ADP-Ribosylation of Actin by the *Clostridium botulinum* C2 Toxin in Mammalian Cells Results in Delayed Caspase-Dependent Apoptotic Cell Death

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The binary C2 toxin from *Clostridium botulinum* mono-ADP-ribosylates G-actin in the cytosol of eukaryotic cells. This modification leads to depolymerization of actin filaments accompanied by cell rounding within 3 h of incubation but does not immediately induce cell death. Here we investigated the long-term responses of mammalian cell lines (HeLa and Vero) following C2 toxin treatment. Cells stayed round even though the toxin was removed from the medium after its internalization into the cells. No unmodified actin reappeared in the C2 toxin-treated cells within 48 h. Despite actin being completely ADP-ribosylated after about 7 h, no obvious decrease in the overall amount of actin was observed for at least 48 h. Therefore, ADP-ribosylation was not a signal for an accelerated degradation of actin in the tested cell lines. C2 toxin treatment resulted in delayed apoptotic cell death that became detectable about 15 to 24 h after toxin application in a portion of the cells. Poly(ADP)-ribosetransferase 1 (PARP-1) was cleaved in C2 toxin-treated cells, an indication of caspase 3 activation and a hallmark of apoptosis. Furthermore, specific caspase inhibitors prevented C2 toxin-induced apoptosis, implying that caspases 8 and 9 were activated in C2 toxin-treated cells. C2I, the ADP-riboisyltransferase component of the C2 toxin, remained active in the cytosol for at least 48 h, and no extensive degradation of C2I was observed. From our data, we conclude that the long-lived nature of C2I in the host cell cytosol was essential for the nonreversible cytotoxic effect of C2 toxin, resulting in delayed apoptosis of the tested mammalian cells.

*Clostridium botulinum* C2 toxin, the prototype of the family of binary actin ADP-ribosylating toxins, mono-ADP-ribosylates G-actin at Arg-177 (1). C2 toxin consists of the enzyme component C2I (431 amino acid residues, 49.3 kDa) and the binding/translocation component C2II (721 amino acid residues, 80.8 kDa). The ADP-riboisyltransferase activity of C2I is located in its C-terminal domain (5), while the enzymatically inactive N-terminal domain (C2IN, amino acid residues 1 to 225) mediates interaction with C2II and translocation of C2I into the cytosol (6). Following limited proteolysis, the active C2IIa protein (~60 kDa) forms ring-shaped heptamers that assemble with C2I and mediate binding of the toxin complex to the cellular receptor, a carbohydrate structure (2, 11, 19). During cellular uptake, the heptamers form pores in endosomal membranes, thereby facilitating translocation of C2I into the cytosol (7). Translocation of C2I requires unfolding (14) and subsequent refolding of the protein in the cytosol by the host cell chaperone Hsp90 (13).

In the cytosol, C2I catalyzes covalent transfer of the ADP-ribose moiety from NAD (NAD⁺) to Arg-177 of G-actin (1). Mono-ADP-riboisylolation of actin induces the depolymerization of F-actin and thereby a complete loss of actin filaments, resulting in rounding of cultured monolayer cells (1). The ADP-riboisyltransferase at Arg-177 turns G-actin monomers into “capping proteins,” which assemble at the barbed ends of F-actin and prevent the addition of nonmodified G-actin molecules to these filament ends (37). ADP-riboisylated G-actin does not affect the pointed end of filaments, leading to an increase of the critical concentration for actin polymerization and thereby to a complete depolymerization of actin filaments (39). Recently, it was found that ADP-riboisylated actin did not show any major conformational alterations compared to nonmodified G-actin; thus, the ADP-riboisylation of actin at Arg-177 most likely leads to a severe steric clash and disruption of the contact sites which hold the actin filaments together (27). Moreover, ADP-riboisylation affects the interface of G-actin with gelsolin, and nucleation activity of the actin-gelsolin complex is reduced (18, 38, 41). In contrast to the described cytopathic effects immediately following ADP-riboisylation of actin by C2 toxin, the long-term responses of toxin-treated cells are not precisely understood. We reported earlier that C2 toxin-treated HeLa cells failed to divide because of blocked transition from G2 phase to mitosis (4). Interestingly, most of the cells were not immediately killed by the toxin and stayed viable for more than 1 day (4). However, the induction of cell death by either C2 toxin or other members of the family of binary actin ADP-riboisylating toxins has not been investigated so far.

Here, we examined C2 toxin-treated HeLa and Vero cells starting from the application of C2 toxin until death. In particular, we focused on the fate of ADP-riboisylated actin in intoxicated cells and the stability of C2I in the cytosol. Treat-
ment with C2 toxin resulted in a nonreversible rounding of cells. Moreover, no unmodified actin reappeared in the cytosol of C2 toxin-treated cells. Actin was rapidly and ultimately modified in C2 toxin-treated cells after ~3 h of incubation, depending on the toxin concentration. However, ADP-ribosylation was not a signal for accelerated degradation of actin in the cells. Active C2I ADP-ribosyltransferase was found in the cytosol of intoxicated cells for at least 48 h, demonstrating the long-lived nature of C2I in the host cell cytosol. Finally, we discovered a delayed apoptosis in the C2 toxin-treated cells and analyzed the role of different caspases in this process.

MATERIALS AND METHODS

Cell culture and cytotoxicity assays. HeLa cells (ATCC CCL-2) were cultivated in minimal essential medium containing 10% heat-inactivated fetal bovine serum, 1.5 g/liter sodium bicarbonate, 1 mM sodium pyruvate, 2 mM l-glutamine, and 0.1 mM nonessential amino acids (Invitrogen, Karlsruhe, Germany). Cells were cultivated at 37°C and 5% CO2. Cells were trypsinized (Sigma-Aldrich, München, Germany) and resuspended in medium less than 15 to 20 times. For cytotoxicity experiments, cells were seeded in culture dishes and incubated in complete medium with toxin. To prevent endosomal acidification, 100 nM bafilomycin A1 (Calbiochem, Bad Soden, Germany) was applied to the cells. At given times, the cells were visualized by using a Zeiss Axiovert 40CFI microscope (Zeiss, Oberkochen, Germany) with a Jenoptik charge-coupled device camera (Jenoptik, Jena, Germany). The cytopathic effects triggered by the toxins were analyzed in terms of morphological changes.

Expression and purification of recombinant proteins. The components of C. botulinum C2 toxin (C2I and C2II) were expressed as glutathione S-transferase fusion proteins in Escherichia coli BL21 cells. The proteins were purified on glutathione beads. Following proteolytic cleavage of the glutathione S-transferase domain (10 NIH units/ml of bead suspension), recombinant proteins were recovered as described previously (2, 5). C2II was activated with trypsin for 30 min at 37°C. For cell-free ADP-ribosylation of actin, 20 to 50 μg of whole-cell lysate protein was incubated for 30 min at 37°C in a buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 5 mM MgCl2, complete protease inhibitor, 0.3 μg of C2I, and 10 μM biotinylated NAD+ (R&D Systems, Wiesbaden, Germany). The reaction was stopped by adding 5% SDS sample buffer (625 mM of Tris-HCl [pH 6.8], 20% SDS, 8.5% glycerol, 0.2% bromophenol blue, 100 mM DTT) and boiling the samples for 5 min at 95°C. The samples were subjected to SDS-PAGE, and subsequently the biotin-ADP-ribosylated proteins were transferred to a nitrocellulose membrane and visualized with peroxidase-coupled streptavidin in a subsequent chemiluminescence reaction.

Detection of apoptotic cells by measurement of plasma membrane asymmetry. HeLa cells were grown in 6-cm dishes to subconfluence and incubated together with the toxins or inhibitors in medium. Subsequently, cells were trypsinized and centrifuged by pelleting. Aliquots of 5 × 105 cells were incubated with 50 μl of annexin V-Fluos labeling solution (Roche, Mannheim, Germany) for 15 min at room temperature. Propidium iodide (PI) staining was performed in order to distinguish nonapoptotic cells. Cells were washed with 1 ml of a PBS-Tris-HCl (0.1 M Tris-HCl, 0.1% [wt/vol] trisodiumcitrate dihydrate and 0.1% [wt/vol] Triton X-100 mixed buffer) to permeabilize the cytoplasmic membrane. Subsequently, the cells were incubated for 30 min at 4°C in digitonin buffer to allow leakage of the cytoplasmic proteins into the supernatant. The separation of the cytosolic proteins from other cellular proteins was confirmed by immunoblot analysis with antibodies against the membrane fraction, immunoblot analysis with antibodies against the β subunit of G proteins (Gβ-T-20; Santa Cruz Biotechnology, Heidelberg, Germany) and against Rho-GDI (K-21; Santa Cruz Biotechnology, Heidelberg, Germany) was performed. This cytosolic fraction was used for detection of enzymatic C2I protein either by in vitro ADP-ribosylation with βγ-subunit of actin as a substrate (Cytoskeleton, Denver, CO) and biotinylated NAD+ as a cosubstrate (R&D Systems, Wiesbaden, Germany) or by performing a bioassy with native HeLa cells. In brief, cytosolic samples were applied to naïve cells together with fresh C2IIa and the number of rounded cells was determined after various incubation periods. In this assay, cell rounding was monitored to indicate C2I activity. To ensure that cell rounding was due to C2I activity, cytosols were incubated together with an antisense raised in rabbits against C2I. To detect C2I protein in the cytosol, cells (3.5 × 106/6-cm dish) were incubated for 40 min at 4°C in serum-free medium together with biotin-labeled C2I (0.4 μg/ml plus C2IIa (0.8 μg/ml)) to enable toxin binding. Subsequently, the medium was removed and the cells were washed with ice-cold PBS and subsequently incubated at 37°C in complete toxin-free medium for the indicated times. The cytosolic fraction was obtained by ultracentrifugation (20,000 × g), and an aliquot (25 μg of protein) was subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed with streptavidin-peroxidase (Roche, Mannheim, Germany) as a marker for cytosolic proteins or against rab-5 (BD Biosciences, Heidelberg, Germany) as a marker for early endosomal proteins.

ADP-ribosylation of actin. For cell-free ADP-ribosylation of actin, 20 to 50 μg of whole-cell lysate protein was incubated for 30 min at 37°C in a buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 5 mM MgCl2, complete protease inhibitor, 0.3 μg of C2I, and 10 μM biotinylated NAD+ (R&D Systems, Wiesbaden, Germany). The membrane was blocked for 60 min with 5% nonfat dry milk in PBS containing 0.05% Tween 20 (PBS-T). For the detection of actin, the samples were probed with a mouse monoclonal anti-β-actin antibody (clone AC-15; Sigma-Aldrich, Seelze, Germany). Detection of poly(ADP-ribosyl)transferase 1 (PARP-1) was performed using the mouse monoclonal antibody C8-H10 (25) kindly provided by A. Bürkle, Konstanz, Germany. Cleaved caspase 3 was detected with rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA). After being washed with PBS-T, the membranes were incubated for 1 h with the corresponding antispecies (mouse or rabbit) antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany). Subsequently the membrane was washed, and proteins were visualized using an enhanced chemiluminescence system according to the manufacturer’s instructions (Millipore, Schwalbach, Germany). Detection of active C2I ADP-ribosyltransferase in the cytosol. HeLa cells were incubated together with 0.2 μg/ml of C2I and 0.4 μg/ml of C2IIa for 3 h until all cells rounded up. Subsequently, the toxin was removed from the medium and cells were further incubated at 37°C. At the given time points cells were washed with ice-cold PBS and scraped in a solution of 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 5 mM MgCl2 supplemented with complete protease inhibitor. Cells were lysed by being transferred 10 times through a syringe (0.45-mm by 12-mm needle). Subsequently, cells were centrifuged (100,000 × g, 1 h, 4°C), and the supernatant (i.e., the cytosolic fraction) was collected. To confirm separation of the cytosolic fraction from the membrane fraction, immunoblot analysis with antibodies against the β subunit of G proteins (Gβ-T-20; Santa Cruz Biotechnology, Heidelberg, Germany) and against Rho-GDI (K-21; Santa Cruz Biotechnology, Heidelberg, Germany) was performed. This cytosolic fraction was used for detection of enzymatic C2I protein either by in vitro ADP-ribosylation with βγ-subunit of actin as a substrate (Cytoskeleton, Denver, CO) and biotinylated NAD+ as a cosubstrate (R&D Systems, Wiesbaden, Germany) or by performing a bioassy with native HeLa cells. In brief, cytosolic samples were applied to naïve cells together with fresh C2IIa and the number of rounded cells was determined after various incubation periods. In this assay, cell rounding was monitored to indicate C2I activity. To ensure that cell rounding was due to C2I activity, cytosols were incubated together with an antisense raised in rabbits against C2I. To detect C2I protein in the cytosol, cells (3.5 × 106/6-cm dish) were incubated for 40 min at 4°C in serum-free medium together with biotin-labeled C2I (0.4 μg/ml plus C2IIa (0.8 μg/ml)) to enable toxin binding. Subsequently, the medium was removed and the cells were washed with ice-cold PBS and subsequently incubated at 37°C in complete toxin-free medium for the indicated times. The cytosolic fraction was obtained by ultracentrifugation (20,000 × g), and an aliquot (25 μg of protein) was subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed with streptavidin-peroxidase (Roche, Mannheim, Germany) for the presence of biotin-C2I. Additionally, the cytosolic proteins were extracted with digitonin by a modified protocol according to the work of Wiedlocha et al. (40). In brief, cells, which were grown in 12-well plates and treated with the toxin, were incubated for 5 min at 25°C with digitonin buffer (20 μg/ml digitonin [Sigma-Aldrich, Seelze, Germany]) in PBS) to permeabilize the cytoplasmic membrane. Subsequently, the cells were incubated for 30 min at 4°C in digitonin buffer to allow leakage of the cytoplasmic proteins into the supernatant. The separation of the cytosolic proteins from other cellular proteins was confirmed by immunoblot analysis with antibodies raised against Hsp90 (Santa Cruz Biotechnology, Heidelberg, Germany) as a marker for cytosolic proteins or against rab-5 (BD Biosciences, Heidelberg, Germany) as a marker for early endosomal proteins.

RESULTS

C2 toxin-mediated ADP-ribosylation does not accelerate the degradation of actin in HeLa and Vero cells. To investigate the long-term effects of C2 toxin on HeLa cells, cells were incu-
in combination with biotin-labeled NAD. To this end, cell lysates were incubated together with fresh C2I and C2IIa for 30 min at 37°C. The morphology was documented by incubating the cell lysate proteins together with biotin-labeled NAD⁺ and C2I or C2IIa. Individual toxin components did not change the morphology of cells within the indicated incubation periods (not shown). In parallel, the ADP-ribosylation status of actin from the cells was analyzed. To this end, cell lysates were incubated together with fresh C2I in combination with biotin-labeled NAD⁺ as cosubstrate. The resulting biotin-labeled, i.e., ADP-ribosylated, actin was shown in Fig. 1B (upper panel). A strong signal in this assay indicates that the actin was not ADP-ribosylated in the intact cells; therefore, actin is a substrate for the subsequent ADP-ribosylation in the cell-free system, as shown for the control cells that had not been incubated together with C2 toxin in the culture medium (Fig. 1B, upper panel). In contrast, actin from cells that had been incubated together with C2 toxin displayed no signal in the ADP-ribosylation assay, indicating that actin was completely ADP-ribosylated in the intact cells by C2I (Fig. 1B, upper panel, C2I + C2IIa).

Next, we investigated the amount of actin from the cells by immunoblot analysis with an antibody against β-actin (Fig. 1B, lower panel). The amount of actin did not decrease during the 48 h of toxin treatment. We confirmed that the anti-β-actin antibody recognized ADP-ribosylated actin as well as unmodified actin (data not shown). A comparable result was obtained using Vero cells instead of HeLa cells (not shown). Taken together, ADP-ribosylation by C2 toxin did not alter the overall amount of actin within the tested incubation periods and thus indicated that ADP-ribosylation did not trigger degradation of actin in HeLa and Vero cells. The nonreversible cytotoxic effect was still existent even when only 1 ng/ml of C2I was applied to the cells and when the toxin was removed from the medium immediately after rounding of the cells (not shown).

**Enzyme-active C2I persists in the host cell cytosol for at least 48 h.** We investigated the stability of C2I in host cells and determined its ADP-ribosyltransferase activity in the cytosol of intoxicated HeLa cells after different periods of incubation. To this end, cells were incubated for 3 h at 37°C together with C2 toxin (0.2 μg/ml of C2I plus 0.4 μg/ml of C2IIa) to allow internalization of C2I into the cytosol. As a control, cells were left untreated. After 3 h of incubation with C2 toxin, the cells were round, indicating that C2I was internalized into the cytosol. At this point in time the toxin was removed from the medium and the cells were further incubated in fresh toxin-free medium for up to 48 h. Cells were lysed either immediately after 3 h of toxin incubation or after the indicated periods of incubation in toxin-free medium. Cytosolic fractions from the cells were obtained by ultracentrifugation and used to monitor any active C2I protein in the cytosol. Successful separation of the cytosolic fraction from the membrane fraction by this method was confirmed with an antibody against the β subunit of G-proteins (Gb T-20) (not shown).

We performed two different assays to detect enzyme-active C2I in the cytosolic fractions from the cells. In the first assay, the ADP-ribosyltransferase activity of C2I was analyzed by incubating aliquots of the C2I-containing cytosols for 30 min together with biotin-NAD⁺ as a cosubstrate and β/γ-actin as a substrate. The biotin-labeled and therefore ADP-ribosylated actin is shown in Fig. 2A. ADP-ribosylated actin was not detected in the cytosol of control cells not treated with toxin (Fig. 2A, con). As a positive control, fresh C2I instead of that in the cytosol was used as an enzyme (Fig. 2A, C2I). However, under these experimental conditions, the amounts of ADP-ribosylated actin were comparable for all cytosolic samples ranging from 3 to 24 h of incubation. Only the cytosol that was obtained from cells after 48 h of toxin treatment revealed a slightly weaker signal for ADP-ribosylated actin, indicating that enzymatically active C2I was still present in the cytosol of intact cells 48 h following application of C2 toxin.

An alternative assay was performed to test whether C2I from the cytosolic fractions still interacted with C2IIa. To this end, HeLa cells were treated with C2 toxin as described above. Additionally, cells were incubated with C2 toxin in the presence of bafilomycin A1, which inhibits the translocation of C2I from acidic endosomes into the cytosol (Fig. 2B, 3 h + Baf + C2IIa). Equal amounts of the cytosolic extracts were applied to naïve HeLa cells supplemented with fresh transport component C2IIa. As a control, either cells were left untreated (neg-
with fresh C2I plus C2IIa were round after ~3 h (C2I + C2IIa). Naïve cells, which were treated with C2IIa in combination with the individual cytosolic fractions containing C2I, because they were obtained from the cells that had been previously incubated with C2 toxin for 3 to 48 h, started to round after ~2 to 3 h. After 8 h of incubation, ~70 to 90% of these cells were round. Thus, as observed in the ADP-ribosylation assay (Fig. 2A), there was comparable ADP-ribosyltransferase activity in the cytosol after 3 to 24 h of incubation, implying comparable amounts of biologically active C2I protein in the cells. The cytosol from cells that had been incubated for 48 h was less active with respect to intoxication of naïve cells, and after 8 h of incubation only ~50% of the cells were round. The cytosolic fractions from cells which had never been incubated with C2 toxin (con + C2IIa) or from cells which had been treated with C2 toxin in the presence of bafilomycin A1 (3 h + Baf + C2IIa) did not induce cell rounding when applied to naïve cells together with C2IIa. This result clearly indicates that cell rounding is due to the presence of C2I activity in the cytosol. In addition, we used an anti-C2I antiserum to confirm that the observed cell rounding was indeed due to the enzyme activity of C2I in the cytosolic fractions. This antiserum neutralizes the cytopathic effect of C2I, i.e., no cell rounding is observed after pretreatment of C2I with the antiserum and subsequent application of this pretreated C2I protein to cells together with C2IIa (not shown). Cytosols from cells which had been incubated with C2I plus C2IIa (0.2 ± 0.4 µg/ml) prior to lysis were incubated either with or without anti-C2I for 30 min and subsequently applied to naïve HeLa cells together with C2IIa. As shown in Fig. 2C, incubation of the cytosols with the antiserum prevented cell rounding while in the absence of the antiserum cell rounding was observed following application of cytosol plus C2IIa to the cells. The antiserum alone had no effect on the morphology of cells within 24 h of incubation (not shown). Taking all these results together, C2I remains catalytically active as an ADP-ribosyltransferase for at least 48 h after translocation into the cytosol of HeLa cells.

Next, we investigated the amount of C2I protein in the cytosol of HeLa cells. To this end, the cells were incubated for 40 min (i.e., 0.67 h) at 4°C together with C2IIa (0.8 µg/ml) plus biotin-labeled C2I (0.4 µg/ml) to allow binding of the toxin to the cell surface. Subsequently, the toxin was removed and cells were further incubated at 37°C in toxin-free medium for up to 48 h. At the indicated time points, cells were lysed and the cytosolic fractions were obtained by either ultracentrifugation (Fig. 3A) or digitonin extraction (Fig. 3B). Equal amounts of protein from the cytosolic fractions were analyzed for the presence of biotin-C2I with streptavidin-peroxidase. In HeLa cells, the amount of biotin-C2I protein showed no obvious decrease within 48 h of incubation. It is worth mentioning that biotin-labeling of C2I did not interfere with enzyme activity and the cellular uptake mechanism of the toxin. Importantly, there was no bleeding of biotin-C2I from the cytoplasmic membrane into the cytosolic fraction during preparation of cytosolic proteins. This was demonstrated with cells that had been incubated with C2IIa plus biotin-C2I, exactly like the other cells used in this experiment, but which had not been incubated at 37°C after the toxin had bound to the membrane. Despite the failure of these cells to internalize the cell-bound toxin, no biotin-labeled C2I protein was detectable in the cytosolic fractions from these
cells (Fig. 3 A and B, indicated as 0.67 h, 4°C). A comparable result was obtained when Vero cells were used; however, there was a slight decrease in the amount of biotin-C2I between 24 and 48 h of incubation. Taken together, the results indicate that a major portion of the C2I protein that reached the cytosol was not degraded but persisted as an active ADP-ribosyltransferase for at least 48 h.

**C2 toxin induces delayed apoptotic cell death.** Based on the observation that C2 toxin-treated cells did not recover, we analyzed toxin-treated HeLa cells by different assays to detect cell death. In the first assay, the loss of membrane asymmetry, a hallmark of early apoptosis, was analyzed by measuring annexin V-stained phosphatidylserine. Another important feature of apoptotic cell death is the preservation of membrane integrity. Thus, double-stained cells (stained with annexin V and PI) were evidence of necrosis in this assay. In C2 toxin-treated HeLa cells, a time- and concentration-dependent increase of annexin V-positive cells was detected (Fig. 4A). When cells were treated with 0.1 µg/ml of C2I plus 0.2 µg/ml of C2IIa, annexin V-stained cells were not detected before 24 h of incubation, strongly suggesting that C2 toxin treatment resulted in apoptotic cell death. When incubated with a higher concentration of C2 toxin (0.2 µg/ml of C2I plus 0.4 µg/ml of C2IIa), annexin V-positive cells appeared after 15 h. However, double-stained cells (stained with annexin V and PI) were detected at both toxin concentrations, too. Therefore, another assay was performed to confirm the C2 toxin-induced apoptosis.

Thus, we investigated the C2-toxin induced DNA fragmentation as another specific feature of apoptosis (Fig. 4B). To this end HeLa cells were treated with C2 toxin (0.2 µg/ml of C2I plus 0.4 µg/ml of C2IIa), annexin V-positive cells appeared after 15 h. However, double-stained cells (stained with annexin V and PI) were detected at both toxin concentrations. Consequently, the assay was performed to confirm the C2 toxin-induced apoptosis.
that treatment of HeLa cells with C2 toxin resulted in apoptotic cell death. The point in time for the first appearance of apoptotic cells was dependent on the toxin concentration and incubation time. In any case, the first significant signs of apoptosis appeared most obvious at ~15 to 24 h after toxin application. Thus, the induction of apoptosis was delayed compared to the rapid toxin-induced disruption of the actin cytoskeleton. Moreover, C2 toxin-mediated apoptosis was linked to activation of caspase 3.

C2 toxin-induced apoptosis correlates with the activation of caspases 3, 8, and 9. To obtain a more detailed view, we further used specific inhibitors to investigate the role of initiator caspases 8 and 9 in C2 toxin-mediated apoptosis. The caspase 8 inhibitor z-IETD-fmk (IETD) and caspase 9 inhibitor z-LEHD-fmk (LEHD), as well as the pan-caspase inhibitor z-VAD-fmk, were applied to the cells 30 min prior to the application of C2 toxin.

We confirmed the activation of caspases 3, 8, and 9 in C2 toxin-treated HeLa cells by detecting apoptotic cells with the assay of Nicoletti et al. (28). Therefore, cells were incubated for 30 min with the caspase inhibitors z-IETD-fmk, z-LEHD-fmk, and z-VAD-fmk, respectively, and subsequently treated with C2 toxin (0.2 μg/ml of C2I plus 0.4 μg/ml of C2IIa). Following an additional 24 h of incubation, the cells were analyzed for DNA fragmentation as described above. As shown in Fig. 6A, each caspase inhibitor significantly reduced the amount of apoptotic cells in comparison to cells treated with C2 toxin alone. It is worth mentioning that none of the caspase inhibitors alone was cytotoxic under the conditions used in these experiments, as the inhibitors neither increased the amount of apoptotic cells nor showed an effect on the cell morphology within 24 h of incubation (not shown). Moreover, the solvent dimethyl sulfoxide (DMSO) did not reduce the percentage of apoptotic cells due to C2 toxin treatment within 24 h of incubation (Fig. 6A, inset). After 15 and 24 h of incubation with C2 toxin, cleavage of PARP-1 was detected in the absence of the inhibitors (Fig. 6B). In contrast, C2 toxin-induced cleavage of PARP-1 was completely prevented by each of the caspase inhibitors (Fig. 6B), indicating that caspases 8 and 9 are activated in C2 toxin-treated HeLa cells. In turn, equal protein loading was confirmed by an actin-immunoblot analysis (Fig. 6B, lower panel).

Taken together, these findings clearly indicate that C2 toxin treatment results in the activation of the initiator caspases 8 and 9, as well as the downstream effector caspase 3 in HeLa cells.

DISCUSSION

ADP-ribosylation of G-actin represents an efficient mechanism used by various bacterial toxins to destroy the cytoskeleton of mammalian cells. The immediate cytopathic effects of such toxins, i.e., the ADP-ribosylation of actin leading to depolymerization of F-actin and rounding of cultured cells, have been studied in detail. In contrast, the terminal fate of cells treated with actin ADP-ribosylated toxins has not been investigated in detail so far; thus, it is still not clear how these toxins kill the cells. Moreover, it is known neither how long the enzyme components of the binary actin ADP-ribosylating toxins persist in the host cell cytosol nor whether the toxin-
and 9 in HeLa cells. Cells were preincubated for 30 min at 37°C together with the indicated concentrations of the specific caspase inhibitors z-IETD-fmk (IETD) for caspase 8 and z-LEHD-fmk (LEHD) for caspase 9 or with the pan-caspase inhibitor z-VAD-fmk (VAD). An equal amount of DMSO was incubated with cells as solvent control (DMSO). Subsequently, the cells were treated at 37°C with C2I (0.2 μg/ml) plus C2IIa (0.4 μg/ml) or left untreated for control (con). (A) After 24 h, the amount of apoptotic cells was determined by the method of Nicoletti et al. (28). Significance was tested against C2 toxin-treated cells by Student’s t test (**, P < 0.005; ***, P < 0.0005). (Inset) Another set of cells were incubated with C2 toxin either in the absence or in the presence of DMSO (0.67%, vol/vol) to test the influence of DMSO on C2 toxin-induced apoptosis of HeLa cells. After 24 h the amount of apoptotic cells (DNA fragmentation) was determined by the method of Nicoletti (28). Values are given as means ± SDs (n = 3). (B) Cells were treated as described above. After 15 and 24 h of incubation with the toxin and the specific inhibitors, cells were lysed and probed with the C-II-10 antibody to detect PARP-1 and cleaved PARP-1 (upper panel). Equal protein loading was confirmed by immunostaining of β-actin (lower panel). Final concentrations of the caspase inhibitors were as follows: z-IETD-fmk (IETD), 100 μM for 15 h/50 μM for 24 h; z-LEHD-fmk (LEHD), 100 μM for 15 h/50 μM for 24 h; z-VAD-fmk (VAD), 60 μM for 15 h/30 μM for 24 h. Reduced ADP-ribosylation of actin triggers degradation of this modified actin in mammalian cells. Therefore, we have focused on the long-term effects of C2 toxin. In the present report we describe a series of experiments which demonstrated that the early C2 toxin-mediated effects were never reversible, most likely due to the long-lived nature of C2I ADP-ribosyltransferase C2I in the host cell cytosol. Interestingly, C2 toxin treatment did not immediately kill the tested mammalian cell lines. Intoxicated cells stayed viable for ~15 to 24 h, depending on the toxin concentration, before delayed cell death was induced. Specific assays clearly indicated that apoptosis was induced by C2 toxin in at least a portion of the intoxicated cells. In detail, the results from the annexin V staining assay revealed 20 to 30% annexin V-single-stained, i.e., apoptotic, cells at 24 h after application of C2 toxin. This finding was confirmed by the DNA fragmentation assay of Nicoletti et al. (28). Therefore, we can clearly state that this portion of the C2 toxin-treated cells is apoptotic. This is in the range of the amount of apoptotic cells induced by treatment of asynchronously growing cells with other bacterial toxins, such as Clostridium difficile TcdB (16). At 24 h after application of the toxin, there was also a portion of ~30 to 50% annexin V/PI-double-stained cells, dependent on the concentration of C2 toxin. For these cells, we cannot state the type of cell death; thus, we cannot exclude the possibility that a certain portion of C2 toxin-treated cells was necrotic. However, we detected an almost complete cleavage of PARP-1, a substrate of caspase 3, after 15 and 24 h of treatment of the cells with C2 toxin. This finding indicates that PARP-1 cleavage occurred in most, if not all, of the C2 toxin-treated cells and suggests that these cells were apoptotic. Moreover, the results obtained with the individual caspase inhibitors clearly demonstrate that C2 toxin treatment induces activation of caspases 3, 8, and 9 in the tested cell lines, indicating that caspase-dependent cell death is at least one mechanism for C2 toxin-mediated cell death.

To elucidate the mechanism for delay between the early cellular reactions induced by C2 toxin and the later-occurring cell death in more detail, we first tested whether the binding/translocation component C2Ia induced apoptosis independently from the enzyme component. The application of C2Ia alone caused a negligible cytotoxic effect after 48 h, which was not comparable to the effect of C2IIa plus C2I. Therefore, C2Ia was not responsible for the C2 toxin-mediated cell death. Moreover, the N-terminal domain of C2I as well as an enzymatically inactive C2I protein (C2IE387/389Q) had no cytotoxic effect when applied together with C2Ia to cultured cells (3, 5), implying that the enzyme activity of C2I was crucial for inducing delayed apoptosis.

Our results suggest that the long-lived nature of C2I in the cytosol of intoxicated cells was an important reason for the nonreversible cytotoxic effect of extremely low amounts of C2 toxin, such as 1 ng/ml. When we monitored C2I in the cytosol of intoxicated HeLa cells, active ADP-ribosyltransferase was detectable for at least 48 h. However, there was a decrease in the ADP-ribosyltransferase activity of C2I between 24 and 48 h while the amounts of C2I protein were comparable. This might be due to conformational changes in a portion of the C2I molecule between 24 and 48 h, for instance induced by cleavage of a few N-terminal amino acid residues, not detectable by immunoblot analysis. We observed earlier that N-terminally truncated C2I lacks ADP-ribosyltransferase activity, most likely due to improper folding of the truncated C2I (unpublished observation). We were able to detect C2I protein in the cytosol of Vero cells at 48 h after toxin application, too, indicating that the persistence of C2I in the cytosol was not restricted to HeLa cells. In Vero cells, however, there was a moderate decrease in the amount of C2I protein in the cytosol between 24 and 48 h of incubation, suggesting that the stability of C2I in the host cell cytosol might be influenced by the individual cell type. In conclusion, this observation indicates that C2I was rather stable and not extensively degraded in the host cell cytosol. Thus, if there is any de novo-synthesized actin it would become ADP-ribosylated by persistent C2I, thereby blocking the reconstitution of F-actin. When we performed the same type of experiments with the recombinant C2IN-C/SpvB fusion toxin instead of C2I, we observed comparable immediate cytopathic effects, i.e., ADP-ribosylation of actin and cell rounding, but completely different long-term effects. This fusion toxin contains the catalytic domain of the Salmonella enterica ADP-ribosyltransferase SpvB (17) and modifies actin...
in intact cells exactly as C2I does, leading to depolymerization of F-actin and cell rounding (31). However, C2IN-C/SpvB is rapidly degraded in the host cell cytosol, and nonmodified actin reappeared in the cells, leading to reconstitution of F-actin and recovery of the intoxicated cells (S. Pust and H. Barth, unpublished observation). This observation suggests that newly synthesized actin was not modified because there was no more ADP-ribosyltransferase activity in the cytosol. Reuner and coworkers reported earlier that microinjection of ADP-ribosylated actin into hepatocyte-hepatoma hybrid cells significantly decreased actin synthesis within 1 h (32). The velocity and degree of inhibition of actin synthesis by ADP-ribosylated G-actin, however, might depend on the individual cell type, as we have experimental evidence that both HeLa and Vero cells synthesize new actin to a certain extent, even when the actin is ADP-ribosylated (S. Pust and H. Barth, unpublished observation). In conclusion, we were able to demonstrate the long-lived nature of the C2I protein in the cytosol of cultured mammalian cell lines, and our observations with the C2IN-C/SpvB fusion toxin promote the hypothesis that this remarkable stability of the C2I ADP-ribosyltransferase might be crucial for the observation that C2I toxin-treated cells never recovered and finally died. Prompted by these findings, it will be interesting to investigate whether the other members of the binary actin ADP-ribosylating toxin family, in particular the Clostridium perfringens iota-toxin and the Clostridium difficile transferase CDT, behave comparably to the C2I toxin concerning the stability of the enzyme components in the host cell cytosol, to learn whether this is a common principle of this toxin family.

In this report we present experiments which demonstrate for the first time that ADP-ribosylation by C2I toxin did not promote the degradation of this modified actin in the cytosol of mammalian cells. Until now, little was known about the fate of ADP-ribosylated actin in the host cell cytosol, and recently Tezcan-Merdol and coworkers reported that ADP-ribosylation by S. enterica SpvB serves as a signal for an accelerated degradation of actin in Acanthamoeba rhysodes (35). The reason underlying this interspecies difference is not known yet. Taken together, C2I toxin did not alter the overall amount of actin in the tested cell lines but influenced the equilibrium between unmodified and ADP-ribosylated actin.

In an earlier study, we reported that C2I toxin treatment of HeLa cells results in an arrest at the G2/M checkpoint of the cell division cycle (4). In those cells, activation of p34\textsuperscript{cdk2} kinase and Cdc25-C phosphatase, which trigger the transition of cells from G2\textsubscript{ph} to mitosis, are blocked. One could speculate that C2I toxin-treated cells survive until they reach the mitotic checkpoint, even though actin is thoroughly ADP-ribosylated. Thus, apoptosis might be triggered by a complete absence of unmodified actin at this checkpoint. Besides its ADP-ribosyltransferase activity, C2I harbors NAD\textsuperscript{+}-hydrolyase activity in the absence of actin (29). The C2I-catalyzed hydrolysis of NAD\textsuperscript{+} might deplete the cellular levels of NAD\textsuperscript{+} and ATP, thereby disturbing the energy metabolism of intoxicated cells (10). Cell death due to the depletion of NAD\textsuperscript{+} and ATP was reported for ricin (22) and for PARP-1 (21, 34). Experiments are under way in our laboratory to test whether the NAD\textsuperscript{+}-hydrolyase activity of C2I contributes to the delayed toxin-induced apoptosis. Furthermore, we will investigate the pathways leading to activation of caspases 8 and 9 in C2I toxin-treated cells in more detail and focus on the mitochondrial pathway. The inhibition of caspase 8 totally abolished caspase 3-mediated proteolysis of PARP-1. If activation of caspase 9 is independent from that of caspase 8, one should expect some residual PARP-1 cleavage following caspase 8 inactivation. Thus, our results give a hint that C2I toxin-induced caspase 9 activation is mediated by active caspase 8. Therefore, we will investigate whether C2I toxin treatment has any effect on the Bcl-2 family members, in particular Bid, which links the activation of caspase 9 to that of caspase 8. Bid is a known substrate for caspase 8, and proteolytically activated Bid mediates cytochrome c release via the proapoptotic Bcl-2 protein Bax, resulting in activation of caspase 9 (for a review, see reference 15).

Interestingly, some cell types do not show the signs of delayed apoptosis despite responding to C2I toxin, as demonstrated for cerebellar granule neurons (26). In human umbilical cord vein endothelial cells, Hippenstiel and coworkers observed a small yet significant increase in programmed cell death following treatment with C2I toxin (16). Therefore, the C2I toxin-mediated induction of apoptosis might depend on the individual cell type.

Furthermore, it is not clear whether the data obtained with cultured cells mirror the situation in intoxicated animals. In vivo studies could help us to better understand the biological relevance of the C2I toxin-mediated cell death. However, our observation might contribute toward better understanding the role of C2I toxin in disease. Simpson reported earlier that the application of 1 to 2 pmol of purified C2I toxin kills mice, rats, guinea pigs, and chickens within 1 h and elicits hypotension plus fluid accumulation in the lungs (33). These effects can be explained by the rapid C2I toxin-induced rounding of endothelial cells leading to disruption of the endothelial tissue. Moreover, it has been demonstrated that C2I toxin causes hemorrhagic lesions in the intestinal wall (30), most likely a consequence of the delayed toxin-induced cell death.

Finally, additional actin ADP-ribosylating toxins have been investigated by other researchers and in our own laboratory for their ability to induce cell death. Browne and coworkers demonstrated earlier that the infection of human macrophages with S. enterica resulted in delayed apoptosis at about 18 to 24 h following infection and that the underlying reason was most likely the ADP-ribosylation of actin by SpvB (9). Strains harboring inactive SpvB do not induce apoptosis (9). In line with these observations, Kurita and coworkers reported that ADP-ribosylation of actin by SpvB triggers cell death of macrophage-like cells with many features of apoptosis (23). However, the mechanism leading to SpvB-mediated apoptosis is still not precisely known. In the latter study SpvB was expressed intracellularly; thus, the SpvB protein was permanently present in the cytosol and all newly synthesized actin became ADP-ribosylated. The observation that C2I toxin-treated cells did not recover is in line with earlier observations made in our laboratory for other binary actin ADP-ribosylating toxins, C. perfringens iota-toxin (8) and the C. difficile transferase CDT (12). The molecular mode of action of these toxins and the early effects on cultured cells are comparable to those of C2I toxin, and we have the first experimental evidence that CDTa also induces delayed apoptosis in HeLa cells (K. Heine and H. Barth, unpublished observation). However, further work will...
be required to characterize the delayed cell death, mediated by bacterial actin ADP-ribosylating toxins, in more detail.

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REFERENCES