Infection and Immunity

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Functional Characterization of an Extended Binding Component of the Actin-ADP-Ribosylating C2 Toxin Detected in *Clostridium botulinum* Strain (C) 2300▽

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***Clostridium botulinum* C2 toxin consists of the binding component C2II and the enzyme component C2I, which ADP-ribosylates G-actin of eukaryotic cells. Trypsin-activated C2II (C2IIa) forms heptamers that mediate cell binding and translocation of C2I from acidic endosomes into the cytosol of target cells. By genome sequencing of *C. botulinum* strain (C) 2300, we found that C2II from this strain carries a C-terminal extension of 129 amino acids, unlike its homologous counterparts from strains (C) 203U28, (C) 468, and (D) 1873. This extension shows a high similarity to the C-terminal receptor-binding domain of C2II and is presumably the result of a duplication of this domain. The C2II extension facilitates the binding to cell surface receptors, which leads to an increased intoxication efficiency compared to that of C2II proteins from other *C. botulinum* strains.**

Clostridium botulinum is a taxonomic collection of several distinct species of anaerobic, Gram-positive, spore-forming bacteria which produce the most poisonous substances known, the botulinum neurotoxins (BoNTs) (9). BoNTs are classified into seven groups (A to G) based on their antigenicity (12). Four distinct phenotypic groups of *C. botulinum* (I to IV) have been identified: group I contains proteolytic strains which are able to produce one or more BoNTs of types A, B, and F; group II comprises nonproteolytic strains producing BoNTs of type B, E, or F; group III includes strains which produce BoNT C and D; and group IV strains produce BoNT G (4). Some strains are able to produce toxins in addition to the neurotoxins. *C. botulinum* type C and D cultures produce a binary C2 toxin in addition to BoNT C and BoNT D (15).

This binary C2 toxin is composed of the enzyme component C2I, which ADP-ribosylates monomeric G-actin, and the binding and translocation component C2II, which is responsible for the interaction with a eukaryotic cell membrane receptor and the ensuing endocytosis (20). Previous studies showed that the enzymatic component C2I is an ~49-kDa protein, whereas the inactive binding component C2II has a mass of ~80 kDa (1, 15). C2II is activated by proteolytic cleavage of an ~20-kDa amino-terminal fragment. The active 60-kDa part of the binding and translocation component forms SDS-stable heptamers, which bind to the surface of target cells through membrane receptors (2). After binding of C2I to C2II heptamers, the C2 toxin receptor complex is endocytosed (3). In acidic endosomes, C2II heptamers undergo major conformational changes. A hydrophobic loop from each C2II subunit inserts into the endosomal membrane and forms a pore, which allows translocation of the enzymatic component into the cytosol. Eventually, C2I-catalyzed ADP-ribosylation of actin leads to redistribution of the cytoskeleton and depolymerization of F-actin resulting in cells becoming rounder (17, 22).

In this work, draft genome sequencing of a *C. botulinum* type C strain, named 2300, and subsequent annotation of the predicted coding regions led to the detection of an unusual gene coding for the C2II toxin subunit. The molecular features of the respective gene and the functional characterization of the C2 toxin subunit are summarized below.

MATERIALS AND METHODS

DNA extraction from *C. botulinum* strain (C) 2300. Genomic DNA of *C. botulinum* strain (C) 2300 was prepared from an overnight culture grown in YTG medium (16). Cells (20-ml suspension) were harvested by centrifugation for 15 min at 2,790 × *g* and 4°C. The cell pellet was washed with 10 mM KH_2PO_4 buffer (pH 7.5) and resuspended in 4 ml STE buffer (6.7% [wt/vol] saccharose, 50 mM Tris, and 1 mM EDTA, pH 8.0) containing 20 mg/ml lysozyme, and the cell suspension was incubated at 37°C for 30 min. Cell lysis was achieved by adding 500 μl 10% (wt/vol) SDS solution and by further incubation at 37°C for 10 min. Subsequently, 430 μl EDTA (0.5 M) and 36 μl Tris-HCl (1.0 M, pH 8.0) and proteinase K solution (Qiagen, Hilden, Germany) were added, followed by incubation for 3 h at 37°C. The genomic DNA was purified by chloroform-isoamylalcohol (24:1) extraction, precipitated with isopropanol, and washed with 70% ethanol.

Draft genome sequencing and annotation of *C. botulinum* strain (C) 2300. Genomic DNA of *C. botulinum* strain (C) 2300 was sequenced by applying the whole-genome shotgun approach, using the Genome Sequencer FLX system (Roche Applied Science, Mannheim, Germany). A single-strand template DNA library was established by using 5 μg of a genomic DNA sample. Library preparation and subsequent pyrosequencing followed standard protocols provided by the manufacturer. In total, 634,808 reads were obtained with a single sequencing run, and 153,418,896 nucleotides of genomic DNA were detected. The sequence reads were assembled with the Genome Sequencer De Novo Assembler software (Roche Applied Science, Mannheim, Germany), resulting in 154 large genomic contigs (≥500 nuclotides) with a draft genome size of 2.88 Mb. The draft genome sequence of *C. botulinum* strain (C) 2300 was uploaded into the database GenDB to perform an automated gene prediction and annotation (13), leading to 2,746 predicted protein-coding sequences.

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TABLE 1. Substitutions of amino acids from C2 toxin component C2I and C2II of *C. botulinum* strains (C) 2300 and (C) 203U28

Component	Strain	Amino acid at position:																			
		19	46	49	97	98	121	128	147	157	160	173	179	231	232	238	243	293	327		
C2I	2300	Lys	Asn	Tyr	Asp	Ala	Asp	Thr	Ile	Asn	Val	Asp	Asp	Ala	Glu	Ile	His	Gln	Gln		
	203U28	Gln	Thr	Asp	Glu	Val	Asn	Ile	Val	Asp	Ile	Asn	Asn	Glu	Lys	Val	Gln	Blu	Thr		
		277	495	496	529	546	556	560	571	576	610	617	619	622	629	631	632	640	645	673	677
C2II	2300	Ala	Pro	Asp	Arg	Ala	Arg	Val	Asn	Asn	Gly	Lys	Ile	Asn	Ser	Lys	Ser	Ile	Phe	Asn	Ile
	203U28	Gly	Ser	Gly	Ala	Lys	Ser	Asn	Asp	His	Gln	Asn	Ser	Gln	Ala	Thr	Ala	Leu	Arg	Ser	Lys

PCR amplification, cloning, and DNA sequencing. The preparation of plasmid DNA from *Escherichia coli* cells was performed by the alkaline lysis technique using the QIAprep Spin miniprep kit (Qiagen, Hilden, Germany). DNA restriction, analysis by agarose gel electrophoresis, and DNA ligation were performed according to standard protocols (19). The transformation of plasmid DNA was carried out by heat shock transformation with competent *E. coli* TOP10 cells. DNA amplification was performed with a PTC-100 thermocycler (MJ Search, Waltham, MA) and BioTaq DNA polymerase (Bioline, Luckenwalde, Germany) or Phusion Hot Start high-fidelity DNA polymerase (Finnzymes, Espoo, Finland). PCR products were purified with the PCR purification Spin Kit (Qiagen, Hilden, Germany). All oligonucleotides used in this study were purchased from Operon Biotechnologies, Ebersberg, Germany. The cloning of PCR products was performed in vector plasmid pCR-Blunt II with the Zero Blunt TOPO PCR cloning kit (Invitrogen, Karlsruhe, Germany) by following the manufacturer's protocol. Cloned PCR products were sequenced by IIT Biotech, Bielefeld, Germany. For expression of recombinant C2II, genomic DNA from strain (C) 2300 was used as the template and C2II was amplified by using the following primers: 5′C2II-BamHI (GGATCCATGTTAGTTTCAAAATTTGAGAACTCT) and 3′C2II-EcoRI (GAATTCCTATAAATCGCCAATCCTAGTCACTT). Afterwards, the PCR product was digested using BamHI and EcoRI and cloned into the pGEX vector system (Pharmacia Biotech, Uppsala, Sweden). The C2II-pGEX-2T plasmid was transformed into chemical competent *E. coli* TG1 cells. Plasmid DNA was prepared using standard procedures and sequenced to confirm the DNA sequence.

Expression, purification, and activation of C2II. C2I and C2II were expressed in *E. coli* BL21(DE3) as recombinant glutathione *S*-transferase fusion proteins and purified by affinity chromatography with glutathione-Sepharose 4B according to the manufacturer's instructions (11). Glutathione *S*-transferase was cleaved off by thrombin (3.25 NIH units/ml of bead suspension) followed by inactivation of thrombin by use of benzamidine beads. C2II was activated with 0.13 μg of trypsin per μg of total protein for 30 min at room temperature. The active forms of C2II (C2IIa and ΔC2IIa, the active recombinant short version of C2II) were used in all experiments.

Cell culture and cytotoxicity assay. CaCo-2 cells were cultivated in Dulbecco's minimum Eagle's medium, HT-29 cells were cultivated in McCoy's 5A medium, HeLa cells were cultivated in Dulbecco's modified Eagle's medium, and Chinese hamster ovary CHO-K1 and CHO-RK14 cells were cultivated in Ham's F-12–Dulbecco's minimum Eagle's medium (MEM) (1:1). All media contained 2 mM L-glutamate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS). Cells were routinely kept in tissue culture flasks at 37°C and 5% CO_2 and were trypsinized and replated twice per week. For cytotoxicity experiments, cells were replated in 12-well dishes. After the dishes were washed with phosphate-buffered saline (PBS), the medium was changed to serum-free medium (for CHO-K1 and CHO-RK14 cells) or medium with FCS (for CaCo-2 and HeLa cells).

TER assay. For the transepithelial resistance (TER) assay, CaCo-2 cells were plated on Falcon cell culture inserts and incubated for 5 to 7 days with medium exchange every 3 days. Assays were performed when TER values reached ~1,800 to 2,000 $\Omega \cdot cm^2$. TER was determined using a resistance system for electrophysiological readings of filter cups (Endohm-12; World Precision Instruments, Sarasota, FL). To avoid a drastic drop in the starting TER, all washing and incubation steps were carried out using medium supplemented with 10% FCS. Activated C2II (500 pM) (ΔC2IIa and C2IIa) and C2I (215 pM) were applied to the upper compartment of the Falcon cell culture inserts, and the resulting decrease in TER was monitored for up to 480 min.

***In vivo* pore-forming assay.** $^{86}Rb^+$ release experiments were performed as described recently (11). HT-29 cells were plated in complete medium containing $^{86}Rb^+$ (1 μCi/ml) at a density of ~2×10^5 cells/well in 24-well cell culture plates. After 48 h, cells were washed twice with PBS and cooled to 4°C. Afterwards, fresh medium (4°C, without serum), containing activated C2II (ΔC2IIa and C2IIa), was added. Proteins were allowed to bind for 45 min at 4°C, followed by washing twice with cold medium to remove unbound toxin. To initiate membrane insertion of the toxins, warm medium (37°C; without serum, pH 4.8) was applied, and cells were incubated for 5 min at 37°C. After a shift back to 4°C, incubation was continued for 40 min at low temperature to allow $^{86}Rb^+$ efflux. Aliquots of the medium were removed, and $^{86}Rb^+$ release was determined by liquid scintillation counting.

Actin staining. Cells were washed with warmed PBS, fixed with 4% formaldehyde, and permeabilized with 0.15% Triton X-100 in PBS. For visualization of the actin cytoskeleton, cells were stained with TRITC (tetramethyl rhodamine isocyanate)-phalloidin (Invitrogen, Paisley, United Kingdom). After staining, cells were washed and embedded in Prolong Gold with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen, Paisley, United Kingdom). Pictures were acquired with a Zeiss Axiocam HRm and a Fluar 20× NA 0.75 objective and a Plan Apo 100× NA 1.4 objective.

Nucleotide sequence accession number. The sequence for the C2II component of C2 toxin from *C. botulinum* strain (C) 2300 was deposited in the GenBank database under accession number GU108606.

RESULTS

Detection of a C2II protein with a C-terminal extension. After sequencing and annotation of the *C. botulinum* strain (C) 2300 draft genome, the genes encoding the C2 toxin were identified and further characterized. The nucleotide sequence of the enzymatic component of C2 toxin, C2I, was compared with previously published sequences deposited in GenBank. As expected, the C2I gene of *C. botulinum* strain (C) 2300 revealed 97 to 99% nucleotide sequence identity with the respective C2I gene from BoNT type C strains 203U28 (8) and 468 (18) and with BoNT type D strain 1873 (18). An amino acid sequence comparison revealed the highest similarity between C2I of *C. botulinum* strains (C) 2300 and 203U28 (8), with only 18 amino acid mismatches in the complete coding region of 431 amino acids (Table 1). This means a similarity of 95%.

Likewise, the C2II gene and the deduced C2II protein of *C. botulinum* strain (C) 2300 were compared with their homologous counterparts from strains (C) 203U28 (10), (C) 468 (18), and (D) 1873 (18) deposited in the GenBank database. Surprisingly, the amino acid sequence alignment of the C2II proteins revealed the presence of a putative 129-amino-acid extension at the C terminus of the C2II protein of *C. botulinum* strain (C) 2300 (Fig. 1), whereas the amino-terminal portions of the C2II proteins were highly similar. When comparing the C2II protein of *C. botulinum* strain (C) 2300 with that of strain 203U28, for instance, an identity of the common part of 98% was determined. Only 20 mismatches were detected (Table 1). Likewise, a comparison of the nucleotide sequences of the C2II coding regions showed a high level of nucleotide sequence identity over the entire length of hitherto known C2II genes (Table 2). Mismatches in this common part of the C2II

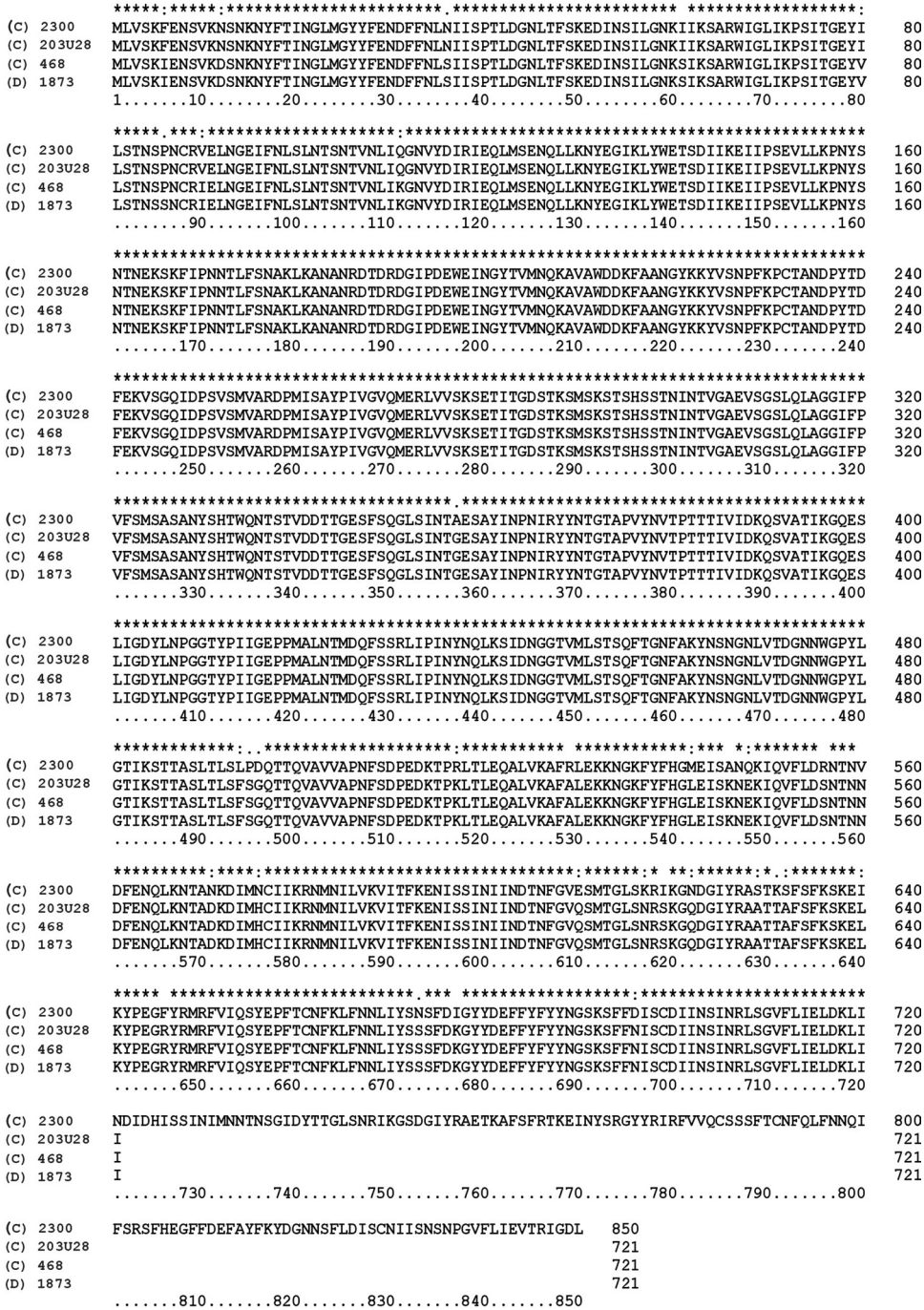

FIG. 1. Alignment of amino acid sequences of C2II from *Clostridium botulinum* strains (C) 2300, (C) 203U28, (C) 468, and (D) 1873. Three characters are used to mark conserved positions: an asterisk indicates positions which have a single, fully conserved residue; a colon indicates that one of the "strong" STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, and FYW groups is fully conserved; a period indicates that one of the "weaker" CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, and HFY groups is fully conserved. These are all the positively scoring groups that occur in the Gonnet Pam 250 matrix. The strong and weak groups are defined by a score of >0.5 and a score of <0.5, respectively. The alignment was done by ClustalX (21).

genes comprise 56 to 68 nucleotides, thus leading to sequence similarities ranging from 96 to 98% in a comparison with nucleotides 1 to 2166 of the C2II gene from *C. botulinum* strain (C) 2300. However, at this position of the C2II gene of *C. botulinum* strain (C) 2300, we observed the presence of four additional nucleotides in the alignment (Fig. 2), apparently resulting in a putative frameshift of the coding region. To verify the nucleotide sequence obtained by pyrosequencing, the C2II gene of *C. botulinum* strain (C) 2300 was amplified by PCR from genomic DNA data, cloned into the plasmid vector pCR-Blunt II,

TABLE 2. Features and differences of the nucleotide sequences from the C2II gene from *Clostridium botulinum* strains (C) 203U28, (C) 468, and (D) 1873 and the common part of the C2II gene from strain (C) 2300

Strain	Sequence length (nt)	No. of:		% identity to the common part of strain 2300
		Matches	Mismatches	
(C) 2300	2,553			100
(C) 203U28	2,166	2,110	56	98
(C) 468	2,166	2,099	67	96
(D) 1873	2,166	2,098	68	96

and resequenced by means of Sanger technology (data not shown). This control assay confirmed the initially obtained sequence of the C2II gene from *C. botulinum* strain (C) 2300.

The C2II extension is similar to the C-terminal domain of the binding and translocation component. To further analyze the differences in length of the C2II gene from *C. botulinum* strain (C) 2300 and that from other strains, we compared the nucleotide sequences detected downstream of the stop codons of C2II genes (Fig. 2). These data indicated clear differences between hitherto described C2II genes and that detected in the genome of *C. botulinum* strain (C) 2300. This observation supports the view that the differences do not represent a frameshift in the *C. botulinum* strain (C) 2300 gene sequence but are indicative of a C-terminal extension. To investigate the role of this putative C-terminal extension of the C2II protein, this protein stretch was compared by BLASTP with proteins deposited in GenBank. Surprisingly, we thereby detected that the extension exhibits significant similarity to the C-terminal part of C2II proteins. We found for instance a 57% amino acid sequence similarity to the C-terminal part of C2II protein from strain (C) 203U28 (Fig. 3). The similarity between the proteins begins at amino acid 586 of the binding and translocation component in strain (C) 203U28. From the 134-amino-acid C terminus of the long version of C2II, 77 amino acids concur with the C-terminal part of the short-version protein. Furthermore, there are 25 amino acid substitutions which probably do not change the chemical properties of the protein. The similarity of the extension in C2II from *Clostridium botulinum* strain (C) 2300 (amino acids 716 to 850) to the region from amino acid 586 to 720 of the same protein is also shown in Fig. 3. Comparison of those internal parts of the long-version protein reveals an identity of 58%. These data suggest that the extended region of the C2II protein from *C. botulinum* strain (C) 2300 can be caused by protein domain duplication.

As no DNA sequence downstream of the C2II gene from *C. botulinum* strain (C) 92-13 was available from the GenBank database entry, the complete C2II coding region of this strain was amplified by PCR from genomic DNA, and the nucleotide sequence of the PCR product was subsequently determined. *C. botulinum* strain (C) 92-13 was previously used for the detailed molecular characterization of the C2 toxin (14). Interestingly, the PCR product generated on (C) 92-13 DNA was the same size as that deduced from the extended C2II gene of *C. botulinum* strain (C) 2300 DNA (~2,500 bp) (data not shown). This observation was confirmed at the nucleotide level after Sanger sequencing of the PCR product, revealing that both genes are almost identical and that both are characterized by a 3′ extension of the C2II coding region (Fig. 2). These data suggest that the extended version of the C2II gene initially detected in the genome of *C. botulinum* strain (C) 2300 is also present in other environmental isolates and that at least two types of the C2II proteins exist in nature. In previous studies, C2II from strain (C) 92-13 was amplified from genomic DNA according to the sequence of C2II from strain (C) 203U28 and therefore only a partial C2II protein was expressed and purified (2). Based on these findings, recombinant C2II from strain (C) 92-13 is termed ΔC2IIa.

Intoxication efficiency of recombinant ΔC2IIa (strain [C] 92-13) and C2IIa (strain [C] 2300). Because the C-terminal extension in C2II of *C. botulinum* strain (C) 2300 is similar to the receptor-binding domain of other C2II proteins (e.g., strain [C] 203U28), we investigated a possible effect of this extension on the intoxication efficiency in cell culture. To compare the impacts of the different versions of C2II on the disruption of the actin cytoskeleton by the toxin, we employed fluorescence microscopy (Fig. 4). HeLa cells were treated with C2I (430 pM) and C2IIa (strain [C] 2300) or ΔC2IIa (strain [C] 92-13) (each 1 nM). The cells were fixed at intervals of 1 h, and the actin cytoskeleton was stained with TRITC-conjugated phalloidin to obtain a time course of the induced morphological changes. After 2 h of intoxication, most C2IIa-treated cells started to become rounder or revealed unstructured dots of F-actin in their cytoplasm. Most ΔC2IIa-treated cells showed no signs of intoxication at this time point. Intoxication of ΔC2IIa-treated cells started with an approximate delay of 1 h. After 3 h, all C2IIa-treated cells became round. A comparable intoxication with ΔC2IIa took 5 h. C2I alone caused no intoxication. These data suggest that the extension in C2II leads to a faster intoxication of HeLa cells.

A more sensitive method for monitoring cell intoxication by clostridial toxins is the measurement of the TER in cell monolayers. For that, CaCo-2 cells were grown on filters until the starting resistance reached around 1,800 to 2,000 $\Omega \cdot cm^2$. C2I (215 pM) and C2IIa or ΔC2IIa (each 500 pM) were applied to the apical reservoir. Cell intoxication (i.e., TER decrease) was monitored for up to 8 h, and TER values are shown as the percentage of starting resistance. As shown in Fig. 5A, ΔC2IIa and C2I abolished TER with a half-time of 190 min, whereas C2IIa and C2I decreased TER with a half-time of 115 min. These data indicate again that the extension in C2II leads to an accelerated cell intoxication. A similar result was obtained by intoxication of wild-type CHO-K1 and C2 toxin-resistant CHO-RK14 cells (Fig. 5B). After 3 h of intoxication, C2IIa induced more rounding of CHO-K1 cells than ΔC2IIa. CHO-RK14 cells are deficient in binding C2 toxin in the 2 nM range (7). Interestingly, ΔC2IIa already induced cell rounding at 1

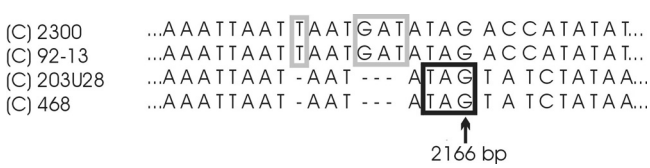

FIG. 2. Nucleotide sequences of C2II genes near the prior stop codon (~position 2166) of *Clostridium botulinum* strains (C) 2300, (C) 92-13, (C) 203U28, and (C) 468. The additional nucleotides are marked by gray boxes. Stop codons are marked by a black box.

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2300ex  716 LDKLINDIDHISSINIMNNTNSGIDYTTGLSNRIKGSDGIYRAETKAFSFRTKEINYSRGYYRIRFVVQCSSSFTCNFQL 795
2300    586 LVKVITFKENISSINIINDTNFGVESMTGLSKRIKGNDGIYRASTKSFSFKSKEIKYPEGFYRMRFVIQSYEPFTCNFKL 665
203U28  586 LVKVITFKENISSINIINDTNFGVQSMTGLSNRSKGQDGIYRAATTAFSFKSKELKYPEGRYRMRFVIQSYEPFTCNFKL 665
            * *:*.  ::******:*:** *::  ****:* **.****** *.:***::**::*..* **:***:*. ..*****:*


2300ex  796 FNNQIFSRSFHEGFFDEFAYFKYDGNNSFLDISCNIISNSN--PGVFLIEVTRI-  847
2300    666 FNNLIYSNSFDIGYYDEFFYFYYNGSKSFFDISCDIINSINRLSGVFLIELDKLI  720
203U28  666 FNNLIYSSSFDKGYYDEFFYFYYNGSKSFFNISCDIINSINRLSGVFLIELDKLI  720
            *** *:* **. *::*** ** *:*.:**::***:**.. *  .******: ::
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FIG. 3. Alignment of three amino acid sequences of C2II from *Clostridium botulinum* strain (C) 2300 and from *Clostridium botulinum* strain (C) 203U28. The amino acid sequence designated 2300ex from C2II of *Clostridium botulinum* strain (C) 2300 (amino acids 716 to 847) represents the C-terminal extension of C2II. The amino acid sequence designated 2300 is derived from the same strain and covers the amino acids 586 to 720. The amino acid sequence designated 203U28 is the C-terminal part of C2II of *Clostridium botulinum* (C) strain 203U28. This alignment was done with ClustalX (21). Three characters are used to mark conserved positions: an asterisk indicates positions which have a single, fully conserved residue; a colon indicates that one of the "strong" STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, and FYW groups is fully conserved; a period indicates that one of the "weaker" CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, and HFY groups is fully conserved. These are all the positively scoring groups that occur in the Gonnet Pam 250 matrix. The strong and weak groups are defined by a score of >0.5 and a score of <0.5, respectively.

nM in this Chinese hamster ovary cell mutant. All together, these data indicate that the extension in C2II caused increased intoxication efficiency.

The C2II extension facilitates release of $^{86}Rb^{+}$ ions. To analyze the pore formation of ΔC2IIa and C2IIa at cell membranes, endosomal conditions can be mimicked by extracellular acidification (5, 11). To this end, HT-29 cells were preloaded overnight with $^{86}Rb^{+}$ ions prior to treatment with various concentrations of ΔC2IIa or C2IIa on ice. Subsequently, a pH shift of the culture medium mimicking the en-

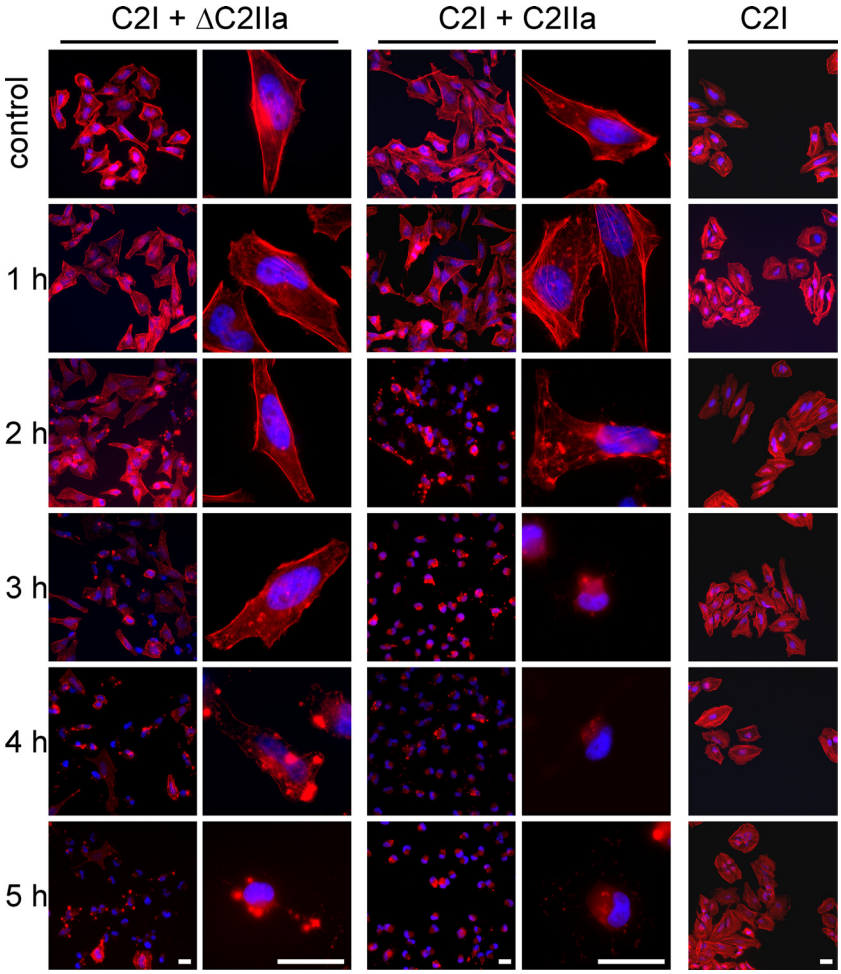

FIG. 4. Time course of the intoxication of HeLa cells with C2 toxin possessing the long (C2IIa) or short (ΔC2IIa) version of the binding component. HeLa cells were treated with 430 pM C2I and 1 nM C2IIa or ΔC2IIa. As a control, HeLa cells were treated with 430 pM C2I alone. Cells were fixed after the indicated times, and the actin cytoskeleton was stained by TRITC-phalloidin (red). The nuclei were stained by DAPI (blue). Bars, 30 μm.

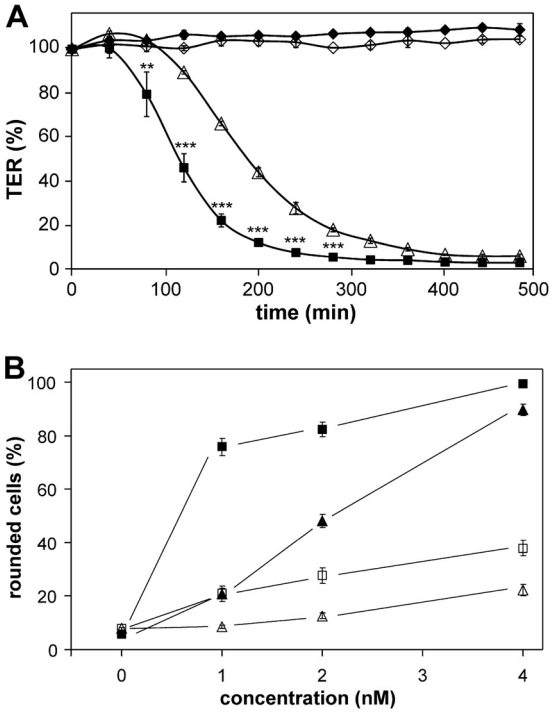

FIG. 5. Cell intoxication by C2 toxin possessing the long (C2IIa) or short (ΔC2IIa) version of the binding component. (A) Human colon carcinoma (CaCo-2) cell monolayers were treated with 215 pM C2I and 500 pM C2IIa (■) or ΔC2IIa (△). Reduction of transepithelial resistance (TER) due to cell rounding over time (min) served as a parameter for intoxication. Results are mean values (±SD) of results for at least three independent experiments and are shown as percentages of starting TER. Controls are buffer (◇) or C2I alone (◆). Asterisks indicate significant differences (**, $P < 0.02$; ***, $P < 0.001$) calculated by analysis of variance (ANOVA). (B) CHO-K1 (■, ▲) and CHO-RK14 (□, △) cells were treated with C2IIa (■, □) or ΔC2IIa (▲, △) at the indicated concentrations. C2I was added to C2IIa or ΔC2IIa at a ratio of 3:7. Cells were further incubated at 37°C, and after 3 h, pictures were taken. The percentage of round cells per field (~300 cells/field) is given as the mean ± SD ($n = 3$).

dosomal conditions allowed the membrane insertion of the heptameric binding component, followed by pore formation and $^{86}Rb^+$ ion release. As expected, C2IIa caused a stronger release than ΔC2IIa (Fig. 6). Whereas 0.5 nM C2IIa induced a significant release of $^{86}Rb^+$ ions, 1 to 2 nM ΔC2IIa was needed to get a similar release.

DISCUSSION

During manual annotation of predicted coding regions in *C. botulinum* strain (C) 2300, the gene for the binding and translocation component of the C2 toxin attracted attention (C2II), because the length of this gene was about 2,553 nucleotides. Different BLAST hits indicated that other strains in GenBank possess only a short version (2,166 nucleotides) of the C2II component. An extended version of the binding and translocation component was identified in *C. botulinum* strain (C) 2300 and (C) 92-13. Further analysis revealed that the additional C-terminal part exhibited significant sequence similarity with the known C terminus of C2II, suggesting a domain duplication as the reason for the extension.

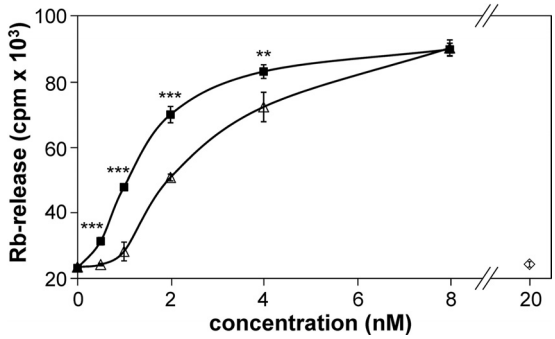

FIG. 6. Comparison of pore formation by C2IIa and ΔC2IIa in HT-29 cells. Cell monolayers in 24-well culture plates were preloaded with $^{86}Rb^+$ (1 μCi/ml) for 48 h and, thereafter, washed twice with PBS. Subsequently, C2IIa (■) or ΔC2IIa (△) was allowed to bind at 4°C for 45 min. As a control, 20 nM C2I (◇) was used. Afterwards, cells were exposed for 5 min to an acidic shift (pH 4.8) at 37°C to initiate membrane insertion of C2II and ΔC2IIa. After an additional incubation of 40 min at 4°C, the radioactivity in the medium was measured by using liquid scintillation counting. Asterisks indicate significant differences (**, $P < 0.02$; ***, $P < 0.001$) calculated by ANOVA.

C2II exhibits significant sequence similarity with the binding components of other actin-ADP-ribosylating toxins like *C. perfringens* iota toxin or *C. difficile* transferase (CDT) (1) and is also highly similar to the protective antigen (PA) of anthrax toxin (23). All of these toxin components consist of four domains which are suggested to be involved in activation (domain 1), pore formation (domain 2), self-association (domain 3), and binding to the cellular receptor (domain 4) (1).

Noteworthy, the amino acid sequences of domains 1 to 3 are highly similar between the toxins, whereas domain 4, which is responsible for receptor interaction, is different. In the present study, this receptor-binding domain was found to be extended in the C2II isoforms described here. Recently, the crystal structure of the short form of C2II has been reported (20). In this report, the receptor domain (domain 4) of C2II was unstructured. Our present study may offer an explanation for this result. The short C2II protein version may contain only a part of the receptor domain, which, therefore, remained unstructured under the crystallization conditions. When the receptor domain is changed, the question arises as to whether the biological activity of the toxin is also affected.

Our studies of the activities of the short and long forms of C2 toxin on intoxication of cells and on the decrease in transepithelial electrical resistance of cell monolayers clearly show that the long form of C2II causes stronger effects. First, the rounding of cells occurred earlier with the long form than with the short form of the toxin, and second, the decrease in TER was more rapidly induced by the long form of the toxin than by the short version.

It has been reported that C2II binds to asparagine-linked complex and hybrid carbohydrates (6). Here we used a mutant Chinese hamster ovary cell line which is deficient in *N*-acetylglucosaminyltransferase I and, therefore, largely resistant to C2 toxin for studies on the sensitivity toward the toxins. Both the short and large forms of C2 toxin were much less toxic toward these mutant cells than to wild-type control cells. Although cell sensitivities toward the toxins were different, these findings suggest that both forms of toxin bind to the same type

of receptor. Binding of C2 toxin to carbohydrate structures is most likely polyvalent (note the heptameric structure of activated C2II). Therefore, we propose that the short and long forms of the toxin can bind to the same receptor but with different affinities, resulting in different biological effects. However, direct receptor-binding studies are necessary to support the view that variation of the receptor domain has consequences for the receptor affinity of the toxin.

Taken together, we report on a novel long version of the binding component of C2 toxin in *C. botulinum* strain (C) 2300. It appears that C2 toxin from strain (C) 92-13, which is frequently used in biological studies of C2 toxin, is also a long-version toxin which is characterized by an extension of the receptor domain, resulting in increased biological activity most likely due to increased receptor binding.

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