The actin-ADP-ribosylating binary *Clostridium botulinum* C2 toxin consists of two individual proteins, the binding/translocation component C2II and the enzyme component C2I. To elicit its cytotoxic action, C2II binds to a receptor on the cell surface and mediates cell entry of C2I via receptor-mediated endocytosis. Here we report that binding of C2II to the surface of target cells requires cleavage of C2II by trypsin. Trypsin cleavage causes oligomerization of the activated C2II (C2IIa) to give SDS-stable heptameric structures, which exhibit a characteristic annular or horseshoe shape and form channels in lipid bilayer membranes. Cytosolic delivery of the enzyme component C2II is blocked by bafilomycin but not by brefeldin A or nocodazole, indicating uptake from an endosomal compartment and requirement of endosomal acidification for cell entry. In the presence of C2IIa and C2I, short term acidification of the extracellular medium (pH 5.4) allows C2I to enter the cytosol directly. Our data indicate that entry of C2 toxin into cells involves (i) activation of C2II by trypsin-cleavage, (ii) oligomerization of cleaved C2IIa to heptamers, (iii) binding of the C2IIa oligomers to the carbohydrate receptor on the cell surface and assembly with C2I, (iv) receptor-mediated endocytosis of both C2 components into endosomes, and finally (v) translocation and release of C2I into the cytosol after acidification of the endosomal compartment.

Bacterial exotoxins that enzymatically modify substrates in eukaryotic target cells have developed delivery systems to transport their active components across lipid membranes into the cytoplasm to mediate their cytotoxic effects. In most cases, this process requires four steps: receptor-binding on the surface of target cells, receptor-mediated endocytosis, translocation of the enzyme domain into the cytosol, and modification of the specific target (1). Bacterial protein toxins of the AB type consist of a binding domain (B domain), which binds to a specific receptor on target cells and mediates translocation of the enzymatic A domain into the cytosol. In some toxins (e.g. diphtheria toxin), the A and B domains are located on the same polypeptide chain (2). In binary bacterial protein toxins, the enzyme component and the binding component are individual and nonlinked proteins that assemble on the target cell (3, 4). Members of this binary toxin family are *e*-toxin from *Clostridium perfringens* (5), the ADP-ribosyltransferase from *Clostridium difficile* (6), *Clostridium spiroforme* toxin (7), the vegetative insecticidal proteins from *Bacillus cereus* (8), the anthrax toxin from *Bacillus anthracis* (9), and *C. botulinum* C2 toxin (10).

C2 toxin consists of the enzyme component C2I, an ADP-ribosyltransferase that modifies G-actin (11, 12) and the binding component C2II, which mediates cell entry of the toxin (13, 14). A prerequisite of the toxin action is the assembly of both components at the surface of the target cells. Recently, we reported that C2II binds to asparagine-linked glycans on the cell surface (15). Subsequent to binding, C2II and C2I are taken up by receptor-mediated endocytosis (16). C2I is translocated into the cytosol, and finally, it ADP-ribosylates G-actin at arginine 177 (17). This modification of G-actin causes inhibition of actin polymerization (11). Moreover, ADP-ribosylated G-actin acts like a capping protein to block polymerization of unmodified actin at the barbed ends of F-actin (18). Finally, these effects cause depolymerization of actin filaments, breakdown of the actin cytoskeleton, and rounding up of target cells (19, 20). While the enzyme component C2I (21, 22) and its action (20) was characterized in detail, much less is known about the binding component C2II and the cellular uptake mechanism for C2 toxin.

Here the activation and oligomerization of C2II and the requirements for toxin entry into mammalian cells were studied. We show that proteolytic activation of the C2II binding component by trypsin (23) is a prerequisite for cellular C2 toxin uptake. Thereby, a 20-kDa peptide is cleaved off from the N-terminus of native C2II, resulting in active C2IIa. C2IIa but not native C2II oligomerizes to heptamers, which form ion-permeable channels in artificial lipid bilayer membranes (24). Moreover, we present evidence for the cell entry of C2 toxin from an acidic compartment.

**EXPERIMENTAL PROCEDURES**

**Materials—**Cell culture medium was from Biochrom (Berlin, Germany), fetal calf serum was from PAN Systems (Aidenbach, Germany), and cell culture materials were from Falcon (Heidelberg, Germany). The C2I enzyme component was purified as recombinant glutathione S-transferase fusion proteins with the glutathione S-transferase Gene Fusion System from Amersham Pharmacia Biotech as described (21). Anti-HB4 antiserum was raised from rabbits against the 15 N-terminal amino acids from trypsin-activated C2IIa coupled to keyhole limpet hemocyanine (synthesized by H. R. Rackwitz, Deutsches Krebsforschungs...
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Activation of C2II by Trypsin Cleavage Leads to Oligomerization of C2II—The C2II binding component from C. botulinum C2 toxin was purified and activated with trypsin as described (10, 23). Recombinant C2II was purified as recombinant glutathione S-transferase fusion protein with the glutathione S-transferase Gene Fusion System from Pharmacia Biotech and cleaved with thrombin as described. For activation, C2II was incubated for 20 min at 37 °C with 0.2 μg of trypsin/μg of C2II and subsequently with 2 μg of trypsin inhibitor for 1 h at 4 °C to block trypsin effects. C2IIa oligomers were isolated by centrifugation at 4 °C with a Vivaspin 4 ML Concentrator device with a cut-off mass of 100 kDa. Activation and oligomerization of C2IIa were analyzed by 3–12.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) with or without prior boiling of the samples, respectively.

Analytical Ultracentrifugation—Molecular mass studies on recombinant C2IIa oligomers were performed using an An-60 Ti rotor in an XL-A type analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA) equipped with UV absorbance optics. Sedimentation equilibrium technique was employed to directly determine the molecular mass. Sedimentation equilibrium was analyzed using externally loaded six-channel centerpieces of 12-mm optical path filled with about 70 μl of liquid. This cell type allows the analysis of three solvent-solution pairs. Three of these cells were used to simultaneously analyze different samples in one run. Sedimentation equilibrium was reached after 2 h of overspeed at 14,000 rpm followed by an equilibrium speed of 10,000 rpm for about 2 h. The radial absorbances of each compartment were recorded at three different wavelengths (275, 280, and 285 nm) using the molar absorbance coefficients. Molecular mass calculations were carried out by simultaneously fitting the sets of three radial absorbance distribution curves described by the equation

\[ A = A_o \exp\left(\frac{MKr^2}{2} - r^2\right) \]

with \( K = \left(1 - \frac{\rho}{\pi \rho_0}\right) \rho_2 / 2RT \), using our computer program Polymole (25). In these equations, \( \rho \) is the solvent density, \( \rho_0 \) is the partial specific volume, \( r \) is the angular velocity, \( R \) is the gas constant, and \( T \) is the absolute temperature. \( A_o \) is the radial absorbance, and \( A_{o0} \) is the corresponding value at the meniscus position. The partial specific volume was calculated from the amino acid composition and the density increments of the individual amino acids.

Black Lipid Membrane Experiments—Channel formation experiments of C2IIa oligomer in black lipid bilayer membranes were performed as described previously (24, 26). In brief, membranes were formed by painting onto a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in n-decane across a 0.5-mm² circular hole, which connects two aequous compartments of a Teflon chamber. The single channel recordings were performed by the use of Golom electrodes (with salt bridges) connected in series to a voltage source and a current amplifier. The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart or tape recorder.

Electronic Microscopy of C2IIa Oligomers—The stock protein solution (0.15 mg/ml) was diluted 20-fold in a buffer containing 10 m M HEPES, 150 mM NaCl, pH 7.4. Samples (5 μl) were deposited on carbon-coated electron microscopy grids rendered hydrophilic by glow discharge in air and negatively stained with 1% uranyl acetate. Electron microscopy was performed with a CM120 Philips microscope operating at 120 kV. Images were recorded under low dose conditions at \( \times \) 60,000 magnification.

Cell Culture and Cytotoxicity Assays—CHO-K1, HeLa, and NIH3T3 cells, respectively, were cultivated in tissue culture flasks at 37 °C and 5% CO₂. CHO cells were grown in Ham’s F-12/Dulbecco’s modified Eagle’s medium (1:1), and HeLa and NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium. All media contained 5% heat-inactivated (30 min, 56 °C) fetal calf serum, 2 mM l-glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were routinely trypsinized and resuspended twice a week. For cytotoxicity experiments, cells were plated in subconfluent dishes and incubated with the concentra-
a faint band of slowly migrating protein was detected almost at the start of the separation gel. When C2II was subjected to 3–12.5% gradient SDS-PAGE without prior heating for 3 min, no monomeric C2IIa but a protein band of a high migrating band was observed when C2II purified from recombinant C2II (100 μg/ml) was incubated with anti-C2II antiserum HB-4 in an immunoblot analysis (B). Lane 1, native C2II (not heated); lane 2, C2IIa (3 min at 95 °C); lane 3, C2IIa (not heated). C, time course of C2II activation by trypsin. Trypsin was added to C2II, and immediately and after the indicated incubation times at 95 °C, aliquots were taken, heated for 3 min at 95 °C (alternatively not heated), and analyzed by 3–12.5% SDS-PAGE and Coomassie Blue staining.

**C2IIa Oligomers Form Ring-shaped Structures in Solution**—To analyze the stoichiometry of the C2IIa oligomers, the molecular mass of the complex was determined by analytical ultracentrifugation. Therefore, the C2IIa protein solution was analyzed at different concentrations between 14 and 70 μg/ml. Since the solutions contained some low molecular weight material besides the oligomers, an equilibrium speed of 10,000 rpm was used. This allowed us to consider the low molecular weight material to be buried in the base line with negligible increase of the radial absorbance at sedimentation equilibrium. Using the Polymol program, the data (Fig. 4) were fitted simultaneously. A mass average molecular mass of 422 ± 78 kDa was determined. Similar results were obtained from solutions with loading concentrations of 50–70 μg/ml. In the more diluted samples, an average molecular mass of nearly 60 kDa was determined. The high molecular mass component of 420 kDa exceeds the monomer molecular mass about 7 times, indicating that the oligomers should form on the average heptamers. Because the calculated molecular mass of trypsin-activated C2IIa monomer is 59.8 kDa, the deviation from average molecular mass (78 kDa) allows us to speculate that hexamers and octamers may be present, too.

**C2IIa Oligomers Form Channels in Artificial Lipid Bilayer Membranes**—As described previously, trypsin-activated C2IIa...
but not full-length C2II forms ion-permeable channels in artificial lipid bilayer membranes (24). Therefore, we tested the channel forming activity of purified C2IIa oligomers. A typical experiment is given in Fig. 5. Ten min after membrane formation, 100 ng/ml C2IIa oligomers were added to the aqueous 1 mM KCl solution (pH 6) on one side of the membrane. Shortly after the addition, the membrane conductance started to increase in a stepwise fashion. Most of the conductance steps had a single channel conductance of about 150 pS at 1 mM KCl.

C2IIa Oligomers Bind to Cells and Mediate Cell Entry of C2I—As shown above, activated C2IIa but not native C2II binds to cells and mediates C2 toxin effects when delivered together with C2I. Because C2IIa forms SDS-stable but heat-sensitive heptamers, which are able to insert into lipid bilayers, we analyzed which species of C2IIa binds to cells. Therefore, CHO cells were incubated with C2II and C2IIa, respectively, and analyzed in an immunoblot without prior heating of the samples. CHO-WT and the C2 receptor-defective CHO-C2RK14 cells were incubated for 1 h at 4 °C in HBSS with native C2II or trypsin-activated C2IIa to allow binding of the proteins. For controls, cells were incubated without C2II. Cells were washed, lysed, and immediately subjected to SDS-PAGE (3–12.5%) without heating. Proteins were blotted onto a nitrocellulose membrane and analyzed with an antisem against C2II. The immunoblot shows that only the oligomeric C2IIa was significantly bound to CHO-WT cells (Fig. 6). Only a weak signal was detected when CHO-C2RK14 cells were incubated with C2IIa oligomers, which may be due to spontaneously reverted cells. This indicated that the oligomeric C2IIa pre-pores bound specifically to the cellular receptor. Moreover, when C2IIa was bound at 4 °C to cells, which were subsequently washed and then shifted to 37 °C, only oligomeric C2IIa was detected after 30 min, indicating that C2II oligomers do not dissociate during endocytosis. Finally, we incubated CHO-WT cells at 4 °C with both C2IIa and C2I. Cells were washed and further incubated at 37 °C. After 2 h, the cells were completely rounded up. Cells were lysed and subjected to immunoblot analysis. Even after 2 h, C2IIa oligomers remained stable, and no C2IIa monomer was detected. This demonstrates that the C2IIa oligomer represents the species of C2II that binds to the carbohydrate receptor for C2II on the surface of target cells and mediates the uptake of C2I.

Cellular Uptake of C2 Toxin Requires an Acidic Compartment—Previously, it was reported that C2 toxin enters the cell via receptor-mediated endocytosis (16). Next, we studied the intracellular route of C2 toxin to reach its final destination in the cytosol. To answer the question of whether C2 toxin uptake requires an acidic cellular compartment, bafilomycin A1 was applied (30). Bafilomycin is a specific inhibitor of the vacuolar-type H+-ATPase (31) and is frequently used to detect toxin uptake from acidic compartments. As shown in Fig. 7A, cells that were incubated with C2 toxin without bafilomycin A1 (C2) were round. In contrast, cells that were treated with C2 toxin in the presence of bafilomycin (Baf + C2) showed control morphology. Bafilomycin itself did not induce any morphological effects within the incubation times indicated. In line with morphological changes, actin was ADP-ribosylated. The cell lysates were subjected to an in vitro ADP-ribosylation assay with [32P]NAD and C2I. The autoradiography of [32P]ADP-ribosylated actin in Fig. 7B shows that only the actin from the cells treated with C2 toxin without bafilomycin was not radiolabeled, indicating entry and action of C2 toxin. By contrast, radiolabeling of actin was observed when cells were previously treated with C2 toxin in the presence of bafilomycin. These results indicate that bafilomycin A1 prevents C2 toxin uptake and suggest that an acidic compartment is required for the translocation of C2 toxin into the cytosol.

C2 Toxin Can Be Delivered into Cells by Extracellular Acidification in the Presence of Bafilomycin A1—Because C2IIa mediates translocation of C2I across the endosomal membrane, we wondered whether cell surface-bound C2 toxin could be delivered directly into the cytosol after extracellular acidification. To block endosomal toxin uptake, experiments were performed in the presence of bafilomycin A1. NIH3T3 cells were preincubated for 15 min at 37 °C with bafilomycin A1, prechilled, and subsequently incubated for 1 h with C2IIa and C2I at 4 °C to allow binding of the toxin. Cells were washed and incubated for 3 min at 37 °C with prewarmed medium containing bafilomycin at pH 7.5 or 5.0, respectively. Thereafter, fresh prewarmed medium (37 °C, pH 7.5) containing bafilomycin A1 was added, and cells were incubated for further 1.5 h at 37 °C. As shown by phase-contrast microscopy in Fig. 8A, only those cells exhibited C2 morphology that were allowed to bind C2 toxin and were subsequently exposed to pH 5.0 medium. Cells that bound C2 toxin but were not shifted to acidic pH did not round up. The observation that C2 toxin was taken up into the cells after an acidic shift was confirmed by subsequent in vitro ADP-ribosylation of the lysates from these cells (Fig. 8B). Only actin from cells that showed C2 morphology was not radioactively labeled. In these cells, all G-actin was ADP-ribosylated by C2I, which entered the cells after the extracellular acidic shift. For a more detailed characterization of the pH dependence of C2 toxin uptake, the effects of pH values between 4.0 and 7.0 were studied. Cells that were exposed to pH 4.0 and 5.0 after C2 toxin binding were completely round. By contrast, cells showed control morphology after exposure to pH 6.0 and 7.0, respectively (data not shown). Testing small pH steps, we observed the typical C2 morphology (rounding up of cells) at pH ≤5.4. By contrast, cells that were exposed to pH >5.6 showed control morphology. Even after 3 h, no C2 toxin effects were observed on cells that were exposed to pH >5.6. The percentage of rounded cells per field after 1.5- and 3-h exposure is shown in Fig. 9A, again indicating the sharp pH step allowing entry of toxin at pH 5.4.

Cells also rounded up when the time of external acidification was decreased from 3 min to 30 s (not shown). Notably, cells did not round up when they were incubated at 4 °C with C2II (in the absence of C2I), shifted to acidic external pH (3 min, pH 4.5), and were subsequently incubated with C2I in complete medium (pH 7.5 plus bafilomycin A1) (data not shown). This indicates that C2I must be bound to C2IIa prior to acidification. Thus, we propose that the C2IIa pre-pore binds C2I and assembles with the receptor on the cell surface (Fig. 9B). After exposure of the extracellular site to acidic pH, the C2IIa pre-pore inserts into the cytoplasmic membrane and forms a pore similar to that in the endosomal membrane upon acidification. Subsequently, C2I translocates to the intracellular site.

Actin Filaments but Not Microtubules Are Involved in the Uptake of C2 Toxin—Microtubules are involved in the trans-
port of vesicles from the early to the late endosomal compartment (32, 33). To test any influence of the cytoskeletal components on uptake of C2 toxin, microtubules and actin filaments were disassembled by nocodazole and cytochalasin D, respectively. Therefore, CHO cells were incubated for 6 h with nocodazole or cytochalasin D, respectively. C2II plus C2I was added, and cells were incubated for further 12 h. Because nocodazole or cytochalasin D changed cell morphology in their own right, the cellular effects of C2 toxin were determined by subsequent in vitro actin ADP-ribosylation (Fig. 9C). Actin from nocodazole-pretreated cells was not radioactively labeled when cells were subsequently treated with C2 toxin. In contrast, a significant amount of [32P]ADP-ribosylated actin was detected when cells were pretreated with cytochalasin D and subsequently incubated with C2 toxin. These data indicate that the microtubule system is not essential for C2 toxin and suggest that C2I is released from the early endosomes into the cytosol. As expected, brefeldin A, which prevents retrograde transport of proteins to the endoplasmatic reticulum, also had no influence on C2 toxin uptake. When CHO cells were preincubated for 30 min with brefeldin A and subsequently C2II plus C2I was added, cells were completely rounded up after a further 3 h (not shown). Cells were lysed, and in vitro ADP-ribosylation was performed. As shown in Fig. 9C, no radiolabeled actin was detected in C2 toxin-treated cells and in cells that were preincubated with brefeldin A prior to the C2 toxin addition.

FIG. 4. Determination of C2IIa heptamers by analytical ultracentrifugation. Radial absorbance distribution of recombinant C2IIa (loading concentration 0.57 mg/ml) at sedimentation equilibrium was recorded at 275 nm (○), 280 nm (●), and 285 nm (□). T = 20 °C. From the simultaneous curve fit, an average molecular mass of 422 ± 78 kDa was determined.

FIG. 5. Single-channel recording of a diphytanoyl phosphatidylcholine/n-decane membrane in the presence of recombinant C2IIa oligomers expressed in Escherichia coli. 10 min after formation of the membrane, 100 ng/ml oligomers were added to the aqueous phase on one side of the membrane. The aqueous phase contained 1 M KCl (pH 6). The applied membrane potential was 20 mV; T = 20 °C.

FIG. 6. Binding of C2IIa oligomers to CHO cells. CHO-WT as well as CHO-C2RK14 cells were incubated for 1 h at 4 °C in HBSS with C2II (200 ng/ml) or C2IIa (200 ng/ml), respectively. Control cells were without C2II or C2IIa. Cells were washed and lysed, and C2II binding was tested by 3–12.5% SDS-PAGE followed by anti-C2II immunoblot analysis. Alternatively, after incubation with C2IIa at 4 °C, CHO-WT cells were incubated for an additional 30 min at 37 °C prior to lysis. In parallel, CHO-WT cells were incubated with C2IIa plus C2I (100 ng/ml) at 4 °C, washed, exposed for 2 h to 37 °C, and lysed.

DISCUSSION

Here we have studied the binding/translocation component C2II of C2 toxin and describe its properties, cell binding, and carrier function to deliver C2I into the target cells. The binding component C2II exhibits significant sequence similarity with the protective antigen (PA), the binding component of anthrax toxin (9, 34). Like PA, C2II has to be activated by partial proteolysis (23, 35). C2II is highly trypsin-sensitive; however,
at present it is not known whether trypsin is the physiological activator. As shown in this communication, trypsin activation was a prerequisite for C2II cell binding. The related PA (83 kDa) of anthrax toxin is cleaved near its N terminus and thereby activated by cellular proteases including furin (9, 36) but reportedly binds to cells even as a full-length protein (37). Similar to activated PA (PA63), activated C2IIa but not full-length C2II formed oligomers (9). The oligomerization of C2II occurred immediately after trypsin cleavage, and the oligomers were SDS-stable but heat-sensitive.

Using electron microscopy, we show that C2IIa oligomers form ring-shaped and horseshoe-shaped structures with outer diameters of 11–13 nm. In the middle, the oligomeric complexes are characterized by a “dark” spot, which might reflect a hole-like structure of 2–4 nm. More insights into the stoichiometry of the oligomeric structure were obtained by analytical ultracentrifugation of the complexes. These studies revealed that the oligomers are predominantly heptameric, a finding that is in correspondence with the electron microscopic data. However, the formation of some hexamers and octamers could not be excluded. Again the similarity to PA63 is evident. Also, PA63 forms heptamers (38).
Recently we have shown that activated C2IIa forms ion-permeable channels in artificial lipid bilayer membranes (24). Using isolated oligomers, we observed a more efficient channel formation than previously reported. Channel formation is also known for the protective antigen (38–40) and for several other toxins including diphtheria toxin (41, 42). Similar to channels formed by anthrax PA63 and diphtheria toxin, the C2IIa channel is cation-selective. This is based on point charges at the channel mouth (24).

In previous studies by Simpson (16), C2 toxin was shown to enter cells by receptor-mediated endocytosis. Recently, using a receptor-deficient mutant CHO cell line (RK14-CHO cells) (29), we identified asparagine-bound complex carbohydrates as toxin receptors or at least part of the receptor (15). After receptor binding, C2 toxin is endocytosed. Endocytosis appears to occur with C2IIa alone or with C2I attached to C2IIa (16). In general, two pathways of intracellular routing exist for bacterial protein toxins (43). One group of toxins is routed by a retrograde transport to the ER, where cellular uptake occurs. These toxins include choler toxin (44), shiga toxin (45), and Pseudomonas exotoxin A (46). The other group of toxins, including diphtheria toxin (47) and anthrax toxin (48), translocate into the cytosol from an endosomal compartment. To get more insights into the cellular routing of C2 toxin, several inhibitors for cellular uptake and transport mechanisms were tested for their effects on C2 toxin-induced intoxication of cells. The drug brefeldin A, which blocks retrograde transport of choler toxin and ricin to the ER (49, 50), did not decrease the C2 toxin effects. In contrast, the fungal metabolite bafilomycin A1 completely inhibited C2 toxin effects on cells. Bafilomycin A1 specifically blocks the vacuolar-type H+ATPase in the endosomal membrane and thereby prevents acidification of endosomal vesicles (30, 51). This observation indicates that an acidic compartment is required for uptake of C2 toxin and that C2 toxin may be taken up in the same way as the anthrax toxins. Moreover, when C2 toxin was bound to whole cells at 4°C in the presence of bafilomycin A1 and cells were subsequently exposed to low pH and incubated at 37°C, the toxin was translocated directly from the cell surface into the cytosol. This system mimics the endosomal environment (52). Similar cell membrane translocation was reported for diphtheria toxin (53) and anthrax toxin (40).

We believe that after acidification of the early endosomal compartment, the oligomeric C2IIa pre-pores insert into the endosomal membrane and mediate subsequent C2I translocation into the cytosol. Fig. 10 shows our actual model for the uptake of C2 toxin into eukaryotic cells summarizing the results discussed above. Based on crystal structure analysis, it has been suggested that the central lumen of the PA heptamer channel has an average diameter of ~2 nm (54). In artificial membranes, a C2IIa channel diameter of about 1 nm has been proposed (24). Thus, in consideration of the sequence homologies between C2II and PA and the findings with black lipid membranes, a similar size of the channels of PA and C2II is likely. So far, the precise mechanism underlying the membrane insertion and translocation of the enzyme component into the cytosol is not clear. For the heptameric PA, it has been proposed that seven amphipathic hairpins each formed by the 2β2–2β3 loop of PA63 result in a 14-stranded β-barrel structure that is basically involved in membrane insertion (54). This amphipathic 2β2–2β3 loop recognized in PA is highly conserved in C2II (amino acid residues 300–330), suggesting a similar function of these sequences. The translocation of C2I into the cytosol is even less clear. Two models are possible. In one case, the channel formed is capable of translocating several enzyme components into the cytosol. The other model implies that the process of membrane insertion and channel formation is also the driving force for the translocation of the enzyme component. Therefore, it is noteworthy that the intoxication of cells by C2 toxin in the presence of bafilomycin was not observed when the pH-dependent step was carried out with C2IIa alone and the enzyme component (C2I) was added after increasing the pH to 7.5 again. This may indicate that C2I must be present when the pH-dependent conformational change and/or the membrane insertion occurs, favoring a model that combines insertion and transport. On the other hand, it has been shown with anthrax toxin that the PA heptamer binds seven enzyme LF components (48), and the intoxication of cells is increased with increasing stoichiometry of PALF (7:1 < 7:2 < 7:3 < 7:5, etc.). It is difficult to believe that several enzyme components undergo the same conformational changes and subsequent translocation concomitantly with the membrane insertion of the binding heptamer. Thus, further studies are necessary to clarify the translocation mechanisms of this family of protein toxins in detail.

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FIG. 10. Model of the uptake of C. botulinum C2 toxin into eukaryotic cells. After activation with trypsin, C2IIa forms heptamers and assembles with C2I. This complex binds to the carbohydrate receptor for C2IIa on the cell surface and is internalized via receptor-mediated endocytosis. After acidification of the early endosomes, C2II forms a channel in the membrane, and C2I escapes into the cytosol. This step can be blocked by bafilomycin A1 (Baf). Transport of vesicles from early to late endosomes is inhibited by nocodazole (Noc). Nocodazole has no influence on the uptake of C2 toxin.
Cellular Uptake of Clostridium botulinum C2 Toxin Requires Oligomerization and Acidification

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