The Host Cell Chaperone Hsp90 Is Necessary for Cytotoxic Action of the Binary Iota-Like Toxins

Gerd Haug, Klaus Aktories, and Holger Barth*

*Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität
Freiburg, D-79104 Freiburg, Germany*

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The heat shock protein Hsp90 is essential for uptake of the binary actin ADP-ribosylating toxins *Clostridium perfringens* iota-toxin and *Clostridium difficile* transferase into eukaryotic cells. Inhibition of Hsp90 by its specific inhibitor radicicol delayed intoxication of Vero cells by these toxins. A common Hsp90-dependent mechanism for their translocation is discussed.

The family of clostridial binary actin ADP-ribosylating toxins includes the iota-like toxins *Clostridium perfringens* iota-toxin (13, 18), *Clostridium spiroforme* toxin (15, 18), and *Clostridium difficile* ADP-ribosyltransferase (CDT) (7, 16), as well as the distinct *Clostridium botulinum* C2 toxin (1, 11). These toxins are composed of two nonlinked proteins, a binding and translocation component and an enzyme component (for a review, see reference 3). The toxins ADP-ribosylate G-actin in the cytosol of eukaryotic cells at arginine-177 (2), which leads to the disassembly of actin filaments and the rounding of cultured monolayer cells (10, 12).

The iota-like toxins share high sequence homology, and their enzyme components and binding components are interchangeable and generate biologically active chimeras (for a review, see reference 14). Although iota-like toxins and C2 toxin share sequence homology and are very closely related in molecular organization, there are important differences between the C2 toxin and the iota-like toxins. C2 toxin ADP-ribosylates β - and γ -actin, whereas iota-like toxins modify all actin isoforms, including muscle actin (9). Moreover, the enzyme component of C2 toxin, C2I, does not interact with the binding components of iota-like toxins. While all tested mammalian cells were sensitive to C2 toxin, the receptor for iota-toxin does not seem to be ubiquitous and is instead distributed at the poles (e.g., iota-toxin binds to the basolateral surfaces of CaCo-2 cells but not to the apical surfaces) (5).

C2 toxin and iota-toxin use similar mechanisms for cell entry. Their proteolytically activated binding and translocation components (designated C2II for C2 toxin and Ib for iota-toxin) form heptamers in solution (4, 5). For C2 toxin, we found that the C2II heptamers insert themselves as pores into the membranes of acidic endosomes and mediate the translocation of the enzyme components into the cytosol (4). Pore formation is absolutely necessary for translocation of the enzyme component C2I (6). Moreover, unfolding of the C2I protein seems to be a prerequisite for its translocation across membranes. Recently, it was shown that the host cell chaperone Hsp90 is essential for translocation of C2I from endo-

somes into the cytosol (8). When Hsp90 was blocked by the specific inhibitor radicicol or geldanamycin, a dramatically reduced intoxication of cells by C2 toxin was observed (8). Both inhibitors bind to the ATP binding site of Hsp90 and thus show the identical mode of action. However, for iota-like toxins, the mechanism of translocation of the enzyme components across endosomal membranes is poorly understood. In this study, we tested the role of Hsp90 in cytotoxicity of the iota-like toxins.

African green monkey kidney (Vero) cells were used to test whether inhibition of Hsp90 had any effect on the cytotoxic action (detected as cell rounding) of iota-toxin. Cells were grown at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (Biochrom, Berlin, Germany) containing 5% heat-inactivated (30 min, 56°C) fetal calf serum (PAN Systems, Aidenbach, Germany), 2 mM L-glutamate, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. To inhibit Hsp90, cells were pretreated in 12-well plates with the specific inhibitor radicicol (Sigma, Deisenhofen, Germany) for 1 h (10, 30, and 50 μ M final concentrations in complete medium). The inhibitor alone had no influence on cell morphology within 5 h (data not shown). When cells were incubated with radicicol prior to the addition of *C. perfringens* iota-toxin (100 ng of Ia per ml and 200 ng of Ib per ml, which were purified as recombinant glutathione S-transferase [GST] proteins and activated as described previously [5]), cell rounding was delayed compared to that of cells which were not treated with radicicol (Fig. 1). After 75 min of incubation, cells which were not treated with radicicol started to become round, while radicicol prevented cells from rounding (Fig. 1, upper panels). After 120 min, i.e., a time when cells without radicicol were strongly intoxicated, fewer cells were intoxicated when radicicol was present. However, no complete protection was observed (Fig. 1, middle panels). After 170 min, nearly all cells were intoxicated, even in the presence of the highest concentration of radicicol (Fig. 1, lower panels). This indicates that inhibition of Hsp90 by radicicol delayed intoxication of cells by iota-toxin but did not completely block it at the given concentrations of the toxin.

There are various possible explanations for how radicicol is able to protect cells from the cytotoxic effects of iota-toxin. We tested whether radicicol interferes with the ADP-ribosylation of actin by Ia by performing a radioactive in vitro ADP-ribosylation of actin from Vero cell lysate with and without radicicol, as described earlier (5). The autoradiographic results,

* Corresponding author. Mailing address: Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Otto-Krayer-Haus, Albert-Ludwigs-Universität Freiburg, Albertstr. 25, D-79104 Freiburg, Germany. Phone: 49-0761-2035308. Fax: 49-0761-2035311. E-mail: holger.barth@pharmakol.uni-freiburg.de.

FIG. 1. Effect of radicicol on cytotoxic action of iota-toxin in Vero cells. Vero cell monolayers were preincubated with or without radicicol (Rad; concentrations as indicated) in complete medium for 1 h at 37°C. Ia (100 ng/ml) and Ib (200 ng/ml) were added, and cells were then kept at 37°C for intoxication. Photos were taken at the indicated times.

which are shown in Fig. 2, demonstrate that the presence of radicicol did not diminish ADP-ribosylation of actin by Ia.

To test whether the involvement of Hsp90 is a general principle for the uptake of actin ADP-ribosylating toxins, we tested whether inhibition of Hsp90 had any effect on the intoxication of cells by the *C. difficile* transferase CDT, another iota-like toxin. Cells were pretreated with various concentrations of radicicol for 1 h at 37°C to block Hsp90 activity and subsequently Ib (100 ng/ml) together with the recombinantly expressed enzyme component GST-CDTa (50 ng/ml). Cells were further incubated, and pictures were taken at the indicated times (Fig. 3). At 75 min of

incubation after toxin addition, cells which were treated with toxin but not with radicicol started to round. In contrast, radicicol completely prevented the cells from rounding after this incubation time (Fig. 3, upper panels). After 120 min, cells without radicicol were almost all round, i.e., completely intoxicated; in the presence of radicicol, the number of round cells was significantly lower (Fig. 3, middle panels). At 170 min after the addition of the toxin, cells showed significant rounding even in the presence of 50 μ M radicicol (Fig. 3, lower panels).

The finding that Hsp90 is involved in cellular uptake of C2 toxin as well as of iota-like toxins suggests that all binary actin ADP-ribosylating toxins may have a common translocation mechanism by which their enzyme components are delivered from acidified endosomes into the cytosol. This is important, because C2 toxin and the iota-like toxins differ in many aspects of action and component interaction (see above). One hypothesis is that Hsp90 acts specifically for translocation of ADP-ribosyltransferases. Ratts et al. reported that Hsp90 is essential for the translocation of diphtheria toxin, another ADP-ribosyltransferase, from endosomes into the cytosol (17). Currently, we are studying the precise role of Hsp90 in translocation of binary actin ADP-ribosylating toxins across endosomal membranes. This includes a close characterization of the interaction of Hsp90 with the toxin components and the identification of further cochaperones.

FIG. 2. Effect of radicicol on ADP-ribosyltransferase activity of Ia. Vero lysate was pretreated with or without 50 μ M radicicol for 1 h at 37°C. Ia (5 nM final concentration) and [³²P]NAD was added, and ADP-ribosylation of actin was performed at 37°C. Samples were taken at the indicated times, the reaction was stopped, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. An autoradiograph is shown.

FIG. 3. Effect of radicicol on cytotoxic action of *C. difficile* transferase CDTa in Vero cells. Vero cell monolayers were preincubated with or without radicicol (concentrations as indicated) in complete medium for 1 h at 37°C. GST-CDTa (50 ng/ml) and Ib (100 ng/ml) were added, and cells were then kept at 37°C for intoxication. Photos were taken at the indicated times.

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