted and the biotin-labelled, i.e. ADP-ribosylated, EF-2 was detected with streptavidin-peroxidase. Intensity of the biotin-labelled EF-2 band was determined by densitometry and is given as mean ± SD ($n = 3$). Significance was tested by using the student's *t*-test ($^{***}P < 0.0005$). Equal protein loading was confirmed with a MEK1-specific antibody (not shown).
D. Anti-CypA has no influence on the enzyme activity of LF_NDTA. Lysate from CHO-K1 cells was pre-incubated for 30 min at 37°C with anti-CypA antibody (2 μl in 10 μl of lysate). For control, lysate was left untreated. LF_NDTA (1 nM) and 10 μM biotin-NAD^+ were added and samples were incubated for 10 min at 37°C. Proteins were separated by SDS-PAGE, blotted and ADP-ribosylated EF-2 was detected with strepatavidin-peroxidase. Intensity of the biotin-labelled EF-2 band was determined by densitometry and is given as mean ± SD ($n = 3$). Significance was tested by using the student's *t*-test (NS = not significant). Equal protein loading was confirmed with a MEK1-specific antibody (not shown).

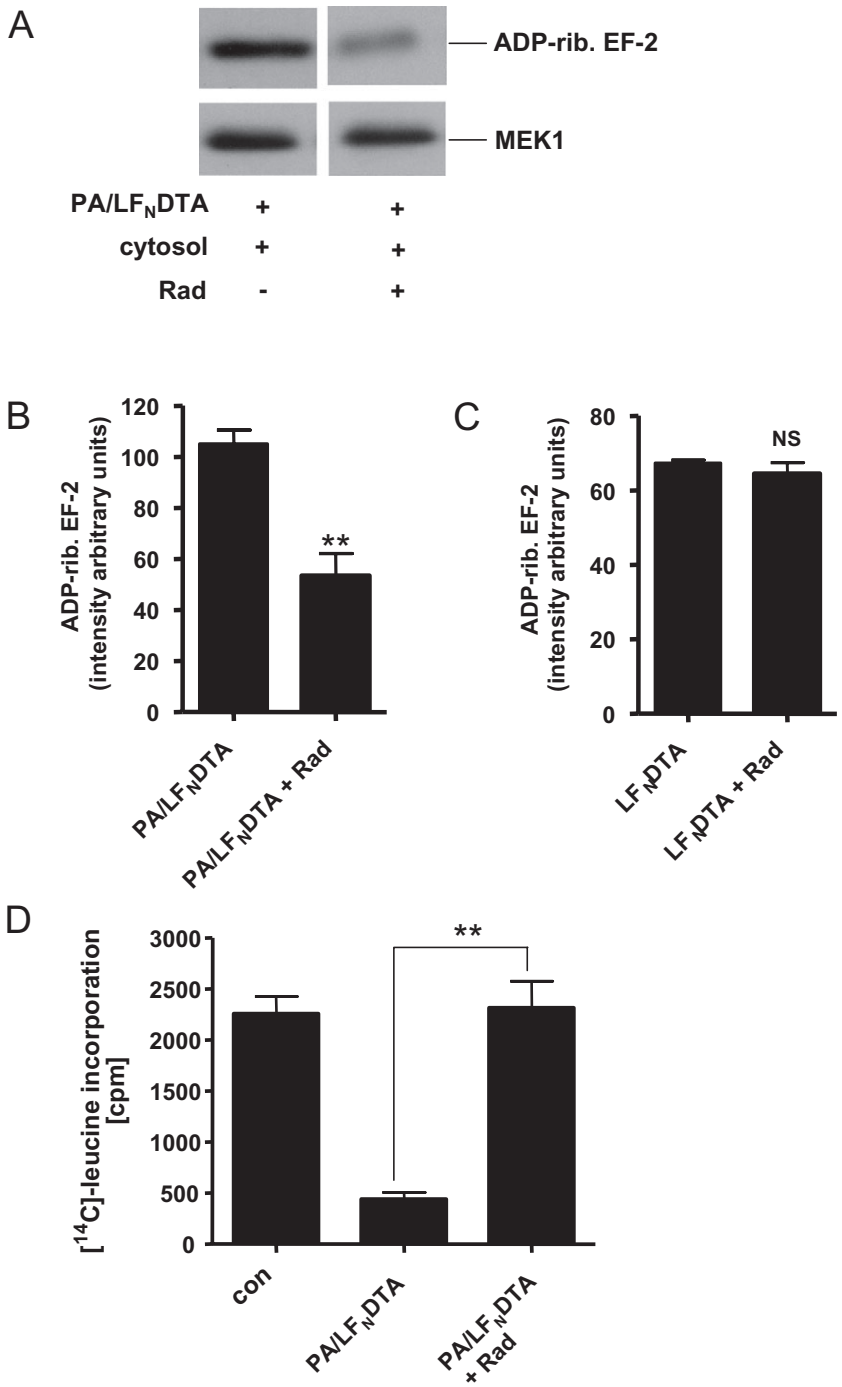
contrast, there was no interaction between LF and CypA or Hsp90 (Fig. 6A). Thus, the DTA portion of LF_NDTA likely mediates binding of this protein to CypA and Hsp90 and therefore we performed this binding assay with biotinylated DTA proteins. As shown in Fig. 6B, there was a specific and concentration dependent binding of both wild-type DTA and DTA-E148S, which lacks ADP-ribosyltransferase activity, to the immobilized CypA and Hsp90 proteins. However, the lack of interaction between LF and CypA or Hsp90 *in vitro* is an indication that PA-mediated uptake of LF into the cytosol of target cells might be independent of these host cell factors.

CypA and Rad do not protect cells from intoxication by LF

Prompted by the observation that both CypA and Hsp90 interact with LF_NDTA but not with LF *in vitro*, we addressed whether inhibition of these host cell factors has an effect on the intoxication of cells by anthrax lethal toxin (LeTx = PA + LF). In a first approach, we tested the influence of CsA and Rad on the LeTx-induced cell death of cultured macrophages. J774A.1 cells were pretreated with the inhibitors alone and in combination, challenged with LeTx, and the viability of the cells was determined by MTS assay. As shown in Fig. 7A, neither CsA nor Rad nor the combination of both had a significant inhibitory effect on the cytotoxic action of the toxin, implying that Cyps and Hsp90 are not necessary for uptake of LF into the cytosol of the macrophages. In line with this result, there was no inhibitory effect of CsA or Rad on the LF-catalysed cleavage of MEK1 in CHO-K1 fibroblasts and Vero epithelial cells as demonstrated in Fig. 7B and C respectively. Taken all together, our results indicate that CsA and Rad inhibit the PA-mediated transport of LF_NDTA but not of LF into the cytosol of mammalian target cells, strongly suggesting that the DTA domain of the fusion toxin is responsible for interaction with both host cell factors.

Discussion

It has become evident that cytosolic host cell factors are required for the intracellular membrane translocation of certain bacterial protein toxins, including diphtheria toxin (DT) (Ratts *et al*., 2003), anthrax lethal toxin (Tamayo *et al*., 2008) and the binary actin ADP-ribosylating C2 toxins from clostridia (Haug *et al*., 2003a; Kaiser *et al*., 2009). The chaperone activity of the heat shock protein Hsp90 facilitates translocation of the catalytic subunits of DT (DTA) (Ratts *et al*., 2003) and C2 toxin (C2I) (Haug *et al*., 2003a) from the lumen of acidified endosomes to the cytosol. In addition to Hsp90, thioredoxin reductase and β-COP from the COPI coatomer complex are required for the productive membrane translocation of DTA (Ratts *et al*., 2003). Interestingly, the conserved peptide motif T1 within the DT transmembrane helix 1, which mediates the interaction with β-COP (Ratts *et al*., 2003), was also identified in anthrax LF and EF as well as in the neurotoxins from *Clostridium botulinum* BoNTs A, C, D (Ratts *et al*., 2005). So far, no host cell chaperones are known for the BoNTs but it was reported that the heavy chain of BoNT/A, which forms a pore in endosomal membranes to achieve translocation of the enzymatically active light chain, serves as an intramolecular chaperone for the latter



(Brunger *et al.*, 2007; Montal, 2010). The N-terminal domain of anthrax LF (LF_N) interacts with COPI coatomer complex proteins and thus Tamayo and co-workers concluded that the facilitated delivery of LF_NDTA into the cytosol of target cells is likely analogous to that of DT entry (Tamayo *et al.*, 2008). However, it is not clear whether chaperones, for instance Hsp90, are also involved in membrane translocation of LF_NDTA.

In the present study, we have performed a series of experiments to investigate whether Hsp90 and Cyps are required for PA-mediated delivery of active LF_NDTA and LF *in vitro* and during uptake into mammalian cells. By using the specific pharmacological inhibitors Rad and CsA, we found that Hsp90 as well as Cyps participate in uptake of LF_NDTA into the host cell cytosol and protected cells from intoxication by LF_NDTA/PA. The data obtained

Fig. 5. Rad has an inhibitory effect on the *in vitro* translocation of LF_NDTA from endosomal vesicles into the cytosol and protects CHO-K1 cells from intoxication by LF_NDTA/PA.
A. Effect of Rad on membrane translocation of LF_NDTA *in vitro*. PA_{63}/LF_NDTA-loaded endosomal vesicles were purified by sucrose density gradient centrifugation from CHO-K1 cells, previously treated with PA_{83} (10 nM) + LF_NDTA (10 nM) in the presence of Baf A1. After gradient centrifugation aliquots (5 μl) of the PA_{83} + LF_NDTA-loaded endosomal vesicles (indicated as EE) were incubated for 30 min at 37°C together with freshly prepared CHO-K1 cytosol (40 μg of protein) in 20 μl translocation buffer containing biotin-NAD^+ (10 μM). To test the influence of Rad on translocation of LF_NDTA, freshly prepared cytosol was incubated for 30 min at 37°C together with 20 μM Rad before application upon the toxin-containing endosomes. Thereafter, the endosomes were collected as pellets by ultracentrifugation, the cytosolic supernatant was subjected to SDS-PAGE, blotted and the biotin-labelled, i.e. ADP-ribosylated, EF-2 (ADP-rib. EF-2 = ADP-ribosylated EF-2) was detected by using streptavidin-peroxidase and the ECL system. Equal amounts of protein from the cytosolic fractions were confirmed by immunoblot analysis with an anti-MEK1 antibody (lower panel).
B. The intensity of the biotin-labelled EF-2 band was determined by densitometry and is given as mean ± SD ($n = 3$). Significance was tested by using the student's *t*-test (**$P < 0.005$).
C. Rad has no effect on the ADP-ribosyltransferase activity of LF_NDTA *in vitro*. Lysate from CHO-K1 cells was pre-incubated for 30 min at 37°C with 10 μM Rad or left untreated for control. After that, LF_NDTA (1 nM) and biotin-NAD^+ (10 μM) were added and samples were incubated for 10 min at 37°C. Proteins were separated by SDS-PAGE, blotted and ADP-ribosylated EF-2 was detected with strepatavidin-peroxidase. Intensity of the biotin-labelled EF-2 band was determined by densitometry and is given as mean ± SD ($n = 3$). Significance was tested by using the student's *t*-test (NS = not significant). Equal protein loading was confirmed with a MEK1-specific antibody (not shown).
D. Rad protects CHO-K1 cells from intoxication by LF_NDTA/PA. CHO-K1 cells were pretreated for 1 h at 37°C with or without Rad (1 μM) at 37°C. Cells were further incubated with PA_{63} (6.4 nM) plus LF_NDTA (3.7 nM) for 2 h at 37°C. Afterwards, the medium was replaced by L-leucine-free medium supplemented with [^{14}C]-leucine to monitor incorporation of radioactive leucine as an indicator for protein synthesis in the cells. After 1 h incubation at 37°C cells were washed three times with ice-cold PBS and lysed by treatment with 0.1% SDS. Proteins were precipitated by 10% trichloroacetic acid and the amounts of incorporated [^{14}C]-leucine was measured by filter assay and liquid scintillation counting. Data are given as mean ± SD ($n = 3$; **$P < 0.005$).

from both an *in vitro* translocation assay with partially purified endosomal vesicles and a cellular intoxication assay clearly revealed that CypA is necessary for efficient translocation of LF_NDTA from the endosome to the cytosol. Similarly, we show that Hsp90 is required for PA-mediated uptake of active LF_NDTA, and our results indicate that the chaperone is likely involved in either translocation from the endosome or a subsequent step such as refolding. Importantly, we excluded that CsA or Rad interfered with other steps of toxin action, such as receptor binding, endocytosis or modification of EF-2.

These findings are in line with our recent observation that Hsp90 and CypA facilitate membrane translocation of C2I, the enzyme component of the binary actin ADP-ribosylating *C. botulinum* C2 toxin (Kaiser *et al*., 2009). It is widely accepted that the binary C2 and the anthrax toxins share an overall comparable mechanism to deliver their enzyme components into the cytosol of target cells [for review see (Barth *et al*., 2004)]. Similar to PA_{63}, the binding and translocation component from C2 toxin, C2IIa, forms ring-shaped heptamers (prepores), which share high structural homology with PA_{63}. First, C2IIa mediates binding of C2I to the cell surface and later, during uptake, the release of C2I from acidified early endosomes. To this end, C2IIa forms pores with an inner diameter of ~1–2 nm that insert into the endosomal membrane; unfolded C2I translocates in a pH-dependent manner through these pores to the cytosol (Barth *et al*., 2000; Blöcker *et al*., 2003a,b; Haug *et al*., 2003b). We have recently demonstrated the essential role of Hsp90 and CypA during uptake of C2I into the host cell cytosol, showing a direct and specific interaction between C2I and both host cell proteins *in vitro* and in intact cells (Haug *et al*., 2003a; Kaiser *et al*., 2009). Although we detected binding of Hsp90 and CypA to the N-terminal adaptor domain of C2I (C2IN) (Kaiser *et al*., 2009), these proteins might also interact with the C-terminal ADP-ribosyltransferase domain of C2I as the crystal structure of C2I revealed very similar folding and overall structures of its N- and the C-terminal domains (Schleberger *et al*., 2006). Taken all together, our data suggest that Hsp90 and CypA may facilitate toxin-mediated delivery of C2I and LF_NDTA through analogous mechanisms.

In the light of our results obtained for LF_NDTA, the observation that the PA-mediated uptake of LF into the cytosol was not influenced by Rad or CsA is remarkable. However, it is consistent with our earlier observations (Haug *et al*., 2003a) and with a recent report by Zornetta *et al*. demonstrating by confocal microscopy that the combination of both inhibitors did not block the delivery of fluorescent LF or EF proteins into the cytosol of BHK cells (Zornetta *et al*., 2010). Because LF_N mediates the interaction of both LF and LF_NDTA with PA_{63}, a comparable mechanism for the PA-dependent translocation of both proteins into the cytosol should be expected. Moreover, Tamayo *et al*. demonstrated that LF_NDTA requires COPI coatomer complex for membrane translocation and that the LF_N domain interacts with the components of this complex, such as ζ-COP and β-COP (Tamayo *et al*., 2008). Therefore, we expected that the interaction of LF_NDTA with Hsp90 and CypA would also occur via the LF_N moiety and therefore should be comparable for LF_NDTA and LF. In contrast, our results clearly demonstrated that *in vitro* Hsp90 and CypA directly interact with DTA, the ADP-ribosyltransferase domain of the LF_NDTA fusion toxin, a plausible explanation for the finding that inhibition of these

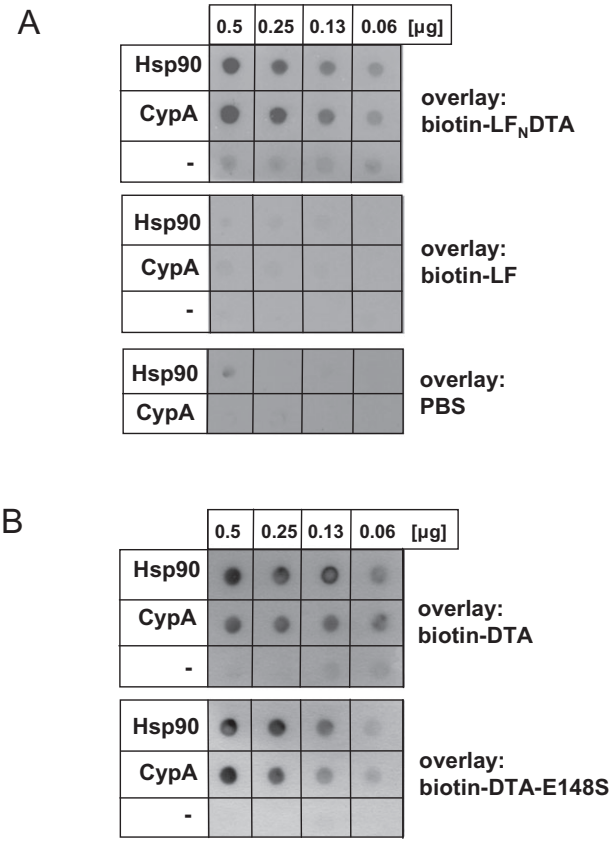


Fig. 6. LF_NDTA but not LF directly interacts with both Hsp90 and CypA *in vitro*.
A. Hsp90 and CypA (0.5, 0.25, 0.13 and 0.06 µg of each protein) were vacuum aspirated onto a nitrocellulose membrane using a dot-blot system. For control, PBS was aspirated instead of protein. The membrane was blocked with 5% non-fat dry milk in PBS-T and subsequently incubated with either biotinylated LF_NDTA (upper panel), biotinylated LF (mid panel, each protein 200 ng ml^{-1} in PBS) or for control with PBS (lower panel). Subsequently, membranes were washed three times with PBS-T followed by incubation with streptavidin-peroxidase to detect bound biotinylated LF_NDTA or LF proteins by the ECL system.
B. DTA directly interacts with both Hsp90 and CypA *in vitro*. Hsp90 and CypA were spotted onto a membrane and the membrane was blocked exactly as described in A. Subsequently, the membrane was incubated with biotinylated wild-type DTA (upper panel) and biotinylated DTA-E148S (mid panel, each protein 200 ng ml^{-1} in PBS). The bound DTA proteins were detected as described in A.

host cell proteins does not affect PA-mediated uptake of LF. This is also in line with our observation that LF_NDTA, but not LF, directly interacts with Hsp90 and CypA *in vitro*.

Moreover, the findings that LF_N does not interact with Hsp90 but does interact with components of the COPI coatomer complex are a hint that during PA-mediated membrane translocation these factors might bind to separate domains of the LF_NDTA protein. Taken together, the results reported here and earlier work suggest that PA-mediated delivery of LF depends on COPI coatomer complex proteins, but not on Hsp90 or CypA, while delivery of LF_NDTA requires both Hsp90 and CypA in addition to COPI coatomer complex. Inhibition of Hsp90 and CypA activity likely prevents proper folding of the DTA domain and may affect release of the protein into the cytosol. In contrast, the enzyme domain of LF seems to refold independent of these factors. However, at present we cannot exclude the involvement of other chaperones or PPIases in the PA-mediated delivery of LF. Thus, we conclude that the enzyme domain of the cargo protein, and not the LF_N domain or the translocation pore, is responsible for the interaction with Hsp90 and CypA during toxin-mediated delivery.

From this perspective, it is interesting that all bacterial toxins that have been found to interact with either Hsp90 or PPIases thus far are mono-ADP-ribosyltransferases, which deliver their catalytic domain from acidified endosomes to the cytosol. Besides DT, PA/LF_NDTA and C2 toxin, other bacterial ADP-ribosylating toxins that require Hsp90 and Cyps for membrane translocation are the *Clostridium perfringens* iota toxin and the *Clostridium difficile* transferase CDT (Kaiser *et al*., in preparation), strongly implying a common chaperone/PPIase-dependent translocation mechanism for the family of binary ADP-ribosylating toxins. Moreover, C2 fusion toxins were also sensitive to CsA and Rad when the catalytic domain of C2I was replaced by a foreign ADP-ribosyltransferase domain, for instance the Rho-inactivating C3 protein from *Clostridium limosum* or the catalytic actin-ADP-ribosylating C/SpvB domain of *Salmonella enterica* (Haug *et al*., 2003a; Pust *et al*., 2007).

Bacterial mono-ADP-ribosyltransferases show a highly conserved overall folding of their catalytic cores (Collier, 1995; Galan, 1998; Masignani *et al*., 2000). Therefore refolding of these domains following translocation across intracellular membranes might be facilitated by Hsp90/CypA and thus could explain why ADP-ribosyltransferases require the assistance of these host cell factors during uptake into the host cell cytosol. Future experiments with new toxin chimeras will be helpful to verify this hypothesis and to characterize the molecular interaction between bacterial ADP-ribosyltransferases and host cell factors in more detail.

Finally, it will be interesting to investigate whether Hsp90 and Cyps also facilitate membrane translocation of bacterial ADP-ribosylating toxins that deliver their enzyme domains to the host cell cytosol by mechanisms other than translocation from acidified endosomes. Such examples are the ADP-ribosyltransferase SpvB from *S. enterica* (Lesnick and Guiney, 2001) and exoenzyme S from *Pseudomonas aeruginosa*, which are delivered by intracellularly located bacteria into the cytosol of host cells (Barbieri and Frank, 2002) or cholera toxin, which delivers its ADP-ribosyltransferase moiety from the endoplasmic reticulum into the cytosol (Majoul *et al*., 1996). Interestingly, the catalytic domain of the plant toxin ricin, which

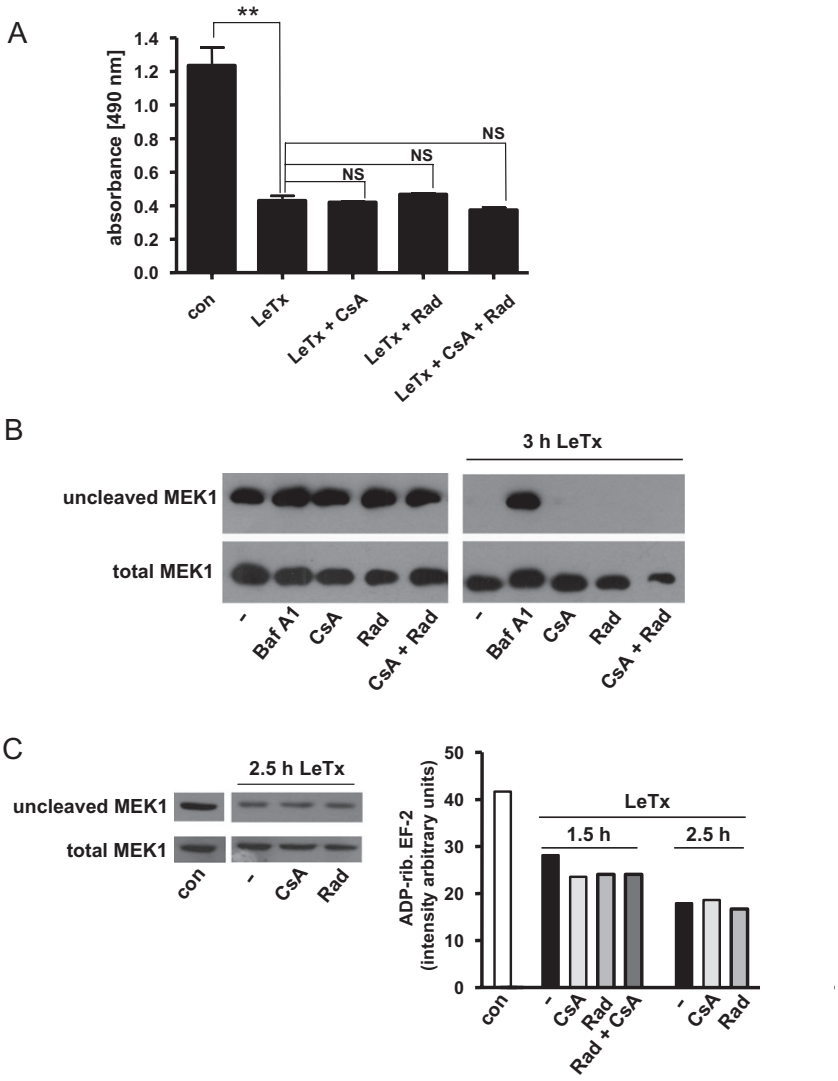


Fig. 7. CsA and Rad do not inhibit the cytotoxic effects of the anthrax lethal toxin (LeTx) in CHO-K1, J774A.1 and Vero cells.
A. J774A.1 macrophages were pretreated for 30 min with either CsA (10 μM) or Rad (1 μM) or with the combination of both inhibitors. For control, cells were left untreated (con). Subsequently PA_{83} (3.6 nM) + LF (3.3 nM) were added, the cells were further incubated at 37°C in the presence of the toxin and the inhibitors and the viability was measured after 3 h by MTS assay. Data are given as mean ± SD ($n = 3$). Significance was tested by using the student's *t*-test (**$P < 0.005$, NS = not significant).
B. Effect of CsA and Rad on the LF-catalysed cleavage of MEK1 in the cytosol of CHO-K1 cells. Cells were pretreated for 30 min with either CsA (10 μM) or Rad (10 μM) or with the combination of both inhibitors. For control, cells were left untreated. As a further control, cells were treated with Baf A1 (100 nM) to inhibit endosomal acidification. PA_{83} (1.2 nM) + LF (1.1 nM) were applied and cells were incubated for 3 h at 37°C, lysed and equal amounts of protein were subjected to SDS-PAGE. Immunoblot analysis was performed with an antibody against the N-terminal peptide of MEK1, which is removed by LF from the MEK1 protein (upper panel) and with an antibody raised against the unmodified C-terminal part of MEK1 (lower panel).
C. Effect of CsA and Rad on the LF-catalysed cleavage of MEK1 in the cytosol of Vero cells. Vero cells were pretreated for 30 min with CsA (10 μM) or Rad (10 μM). Cells were incubated for 1.5 and 2.5 h with PA_{83} (1.2 nM) + LF (1.1 nM). Cells were lysed and MEK1 cleavage was analysed exactly as described in B (Western blot shown for the 2.5 h values). The intensity of the uncleaved MEK1 bands was determined by densitometry.

translocates as an unfolded protein from the endoplasmic reticulum to the cytosol, also interacts with cytosolic host cell chaperones Hsc70 and Hsp90, which likely refold plant toxin ricin after translocation and prevent its degradation and aggregation (Spooner *et al*., 2008).

Taken all together, we have demonstrated that, although the LF_NDTA fusion toxin and LF bind to PA via the same domain and only differ in their C-terminal catalytic domains, LF_NDTA requires Hsp90 and CypA for PA-mediated delivery into the host cell cytosol, while LF does not require these factors. Moreover, we provided evidence that DTA directly interacts with both Hsp90 and CypA *in vitro*. The ADP-ribosyltransferase activity of DTA is not required for this interaction, as wild-type DTA and DTA-E148S, which lacks the active-site residue glutamic acid-148 and shows drastically reduced (~300-fold) ADP-ribosyltransferase activity (Wilson *et al*., 1990) bound to immobilized CypA and Hsp90 proteins, too. These findings, along with results reported elsewhere, suggest that the ADP-ribosyltransferase domains of bacterial toxins recruit Hsp90 and/or PPIases to facilitate their membrane translocation and/or refolding during uptake into the cytosol. Overall, our findings strongly suggest that the catalytic domains of bacterial toxins are of greater importance than previously recognized in the recruitment of host cell factors necessary for efficient cellular uptake.

Experimental procedures

Materials

Cell culture media DMEM and fetal calf serum were obtained from Invitrogen (Karlsruhe, Germany) and HAM's F12 from Gibco (Karlsruhe, Germany). Cell culture materials were obtained from TPP (Trasadingen, Switzerland). Complete® protease inhibitor was purchased from Roche (Mannheim, Germany). The protein molecular weight marker Page Ruler prestained Protein ladder®

was from Fermentas (St. Leon-Rot, Germany). Biotinylated NAD^+ was supplied by R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany). Baf A1 was obtained from Calbiochem, CsA was obtained from Fluka (Munich, Germany) and Rad from Sigma-Aldrich (Munich, Germany). Streptavidin-peroxidase was bought from Roche and the enhanced chemiluminescence (ECL) system from Millipore (Schwalbach, Germany).

Purification and biotinylation of proteins

CypA was purified as described (Fanghänel and Fischer, 2003). Human Hsp90β was purified as described (Richter *et al.*, 2008). Anthrax proteins and fusion toxin LF_NDTA were expressed and purified as described (Wesche *et al.*, 1998). DTA-E148S construct was generated by mutating residue 148 of DTA using QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Both, DTA and DTA-E148S genes, were then PCR amplified and cloned into pET-SUMO vector (Invitrogen) using the manufacturer provided protocol so that an in frame SUMO–DTA fusion protein is produced. The *E. coli* BL21(DE3) strain was used as the host strain for expression of the recombinant proteins. Overexpressed SUMO–DTA fusion proteins were purified using a nickel-chelating agarose column and then cleaved with SUMO protease. Subsequently, the recombinant DTA proteins were separated from SUMO and SUMO protease using a nickel-chelating agarose column. The biotinylation of LF_NDTA, LF, DTA and DTA-E148S was performed with sulfo-NHS-biotin (Pierce, Rockford, Illinois, USA) according to the manufacturer's instructions.

Cell culture and intoxication assays

CHO-K1 (Chinese hamster ovary) cells were cultivated in DMEM and HAM's F12 containing 5% heat-inactivated fetal calf serum, 1 mM sodium-pyruvate and Penicillin-Streptomycin (1:100). J774A.1 macrophage-like cells were cultivated at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM), containing 10% heat-inactivated fetal calf serum, L-glutamate (4 mM), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}). 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. The cell lines were cultivated at 37°C and 5% CO_2. Cells were trypsinized and reseeded for at most 15–20 times. For intoxication experiments, cells were seeded in culture dishes and for inhibition of the PPIase activity of Cyps, the cells were pre-incubated for 30 min with 20 μM CsA. Control cells were incubated without any inhibitor. Subsequently, the indicated concentrations of PA_{63} + LF_NDTA were added and cells were further incubated at 37°C with toxin plus inhibitor. After the given incubation periods the cells were lysed, followed by an incubation with LF_NDTA (1 nM) and biotin-labelled NAD^+ (10 μM) for 30 min at 37°C for an *in vitro* ADP-ribosylation of the EF-2, which is not yet ADP-ribosylated by the toxin. Samples were subjected to SDS-PAGE, blotted and ADP-ribosylation status of EF-2 was analysed. Equal amounts of protein were confirmed by immunoblot analysis with an anti-β-actin antibody. Intensity of ADP-ribosylated EF-2 was determined by densitometry using the Adobe Photoshop 7.0 software. To determine the cytotoxic effects of lethal toxin, J774A.1 macrophages were pretreated with PA_{63} + LF at 37°C and the viability was measured by Cell Titer 96® Aqueous One Solution cell proliferation assay (MTS assay) (Promega, Mannheim, Germany) according to the manufacturer's instructions. Alternatively, the LF-catalysed cleavage of MEK1 in the cytosol of CHO-K1 and Vero cells was detected by immunoblot analysis with an antibody raised against MEK1 (Nanotools, Teningen, Germany).

Measurement of protein biosynthesis by incorporation of radioactively labelled amino acids

Rad studies. CHO-K1 cells were pretreated with or without 1 μM Rad for 1 h at 37°C. Cells were further incubated with the indicated concentrations of PA_{63} + LF_NDTA for 2 h at 37°C. Afterwards, the medium was replaced with L-leucine-free medium supplemented with [^{14}C]-leucine to monitor protein synthesis. After 1 h incubation at 37°C, cells were washed three times with cold PBS and lysed by 0.1% SDS. Proteins were precipitated by 10% trichloroacetic acid and the amount of incorporated [^{14}C]-leucine was measured by filter assay and liquid scintillation counting.

CsA studies. CHO-K1 cells were incubated with or without 1 M CsA for 30 min at 37°C, after which time PA_{83} and LF_NDTA were each added to a final concentration of 10 nM. As a control, buffer alone was added. Cells were allowed to incubate for an additional 4 h at 37°C. At this time, the buffer was removed and replaced with leucine-free media supplemented with [^{3}H]-leucine. Cells were incubated for an additional 1 h at 37°C, at which time the media was removed, cells were washed twice with PBS and trypsin was added to lift the cells from the plate. Cells were transferred to vials containing scintillation fluid, and the radioactivity was measured using scintillation counting.

ADP-ribosylation of EF-2 by LF_NDTA in a cell-free system

CHO-K1 lysate (30 μg of protein) was incubated for 30 min at 37°C with 20 μM CsA or 10 μM Rad or anti-CypA antibody (2 μl). As a control, cell lysate was incubated with buffer only. After 30 min, a final concentration of 1 nM LF_NDTA was added together with 10 μM biotin-labelled NAD^+ and incubated for 10 min at 37°C. Samples were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane. ADP-ribosylated, i.e. biotin-labelled, EF-2 was detected with streptavidin-peroxidase.

Detection of LF_NDTA in the cytosolic fractions of CHO-K1 cells

The cytosolic fraction from cultured CHO-K1 cells was obtained by treatment of cells with digitonin according to the method originally described by Wiedlocha *et al.* (Wiedlocha *et al.*, 2005; Kaiser *et al.*, 2009). CHO-K1 cells, grown in 12-well plates, were pre-incubated at 37°C with CsA (20 μM) for 30 min. Control cells were incubated without any inhibitor. Subsequently, the indicated amounts of PA_{63} and biotin-labelled LF_NDTA were applied to cells, which were incubated for an additional 1.5 h with toxin and the inhibitors. For a control, cells were incubated without PA_{63} and LF_NDTA. Afterwards, cells were washed twice with ice-cold PBS to remove unbound toxin from the surface, then they 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 for an additional 30 min at 4°C to allow extraction of the cytosolic proteins, including biotin-labelled LF_NDTA. The supernatant was collected, equal amounts of protein were subjected to SDS-PAGE and biotin-labelled LF_NDTA was detected with streptavidin-peroxidase using the ECL system. To confirm equal amounts of protein, immunoblot analysis with antibodies raised against MEK1 was performed. The absence of early endosomes in the cytosolic fraction was confirmed in an immunoblot with an antibody raised against EEA1, a marker protein for early endosomes. Intensity of the biotin-labelled LF_NDTA was determined by densitometry using the Adobe Photoshop 7.0 software.

In vitro *translocation of LF_NDTA from enriched endosomal vesicles*

The partial purification of toxin-preloaded endosomal vesicles was performed according to a modified flotation protocol from Lemichez *et al.* (Lemichez *et al.*, 1997). In brief, CHO-K1 cells were seeded on 10 cm culture dishes. Cells were carefully scraped off from 30 dishes, collected and incubated in 6 ml cell culture medium for 2 h with PA_{83} (10 nM) plus LF_NDTA (10 nM) in the presence of Baf A1 (100 nM). Then the toxin-containing medium was removed and fresh toxin-free medium containing Baf A1 was added to the cells for an additional hour. Cells were washed and resuspended in 1 ml 11.4% sucrose buffer and discontinuous sucrose density gradient ultracentrifugation was performed with a TH461 rotor at 31 000 r.p.m. for 2 h at 4°C. The phase containing early endosomes was collected as a pellet by ultracentrifugation (70 000 r.p.m., 20 min, 4°C). The pellet was resuspended in 100 μl translocation buffer (100 mM Tris-HCl pH 7.4, 25 mM EDTA, complete protease inhibitor, 5 mM ATP and 5 mM $MgCl_2$).

Translocation of LF_NDTA from the enriched endosomal vesicles was performed according to the method of Tamayo *et al.* (Tamayo *et al.*, 2008) with modifications. Toxin-loaded endosomes (5 μl) were incubated for 30 min at 37°C together with freshly prepared CHO-K1 cytosol (40 μg of protein) in 20 μl translocation buffer containing biotin-NAD$^+$ (10 μM). To test the influence of Cyps or Hsp90 on the translocation of LF_NDTA from the endosomal vesicles into the cytosol, aliquots from the cytosol were incubated for 30 min at 37°C together with 20 μM CsA or 20 μM Rad before application upon the toxin-containing endosomal vesicles. To confirm the influence of CypA on the translocation of LF_NDTA, the cytosol was incubated for 30 min at 37°C together with 4 μl of anti-CypA antibody (Calbiochem) before addition of the cytosol to toxin-containing endosomes. Thereafter, the supernatant (cytosol) and the pellet fraction (endosomal vesicles) were separated by ultracentrifugation (70 000 r.p.m., 20 min, 4°C). The cytosolic supernatant containing biotin-labelled, ADP-ribosylated, EF-2 was subjected to SDS-PAGE and biotin-labelled EF-2 was detected on a nitrocellulose membrane using streptavidin-peroxidase and the ECL system.

As controls, heat-inactivated cytosol (30 min 95°C) and translocation buffer (without cytosol and without biotin-NAD$^+$) were applied to the endosomes. In these samples the endosomes were collected as pellets after an incubation of 30 min at 37°C by ultracentrifugation (70 000 r.p.m., 20 min, 4°C). The supernatant was subsequently incubated with fresh CHO-K1 lysate plus biotin-NAD$^+$ (30 min, 37°C), and subjected to SDS-PAGE and subsequent immunoblot analysis. The biotin-labelled EF-2 was detected by using streptavidin-peroxidase and the ECL system.

Equal amounts of protein from the cytosolic fractions were confirmed by immunoblot analysis with an anti-MEK1 antibody. Intensity of the biotin-labelled LF_NDTA was determined by densitometry using the Adobe Photoshop 7.0 software.

SDS-PAGE and immunoblot analysis. For immunoblot analysis, equal amounts of protein were subjected to SDS-PAGE according to the method of Laemmli (Laemmli, 1970). Subsequently, the proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany). The membrane was blocked for 30 min with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T). For the detection of the different biotin-labelled substrate molecules (LF_NDTA or EF-2), the samples were probed with streptavidin-peroxidase. Subsequently, the membrane was washed and proteins visualized using a chemiluminescence (ECL) system according to the manufacturer's instructions. For the detection of actin as a loading-control, the samples were probed with a mouse monoclonal anti-β-actin antibody (clone AC-15; Sigma-Aldrich). After washing with PBS-T, the membrane was incubated for 1 h with an anti-mouse antibody coupled to horseradish-peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany). The membrane was washed and the proteins visualized in a subsequent chemiluminescence reaction. For detection of cleaved MEK1, an antibody from Santa Cruz Biotechnology was taken. Uncleaved MEK1 was detected using a monoclonal antibody from Nanotools.

Dot-blot analysis of the interaction between immobilized Hsp90 and CypA with LF_NDTA. Different amounts of Hsp90 and CypA were vacuum aspirated onto a nitrocellulose membrane using a dot-blot system (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. Subsequently the membrane was blocked for 1 h with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T). The membrane was incubated with the biotinylated proteins LF_NDTA, LF, DTA and DTA-E148S (200 ng ml^{-1} each), washed three times with PBS-T (5 min each) and the bound biotinylated proteins were detected with streptavidin-peroxidase using the ECL system.

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

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