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Clostridium botulinum C2 Toxin Delays Entry into Mitosis and Activation of p34^{cdc2} Kinase and cdc25-C Phosphatase in HeLa cells

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The *Clostridium botulinum* C2 toxin ADP-ribosylates monomeric actin, thereby inducing disassembly of actin filaments, alteration of focal adhesions, and rounding of cells. After treatment with C2 toxin, cells stop to proliferate but remain viable for about 2 days. In view of reported correlations between the structure of the actin cytoskeleton and cell cycle transition, the effects of C2 toxin on the G_2/M phase transition of the cell division cycle were studied. Since C2 toxin delayed entry into mitosis in HeLa cells, those enzymes which control entry into mitosis, the cyclin-dependent protein kinase mitosis-promoting factor (MPF) and the phosphatase cdc25-C were examined after treatment of synchronized cells with C2 toxin. MPF is composed of the regulatory cyclin B and the enzymatic p34^{cdc2} kinase subunits. For its activation at the G_2/M border, p34^{cdc2} needs to be associated with cyclin B and additionally dephosphorylated at Tyr-15 by the specific phosphatase cdc25-C. Treatment of synchronized cells in S or G_2 phase with *C. botulinum* C2 toxin prevented p34^{cdc2} protein kinase activation by inhibiting its tyrosine dephosphorylation at the G_2/M border. Furthermore, the activity of cdc25-C phosphatase was decreased after treatment of cells with C2 toxin. Our results suggest that the prevented activation of the mitotic inducers p34^{cdc2} kinase and cdc25-C phosphatase represents the final downstream events in the action of C2 toxin resulting in a G_2 phase cell cycle delay in synchronized HeLa cells.

The eukaryotic cell division cycle is driven by the precisely coordinated and controlled action of cyclin-dependent kinases (31). Entry into mitosis is under control of the cyclin-dependent kinase mitosis-promoting factor (MPF), which is composed of the enzymatic subunit p34^{cdc2}, harboring serine/threonine kinase activity, and the regulatory subunit cyclin B (3, 13). For activation of p34^{cdc2} kinase at the G_2/M border of the cell cycle, its assembly with cyclin B and subsequent dephosphorylation at Thr-14 and Tyr-15 by the specific phosphatase cdc25-C are essential (17, 23). Activated MPF phosphorylates a variety of substrate proteins which play key roles in the mechanism of cell division. Thus, active MPF is essential for entry into mitosis, and so its activation represents an important endogenous cell cycle control system (19).

Before activation of MPF at the G_2/M border, the cell experiences a physiological restriction point in the G_2 phase of the cell division cycle. At this cell cycle checkpoint, the necessary prerequisites for subsequent mitosis are controlled (for reviews, see references 8 and 39). At this point the cell can also integrate exogenous growth control signals from its environment—mediated by, for example, growth factors or cell-cell interaction and matrix attachment—with the endogenous key regulator of cell division, i.e., the superimposed activation machinery of MPF (18). Inhibition of the G_2/M transition of the eukaryotic cell cycle seems to represent a protective mechanism, allowing the cell to react to various extracellular influences such as ionizing radiation (7, 33) or other DNA-damaging agents (32).

In recent years, correlations between the structure of the actin cytoskeleton and cell cycle transition have been reported. The *Escherichia coli* toxins cytotoxic necrotizing factor 1 (CNF-1) and cytolethal distending toxin both lead to a stabilization of actin filaments and, in parallel, inhibit the G_2/M transition in HeLa cells (12, 16). In contrast, the F-actin-destroying drug dihydrocytochalasin B inhibits cell division by blocking cleavage into interphase but has no influence on mitotic processes (34).

In this study, we investigated the effects of the actin-ADP-ribosylating *Clostridium botulinum* C2 toxin on the G_2/M transition of eukaryotic cells. The binary C2 toxin consists of the enzymatic component C2I (49 kDa) and the binding component C2II (activated form about 60 kDa [40]). The two components represent separate proteins. When exhibiting its cytotoxic effects, C2II binds to the cell surface, thereby creating a binding site for C2I. Subsequently, the proteins enter the cell via receptor-mediated endocytosis and C2I is released into the cytosol (46), where it ADP-ribosylates monomeric actin at arginine-177 (1, 49). This ADP-ribosylation of G-actin leads to a complete disassembly of the actin filaments and thereby to a total breakdown of the actin cytoskeleton (51). Consequently, cells round up and focal adhesions are altered. After C2 toxin treatment, a significant decrease of cell division was observed. The destruction of actin filaments could be the underlying mechanism for inhibition of cytokinesis, as in the case of cytochalasin treatment (34). Using synchronized HeLa cells, we show that destruction of the actin cytoskeleton induced by *C. botulinum* C2 toxin is accompanied by a transiently delayed entry of cells into mitosis. The activating tyrosine dephosphorylation of the p34^{cdc2} protein kinase at the G_2/M border was prevented after C2 toxin incubation, and the kinase remained inactive. Furthermore, the cdc25-C phosphatase activity was decreased after treatment of synchronized cells with C2 toxin.

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MATERIALS AND METHODS

Materials. Cell culture medium and trypan blue were obtained from Biochrom (Berlin, Germany), fetal calf serum was obtained from PAN Systems (Aidenbach, Germany), and cell culture materials were obtained from Falcon (Heidelberg, Germany). Amethopterin and thymidine were from Calbiochem (Frankfurt, Germany). Paraformaldehyde was from Merck (Darmstadt, Germany). The C2II binding component from *C. botulinum* C2 toxin was purified and activated with trypsin as described previously (40, 42). The C2I enzyme component was purified as a recombinant glutathione *S*-transferase fusion protein as described (6). The pGEX2T vector (included in the glutathione *S*-transferase gene fusion system) and glutathione-Sepharose 4B were purchased from Pharmacia Biotech (Uppsala, Sweden). The low-molecular-mass protein marker was from Bio-Rad (Hercules, Calif.), and the nitrocellulose blotting membrane was from Schleicher & Schuell (Dassel, Germany). Protein A/G PLUS-agarose beads and anti-cyclin B- and antiphosphotyrosine antibodies were from Santa Cruz (Heidelberg, Germany). Anti-p34^{cdc2} antibody was from Gibco (Karlsruhe, Germany). Anti-mouse antibody coupled to peroxidase was from Dianova (Hamburg, Germany), and donkey anti-rabbit antibody coupled to peroxidase and the enhanced chemiluminescence detection kit were obtained from Amersham (Braunschweig, Germany). Thrombin and phalloidin-rhodamine were purchased from Sigma (Deisenhofen, Germany), [^{32}P]ATP (specific activity, 3 Ci/mmol) was from Amersham Buchler (Braunschweig, Germany), and [^{32}P]NAD (30 Ci/mmol) was from DuPont NEN (Bad Homburg, Germany). Histone H1 was obtained from Boehringer (Mannheim, Germany). Aquasafe 500 scintillation cocktail was from Zinsser Analytic (Frankfurt, Germany). The basic fuchsin for Feulgen staining was from Janssen-Pharma (Geel, Belgium).

Cell culture, synchronization, and cell cycle analysis. HeLa cells were cultivated in tissue culture flasks as monolayers at 37°C and 5% CO_2 in Eagle's minimal essential medium with Earl's salts containing 10% fetal calf serum, 2 mM L-glutamate, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were routinely trypsinized and reseeded twice a week. For experiments, subconfluent growing monolayer cells (about 10^5 cells/cm^2) in 3-cm-diameter plastic dishes were synchronized as described by Mueller and Kajiwara by blockage with 10^{-6} M amethopterin for 16 h in complete medium and subsequent release by thymidine (10 μg/10^6 cells) (37). Because the *C. botulinum* C2 toxin exhibits its full effects on the actin cytoskeleton of HeLa cells after about 2 to 3 h, at 4 or 6 h after release, i.e., when most of the cells were in the S phase, the C2 toxin was added to the synchronized cells (200 ng of activated C2II and 100 ng of C2I per ml) and cells were incubated at 37°C. The degree of cell synchrony was analyzed by flow cytometric measurements of DNA distribution (26) and by counting of mitotic figures, i.e., rounded cells (25). Viability of the cells was tested with a 30-min incubation at 37°C with trypan blue. The cell number was determined with a Neubauer chamber. For a detailed cell cycle analysis, a combined morphological and flow cytometric determination was carried out. For the former, the monolayer cells were removed from the dishes with 0.05% trypsin and heat fixed on glass slides. The chromatin was stained with the Feulgen reagent (43). This procedure allows analysis of mitotic cells among the cell fraction in which rounding was induced by the action of C2 toxin.

Fluorescence staining of F-actin. For fluorescence staining of F-actin, HeLa cells treated with or without C2 toxin were fixed for 30 min at 25°C in phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 0.1% Triton X-100. Thereafter, cells were briefly washed and incubated for 30 min with phalloidin-rhodamine (600 ng/ml) at room temperature in the dark (6).

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (29). For kinase assays, gels were stained with 0.1% Coomassie brilliant blue R-250 in methanol-acetic acid-water (40:10:50), destained in this solution without dye, and dried for autoradiography. For immunoblot analysis, the proteins (100 μg per lane; determined by the method of Bradford [9]) were transferred from the gel onto a nitrocellulose membrane by using a semidry transfer cell (Bio-Rad, Munich, Germany). The membrane was blocked for 30 min with 5% non fat dry milk in PBS containing 0.05% Tween 20 (PBS-T), and then the proteins were probed with either anti-p34^{cdc2} antibody (rabbit; 1:2,000 in PBS-T), anti-cyclin B antibody (rabbit; 1:2,000), or antiphosphotyrosine (anti-P-Tyr) antibody (mouse, 1:2,000). After washing with PBS-T, the blots were incubated for 1 h with donkey anti-rabbit antibody coupled to horseradish peroxidase (1:2,000 in PBS-T) or with anti-mouse antibody coupled to peroxidase (1:2,000), respectively. The membrane was washed again, and proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions.

ADP-ribosylation assay. To test the C2 toxin effect on cells, in vitro analysis of the ADP-ribosylation state of cellular actin was done as described previously (1). Cells were washed with cold PBS, scraped into 500 μl of lysis buffer (50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT]), and sonicated, and 100 μg of protein (determined by the method of Bradford [9]) was incubated with 500 nM [*adenylate*-^{32}P]NAD (about 25 nCi) and 50 ng of C2I toxin for 15 min at 37°C. The reaction was stopped by addition of Laemmli buffer, aliquots (50 μg protein of the reaction mixture) were subjected to SDS-PAGE in a 12.5% gel, and [^{32}P]ADP-ribosylated proteins were detected by autoradiography with a PhosphorImager from Molecular Dynamics (Krefeld, Germany).

Immunoprecipitation of p34^{cdc2} and histone H1 kinase assay. Immunoprecipitation was performed as described previously (4). Cells were washed with cold PBS, scraped into 1 ml of cold lysis buffer (50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM sodium orthovanadate, 50 mM NaF, 50 μg of phenylmethylsulfonyl fluoride per ml), and gently sonicated on ice. After protein determination, p34^{cdc2} was immunoprecipitated from 100 μg of cell lysate protein in 1 ml of lysis buffer with 2 μl of anti-p34^{cdc2} antibody (1 mg/ml) and 50 μl of a 1:1 slurry of protein A/G-agarose beads for 2 h at 4°C. The immunoprecipitates were pelleted (2,000 rpm in an Eppendorf centrifuge) and washed three times with cold lysis buffer. The immunoprecipitates were used for histone H1 kinase assay or immunoblot analysis. Histone kinase assays were carried out by addition of 10 μl of a buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 3.3 μM ATP, 16 μg of histone H1, and 5 μCi of [γ-^{32}P]ATP (specific activity, 3 Ci/mmol) to the immunoprecipitated p34^{cdc2}. The reaction mixture was incubated for 10 min at 37°C, and the reaction was stopped by addition of 10 μl of 2× Laemmli sample buffer and heating for 2 min at 95°C. The agarose beads were pelleted at 420 × *g* (Eppendorf centrifuge model 5417R) for 3 min, and the proteins were separated by SDS-PAGE on a 12.5% gel. The ^{32}P-labeled histone H1 proteins were excised and incubated overnight with 2 ml of a scintillation cocktail at 25°C, and radioactivity was determined by scintillation counting (4).

Immunoprecipitation and phosphatase assay of cdc25-C. Immunoprecipitation of cdc25-C and the subsequent phosphatase assay were performed as described earlier (23). In brief, cells were lysed in the buffer described above (without sodium orthovanadate) and sonicated, and 3 mg of lysate protein was incubated for 2 h at 4°C with cdc25-C antiserum (IH37) and for additional 2 h at 4°C with 50 μl of a 1:1 slurry of protein A/G-agarose beads. The collected immunoprecipitates were washed three times and used for cdc25-C phosphatase assay with inactive p34^{cdc2} as the substrate. Therefore, inactive p34^{cdc2} was immunoprecipitated from 500 μg of S-phase HeLa lysate protein (without sodium orthovanadate) as described above. Immunoprecipitated p34^{cdc2} kinase and cdc25-C phosphatase were mixed and incubated together for 15 min at 30°C. The reaction was stopped on ice, and the samples were washed three times with buffer (50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM sodium orthovanadate). Subsequently, the samples were assayed for p34^{cdc2} kinase activity at 37°C for 10 min as described above.

All experiments were carried out at least two times. Data from representative experiments are presented.

TABLE 1. Cell number and viability of HeLa cells treated with *C. botulinum* C2 toxin[a]

Cells	No. of cells/dish (dead cells as % of total cell no.) after incubation for:		
	24 h	48 h	72 h
Control	1.7×10^5 (<10)	3.2×10^5 (<10)	6.0×10^5 (<10)
C2 treated	1.0×10^5 (<10)	1.0×10^5 (<10)	1.2×10^5 (30)

[a] HeLa cells were grown in complete medium at 37°C in the absence (control) or in the presence of C2 toxin (200 ng of C2II and 100 ng of C2I per ml). After 24, 48, and 72 h, the cells were incubated for 30 min with 0.36% trypan blue in complete medium, and the total cell number and the number of cells unable to exclude trypan blue were determined with a Neubauer chamber.

RESULTS

***C. botulinum* C2 toxin delays the G_2/M phase transition in synchronized HeLa cells.** *C. botulinum* C2 toxin ADP-ribosylates monomeric actin in eukaryotic cells, thereby leading to disassembly of actin filaments and breakdown of the actin cytoskeleton (51). As a consequence, C2 toxin-treated cells round up and their substrate attachment is altered. We observed that C2 toxin-treated logarithmically growing HeLa cells stopped proliferating. Up to 72 h after C2 toxin addition, no significant increase in cell number was detectable (compared with control cells), while the majority of cells appeared to be viable as indicated by trypan blue exclusion even after a 48-h C2 toxin treatment (Table 1). Cells treated with C2 toxin did not recover and did not start to proliferate again. Most of the cells exposed to C2 toxin for longer than 3 days became detached from the substrate and were no more able to exclude trypan blue. Based on these findings, we tested the influence of C2 toxin on the division of synchronized HeLa cells. Cells were blocked in the S phase of the cell cycle with amethopterin and subsequently released from this block with thymidine (37). The

degree of cell cycle synchrony achieved by this method is demonstrated in Fig. 1 by DNA histograms obtained by flow cytometry (26). Compared with an asynchronously growing culture (Fig. 1A), a high proportion (about 98%) of cells treated for 16 h with amethopterin accumulated in S phase (inclusive of cells at the G_1/S border) (Fig. 1B). At 9 h after release from the block with thymidine, the majority (50 to 70%) of cells were in the G_2/M phase of the cell cycle (Fig. 1C). Because cell cycle analysis by fluorescence-activated cell sorting does not allow one to distinguish between cells in G_2 phase and cells in mitosis, we determined the amount of cells in mitosis by microscopic counting of mitotic figures, i.e., rounded cells with the typical condensed chromosomes (25, 47). Figure 1D shows the time course of mitotic figures from 7 to 12 h after release of the cells from amethopterin blockage. The majority of cells entered mitosis between 9 and 10 h after release from the S-phase block. The experiment is representative of more than 20 similar control experiments in which the number of mitotic figures per field determined in viable cultures increased between 7 and 10 h after release at least seven times.

To demonstrate that the C2 toxin exerted its cytotoxic effects even on cells synchronized with the described procedure, C2 toxin was added to the medium 6 h after release from the amethopterin block, and cells were further incubated at 37°C. Every 30 min, a culture was lysed and subjected to an in vitro ADP-ribosylation assay with C2I. The autoradiogram in Fig. 2A shows a significantly decreased signal of [^{32}P]ADP-ribosylated G-actin in the lysates after a 2 to 3 h incubation of cells with C2 toxin. This indicates that after about 3 h, the majority of actin was ADP-ribosylated by the C2 toxin in intact cells and no longer constituted a substrate for subsequent in vitro ADP-ribosylation by C2I. This observation is confirmed by F-actin staining of synchronized cells treated with C2 toxin for 2 h. C2 toxin was added at 6 h after release from the amethopterin block to the cells (for control without toxin), cells were fixed and the F-actin was stained with phalloidin-rhodamine. Figure 2B shows the C2 toxin caused disassembly of the actin filaments.

To test whether the C2 toxin inhibits the division of HeLa cells, C2 toxin was added to synchronized cells 4 h after release from the block. The toxin needs 2 to 3 h to exhibit its full effects. During this time, most of the cells were in late S phase. The cells were further incubated in the presence of the toxin (200 ng of C2II and 100 ng of C2I per ml) in complete medium at 37°C. To determine their cell cycle progression, control cells and C2 toxin-treated cells were analyzed at 1-h intervals by flow cytometry starting at 7 h after release from the block. The passage of cells through S phase was apparently not altered by the toxin; however, that through G_2 phase was affected. Figure 3 shows the DNA histograms of control cells (Fig. 3A) and C2-treated cells (Fig. 3B) at 11.5 h after release from the amethopterin block, i.e., 7.5 h after addition of C2 toxin. While in the control culture only 16% of the cells were in G_2/M but 74% were in G_1, after C2 treatment 54% of the cells were in G_2/M and only 25% were in the G_1. This result indicates that C2 toxin treatment delayed the cell division and thereby entry into the G_1 phase of the cell cycle. These findings were confirmed by the time course of cells in the G_2/M phase, determined by flow cytometry (Fig. 3C). While the control cells started to leave the G_2/M phase at 9 h after release from the S-phase block, cells treated at 4 h after release with C2 toxin remained in G_2/M. Because C2 toxin-treated cells rounded up after about 2 to 3 h (Fig. 2B), it was not possible to determine the amount of mitotic figures in these cultures through morphological criteria by microscopic counting. From the results obtained by flow cytometry described above, it was not clear

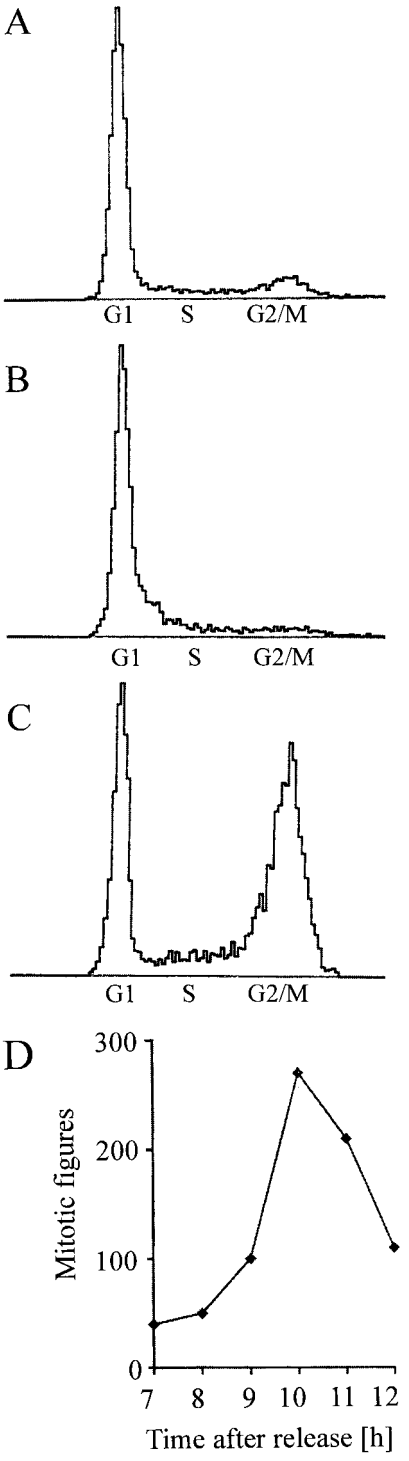

FIG. 1. Cell cycle phase distribution of synchronized HeLa cells. HeLa cells were synchronized with amethopterin and thymidine as described in the text. At the indicated times, cells were fixed and analyzed by flow cytometry. DNA histograms represent asynchronous cells (A), amethopterin-blocked cells (16 h; B) and cells 9 h after release from the amethopterin block (C). Abscissa, relative fluorescence; ordinate, relative cell number. (D) Time course of mitotic figures of amethopterin-thymidine-synchronized HeLa cells starting 7 h after release from the block.

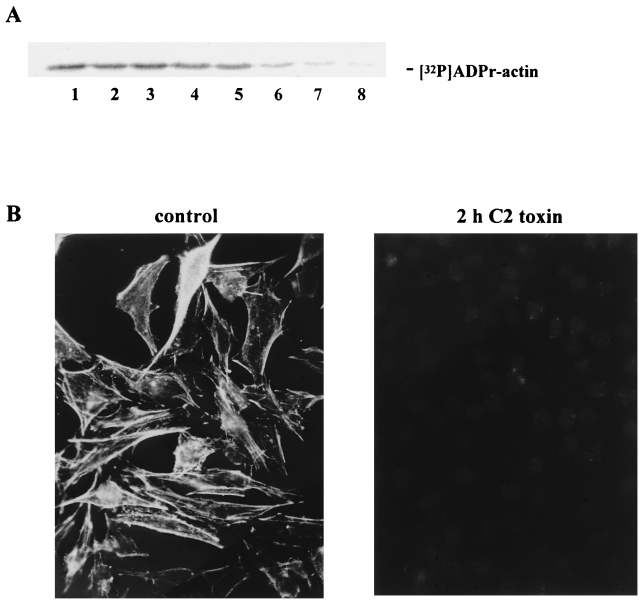

FIG. 2. Cytotoxic effect of *C. botulinum* C2 toxin on synchronous HeLa cells. (A) Time course of C2 toxin-induced actin ADP-ribosylation. At 6 h after release from the amethopterin block, C2 toxin (200 ng of C2II and 100 ng of C2I per ml) was added to synchronized HeLa cells. Cells were incubated at 37°C; immediately and every 30 min after toxin addition, cells were lysed and lysate proteins (100 μg) were subjected to an in vitro ADP-ribosylation assay with C2I. The autoradiogram of [^{32}P]ADP-ribosylated actin is shown. Lane 1, control (without C2 toxin); lanes 2 to 8, incubation for 30 min with C2 toxin, 60 min with C2, 90 min with C2, 120 min with C2, 150 min with C2, 180 min with C2, and 210 min with C2, respectively. (B) C2 toxin-induced morphological changes and F-actin redistribution. Synchronized control cells (8 h after release from the amethopterin block) as well as synchronized cells treated with C2 toxin for 2 h (6 to 8 h after release from the block; 200 ng of C2II and 100 ng of C2I per ml) were fixed, and F-actin was stained with phalloidin-rhodamine.

TABLE 2. Cell cycle distribution of synchronized HeLa cells treated with *C. botulinum* C2 toxin[a]

Cells	% of cells (mean ± SD [n = 6])					
	9.75 h after release			11.25 h after release		
	G_2	M	G_1/S	G_2	M	G_1/S
Control	62 ± 8	19 ± 5	19 ± 6	7 ± 5	10 ± 4	83 ± 8
C2 treated	85 ± 4	9 ± 3	6 ± 3	54 ± 4	38 ± 5	8 ± 3

[a] HeLa cells were synchronized with amethopterin and thymidine as described in the text, and at 4 h after release from the amethopterin block were treated with C2 toxin (200 ng of C2II and 100 ng of C2I per ml). At 9.75 and 11.25 h after release, toxin-treated and untreated control cells were collected from the dish by trypsin treatment and subsequent centrifugation. Part of the cells were fixed on glass slides, and their DNA was stained with the Feulgen reagent. Morphological analysis was carried out on enlarged photographic prints. The other part of the cells was analyzed by flow cytometry.

whether the C2 toxin-treated cells were blocked in mitosis, or whether C2 toxin treatment of cells in late S or early G_2 phase, respectively, prevented their subsequent entry into mitosis and delayed the cells at the G_2/M border. For a more detailed characterization of that topic, cells were analyzed with respect to their cell cycle phase and especially their mitotic phase. Cells were treated at 4 h after release from the amethopterin block with C2 toxin. At 9.75 and 11.25 h after release (i.e., at 5.75 and 7.25 h after C2 toxin addition), C2 toxin-treated and control cells were collected from the dish and fixed on glass slides, and their DNA was stained with Feulgen reagent. The cell cycle phases of these cells are given in Table 2. The data revealed that C2 toxin treatment provoked a significant but transient delay of entry into mitosis. Subsequently cytokinesis might be blocked because of the destruction of the actin cytoskeleton. In the following experiments, we focused on mechanistic aspects of this C2 toxin-induced delay of the G_2/M transition.

C2 toxin treatment prevents the activation of p34^{cdc2} kinase at the G_2/M border. Since C2 toxin treatment of cells delayed their entry into mitosis prior to G_2/M transition, i.e., at a physiological restriction point of the cell cycle, the p34^{cdc2} kinase activity from C2 toxin-treated cells was analyzed. This enzyme normally becomes activated at the G_2/M border and catalyzes entry into mitosis (13). The p34^{cdc2} kinase activity was measured by immunoprecipitation of p34^{cdc2} and subsequent in vitro phosphorylation assay with histone H1 as the substrate (3, 36). To test whether p34^{cdc2} kinase activation of C2 toxin-treated cells is prevented, a time course of p34^{cdc2} protein kinase activity of synchronized HeLa cells treated with or without C2 toxin was performed. For this purpose, C2 toxin was added at 6 h after release from the amethopterin block to the cells (200 ng of C2II and 100 ng of C2I per ml), which were further incubated at 37°C. Starting at 9 h after release from the

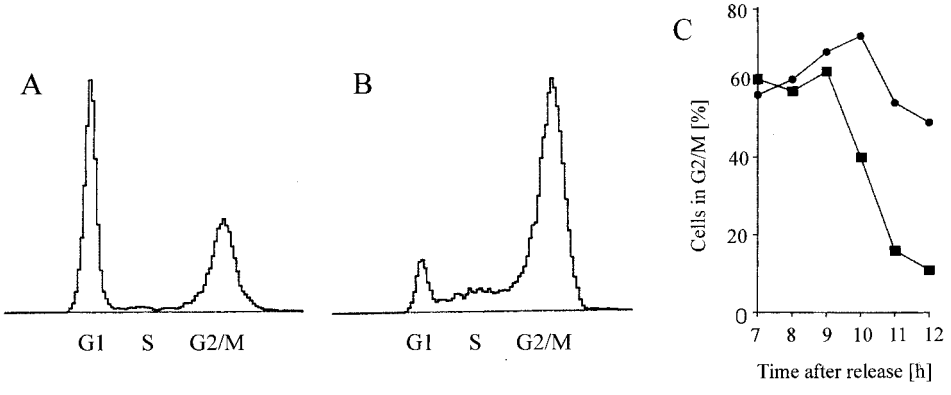

FIG. 3. G_2 delay of synchronous HeLa cells induced by the *C. botulinum* C2 toxin. Four hours after release from the amethopterin block, the cells were treated with C2 toxin (200 ng of C2II and 100 ng of C2I per ml). Starting at 3 h after addition of the C2 toxin (i.e., 7 h after release from the block), cells were fixed and analyzed by flow cytometry. DNA histograms represent control cells (A) and C2 toxin-treated cells (B) at 11.5 h after release from the block (i.e., after 7.5 h of C2 toxin treatment). (C) Time course of the percentage of control (■) and C2-treated (●) cells in G_2/M phase, determined by flow cytometry.

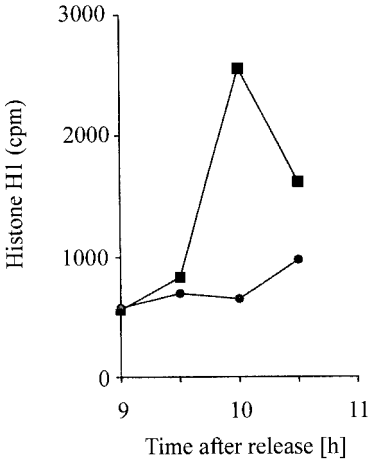

FIG. 4. Time course of p34^{cdc2} protein kinase activity of synchronized HeLa cells untreated (control) or treated with *C. botulinum* C2 toxin. At 6 h after release from the amethopterin block, C2 toxin (200 ng of C2II and 100 ng of C2I per ml) was added to the medium, and the cells were further incubated at 37°C. Starting at 9 h after release from the block, every 30 min control cells (■) and cells treated with C2 toxin (●) were lysed, and p34^{cdc2} was immunoprecipitated and analyzed for histone H1 kinase activity.

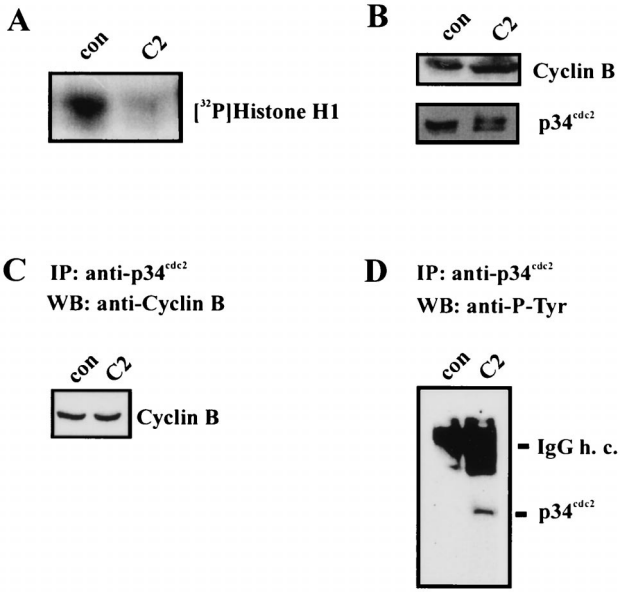

FIG. 5. Effect of *C. botulinum* C2 toxin on the MPF complex of synchronized HeLa cells. At 6 h after release from the amethopterin block, C2 toxin (200 ng of C2II and 100 ng of C2I per ml) was added to the cells. After 4 h at 37°C, cells were lysed and p34^{cdc2} was immunoprecipitated for determination of histone kinase (A). Lysate proteins (100 μg) were subjected to immunoblot analysis with anti-p34^{cdc2} antibody and anti-cyclin B antibody (B). Anti-p34^{cdc2} immunoprecipitates (IP) from the same lysates were probed by Western blotting (WB) with anti-cyclin B antibody (C). The influence of C2 toxin on the tyrosine phosphorylation of p34^{cdc2} was analyzed by immunoblot analysis of p34^{cdc2} immunoprecipitates with anti-P-Tyr (D). con, control; IgG h. c., immunoglobulin G heavy chain.

block, i.e., when the toxin had been present in the medium for 3 h, every 30 min control cells or C2 toxin-treated cells were lysed. Thereafter, p34^{cdc2} was immunoprecipitated from 100 μg of cell lysate protein and analyzed for histone kinase activity. As shown in Fig. 4, p34^{cdc2} kinase activity from control cells dramatically increased between 9.5 and 10 h after release from the S-phase block, reflecting the time course of mitotic figures (Fig. 1D). The kinase activity rapidly decreased after the mitotic peak due to the rapid inactivation of this enzyme after mitotic metaphase. In contrast, p34^{cdc2} kinase activity from C2 toxin-treated cells showed no significant increase. This means that C2 toxin treatment of synchronized HeLa cells in the late S or early G_2 phase of the cell cycle inhibits the subsequent activation of the mitotic inducer p34^{cdc2} kinase.

C2 toxin prevents the activating tyrosine dephosphorylation of p34^{cdc2}. Based on the finding that C2 toxin delays cells at the G_2/M border by preventing activation of p34^{cdc2} kinase, we attempted to analyze the underlying mechanism. MPF, which is composed of the enzymatic Ser/Thr kinase p34^{cdc2} and the regulatory protein cyclin B, is activated after assembly of these two subunits at the G_2/M border by dephosphorylation of tyrosine-15 (and threonine-14) of the p34^{cdc2} subunit (23). It is feasible that the prevention of MPF activation by C2 toxin is caused by (i) effects on the cellular amounts of p34^{cdc2} and/or cyclin B, (ii) alteration of complex assembly, or (iii) prevention of tyrosine dephosphorylation of p34^{cdc2}.

To elucidate the effects of C2 toxin on activation of the MPF complex of synchronized HeLa cells, C2 toxin (200 ng of C2II and 100 ng of C2I per ml) was added to the cells at 6 h after release from the amethopterin block. After incubation at 37°C for a further 4 h, p34^{cdc2} was immunoprecipitated for histone kinase assay. The autoradiogram of the phosphorylated histone H1 is shown in Fig. 5A. To estimate the amounts of the p34^{cdc2} and cyclin B subunits, lysate proteins were subjected to immunoblot analysis with anti-p34^{cdc2} and anti-cyclin B antibodies, respectively. As shown in Fig. 5B, C2 toxin treatment did not affect the amounts of the MPF subunits. Furthermore, C2 did not disturb the assembly of p34^{cdc2} and cyclin B because the complete complex was immunoprecipitated by anti-p34^{cdc2} antibody and protein A/G-agarose beads (Fig. 5C). After toxin treatment, the higher-migrating, i.e., tyrosine-phosphorylated inactive, form of p34^{cdc2} was still detectable, while in control cells the complete p34^{cdc2} was dephosphorylated, i.e., activated (Fig. 5C). To test any effect of C2 toxin treatment on tyrosine phosphorylation of p34^{cdc2}, a blot of p34^{cdc2} immunoprecipitates was probed with anti-P-Tyr antibody. Figure 5D shows that after C2 toxin treatment, p34^{cdc2} was tyrosine phosphorylated to a significantly higher degree than p34^{cdc2} from control cells. These results indicate that *C. botulinum* C2 toxin prevents tyrosine dephosphorylation and activation of the catalytic p34^{cdc2} subunit of MPF. The lack of MPF activation seems to represent the reason for the C2 toxin-induced G_2 delay of synchronized HeLa cells.

C2 toxin treatment prevents the activation of cdc25-C phosphatase at the G_2/M border. The finding that C2 toxin treatment of cells prevented the activating tyrosine dephosphorylation of p34^{cdc2} kinase led us to investigate the influence of the toxin on the activity of cdc25-C phosphatase. Synchronized cells were treated at 6 h after release from the amethopterin block with or without C2 toxin and incubated for further 4 h, i.e., until 10 h after release. Then the cells were lysed, and cdc25-C was immunoprecipitated for phosphatase assay. The cdc25-C immunoprecipitates were incubated for 15 min at 30°C with immunoprecipitated inactive p34^{cdc2} kinase as the substrate. The inactive kinase was immunoprecipitated from S-phase HeLa cells. Finally, the activity of the activated p34^{cdc2} kinase was measured by histone H1 phosphorylation assay. The data shown in Fig. 6 demonstrate that the activity of the inactive p34^{cdc2} kinase (bar 1) was significantly increased by treatment of the kinase with cdc25-C phosphatase from control cells (bar 2). In contrast, a weaker activation was measured on incubation with cdc25-C isolated from C2 toxin-treated cells.

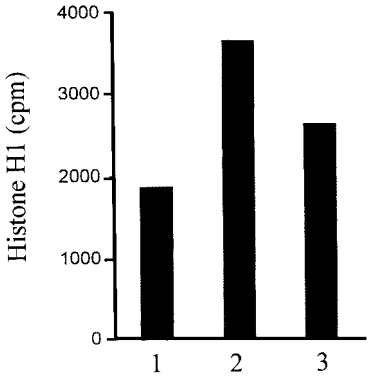

FIG. 6. Effect of *C. botulinum* C2 toxin on cdc25-C phosphatase of synchronized HeLa cells. At 6 h after release from the amethopterin block, C2 toxin (200 ng of C2II and 100 ng of C2I per ml) was given to the cells (for control without toxin). After further 4 h at 37°C, i.e., at 10 h after release, cells were lysed and cdc25-C was immunoprecipitated for determination of phosphatase activity. The cdc25-C immunoprecipitates were incubated for 15 min at 30°C with inactive $p34^{cdc2}$ immunoprecipitated from 500 μg of S-phase HeLa lysate protein. Activity of $p34^{cdc2}$ kinase was measured by histone H1 phosphorylation assay. Histone H1 bands were measured by scintillation counting. Bar 1, S-phase $p34^{cdc2}$ kinase (without cdc25-C); bar 2, S-phase $p34^{cdc2}$ kinase plus cdc25-C from control cells; bar 3, S-phase $p34^{cdc2}$ kinase plus cdc25-C from C2 toxin-treated cells.

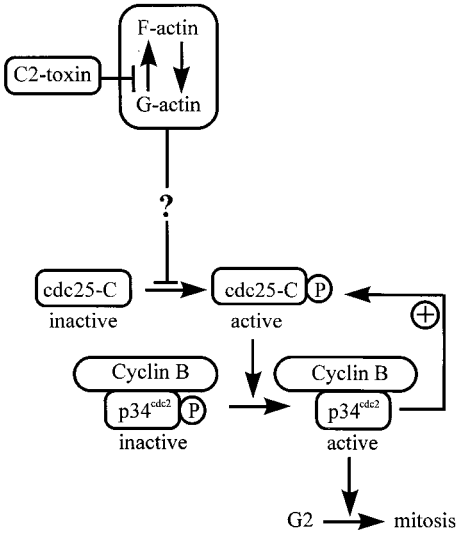

FIG. 7. Model for the G_2 phase cell cycle arrest induced by *C. botulinum* C2 toxin. Cyclin B/$p34^{cdc2}$ is activated at the G_2/M border by dephosphorylation of $p34^{cdc2}$ by the phosphatase cdc25-C. Active $p34^{cdc2}$ kinase drives the cell into mitosis. Furthermore, active $p34^{cdc2}$ kinase activates cdc25-C phosphatase via an autocatalytic loop. Treatment of HeLa cells with C2 toxin delays the G_2/M transition by preventing tyrosine dephosphorylation and thereby activation of the $p34^{cdc2}$ protein kinase. C2 toxin treatment also prevents activation of cdc25-C phosphatase, which indicates that C2 toxin may affect intracellular signal cascades upstream of the mitotic inducers $p34^{cdc2}$ and cdc25-C.

This result indicates that C2 toxin treatment of cells prevents activation of cdc25-C phosphatase at the G_2/M border.

DISCUSSION

A variety of extracellular signals can delay proliferating cells either reversibly or irreversibly at one of two major physiological restriction points of the cell division cycle (24). One of these checkpoints is in G_1 phase; the other is in G_2 phase prior to mitosis. At these restriction points, the cell can integrate exogenous growth-controlling signals from its environment via various signal cascades with the endogenous control systems of cell division (18, 20). The endogenous cell cycle control system is represented by the enzyme family of cyclin-dependent protein kinases, which drive the cell through the individual phases of the division cycle. While much progress has been made in detailed elucidation of the G_1-phase cell cycle checkpoint, the mechanisms leading to a G_2 arrest are less understood.

In this study, we addressed the question of whether the actin ADP-ribosylating *C. botulinum* C2 toxin has any influence on the cell cycle transition of eukaryotic cells since cells treated with C2 toxin seem to stop their proliferation, even when the toxin is removed from the medium. C2 toxin is known to disassemble the cellular actin filaments within a few hours of cell treatment, leading to a breakdown of the actin cytoskeleton and alterations in adhesion of the cell to the substrate. Cytokinesis itself might be influenced because of the disrupted actin cytoskeleton, as observed after cytochalasin treatment (34). Synchronized HeLa cells were treated with C2 toxin during S phase, and analysis of their passage through G_2/M by flow cytometry showed a significant but transient delay in G_2. After the G_2 delay, the C2 toxin-treated cells entered mitotic prophase.

With respect to the mechanism of the G_2 delay caused by C2 toxin, we analyzed its influence on the cyclin-dependent protein kinase mitosis-promoting factor, MPF. This enzyme controls entry into mitosis by phosphorylating specific proteins involved in cell division, such as histone H1 or the lamins. MPF consists of the catalytic subunit $p34^{cdc2}$, which associates with the regulatory protein cyclin B to form the inactive pre-MPF complex during G_2 phase (17). Pre-MPF is activated at the G_2/M border by dephosphorylation of $p34^{cdc2}$ at Thr-14 and Tyr-15, catalyzed by the specific phosphatase cdc25-C. Cells treated with C2 toxin failed to activate $p34^{cdc2}$ kinase by dephosphorylation, indicating that the C2 toxin-induced inhibition of cell division is due to a block prior to mitosis, i.e., in late G_2 phase of the cell cycle. Furthermore, C2 toxin treatment prevented the activation of cdc25-C phosphatase at the G_2/M border. Since $p34^{cdc2}$ kinase and cdc25-C phosphatase are thought to activate each other via an autocatalytic loop (23), one cannot distinguish which of the two enzymes may act as a primary target for toxin-induced signals leading to the G_2-phase delay. As shown in Fig. 7, C2 toxin may induce intracellular signals acting upstream of MPF or cdc25-C.

The phosphatase cdc25-C itself is active in the phosphorylated form and becomes inactivated by dephosphorylation through protein phosphatase 2A (PP2A) (11, 23). The lack of active (i.e., phosphorylated) cdc25-C caused by C2 toxin might be due to an activated PP2A. It has been reported that active PP2A (called INH) may inhibit cells in G_2 phase (30), while inhibition of PP2A leads to a mitosis-like state of cells (21). Since on the one hand PP2A can be inactivated by tyrosine phosphorylation of the catalytic subunit (10) and on the other hand C2 toxin treatment of cells was shown to lead to an alteration in tyrosine phosphorylation of various proteins, probably due to an activation of protein tyrosine phosphatases (45), the superimposed machinery of MPF activation, including PP2A, could represent a target for the C2 toxin-induced effects. At present, however, the pathway connecting the destruction of the actin cytoskeleton to the enzymatic machinery responsible for mitotic control is not clear. A possible link between the actin cytoskeleton and PP2A may be indicated by the observation that actin can be specifically coprecipitated by the use of monoclonal antibodies directed toward the amino-

terminal domain of PR65, the conserved regulatory subunit of PP2A (28). The in vivo relevance of this observation, however, is not yet known.

An involvement of cytoskeletal structures and cell adhesion molecules such as integrins in cell cycle control has been discussed (44, 48, 50, 52). In the case of destruction of the actin cytoskeleton by dihydrocytochalasin B, a G_2 delay was not observed but there was an inhibition of the division of synchronized HeLa cells. These cells are arrested in mitosis because their cleavage furrow is blocked. While the machinery for cell cleavage was disturbed, no influence on the mitotic processes could be observed (34).

Recently other bacterial toxins which act on the actin cytoskeleton of the cell have been analyzed for their influence on the cell cycle transition. Cytolethal distending toxin, produced by *E. coli* and *Campylobacter jejuni* (2, 12), and CNF-1, from *E. coli* (15, 16), both stabilize the actin filaments and, in parallel, induce a G_2 arrest in eukaryotic cells. Cytolethal distending toxin thereby prevents $p34^{cdc2}$ protein kinase dephosphorylation and activation (2). That means that the actin-disrupting C2 toxin shows similar effects with respect to the G_2/M transition as the actin-stabilizing bacterial toxins CNF-1 and cytolethal distending toxin and acts via a different mechanism than dihydrocytochalasin D, which disassembles the actin cytoskeleton as C2 toxin does. In no case so far has a mechanistic link between changes of the cytoskeleton and the cell cycle regulatory enzymes been established. The results point, however, to the same molecular targets for C2 toxin and cytolethal distending toxin from *E. coli*. Genetic evidence for a connection of the actin cytoskeleton and the G_2/M cell cycle control comes from studies in yeast. *Saccharomyces cerevisiae* lacking the TOR2-unique function which mediates the cell cycle-dependent organization of the actin cytoskeleton may arrest in G_2/M of the cell cycle (22).

A delay of cells at the G_2/M border can be caused by exposure to exogenous influences inducing DNA damage, as reported in detail for ionizing radiation, where signal transduction leads to failure of MPF activation at the G_2/M border (7, 14, 32, 36, 38). Since in the present study HeLa cells were treated with C2 toxin during S phase, a DNA-damaging effect cannot be excluded. A delay in the traverse through S phase, however, could not be observed. On the other hand, damage to DNA seems not to be a prerequisite for inducing a block in G_2 phase. Synchronized HeLa cells are reversibly delayed at the G_2/M boundary when they are treated with epidermal growth factor (27). Here too, a failure to activate MPF, due to a lack of tyrosine dephosphorylation of $p34^{cdc2}$, was observed (5).

Thus, our results leave unresolved whether the C2 toxin-induced G_2 arrest and the prevented activation of $p34^{cdc2}$ kinase and cdc25-C phosphatase are directly caused by destruction of the actin cytoskeleton and decrease of focal adhesions or, on the other hand, whether the toxin triggers intracellular signal cascades, such as activation or inactivation of protein kinases and phosphatases, or even DNA damage independent from its effects on actin filaments (Fig. 7).

In conclusion, we have found that (i) *C. botulinum* C2 toxin treatment causes a transient delay of the G_2/M transition of synchronized HeLa cells; (ii) the action of the toxin involves a prevented activation of $p34^{cdc2}$ protein kinase; (iii) the prevented activation of the $p34^{cdc2}$ kinase is based on the lack of an activating tyrosine dephosphorylation of that protein; and (iv) C2 toxin treatment lowers the activation of cdc25-C phosphatase, which is responsible for the activation of the $p34^{cdc2}$ protein kinase. The underlying direct mechanism remains to be elucidated.

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