**The Host Cell Chaperone Hsp90 Is Essential for Translocation of the Binary *Clostridium botulinum* C2 Toxin into the Cytosol***

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*Clostridium botulinum* C2 toxin is the prototype of the binary actin-ADP-ribosylating toxins and consists of the binding component C2II and the enzyme component C2I. The activated binding component C2IIa forms heptamers, which bind to carbohydrates on the cell surface and interact with the enzyme component C2I. This toxin complex is taken up by receptor-mediated endocytosis. In acidic endosomes, heptameric C2IIa forms pores and mediates the translocation of C2I into the cytosol. We report that the heat shock protein (Hsp) 90-specific inhibitors, geldanamycin or radicicol, block intoxication by C2I. Geldanamycin did not affect toxin binding, endocytosis, and pore formation by C2IIa. The ADP-ribosylation of actin in the cytosol of toxin-treated cells revealed that less active C2I was translocated into the cytosol after treatment with Hsp90 inhibitors. Under cellular pH (pH 4.5), which allows the direct translocation of C2I via C2IIa heptamers across the cell membrane and form pores with an inner diameter of about 1–2 nm (10). Recently, we reported that this step could be mimicked on the cell surface, when cells were exposed to an acidic pulse after binding of both toxin components to the cell surface (10). It was proposed that at low pH C2IIa heptamers insert into the endosomal membrane and form pores with an inner diameter of about 1–2 nm (10). Similarly, C2IIa forms pores in cell membranes of intact cells under acidic conditions.2 The binding component C2I was not affected by Hsp90 inhibitors in vitro. The cytotoxic actions of the actin-ADP-ribosylating *Clostridium perfringens* iota toxin and the Rho-ADP-ribosylating C2-C3 fusion toxin was similarly blocked by Hsp90 inhibitors. In contrast, radicicol and geldanamycin had no effect on anthrax lethal toxin-induced cytotoxicity of J774-A1 macrophage-like cells or on cytotoxic effects of the glucosylating *Clostridium difficile* toxin B in Vero cells. The data indicate that Hsp90 is essential for the membrane translocation of ADP-ribosylating toxins delivered by C2II.2

Bacterial AB-toxins are composed of an enzyme domain (A) and a binding domain (B) that mediates cell binding and uptake of the A domain (1). In the family of binary toxins, the A and B domains are located on separated proteins, the enzyme component and binding component. Members of the family of binary actin-ADP-ribosylating toxins are the *Clostridium botulinum* C2 toxin (2), the *Clostridium perfringens* iota toxin (3), *Clostridium spiroforme* toxin (4, 5), *Clostridium difficile* ADP-ribosyltransferase (6), and the VIP toxins (vegetative insecticidal proteins) from *Bacillus cereus* (7). These toxins ADP-ribosylate G-actin at arginine-177 (2), a modification that leads to disassembly of actin filaments, breakdown of the actin cytoskeleton, and rounding up of cultured monolayer cells (8, 9). Over the past few years, the uptake mechanism of the C2 toxin from *C. botulinum* has been studied in detail (10). C2 toxin is composed of the enzyme component C2I (49 kDa) and the binding/translocation component C2II that delivers C2I into the cytosol of target cells. C2II (80 kDa) must be activated by trypsin cleavage (11). An ~20-kDa peptide is cleaved from the N terminus. The resulting C2IIa (~60 kDa) forms ring-shaped heptamers, which assemble with C2I and bind to complex and hybrid carbohydrate structures on the cell surface (12). The C2 toxin-receptor complex is taken up via receptor-mediated endocytosis, and C2I translocates from the early acidic endosomal compartment into the cytosol (10). This step can be blocked by bafilomycin A1, a specific inhibitor of the vesicular H+ ATPase (10). Recently, we reported that this step could be mimicked on the cell surface, when cells were exposed to an acidic pulse after binding of both toxin components to the cell surface (10). It was proposed that at low pH C2IIa heptamers insert into the endosomal membrane and form pores with an inner diameter of about 1–2 nm (10). Similarly, C2IIa forms pores in cell membranes of intact cells under acidic conditions.2 The binding component C2II has high homology to the protective antigen (PA) of anthrax toxins (13), and the uptake mechanism of anthrax toxins appears to be very similar to that of *C. botulinum* C2 toxin and the other actin-ADP-ribosylating toxins (14). Anthrax toxin is a tripartite toxin and consists of the binding component PA and of the enzyme components lethal factor (LF) and edema factor (EF), which are both unrelated to the enzyme component of C2 toxin. Whereas lethal factor LF is a metalloprotease that cleaves mitogen-activated protein kinases (15), edema factor is a calmodulin-dependent adenyl cyclase (16). It was first shown for anthrax toxin that the binding component (PA) forms heptamers and pores that are involved in the delivery of the anthrax enzymes EF and LF into the cytosol (17, 18). However, the precise mechanism of translocation across the endosomal membrane is not known for actin-ADP-ribosylating toxins or for anthrax toxin. One model suggests that the enzyme components are delivered through the pore formed by the binding components. Therefore, it may be expected that the

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† The abbreviations used are: C2I, enzyme component of *C. botulinum* C2 toxin; C2II, binding component of *C. botulinum* C2 toxin; C3, *C. limosum* C3-like exoenzyme; Hep, heat shock protein; LF, lethal factor from *B. anthracis*; PA, protective antigen from *B. anthracis*; TRITC, tetramethylrhodamine isothiocyanate; EF, edema factor; EBL, embryonic bovine lung cells; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

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enzyme components become partially unfolded during the translocation step and refolded in the cytosol. For LF, it was reported that unfolding of the protein was necessary for translocation (19). Recently we obtained similar results for C2I.2 These findings imply that refolding of proteins occurs in the cytosol and that Hsp90 is essential for cytotoxic action of C2 toxin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture medium was obtained from Biochrom (Berlin, Germany), fetal calf serum was from PAN Systems (Aidenbach, Germany), and cell culture materials were obtained from Falcon (Heidelberg, Germany), and cell culture materials were obtained from Falcon (Heidelberg, Germany). The components of C2 toxin (C2I, C2IS16C, C2II) and the components of iota toxin (Ia and Ib) were purified as recombinant glutathione S-transferase proteins as described previously (21, 22). C2II was activated with trypsin as described (10). Recombinant anthrax lethal factor and protective antigen were purchased from Quadratree (Surrey, UK). 

**Cell Culture and Cytotoxicity Assays**—African green monkey kidney (Vero) cells, HeLa, and J774.A1 macrophage-like cells were cultivated in Dulbecco’s modified Eagle’s medium containing 5% heat-inactivated (30 min, 56 °C) fetal calf serum at 37 °C and 5% CO2. Embryonic bovine lung (EBL) cells were grown in minimum Eagle’s medium containing...
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**RESULTS**

**Hsp90 Inhibitors Geldanamycin and Radicicol Protect Eukaryotic Cells from C2 Cytotoxic Effects**—We tested whether Hsp90 was necessary for the cytotoxic action of the C2 toxin on various eukaryotic cell lines. Therefore, Hsp90 was blocked by the specific inhibitors geldanamycin or radicicol. When African green monkey kidney (Vero) cells were treated with C2 toxin (50 ng/ml C2I and 100 ng/ml C2IIa) for 3 h, cells rounded up. When cells were pretreated for 1 h with geldanamycin (1 μM) or radicicol (1 μM) before C2 toxin addition, cell rounding was blocked (Fig. 1A). However, neither geldanamycin nor radicicol blocked C2 toxin effects completely but instead delayed intoxication of cells (Fig. 1B). Higher concentrations of geldanamycin (10 μM) protected Vero cells for more than 5 h from cytotoxic C2 toxin effects. The combination of geldanamycin plus radicicol showed similar effects as the individual components but did not act synergistically. The inhibitors alone did not influence cell morphology within 5 h (not shown).

Less actin was ADP-ribosylated in cells pretreated for 1 h with geldanamycin before C2 toxin addition, as detected by sequential ADP-ribosylation with C2I in the presence of [32P]NAD. Actin, previously ADP-ribosylated by C2I in intact cells, was no longer a substrate for in vitro ADP-ribosylation. The autoradiograph of [32P]ADP-ribosylated proteins was detected by autoradiography with a PhosphoImager from Molecular Dynamics/Amer sham Biosciences.

**Pore-forming Assay—**86Rb efflux experiments were performed as previously described (24). In brief, CHO cells were plated in 24-well culture plates and loaded with 86Rb (1 μCi/ml) for 20 h. C2IIa was added to serum-free medium, and cells were incubated on ice for 1 h to allow for toxin binding. Subsequently, the cells were washed twice with cold medium to remove unbound toxin. To initiate membrane insertion of the toxins, cells were treated with serum-free medium (pH 4.5 and for control pH 7.5) for 5 min at 37 °C. Cells were further incubated at 4 °C, and 86Rb release into the medium was determined by liquid scintillation counting.

**Binding of C2 Toxin by Flow Cytometry—**C2IS16C was labeled with Alexa-488, and binding of C2 toxin to CHO cells was determined as described earlier in detail (21). Cell-associated fluorescence was detected by using a FACSCalibur fluorescence-activated cytometer (BD Biosciences).

**ADP-ribosylation Assay—**Cells were incubated with [32P]NAD in the presence of C2I and C2IIa for 10 min, washed with PBS, and permeabilized with 0.1% (v/v) Triton X-100. Normal goat serum was used to block unspecific reactions. Immunocytochemistry—Cells were fixed with methanol (−20 °C) for 10 min, washed with PBS, and permeabilized with 0.1% (v/v) Triton X-100. Normal goat serum was used to block unspecific reactions.

**RESULTS**

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Inhibition of Hsp90 by geldanamycin also protected human HeLa cells and EBL cells from C2 toxin (data not shown).

We used the recently described C2-C3 fusion toxin (21) to test whether Hsp90 is essential for transport of the fusion toxins, which are based on C2IIa as a delivery system. In this fusion toxin, the C3 transferase from Clostridium limosum, which ADP-ribosylates and inhibits Rho GTPase (25), was fused to the C211-225 adaptor domain of C2I. C2I-C3 is transported into cells via C2IIa (26). Geldanamycin blocked intoxication of cells with the C2-C3 fusion toxin/C2IIa complex, as shown for EBL cells in Fig. 3. This observation suggests that the C211-225-C3 fusion toxin is delivered by the same mechanism into the cytosol as C2I, which also depends on Hsp90 for effective transport of active enzyme component into the cytosol.

Effects of Inhibitors of Hsp90 on Binding, Endocytosis, and Pore Formation of C2 Toxin—To elucidate the mechanisms underlying the inhibition of the cytotoxic action of C2 toxin by Hsp90, we studied the influence of geldanamycin pretreatment on various steps of the cellular uptake of C2 toxin. We tested whether geldanamycin influenced (i) binding of C2 toxin, (ii) endocytosis, (iii) pore formation of C2IIa, and/or (iv) the C2IIa-mediated translocation of C2I across the cell membrane.

First we used flow cytometry to analyze binding of fluorescently labeled C2I (C2I-Alexa-488) to C2IIa on Vero cells (21). Cells were treated with geldanamycin (10 μM) for 1 h at 37 °C and subsequently incubated at 4 °C with C2IIa plus C2I-Alexa-488. Under these conditions, geldanamycin did not decrease the binding of C2 toxin to the cells (Fig. 4A). To test whether geldanamycin blocked endocytotic mechanisms in general, Vero cells were treated with geldanamycin (10 μM) for 1 h, and thereafter, C. difficile toxin B was added. Toxin B inactivates small GTPases of the Rho family by glucosylation and is taken up into cells by a similar pathway as C2 toxin (i.e. the toxin is endocytosed and translocated from the acidic endosomal compartment into the cytosol) (24). Neither geldanamycin (10 μM) nor radicicol (1 μM), alone or in combination, inhibited intoxication of cells with C. difficile toxin B (not shown). These findings suggest that the Hsp90 inhibitors did not block the uptake of toxins by general inhibition of endocytosis. Next, we focused on the role of C2IIa and Hsp90 in translocation of C2I from acidic endosomes into the cytosol of cells. To address whether C2IIa pores are directly involved in translocation of C2I across the endosomal membrane, we mimicked the conditions of the acidic endosomes on the surface of intact cells. Recently, we reported that an acidic pulse on the cell surface is required for translocation of the acidic endosomes on the surface of intact cells. This observation suggests that the Hsp90 inhibitors did not block the uptake of toxins, which are based on C2IIa as a delivery system. In this fusion toxin, the C3 transferase from Clostridium limosum, which ADP-ribosylates and inhibits Rho GTPase (25), was fused to the C211-225 adaptor domain of C2I. C2I-C3 is transported into cells via C2IIa (26). Geldanamycin blocked intoxication of cells with the C2-C3 fusion toxin/C2IIa complex, as shown for EBL cells in Fig. 3. This observation suggests that the C211-225-C3 fusion toxin is delivered by the same mechanism into the cytosol as C2I, which also depends on Hsp90 for effective transport of active enzyme component into the cytosol.

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To exclude the possibility that proteolytic degradation of C2I is involved in the protective effect of the Hsp90 inhibitors, we tested the effect of the proteasome inhibitor MG-132. Vero cells were preincubated for 1 h with MG-132 (MG, 30 μM), and subsequently geldanamycin (1 μM) or radicicol (1 μM) was added for an additional hour. Then C2 toxin was added, and the cells were incubated at 37 °C in the presence of the inhibitors. After 3 h of toxin treatment pictures were taken. MG-132 did not prevent the effects of Hsp90 inhibitors (Fig. 5).

Inhibition of Hsp90 Blocks Translocation of Active C2I ADP-ribose transferase into the Cytosol—If C2I is partially unfolded during translocation through the C2IIa pore, Hsp90 might be essential for proper folding and recovery of enzyme activity of C2I in the cytosol. We tested whether pretreatment of cells with geldanamycin influenced the direct delivery of C2I via C2IIa across the cell membrane induced by an extracellular acidic pulse. For these experiments, cells were preincubated with bafilomycin to block normal uptake of C2 toxin. Cells were incubated for 1 h at 4 °C with C2 toxin in the presence of the inhibitors to allow binding of the toxin on the cell surface. The cells were then shifted to warm acidic medium (pH 4.5, 37 °C)
to induce insertion of C2IIa pores into cell membranes and to allow C2IIa-mediated translocation of C2I into the cytosol. After 5 min, the acidic medium was replaced with neutral medium (37 °C, containing bafilomycin A1 (Baf) and geldanamycin (GA), and cells were further incubated at 37 °C. After 2–4 h, fewer cells exhibited C2 toxin-induced cell rounding when they were treated with geldanamycin compared with control cells (Fig. 6A). Fig. 6B shows the percentage of rounded cells obtained from these experiments.

Next, we made use of the ADP-ribosyltransferase activity of C2I to determine whether less active enzyme was delivered into the cytosol after treatment with inhibitors of Hsp90. After toxin binding and acidic pulse, lysates of Vero cells that were treated with C2 toxin (in the presence or absence of Hsp90 inhibitor) were analyzed by sequential ADP-ribosylation of actin. In this assay, only actin, which was not ADP-ribosylated by C2 toxin in intact cells, served as a substrate for in vitro ADP-ribosylation with [32P]NAD as a cosubstrate. Radiolabeled (i.e., ADP-ribosylated) actin was determined by autoradiography. More [32P]ADP-ribosylated actin was detected when cells were pretreated with geldanamycin (10 μM) or radicicol (1 μM) (Fig. 6C). These results indicated that Hsp90 inhibitors decreased C2IIa-mediated delivery of active C2I ADP-ribosyltransferase from the cell surface into the cytosol directly across cell membranes induced by an extracellular acidic pulse.

**Inhibitors of Hsp90 Prevent Translocation of C2I into the Cytosol**—For intracellular detection of C2I by confocal microscopy, we used rat astrocytes because these cells are larger and show a clearer distribution of cell compartments than Vero cells. When rat astrocytes were treated with C2 toxin (50 ng/ml C2I and 100 ng/ml C2IIa) for 6 h, cells rounded up, and the actin cytoskeleton was destroyed (Fig. 7A). When cells were pretreated for 1 h with geldanamycin (1 μM) or radicicol (1 μM) before C2 toxin addition, cell rounding was blocked (data not shown). As was shown for Vero cells, geldanamycin or radicicol did not block C2 toxin action completely but delayed intoxication of the rat astrocytes.

Next, astrocytes were treated with C2 toxin (50 ng/ml C2I and 100 ng/ml C2IIa) for 2, 4, or 6 h and stained with an antibody against C2I. We detected the C2I toxin in endosomal-like compartments after 2 h. These compartments fused after 4 h, and a clearly detectable, diffuse cytosolic localization of C2I was observed by 6 h (arrow in Fig. 7B). Because of the destruction of the actin cytoskeleton by C2 toxin, the endosomes fused more easily than in the groups pretreated with geldanamycin, radicicol, or bafilomycin alone. When cells were pretreated for 1 h with geldanamycin (1 μM) or radicicol (1 μM) before C2 toxin addition for 6 h, there was less fusion of the endosomal-like compartments, and no C2I was detectable in the cytosol (Fig. 7B; a single astrocyte is shown in each picture). No cross-reactivity of the anti-C2I antibody with the C2IIa component was detectable (data not shown). To demonstrate that these endosomal-like structures were endosomes, we pretreated the astrocytes for 1 h with bafilomycin A1 (100 nM) before the addition of C2 toxin and detected the intracellular distribution of C2I (Fig. 7B). Bafilomycin A1 blocks the vesicular H⁺-ATPase in the endosomal membranes and thereby prevents acidification of endosomes and translocation of C2I toxin from endosomes into the cytosol (10). Colocalization studies of C2I with the early endosomal marker Rab5 in cells that were pretreated with bafilomycin A1 (i.e., C2I was trapped in endosomes) or radicicol revealed a colocalization of C2I toxin with Rab5 (Fig. 8). This finding suggests that treatment of cells with Hsp90 inhibitors trapped C2I in endosomes. Next, we used the Metamorph software to measure the intensity of the C2I toxin staining in the endosomal compartments and compared it to its intensity in the cytosol. The intensity in the endosomes was set as 100%. Compared with the endosomes, the intensity of C2I toxin in the cytosol of the treated cells was 1.7 ± 0.7% after 2 h, 3.7 ± 1.6% after 4 h, and 19.5 ± 7.7% after 6 h. Geldanamycin (2.3 ± 0.6%), radicicol (2.5 ± 0.6%), or bafilomycin (2.1 ± 0.8%) blocked this increase in intensity of C2I toxin staining after 6 h (mean ± S.E.; n = 10).

**Effect of Inhibitors of Hsp90 on Iota Toxin and on Anthrax Lethal Toxin**—Based on our results with the C2 toxin, we tested whether inhibition of Hsp90 might influence the cytotoxic effects of *C. perfringens* iota toxin, another member of the family of binary actin-ADP-ribosylating toxins. As was found for C2 toxin, pretreatment of cells with geldanamycin diminished intoxication of cultured Vero cells by iota toxin. Next, we studied whether Hsp90 is also required for delivery of active LF or EF, respectively, into the cytosol of target cells. For this purpose, we tested whether Hsp90 inhibitors had any effect on the cytotoxic action of anthrax lethal toxin (PA +
LF) on J774.A1 macrophage-like cells. Cells were pretreated with geldanamycin or radicicol. Bafilomycin A1, which is known to protect J774.A1 cells from anthrax lethal toxin, was used as a control. Subsequently, the cells were incubated for another 3 h with PA plus LF in the presence of the inhibitors, pictures were taken, and cell viability was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt-cytotoxicity assay (27). Cells treated with PA plus LF showed dramatic morphological alterations, but neither geldanamycin nor radicicol had any protective effect (Fig. 9A). In contrast, bafilomycin A1 protected cells completely from the cytotoxic effects of lethal factor. The morphological observation was confirmed by the cytotoxicity assay. In contrast to bafilomycin A1, pretreatment of cells with geldanamycin or radicicol before lethal factor did not protect cells from the cytotoxic action of lethal factor (Fig. 9B).

Taken together, our findings demonstrate that inhibition of Hsp90 by geldanamycin or radicicol blocked cytotoxic effects of the binary C. botulinum C2 toxin most likely by preventing translocation and/or refolding of the enzymatic component C2I into an active form. However, these inhibitors had no effect on
the cytotoxic action of the anthrax toxin (PA plus LF) on J774.A1 cells.

DISCUSSION

Substantial work has been done to study the underlying mechanisms by which bacterial AB toxins deliver their enzymatically active domain across membranes into the cytosol of host cells. For some toxins, it appears that pore formation by the binding/translocation domain plays an essential role for translocation of the active domain into the cytosol. If the enzyme domain of a toxin translocates through the pores, a partial unfolding of these proteins is expected. For example, it was reported recently that the enzymatically active light chain (H1101150 kDa) of C. botulinum neurotoxin (BoNT) translocates across artificial lipid membranes, most likely through channels that are formed by the heavy chain (28). Moreover, it was shown that acidic conditions, which are essential for translocation, induced unfolding of the light chain (28).

However, the precise mechanism by which binary toxins deliver their enzyme components into the host cell cytosol is still unclear. It was shown for anthrax toxin (29) as well as for the family of actin-ADP-ribosylating toxins (10, 30, 31) that the binding components form pores in artificial lipid membranes. These pores were proposed to interact with the enzyme components (31, 32) and to be involved in their translocation. Moreover, it was reported that anthrax lethal factor (LF) must be unfolded for translocation (19), and we have obtained similar results for C2I.2

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Presumably, the unfolded enzyme components translocate across the endosomal membrane through the pore that is formed by the binding components. So far it is not clear which molecular mechanisms are involved in unfolding, translocation into the cytosol or refolding of the enzyme in the cytosol.

Here, we report that radicicol and geldanamycin, both of which are specific inhibitors of the heat shock protein Hsp90, protected various cell types from the cytotoxic effects of C2 toxin. Radicicol and geldanamycin both block the intrinsic ATPase activity of Hsp90. To determine the mode and localization of action of the Hsp90 inhibitors in protecting cells against C2 toxin, we studied the various steps involved in the intoxication process of C2 toxin. First, by using Alexa-labeled C2I, we ruled out that the Hsp90 inhibitors affected the binding of the toxin to the target cell membrane. Second, we studied whether the inhibitors interfered with endocytosis, which is a prerequisite of cellular toxin uptake after receptor-binding. For this purpose, the effects of Hsp90 inhibitors on the cellular uptake of the glucosylating C. difficile toxin B was tested. This toxin is endocytosed after receptor binding and also enters the cytosol from acidic endosomes (24). Because neither geldanamycin nor...
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radicicol blocked the action of toxin B, it is unlikely that the Hsp90 inhibitors caused a general blockade of endocytosis. The third step of the uptake process of toxins is the insertion into the membrane of acidic endosomes and subsequent pore formation. To exclude any effects of the Hsp90 inhibitors at this level of the intoxication process, we tested the effect of the compounds on toxin-mediated $^{86}$Rb$^+$ release. By exploiting the recent observation that pore formation by C2 toxin can be mimicked at the cell surface by a short term acidic shift of the culture medium (10), we were able to measure the channel function of the binding component of C2 toxin after membrane insertion. Again, the Hsp90 inhibitors had no effect on this process. All together, these findings showed that inhibition of Hsp90 did not affect binding, endocytosis, or pore formation of C2 toxin and suggested that the inhibitors acted either on the translocation process or on the cytosolic activity of C2I. The latter possibility was excluded by demonstrating that Hsp90 inhibitors did not affect C2I-mediated ADP-ribosylation of actin in vitro. Moreover, the proteasome inhibitor MG132 had no effect, excluding the possibility of enhanced removal of C2 toxin through proteasomal degradation. Thus, our data indicated that the Hsp90 inhibitors affected the translocation step of the toxin, thereby blocking the accumulation of active toxin (C2I) in the cytosol. This hypothesis is supported by studies using the direct pH-induced translocation of C2I across cytoplasmic membranes into the cytosol. Accordingly, we observed that less actin was ADP-ribosylated in cells pretreated with Hsp90 inhibitor, suggesting that less active C2I was delivered into the cytosol of target cells when Hsp90 was inhibited by geldanamycin or radicicol. Moreover, the Hsp90 inhibitors blocked the appearance of C2I in the cytosol, as determined by anti-C2I antibody and fluorescence microscopy.

The function of Hsp90 in translocation of C2I remains unclear. Hsp90 has been shown to interact with several “client” proteins, including several cytoplasmic receptors and kinases. Many of the Hsp90 targets appear to be signaling proteins, and it is suggested that the interaction occurs at a rather late stage during protein refolding. Moreover, the function of Hsp90 depends on the dynamic formation of heteroprotein complexes. The best established partners of Hsp90 are Hsp70, Hop, Hsp40, and p23 (20, 33). Therefore, it is likely that the action of Hsp90 in facilitating the transport of C2I also depends on the cooperation of a chaperone complex. This hypothesis is supported by recent data reported by Ratts et al. (34), in which they attempted to identify cytosolic factors necessary for the translocation of diphtheria toxin from endosomes into the cytosol. Using a cell-free system with isolated endosomes, they observed that a cytosolic translocation factor complex was essential for the translocation of the enzyme domain of diphtheria toxin across membranes. When this cytosolic translocation factor complex from human T cells was analyzed, Hsp90 and thioredoxin reductase (TrR-1) were identified. They observed that Hsp90 and TrR-1 are necessary but not sufficient for translocation, which implies the involvement of other essential factors. Moreover, they observed that the Hsp90 inhibitors geldanamycin and radicicol inhibited the translocation of the enzyme domain of diphtheria toxin. However, in contrast to our findings, they reported that neither geldanamycin nor radicicol blocked the translocation of toxin when applied alone but did inhibit the process when added together (34). This discrepancy with our findings might be explained by the different properties of the two toxins studied and by the different models used. Similar to that observed with C2 toxin, we found that the Hsp90 inhibitors geldanamycin and radicicol retarded the cytotoxic effects of C. perfringens iota toxin. Iota toxin is highly related to C2 toxin and shares a binding domain that forms heptamers and an enzyme domain that modifies actin. Also, the chimeric toxin C2IN-C3 was similarly affected by the Hsp90 inhibitors. C2IN-C3 consists of the adaptor part of C2I, which interacts with the heptameric C2IIa, and of the Rho-ADP-ribosylating C3 exoenzyme. The finding that the Hsp90 inhibitors also delayed the cytotoxic effects of C2IN-C3 indicates that uptake of the chimeric toxin is Hsp90-dependent and that the toxin substrate specificity is not crucial for this dependence.

The binding component of anthrax toxin is very similar to C2I, and a similar translocation process for the active components has been proposed (see "Introduction"). Therefore, we studied the effects of geldanamycin and radicicol on the cytotoxic effects of the anthrax lethal toxin (PA plus LF of B. anthracis). Surprisingly, we did not detect any delay in the toxic effect of this anthrax toxin on J774.A1 macrophage-like cells in the presence of the Hsp90 inhibitors. Various explanations are possible. First, the failure of the Hsp90 inhibitors to protect cells from anthrax toxin depends on the cell type used (e.g. J774.A1). Second, the effect of the chaperone Hsp90 (and/or the Hsp90 complex) is highly specific for a certain toxin. The translocation of the respective enzyme domains by C2IIa and iota b but not that by the protective antigen depends on the presence of active Hsp90. Third, our findings might indicate that it is not the binding component but the type of enzyme component to be translocated that determines the requirement for a chaperone complex. The enzyme domains of C2I toxin, iota toxin, and C2IN-C3 are all ADP-ribose transferases. In contrast, anthrax LF is a metalllopeptate, which cleaves mitogen-activated protein kinase kinase (15). The data so far available are consistent with the hypothesis that the Hsp90 inhibitors block ADP-ribosylating toxins but not all types of enzymes transported by toxins. In line with this notion is the recent finding by Ratts et al. (34), in which the transport of diphtheria toxin (also an ADP-ribosylating toxin) depends on chaperones. Many questions remain open. For instance, it is not clear whether Hsp90 complexes are only involved in the translocation process or whether they play also a role in the refolding of the enzyme domains of toxins. Studies with chimeric toxins, especially with chimeric anthrax toxins, may help to clarify this issue.

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