Formation of a Biologically Active Toxin Complex of the Binary *Clostridium botulinum* C2 Toxin without Cell Membrane Interaction†

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ABSTRACT: *Clostridium botulinum* produces a binary toxin, which is composed of two separate proteins. The enzyme component, C2I, is an ADP-ribosyltransferase which modifies G-actin of eukaryotic cells. The proteolytically activated binding/translocation component, C2IIa, forms ring-shaped heptamers, which bind to cell receptors and mediate the transport of C2I into the cytosol of target cells. According to the current model, receptor-bound C2IIa serves as a docking platform for C2I on the cell surface. Following assembly of C2I, the toxin complex is taken up via receptor-mediated endocytosis, and finally, C2IIa facilitates translocation of C2I from acidic endosomes into the cytosol. Our data support an alternative scenario for the early steps of interaction of the C2 toxin and eukaryotic cells, due to the fact that C2IIa and C2I can interact prior to binding of the toxin to the cell surface. The C2IIa−C2I complex, which was formed in a cell-free system, was detected by native gel electrophoresis and subsequent immunoblot analysis or radiolabeling methods. The preformed C2 toxin complex ADP-ribosylated actin in vitro and induced cell rounding. The interaction of C2I with C2IIa did not enhance the binding of C2IIa to the cellular receptor. Intoxication of Vero cells and of human colon carcinoma cells (CaCo-2) was significantly enhanced when the preformed toxin complex was added to cultured cells as compared to addition of the single components.

Besides the neurotoxins, the pathogenic bacterium *Clostridium botulinum* produces the binary C2 toxin, which consists of two nonlinked proteins, called C2I[1] and C2II (*1*). The enzyme component, C2I (49 kDa), modifies G-actin by ADP ribosylation at arginine 177 (*2*). This modification inhibits further polymerization of actin filaments (*3*), blocks the intrinsic ATPase activity of G-actin (*4*), and interferes with the gelsolin-mediated nucleation of actin filaments (*5*). To reach the cytosol, C2I essentially requires the binding/translocation component, C2II. Only the applied combination of both toxin components has a cytopathic effect on cells (*6*). *C. botulinum* C2 toxin represents the prototype of the family of binary actin-ADP-ribosylating toxins, which comprises besides the C2 toxin, the *Clostridium perfringens* iota toxin, the transferase CDT from *Clostridium difficile*, the toxin from *Clostridium spiroforme*, and the VIP (vegetative insecticidal proteins) from *Bacillus cereus* (*7*). Over the past several years, we and others focused on the cellular uptake mechanism of binary actin-ADP-ribosylating toxins and especially on the role of the binding/translocation component in toxin translocation (for a review, see ref *8*). We reported previously that binding of C2II to carbohydrate receptors on the cell surface requires proteolytic activation (*9*). The nonactivated 80 kDa protein, C2II, is cleaved at amino acid position 182 prior to cell surface binding, leading to self-assembly of C2IIa heptamers (∼420 kDa), ring-shaped structures with an inner diameter of ∼2 nm (*9*). This SDS-stable heptameric form represents the only species of C2II which binds to the cell surface receptor and mediates binding of C2I to cells as demonstrated earlier by flow-cytometry methods (*10*). C2IIa seems to have two different functions during cellular uptake of the toxin. At first, the so-called "prepore" form of C2IIa binds to cell surface receptors and serves as a docking platform for C2I molecules. Following receptor-mediated endocytosis, in acidic endosomes C2IIa heptamers convert from the prepore form to the "pore" form, which inserts into endosomal membranes (*9*). Subsequently, C2I translocates across the membrane into the cytosol, most likely through the C2IIa pores. This translocation step requires an unfolding of the C2I protein (*11*) and is facilitated by the cellular chaperone Hsp90 (*12*).

However, the individual steps leading to receptor binding and formation of the toxin complex are currently unknown. The stage at which the assembly of the toxin components occurs is still unclear, and whether binding of C2IIa to the cellular receptor facilitates subsequent binding of C2I to C2IIa is also still unclear. Here we report that an enzymatically active C2I−C2IIa complex was formed by an in vitro

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[1] Abbreviations: C2I, enzyme component of C2 toxin; EF, *Bacillus anthracis* edema factor; LF, *B. anthracis* lethal factor; PA, *B. anthracis* protective antigen; PBS, phosphate-buffered saline.

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incubation of C2IIa with C2I. The incubation of C2IIa with C2I in a cell-free system prior to application to cultured cells resulted in a more efficient intoxication compared with the applied combination of the single components in the cell medium. Proteolytic cleavage of the C2II protein and subsequent heptamer formation of C2IIa were sufficient for creation of the binding site for C2I.

EXPERIMENTAL PROCEDURES

Materials. Cell culture medium was obtained from Biochrom (Berlin, Germany) and fetal calf serum from PAN Systems (Aidenbach, Germany), and cell culture materials were obtained from Falcon (Heidelberg, Germany). IODO-BEADS were obtained from Pierce (Rockford, IL). $Na^{125}I$ and [^{32}P]NAD were from Hartmann Analytics (Braunschweig, Germany). ECL was purchased from Roche Diagnostics (Mannheim, Germany). C2I and C2II proteins were purified as recombinant GST proteins as described previously (*13*, *14*), and C2II was activated with trypsin as described previously (*9*). The antisera against C2I and C2II were raised from rabbits and characterized previously (*13*, *14*).

Cell Culture and Assays for Cytopathic Effects of the Toxin. African green monkey kidney (Vero) cells and human CaCo-2 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM), containing 5% heat-inactivated (30 min, 56 °C) fetal calf serum, at 37 °C and 5% CO_2. Cells were routinely trypsinized and reseeded three times per week. To test any cytopathic effects of the toxin, cells were grown as subconfluent monolayers and treated with the toxin components at 37 °C in complete medium. For measurement of transepithelial resistance, CaCo-2 cells were grown on filter inserts which were inserted into the EndOhm-12 chamber. A current was applied, and the transepithelial resistance across the cell monolayer was measured as described previously (*14*).

Labeling of the C2I Protein with ^{125}I. Iodination of C2I was performed with IODO-BEADS according to the manufacturer's (Pierce) protocol using 100 μCi of $Na^{125}I$ per 100 μg of protein. Incubation was performed for 15 min at room temperature and subsequently for 1 h on ice. Excess $Na^{125}I$ was removed by filtration.

SDS−PAGE and Immunoblot Analysis. SDS−PAGE was performed according to the methods of Laemmli (*15*). Native 3 to 15% gradient gels were run for 1 h at 140 V prior to application of the protein samples, and proteins were separated by running the native gels at 140 V. For immunoblot analysis of C2I and C2II, the proteins were transferred from the gel onto a nitrocellulose membrane by electroblotting using the semi-dry system. The membranes were blocked for 30 min with 5% nonfat dry milk in PBS containing 0.05% Tween 20 (PBS-T) followed by a 1 h incubation with the respective antiserum (anti-C2I and anti-C2II, both from rabbits). After being washed with PBS-T, the blots were probed for 1 h with donkey anti-rabbit antibody coupled to horseradish peroxidase (1:3000 in PBS-T) and washed, and proteins were detected using the ECL system according to the manufacturer's instructions.

Analysis of the Cell-Bound C2IIa Protein. C2IIa was incubated either with C2I or with buffer (PBS) for control at 4 °C for 15 min. The preincubated proteins were added to the precooled medium (4 °C, no serum) of confluently growing Vero and HeLa cells. Cells were incubated with C2II, C2IIa, and the C2I−C2IIa complex or without any protein for control at 4 °C. The medium was removed and analyzed for the presence of C2IIa by immunoblot analysis. Cells were washed three times with cold serum-free medium, scraped off, and lysed by sonication. Equal amounts of lysate proteins were subjected to SDS−PAGE, and subsequently, the proteins were blotted onto nitrocellulose membranes. C2IIa was detected with a specific antibody as described above.

ADP Ribosylation Assay. The in vitro ADP ribosylation assay of actin by the C2 toxin was performed with CHO cell lysate protein as a source of actin as described previously (*2*, *16*, *17*). Radioactively labeled proteins were detected by phosphorimaging.

All experiments were performed at least three times. Data from representative experiments are given.

RESULTS

Detection of the in Vitro-Formed C2 Toxin Complex. To analyze the in vitro interaction of the toxin components, [^{125}I]-C2I was incubated together with nonactivated C2II (~80 kDa) or with activated C2IIa (~420 kDa) for 40 min. The proteins were subjected to a native gradient gel electrophoresis, and for detection of a [^{125}I]C2I-containing toxin complex, an autoradiography of the gel was performed (Figure 1A). [^{125}I]C2I which was incubated together with C2II (Figure 1A) migrated like [^{125}I]C2I alone (Figure 1A, bottom arrow). In contrast, no signal of free [^{125}I]C2I was detected after preincubation of [^{125}I]C2I with C2IIa (Figure 1A). Instead, a slowly migrating radioactive signal was detected (top arrow). To exclude any nonspecific binding of [^{125}I]C2I to other proteins, we used the *C. difficile* toxin B (~270 kDa) as a control. When [^{125}I]C2I was incubated together with toxin B, only the signal of the free [^{125}I]C2I protein but no shift of [^{125}I]C2I was detected, even after a prolonged exposure time of the radiography (Figure 1A). The next step was to confirm that this slowly migrating signal corresponded to a [^{125}I]C2I−C2IIa complex. Therefore, an immunoblot analysis of this complex with an antiserum raised against the C2II protein was performed in parallel with autoradiography of the same sample (Figure 1B). [^{125}I]C2I was incubated without (Figure 1B, lane 1) or with C2IIa (Figure 1B, lane 2) for 40 min; a native gradient gel electrophoresis was performed, and the gel was blotted onto a nitrocellulose membrane. Autoradiography shows the signal of free [^{125}I]-C2I protein in the absence of C2IIa protein (lane 1). This signal completely disappeared after preincubation of [^{125}I]-C2I with C2IIa (lane 2), and instead, the slowly migrating protein appeared, which contained the total amount of [^{125}I]-C2I. This complex was recognized by the C2II-specific antiserum. Since the antiserum did not cross-react with [^{125}I]-C2I (lane 1), this result clearly indicates that the slowly migrating protein contained both proteins [^{125}I]C2I and C2IIa. Moreover, the complex contained the total amount of [^{125}I]-C2I protein that was present in the reaction mixture. To confirm the formation of a complex of C2IIa and non-radiolabeled C2I in a cell-free system, we incubated 100 ng of C2I with an excess of C2IIa (900 ng of protein) for 15 min. As a control, C2I was incubated without C2IIa protein. The proteins were separated by native gradient gel electro-

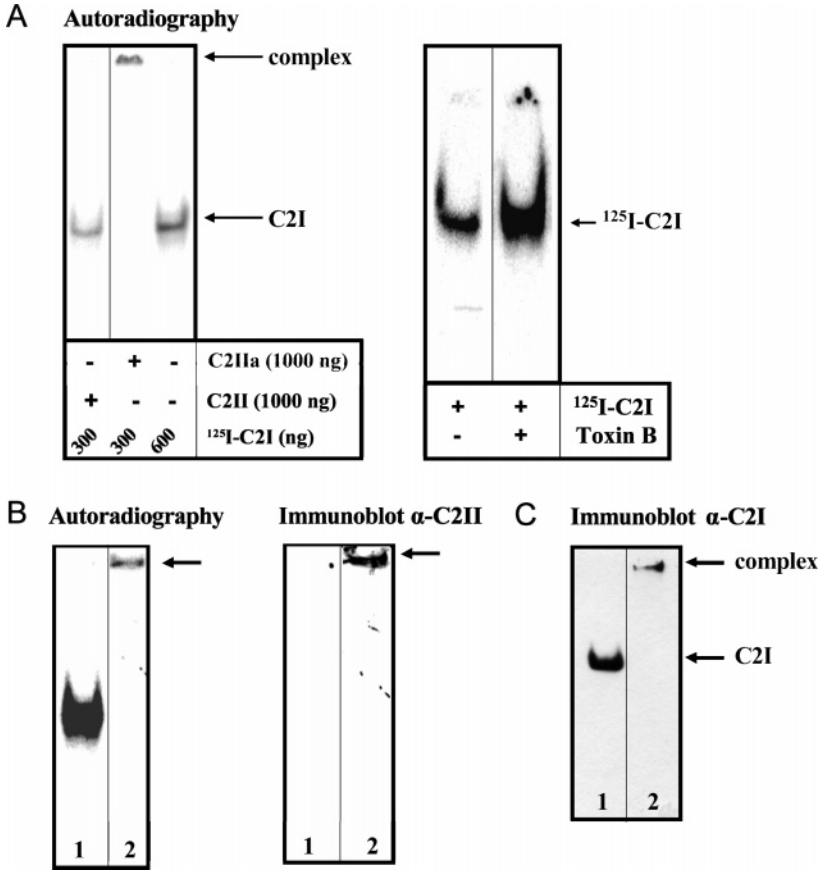

FIGURE 1: Detection of a [^{125}I]C2I−C2IIa complex by native gel electrophoresis. (A) [^{125}I]C2I was incubated alone or together with C2IIa or C2II at room temperature for 40 min (total volume of each sample of 25 μL, adapted with PBS). Native sample buffer was added, and a native gel electrophoresis (3 to 15%) was performed. [^{125}I]C2I was detected via autoradiography which is shown (bottom arrow, free [^{125}I]C2I; top arrow, [^{125}I]C2I in complex with C2IIa). Formation of a complex of C2I and *C. difficile* toxin B was tested by incubation of [^{125}I]C2I (360 ng of protein) alone or together with toxin B (1 μg of protein). Following native gel electrophoresis (3 to 15%), [^{125}I]C2I was detected in an autoradiography (arrow). (B) Comigration of [^{125}I]C2I and C2IIa. [^{125}I]C2I (600 ng of protein) was incubated without (lane 1) or with C2IIa (1 μg of protein, lane 2) at room temperature for 40 min (total volume of each sample of 25 μL, adapted with PBS). Native sample buffer was added, and a native gel electrophoresis (3 to 15%) with a subsequent immunoblot analysis was performed. [^{125}I]C2I was detected by autoradiography of the nitrocellulose membrane, which is shown. In addition, C2IIa was detected with a polyclonal anti-C2II antibody. (C) Anti-C2I immunoblot analysis of a non-radiolabeled C2I−C2IIa complex. C2I (100 ng of protein) was incubated with C2IIa (900 ng of protein) for 15 min at room temperature (lane 2). As a control, C2I was incubated without C2IIa (lane 1). A native gel electrophoresis (3 to 15%) was performed, and C2I-containing proteins were detected with a polyclonal antiserum raised against C2I.

phoresis, blotted onto nitrocellulose, and analyzed with an antiserum raised against C2I (Figure 1C). In the absence of C2IIa, the free C2I was detected (lane 1). In the presence of C2IIa, the toxin complex but no free C2I protein was recognized by the antiserum (lane 2), indicating that the total amount of the C2I protein interacted with C2IIa.

Specific Interaction and Formation of a Complex of [^{125}I]-C2I with C2IIa. The specificity of the interaction of [^{125}I]-C2I with the C2IIa heptamer was demonstrated by competition of radiolabeled C2I with nonlabeled C2I. For this purpose, C2IIa (1000 ng of protein) was incubated with 360 ng of [^{125}I]C2I protein with or without 1500 ng of C2I protein for 30 min to allow binding of C2I to C2IIa. A native gel electrophoresis was performed, and the [^{125}I]C2I protein was detected by subsequent autoradiography (Figure 2, arrow, free [^{125}I]C2I). In the absence of nonlabeled C2I, the total amount of [^{125}I]C2I migrated in the complex with C2IIa (left lane). With the excess of C2I, free [^{125}I]C2I protein was detected, indicating a competition of [^{125}I]C2I with C2I for the binding sites in the C2IIa heptamer. We performed a second experiment for the specificity of the interaction of [^{125}I]C2I with C2IIa and tested whether more than one molecule of [^{125}I]C2I can bind to one C2IIa heptamer. A specific interaction would suggest a limited number of binding sites for C2I on the surface of the C2IIa heptamers. If one hypothetical binding site is postulated for each C2IIa monomer, one would expect at most seven binding sites per C2IIa heptamer. A further possibility is that two monomers of C2IIa create a docking site for one molecule of C2I. A constant amount of C2IIa (1000 ng of protein) was incubated with an increasing amount of [^{125}I]C2I protein to test the saturation of the binding sites for C2I. Samples were run on a native gradient gel, and the [^{125}I]C2I protein was detected by autoradiography (Figure 3A). As a control, [^{125}I]C2I alone (lane 1, bottom arrow) and C2IIa alone (lane 2) were run on the same gel. The ratio between C2I and heptameric C2IIa was 1:1, 3:1, 7:1, and 10:1. In all cases, the [^{125}I]C2I−C2IIa complex was present (lanes 3−6, top arrow). As shown in Figure 3A, no single [^{125}I]C2I protein was detectable in the 1:1 (lane 3) and 3:1 ratios (lane 4). When [^{125}I]C2I was present in a $\geq$7-fold excess with respect to C2IIa heptamers, free [^{125}I]C2I protein appeared (lanes 5 and 6). This result suggests that more than one but less than seven molecules of C2I interacted with one C2IIa heptamer. Studies on the

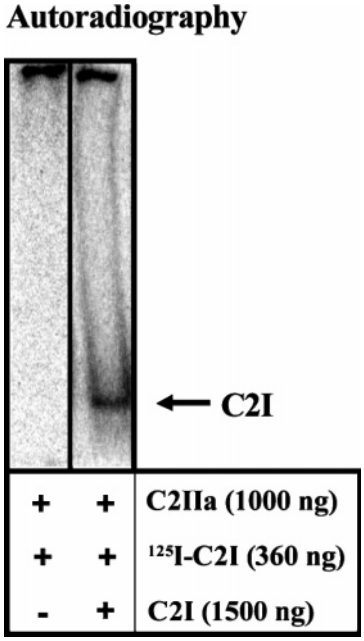

FIGURE 2: Competition of [125I]C2I and C2I for binding to C2IIa. C2IIa (1 μg of protein) was incubated with 360 ng of [125I]C2I protein with or without 1500 ng of C2I protein at room temperature for 30 min (total volume of each sample of 30 μL, adapted with PBS). Six microliters of native sample buffer was added, and a native gel electrophoresis (3 to 15%) was performed as described. [125I]C2I was detected via autoradiography which is shown (arrow, free [125I]C2I).

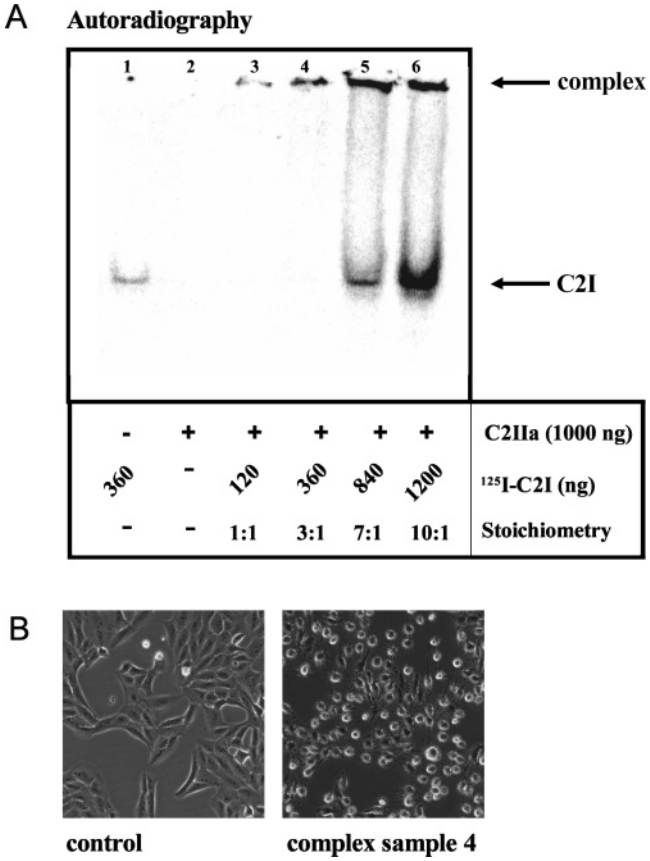

FIGURE 3: Characterization of the C2I−C2IIa toxin complex. (A) C2IIa protein (1 μg) was incubated with increasing amounts of [125I]-C2I (as indicated, sample 3−6) at 37 °C for 40 min (volume adapted to 30 μL with PBS). Native sample buffer was added, and native gel electrophoresis was performed. [125I]C2I was detected via autoradiography which is shown (bottom arrow, free [125I]C2I; top arrow, [125I]C2I in complex with C2IIa). (B) Cytopathic activity of the C2I−C2IIa complex. The preformed complex of sample 4 from Figure 3A (15 μL) was applied to Vero cells (1 mL of complete medium per well), and cells were incubated at 37 °C. Photos were taken after 60 min.

precise stoichiometry of the toxin complex with fluorescent toxin components are underway in our laboratory.

Characterization of the Biological Activity of the C2I−C2IIa Complex. To test whether the toxin complex was able to intoxicate cells, the complex which was formed by the 3:1 [125I]C2I:C2IIa ratio (Figure 3A, lane 4) was added to Vero cells and cells were incubated for 1 h at 37 °C. As a control, cells were incubated without any toxin. The result of the intoxication experiment is shown in Figure 3B. Cells which were treated with the sample of the preformed toxin complex rounded up, indicating that the toxin complex is active. Next, we compared if there are differences in the efficiency of cell intoxication when the in vitro-preincubated C2 toxin components (i.e., preformed C2 toxin complex) or the combination of single proteins C2I and C2IIa was added to the medium. To form the toxin complex in vitro, 400 ng of C2IIa protein and 200 ng of C2I protein were incubated together for 15 min at 4 °C. The total amount of C2I was included in the formation of the C2I−C2IIa complex as confirmed by gel electrophoresis and immunoblot analysis (data not shown). Vero cells were treated with the preformed toxin complex or with 400 ng of C2IIa and 200 ng of C2I which were added into the cell medium without preincubation. After incubation for 3.5 h at 37 °C, pictures were taken (data not shown). The culture which was incubated with the preformed toxin complex contained more round cells than the culture which was exposed to the combination of the single components, C2I and C2II, indicating that the preformed complex was more active.

Cytopathic Activity of the Preformed Toxin Complex versus Single Components. On the basis of this qualitative observation, a quantitative analysis of the toxin effects was performed by measurement of the transepithelial resistance of the human colon cancer CaCo-2 cell line. CaCo-2 cells were grown confluently on filter inserts which were inserted into an EndOhm-12 chamber. After a current had been applied, the transepithelial resistance across the cell layer was measured. An increasing level of intoxication of cells correlated with a decrease in the transepithelial resistance. The preformed C2 toxin complex (37.5 ng of C2I protein and 75 ng of C2IIa protein, incubated for 15 min at 4 °C) was added to cells, and the transepithelial resistance was measured at the indicated time points [Figure 4 (●)]. Alternatively, the combination of the single components, C2I and C2IIa, was added to cells [Figure 4 (○)]. The transepithelial resistance decreased more rapidly when the preformed C2 toxin complex was applied, indicating that the intoxication of cells was more effective than the effect of the combination of both components.

In Vitro ADP Ribosylation of Actin by the in Vitro-Preincubated C2I−C2IIa Complex versus C2I. An in vitro ADP ribosylation of actin was performed to compare the enzymatic activity between free and C2IIa-associated C2I. The C2I protein was incubated with or without C2IIa for 15 min at 4 °C (i.e., under these conditions, the total amount of C2I has bound to C2IIa) and subsequently incubated at 37 °C for 30, 60, and 120 s and 30 min with CHO cell lysate

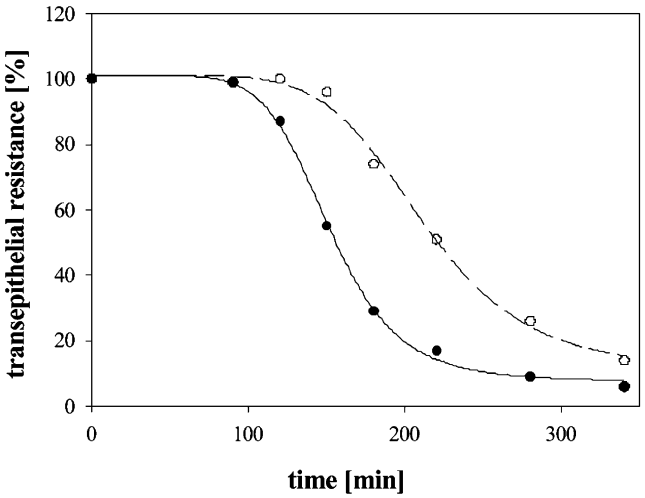

FIGURE 4: Cytopathic activity of the preformed C2I−C2IIa toxin complex vs the single components. C2I (37.5 ng of protein) and C2IIa (75 ng of protein) were incubated together (sample 1, ●) or separately (sample 2, ○) for 15 min at 4 °C (total volume of 50 μL per tube adjusted with PBS). The preincubated C2I−C2IIa complex (sample 1, 50 μL) was given to the CaCo-2 cells together with 50 μL of PBS. The single components, C2I and C2IIa (sample 2, 50 μL each), were added to the cells, and the transepithelial resistance was measured at the indicated time points. Values are given as a percentage of starting transepithelial resistance, which was taken to be 100%.

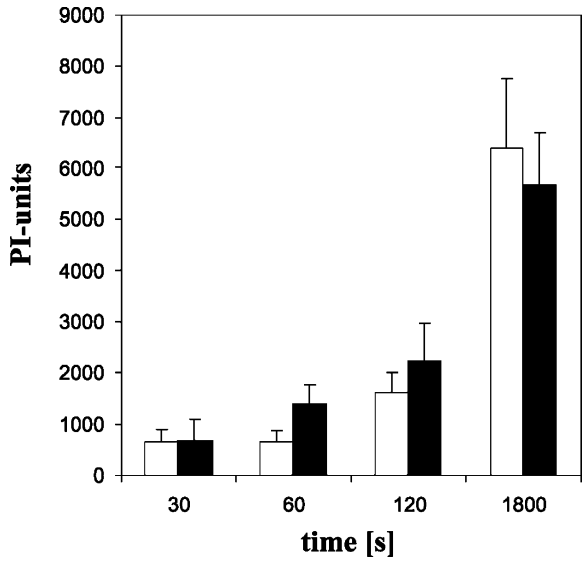

FIGURE 5: In vitro ADP ribosylation of actin by the preformed C2IIa−C2I toxin complex vs C2I. C2I (200 ng of protein) was incubated with or without C2IIa (1800 ng of protein) for 15 min at 4 °C. Subsequently, C2I and the C2I−C2IIa complex were incubated at 37 °C with CHO cell lysate protein and with [^{32}P]-NAD as a cosubstrate. After 30, 60, and 120 s and 30 min, the enzyme reaction was stopped by the addition of SDS sample buffer and heating of the samples (5 min at 95 °C). Following SDS−PAGE and autoradiography, the ADP-ribosylated actin was quantified by phosphorimaging. The data are means of three experiments ± the standard deviation (PI, phosphorimaging units).

protein as a source of actin and with [^{32}P]NAD as a cosubstrate. After the indicated incubation periods, the enzymatic reaction was stopped by the addition of sample buffer and heating of the samples. Following SDS−PAGE and autoradiography, the [^{32}P]ADP-ribosylated actin was quantified by phosphorimaging (Figure 5). There was no dramatic difference in enzymatic activity between C2I and C2IIa-associated C2I. Therefore, an enhanced enzyme activity due to the interaction of C2I with C2IIa was not the reason for the elevated cytopathic activity following in vitro preincubation of C2I with C2IIa.

Does the Interaction of C2I with C2IIa Facilitate the Binding of C2IIa Heptamers to Cells? To address this question, C2IIa was incubated either with or without C2I in solution for 15 min to allow formation of the C2I−C2IIa complex. Subsequently, proteins were allowed to bind to confluently grown Vero cells at 4 °C for various times (1, 2, and 5 min). After the incubation period, the cells were lysed and cell-bound C2IIa was identified by an immunoblot analysis with a specific antibody against C2II. No significant differences in the amounts of cell-bound C2IIa protein were detected (Figure 6A) when comparing preincubated C2IIa alone and the mixture of C2I and C2IIa. This result clearly indicates that preformation of the C2I−C2IIa toxin complex did not enhance the affinity of C2IIa for the cellular receptor. As a control, cells were treated with PBS (Figure 6, lanes 1, 4, and 7). To ensure that equal amounts of C2IIa were added to the cells, the medium was analyzed by immunoblotting for free C2IIa (data not shown). As shown in Figure 6A, the amount of C2IIa protein binding to the cell surface receptor increased over time. When nonactivated C2II was used in this assay instead of C2IIa, no signal was detected (Figure 6B), indicating that only C2IIa, but not C2II, binds to cells.

DISCUSSION

Most of the known AB-type toxins harbor their enzymatic active A domain and their binding domain (B domain) on one single peptide chain, or the two moieties stick together by either disulfide bonds or hydrophobic interaction. In contrast, the binary toxins are secreted by the producing bacteria as two individual proteins, which exhibit only their cytopathic effects after assembly of the two subunits (for a review, see ref *7*).

For intoxication of eukaryotic cells with the binary *C. botulinum* C2 toxin, the presence of both components C2I and C2II in the cell medium is required. In earlier experiments, cytopathic effects of the C2 toxin have been detected when both components were added sequentially to cells. Incubation of cells with C2IIa followed by a washing step to remove unbound C2IIa and a subsequent incubation of these C2IIa-loaded cells with C2I led to cell rounding, indicating that C2I interacts with cell-bound C2IIa (*18*). It was assumed that binding of C2IIa to its receptor might be an essential prerequisite for the creation or optimization of the docking site for C2I within the C2IIa protein, leading to the hypothesis that the assembly of the toxin components occurs on the cell surface. Thus, no or only marginal interaction between C2IIa and C2I was expected in a cell-free system. We demonstrated that a C2I−C2IIa complex was formed in vitro. C2I in the C2I−C2IIa complex ADP-ribosylated actin in vitro and showed no decreased activity compared to free C2I. Moreover, the preformed toxin complex exhibited an enhanced cytopathic activity in comparison to the combination of the single components. Therefore, an alternative scenario for the interaction of C2I with C2IIa prior to cell binding must be discussed. The question of whether binding of the preformed C2 toxin

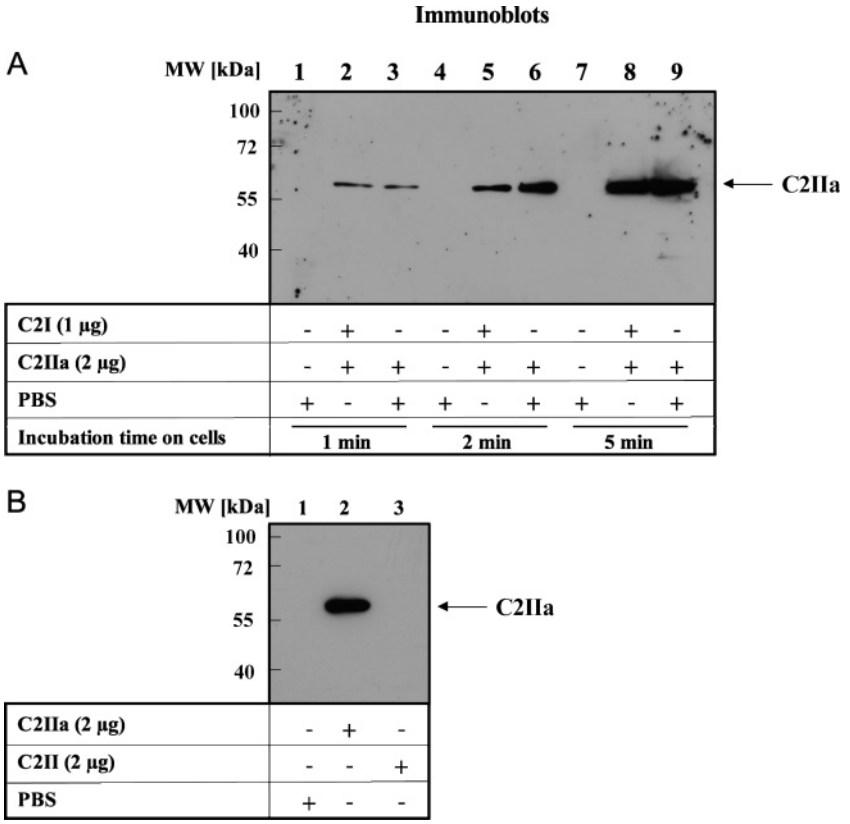

FIGURE 6: Immunoblot analysis of cell-bound C2IIa protein. C2IIa (2 μg of protein) was incubated in solution with either C2I (1 μg of protein) or phosphate buffer for a control at 4 °C for 15 min. The preincubated proteins were added to the precooled medium (4 °C, no serum) of confluently grown Vero cells (without any toxin as a control), and the cells were incubated for further 1, 2, or 5 min at 4 °C. Cells were washed with ice-cold PBS, scraped off into RIPA buffer, and lysed by sonication. (A) Cell lysate proteins were subjected to SDS−PAGE; subsequently, the proteins were blotted onto a nitrocellulose membrane, and C2IIa was detected with a specific antibody raised against C2II (rabbit, 1:2000 dilution) and a peroxidase-coupled antibody (goat anti-rabbit). (B) To exclude any nonspecific binding of C2IIa to the cells or the substratum, nonactivated C2II was used as a control.

complex to the cell is the predominant pathogenetic pathway for C2 toxin or whether it represents an alternative way used by the toxin arises. Under physiological conditions, both toxin components are present in the medium since both proteins are produced and secreted by *C. botulinum*. We reported earlier that the activation of C2II occurs under experimental conditions with trypsin. Nonactivated C2II interacts with neither the cellular receptor nor C2I. Activation of C2II enables C2IIa to interact with the receptor and C2I. At low toxin concentrations, complex formation might enhance the efficiency of the binary toxin. When C2IIa has bound to its receptor but no C2I assembles to the cell-bound C2IIa heptamer, the heptamer is taken up but no C2I protein is delivered into the cytosol. In contrast, when C2IIa assembles with C2I prior to binding to the cellular receptor, the C2I−C2IIa complex is endocytosed; i.e., there is a guarantee that C2I is taken up together with C2IIa and reaches the cytosol. However, we have no experimental evidence that the preformed toxin complex does not dissociate when it is applied to the cell medium. In this case, the reassembly of both toxin components could occur either in the medium or on the surface of cells. The observation that the preformed toxin complex exhibited an enhanced cytopathic activity compared to the combination of the single components argues against this hypothesis.

Does the binding of C2I to C2IIa induce a conformational change within the C2IIa heptamer, which causes an enhanced affinity of C2IIa for its receptor? When we compared the time-dependent cell binding of C2IIa protein with that of the preformed C2I−C2IIa complex, we did not observe any differences. Therefore, the binding of C2I to C2IIa has no influence on binding of C2IIa to its receptor.

There are two binary toxins which are produced by *Bacillus anthracis* and which are causative for anthrax. The central part of the two bipartite anthrax toxins is the protective antigen (PA), which delivers two different enzyme components into the cytosol of eukaryotic cells, the lethal factor (LF), a metalloprotease, and the edema factor (EF), an adenylylcyclase (for a review, see ref *19*). The sequence of *C. botulinum* C2II protein is significantly homologous with that of PA, and like C2II, the PA protein (83 kDa) requires proteolytic activation. After removal of an N-terminal peptide, the resulting PA-63 protein, but not PA-83, forms SDS-resistant ring-shaped heptamers (*20*) with a central lumen of ~3.5 nm (*21*). The cellular uptake mechanism of the anthrax toxins and of the C2 toxin is very similar because both transport components, C2IIa and PA-63, form pores in endosomal membranes and facilitate translocation of the enzyme components into the cytosol (for a review, see ref *7*). According to the current model, PA-83 binds to the cellular receptor followed by its cleavage by the cellular protease furin (*22*). This is in contrast to the C2 toxin because only activated C2IIa but not C2II binds to cell receptors (*10*). Only the PA-63 heptamers serve as docking sites for LF or EF (*23*). This is in perfect agreement with the C2 toxin because only activated C2IIa interacts with C2I (*10*). It was

demonstrated that a PA-63−LF complex was formed in vitro (*23*, *24*). These findings imply that binding of PA-63 to its receptor is not essential for interaction of PA-63 with LF.

One important difference between the C2 toxin and the anthrax toxins is the uptake of C2 toxin via the intestinal system. Most likely, C2II becomes activated by proteases within the gut, while PA-83 is nicked by furin on the cell surface. Therefore, the time point and the place of activation of the binding component might be critical for any complex formation with the toxin because C2IIa heptamers can assemble with C2I molecules prior to binding of C2IIa to the target cells.

Under experimental conditions, one PA-63 heptamer binds to three molecules of LF under saturated conditions (*24*). Actually, it is not known how many C2I molecules assemble to form a C2IIa heptamer. Here we used the radiolabeled C2I protein to determine whether one or more molecules of C2I can bind to C2IIa. If only one molecule of [^{125}I]C2I had bound to one heptamer of C2IIa, a signal resulting from free running [^{125}I]C2I protein would have been expected in the autoradiography results. However, we did not detect any free [^{125}I]C2I protein at a [^{125}I]C2I:C2IIa heptamer ratio of up to 3:1. Further studies on the stoichiometry of the C2I−C2IIa complex with fluorescent proteins are currently underway in our laboratory.

PA does not bind or transport the enzyme components of other binary toxins (*25*). The only functional complementation within the binary toxins occurs among the *C. perfringens* iota toxin, *C. difficile* CDT, and the *C. spiroforme* toxin, further members of the family of clostridial binary actin-ADP-ribosylating toxins (for a review, see ref *7*). The binding components of these toxins are interchangeable and facilitate the translocation of each enzyme component of this toxin group. For iota toxin, it was demonstrated that proteolytic activation of the binding/translocation component Ib facilitates the interaction with Ia (*26*). Activated Ib forms SDS-stable heptamers in solution (*14*, *27*) and on the cell surface (*27*, *28*). Ib oligomers form voltage-dependent ion-selective channels in membranes (*29*). The cellular receptor for Ib has not yet been identified, but it was reported that detergent-resistant domains on the surface of target cells facilitate the oligomerization of Ib (*30*). However, despite the similarities between Ib and C2IIa, there is a remarkable difference between both proteins. In vitro oligomerization of activated Ib results in heptamers, which are less stable than heptamers formed on the cell surface (*28*). Moreover, Ib heptamers generated in solution do not form functional ion channels in lipid membranes and do not promote any cytopathic effect (*28*). As a consequence of these findings, the formation of a biologically active iota toxin complex consisting of Ib heptamers and Ia molecules in a cell-free system remains unlikely. The observation that the formation of functional Ib heptamers requires the cell surface is in contrast to our findings for the C2 toxin and confirms that there are variations in the mode of action between the individual members of the toxin family of clostridial binary actin-ADP-ribosylating toxins. In summary, from our study, it becomes evident that the C2 toxin is more similar to the anthrax system than to the iota toxin with regard to the spatiotemporal interaction of the toxin components.

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