Role of Host Cell Chaperones in Cellular Uptake of *Clostridium Botulinum* C2 Toxin

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Abstract: The binary C2 toxin from *Clostridium botulinum* consists of two separate proteins: the transport component C2IIa delivers the enzyme component C2I into the cytosol of eukaryotic host cells. In the cytosol, C2I mono-ADP-ribosylates actin, thereby inducing depolymerization of actin filaments resulting in delayed caspase-dependent cell death. The sophisticated cellular uptake mechanism of C2 toxin, in particular our new results regarding the role of host cell chaperones and protein-folding helper enzymes during intracellular membrane translocation of C2I, are focused upon in this minireview.

We discovered earlier that translocation of C2I across endosomal membranes in mammalian cells depends on the chaperone activity of the heat shock protein Hsp90. Recently we have demonstrated that cyclosporin A (CsA), an inhibitor of peptidyl-prolyl *cis/trans* isomerase (PPIase) activity of cyclophilins, inhibited intoxication of various mammalian cell lines with C2 toxin. The underlying reason for this effect was the prevented uptake of C2I into the host cell cytosol. CsA, as well as a specific antibody against cyclophilin A, blocked the pH-dependent translocation of C2I-ADP-ribosyltransferase activity across membranes of intact cells and of partially-purified early endosomes *in vitro*. In conclusion, our results imply that the activities of Hsp90 and cyclophilin A are crucial for translocation of the C2I ADP-ribosyltransferase from early endosomes into the cytosol of mammalian cells. This is the first observation that a host cell PPIase, in concert with a heat shock protein, facilitates intracellular membrane translocation of a bacterial protein toxin.

Keywords: C2 toxin, ADP-ribosyltransferase, hsp90, cyclophilin A, cyclosporin A.

GENERAL MODE OF ACTION OF BACTERIAL AB-TYPE EXOTOXINS

Bacterial exotoxins are the causative agents for severe human and animal diseases. Most of these protein toxins are clearly associated with a particular disease as well as a certain bacterium, which produces the toxin. Exotoxins from the AB-type are extremely potent virulence factors due to their ability to enter mammalian cells and to act as enzymes in the host cell cytosol. AB-type toxins contain functionally different domains: the B- (binding) domain binds to its cellular receptor and mediates endocytosis of the toxin and the A- (enzymatic active) domain modifies its specific substrate molecules in the cytosol. To reach the cytosol, the A-domain has to translocate across intracellular membranes and in many AB-toxins, this step is facilitated by the B-domain.

It was discovered that bacterial AB-type toxins exploit intracellular vesicular protein traffic pathways to deliver their A-domain into the host cell cytosol [1-4]. The toxins can be divided into two major groups, depending on which intracellular route they take. "Short-trip toxins", such as diphtheria or anthrax toxins [5-7], translocate from the lumen of acidified endosomes into the cytosol while "long-trip toxins" (i. e. cholera toxin [8, 9]) undergo a retrograde vesicular transport through their target cells and deliver their A-domain from the endoplasmic reticulum into the cytosol.

BINARY ACTIN-ADP-RIBOSYLATING TOXINS

Most often the A- and B-domains are located on the same polypeptide chain; however, there are the so-called binary toxins from *Bacillus* and *Clostridium* species that have the A- and B-domains located on two different non-linked proteins, commonly called components. The single components are individually not toxic when applied to cells or animals. The A- and B-components of binary toxins must assemble to form a biologically functional toxin complex, which is internalized into and intoxicates eukaryotic cells. Apart from the two toxins produced by *Bacillus anthracis*, lethal toxin and edema toxin, members of the family of actin-ADP-ribosylating toxins possess this particular binary structure (for review see [10]).

The family of binary actin-ADP-ribosylating toxins is comprised of the *Clostridium botulinum* C2 toxin, *C. perfringens* iota toxin, *C. difficile* toxin (CDT), *C. spiroforme* toxin, as well as the vegetative insecticidal proteins (VIP) from *Bacillus cereus*. The enzyme components of these toxins are mono-ADP-ribosyltransferases that covalently transfer an ADP-ribose moiety from NAD^+ onto arginine-177 of G-actin. This modification induces depolymerization of actin filaments and complete destruction of the actin cytoskeleton in eukaryotic cells. Recently, we have discovered that these events finally result in delayed caspase-dependent apoptosis of epithelial cells and macrophages [11].

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CLOSTRIDIUM BOTULINUM C2 TOXIN

In 1980, Ohishi *et al.* discovered the binary nature of the C2 toxin [12], which is produced by *C. botulinum* types C and D. Production of C2 toxin correlates with spore formation by the bacteria [13]. C2 toxin consists of enzyme component C2I and binding/translocation component C2II [12], which must interact in a synergistic manner to exhibit a cytotoxic effect [14]. In 1986, Aktories and co-workers demonstrated that the ADP-ribosyltransferase C2I selectively mono-ADP-ribosylates G-actin at arginine-177 [15]. Based on this pioneering work, the new toxin family of binary bacterial actin-ADP-ribosylating toxins was introduced. Later, Barth and co-workers unravelled the cellular uptake mechanism of C2 toxin and took advantage of the binary toxin to deliver foreign cargo proteins into the cytosol of eukaryotic cells [16]. In past years, cell-permeable C2 fusion toxins have been used as valuable tools in pharmacological studies with intact mammalian cells [17].

Although isolated C2 toxin is a very potent enterotoxin that kills mice, rats, guinea pigs, and chickens within 1 hour after application of 1-2 pmoles [14] and causes necrotic, hemorrhagic lesions in the intestinal wall [18], the role of C2 toxin in disease is still not known. All strains of *C. botulinum* that synthesize C2 toxin also produce the extremely potent neurotoxins, which are the causative agents of the very severe disease botulism. Thus, clinical symptoms caused by the neurotoxins dominate the disease and symptoms caused by C2 toxins might not be recognized in these patients.

The enzyme component C2I (49.3 kDa) harbours mono-ADP-ribosyltransferase activity. The catalytic site of C2I, containing highly conserved amino acid residues, found among all bacterial mono-ADP-ribosyltransferases [19], is located in the C-terminal domain of the protein. The N-terminal domain of C2I (C2IN, amino acid residues 1-225) interacts with the activated transport component C2IIa and mediates translocation of the C2I protein into the host cell cytosol [20]. Thus, the enzyme inactive C2IN domain was used as a molecular adaptor for the C2IIa-mediated transport of foreign cargo proteins into cells.

The transport component C2II (80.8 kDa) requires nicking to become biologically active [21]. Proteolytic activation of C2II occurs between residues Lys181 and Ala182 [22]. Nicked C2IIa rapidly and spontaneously forms ring-shaped heptamers (~ 420 kDa), [23]. The structure of C2IIa revealed that the narrowest diameter of the hepatmers is 2.7 nm [24]. Importantly, only C2IIa but not C2II can bind to the cell surface and to C2I [23, 25].

The C-terminal region of C2IIa binds to Asn-linked complex and hybrid carbohydrates, which are present on the surface of all yet tested mammalian cell types, explaining the sensitivity of all cell types to C2 toxin [26].

INTERNALIZATION AND MEMBRANE TRANSLOCATION OF C2 TOXIN

The first steps during internalization of C2 toxin include formation of the C2IIa/C2I complex and binding to its cellular receptor. It is supposed that three molecules of C2I might assemble with one C2IIa heptamer [27]. Interestingly, either the pre-formed C2IIa/C2I complex can bind to the receptor or, C2IIa binds to the receptor first, followed by an assembly of C2I to now cell-bound C2IIa. It has also been suggested that toxin complex formation prior to receptor binding might enhance C2 toxin efficiency when low concentrations of the toxin are applied to cells.

As seen in Fig. (**1**), receptor-bound C2IIa/C2I complexes are internalized by receptor-mediated endocytosis and transported by exploiting the vesicular protein traffic system of host cells. C2I translocates from early acidic endosomes into the cytosol and this step is facilitated by C2IIa. Acidification of the endosomal lumen triggers a major conformational change of C2IIa. As a consequence, C2IIa heptamers expose hydrophobic residues on their surface [28], insert into the membranes of the endosomes and thereby form pores. Importantly, membrane insertion and pore formation of C2IIa is absolutely necessary for translocation of C2I across endosomal membranes [28].

The C2IIa translocation pore is between 27 and 32 Å wide and more than 70 Å long, implying that the pore lumen is too narrow to allow passage of C2I [24]. Therefore, C2I translocates in a partially unfolded conformation through the lumen of C2IIa pores. This was discovered by using a dihydrofolate reductase (DHFR)-C2I fusion protein [29]. In the presence of methotrexate (MTX), which binds to the DHFR portion of that chimera, C2IIa-dependent translocation of the fusion toxin was reduced. DHFR has a linear conformation but after binding of its substrate MTX, DHFR is stabilized into a tightly folded conformation and thus can not efficiently pass through the narrow lumen of C2IIa pores. These findings strongly suggested that C2I must unfold for translocation through C2IIa pores and thus the question arose, how was the ADP-ribosyltransferase activity of C2I recovered in the host cell cytosol following membrane translocation? Thus, we have investigated whether host cell chaperones and/or protein-folding helper enzymes were involved in membrane translocation and/or refolding of the ADP-ribosyltransferase C2I in the cytosol of mammalian cells.

ROLE OF HOST CELL CHAPERONES AND PPIASES DURING INTERNALIZATION OF C2 TOXIN

In 2003, participation of the cellular chaperon heat shock protein (Hsp) 90 on the cellular uptake of bacterial toxins was described for the first time. Ratts *et al.* demonstrated that Hsp90 mediates the membrane translocation of diphtheria toxin [30]. At the same time it was shown by our group that Hsp90 is decisively involved in the translocation of C2I [31]. Pharmacological Hsp90 inhibitors, radicicol (Rad) or geldanamycin (GA), prevented uptake of C2I into the cytosol of mammalian cells and ADP-ribosylation of actin in these cells. We could exclude an influence of the inhibitors on the enzymatic activity of C2I, receptor binding, or endocytosis. The inhibitors prevented escape of C2I from early endosomes into the cytosol [31], showing that Hsp90 is decisively involved in the membrane translocation of C2I. The same was also shown for iota a, the enzyme component of the binary Iota toxin produced by *Clostridium perfringens*, which also ADP-ribosylates G-actin [32].

Recently we could show that membrane translocation of C2I from early acidic endosomes is also dependent on cyclophilin A (CyPA) [33], which is a protein-folding helper

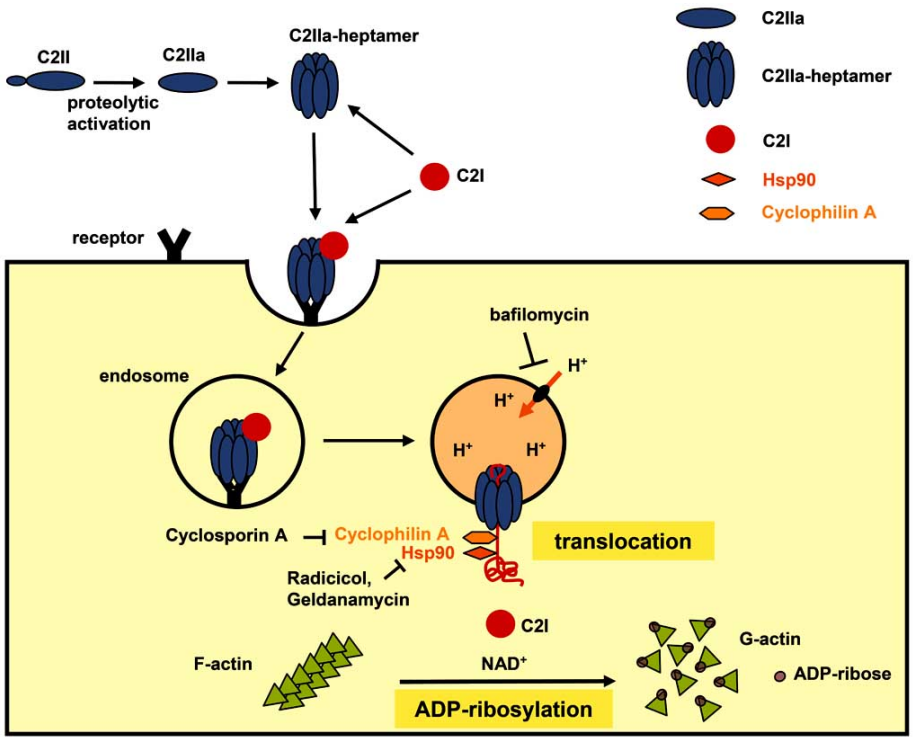

Fig. (1). Current model of the cellular uptake of *C. botulinum* C2 toxin. After binding of toxin components to the receptor and endocytosis, translocation of the enzyme component C2I from early acidic endosomes occurs to the cytosol. C2I then ADP-ribosylates actin leading to depolymerisation of the actin filaments. Translocation of C2I requires the C2IIa pore in the endosomal membrane, as well as the activity of Hsp90 and the prolyl isomerase cylophilin A. Hence, the specific inhibitors cyclosporin A and radicicol or geldanamycin inhibit this step.

enzyme. Cyclophilins are peptiyl/prolyl *cis/trans* isomerases (PPIases) which accelerate the *cis/trans* isomerization of peptide bonds after proline residues (for review see [34]).

Cyclosporin A (CsA) is a specific pharmacological inhibitor of isomerase activity of cyclophilins [35]. It prevents the intoxication of different epithelial cell lines (HeLa, Vero, CaCo-2) with C2 toxin in a time- and concentration- dependent manner (Fig. **2A, B**). In the presence of CsA, the amount of ADP-ribosylated actin in the cells was strongly reduced. CsA did not influence ADP-ribosyltransferase activity of C2I, binding of the toxin to the cell surface, or endocytosis. However, CsA inhibited uptake of C2I into the cytosol [33].

The reason for such results is the CsA-provided inhibition of the membrane translocation of C2I from early acidic endosomes (Fig. **2C**). In addition to Hsp90, cyclophilins are decisively involved in translocating C2I across membranes of early acidic endosomes into the cytosol. In addition, combined application of the inhibitors Rad and CsA showed that cyclophilins and Hsp90 work synergistically [33].

The role of host cell proteins regarding membrane translocation of C2I was characterized *in vitro* with the help of isolated early endosomes (33). If C2 toxin-loaded early endosomes were incubated with fresh cytosol, C2I was released from these endosomes. This could be shown by the ADP-ribosylation of actin [33]. If cytosol was pretreated with CsA before addition to the endosomes, translocation of C2I was not detectable [33]. Translocation of C2I from the endosomes was also prevented if cytosol was preincubated with a specific antibody against already defined CyPA (Fig. **3**). From these results we could conclude that CyPA is necessary for membrane translocation of C2I. Pull down experiments could finally show that in intact cells the N-terminal domain of C2I interacts with CyPA [33]. This domain mediates translocation of C2I through the C2IIa pore.

OVERVIEW: PARTICIPATION OF CYTOSOLIC PROTEINS ON UPTAKE OF BACTERIAL TOXINS

Not much is known about the involvement of cytosolic factors on the uptake of bacterial toxins into their host cells. To our knowledge there have been six publications regarding this topic. In 1997 Lemichez *et al.* showed for the first time that a host cell protein (β-COP) participates in the uptake of a bacterial toxin (i.e. diphtheria toxin) [36]. In 2003 the knowledge about diphtheria toxin was extended, and it was shown that Hsp90 and thioredoxin reductase are also involved in the translocation process of this toxin [30]. For anthrax lethal factor it was shown in 2008 that β-COP facilitates translocation across vesicular membranes [37]. Last but not least there has been the work of our own group, showing the participation of Hsp90 on the uptake of C2 and iota toxins [31, 32]. Most recently, as described in this review, the participation of cyclophilin A on membrane translocation of C2I is also important [33]. We expect more research in the years ahead on this interesting topic, elucidating the ingenious mechanisms that bacterial toxins have evolved to exploit the cellular machinery of their host cells to mediate their own uptake.

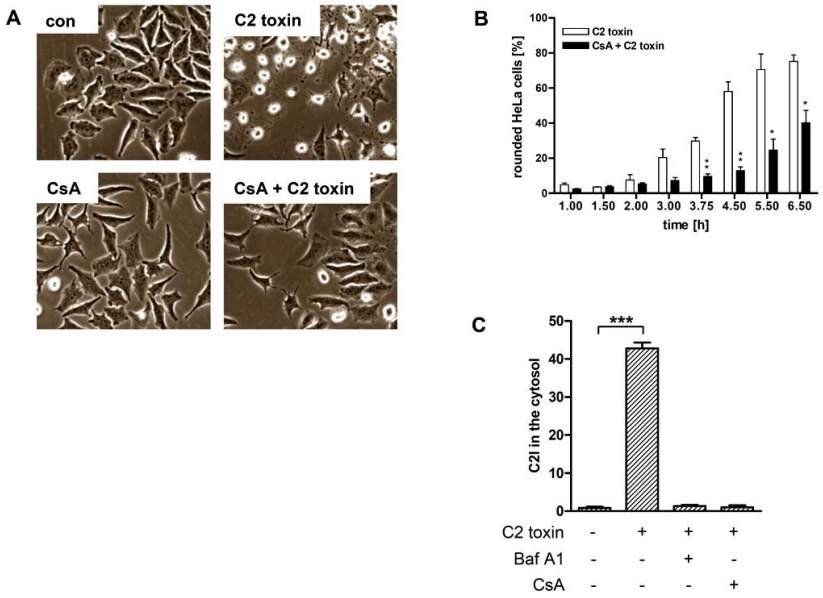

Fig. (2). Influence of CsA on intoxication of HeLa cells with the *C. botulinum* C2 toxin. **A.** Morphology of HeLa cells after a 135-minute incubation with C2 toxin in the absence or presence of CsA. **B.** Quantitative analysis of the C2 toxin-induced cell rounding in the absence or presence of CsA (n = 3 ± SD, significance determined with student t test). **C.** Detection of C2 toxin in the cytosolic fraction of HeLa cells which were incubated with toxin in the presence or absence of bafilomycin A1 (Baf) or cyclosporin A (CsA). n = 3 ± SD, with significance determined by a student t test.

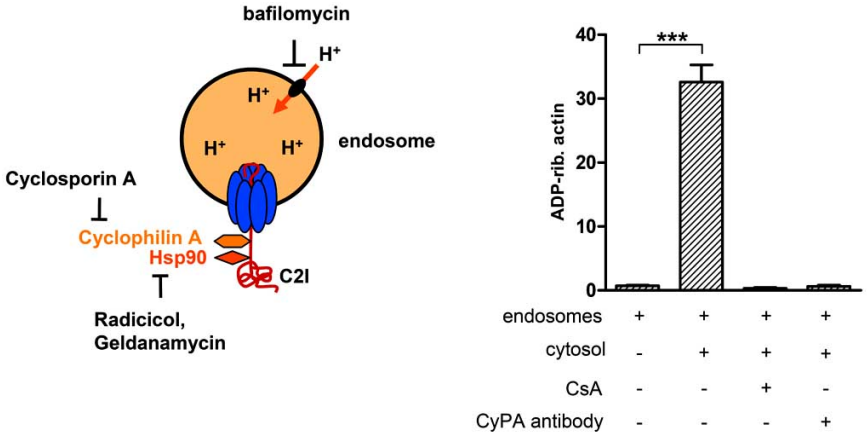

Fig. (3). The translocation of C2I from isolated early endosomes requires Hsp90 and cyclophilin A. The translocation of C2I from the C2 toxin-loaded endosomes was triggered by the addition of HeLa-cell cytosol. Cyclosporin A (CsA) or a specific antibody against cyclophilin A (CyPA) prevented the translocation of C2I. The presence of C2I in the cytosol was registered by ADP-ribosylation of actin. n = 3 ± SD, with significance determined by a student t test.

SUMMARY AND PERSPECTIVES

The results reported in this minireview show for the first time a participation of host cell PPIases on membrane translocation of a bacterial toxin and contribute basically to an understanding of the cellular uptake mechanisms of bacterial protein toxins.

The focus of our future work will be i) to prove whether additional PPIases, for instance FK506-binding proteins, are involved in membrane translocation of bacterial toxins, too ii) to investigate whether internalization of other bacterial toxins also depends on host cell chaperones and PPIases.

Finally, our results might be a potential basis for future clinical implications. The targeted pharmacological inhibition of specific chaperones or PPIases in mammalian cells could be therapeutically considered to restrain the internalization of certain bacterial toxins into body-cells.

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ABBREVIATIONS

CypA = Cyclophilin A;

Hsp90 = Heat shock protein 90;

CsA = Cyclosporin A;

Rad = Radicicol;

GA = Geldanamycin;

PPIases = Peptidyl/prolyl *cis/trans* isomerases

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