The Binary *Clostridium botulinum* C2 Toxin as a Protein Delivery System - Identification of the Minimal Protein Region Necessary for Interaction of Toxin Components§

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Running title: Residues 1-87 mediate cell delivery of *Clostridium botulinum* C2I

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SUMMARY

The binary Clostridium botulinum C2 toxin is composed of the enzyme component C2I and the binding component C2II, which are individual and non-linked proteins. Activated C2IIa mediates cell binding and translocation of C2I into the cytoplasm. C2I ADP-ribosylates G-actin at Arg-177 to depolymerize actin filaments. A fusion toxin, containing the N-terminal domain of C2I (residues 1-225), transports C3 ADP-ribosyltransferase from Clostridium limosum into cells (Barth, H., Hofmann, F., Olenik, C., Just, I., and Aktories, K. (1998) Infect. Immun. 66, 1364-1369). We characterized the adaptor function of C2I and its interaction with C2IIa. The fusion toxin GST-C2I\textsuperscript{1-225}-C3 was efficiently transported by C2IIa, indicating that C2IIa translocates proteins into the cytosol even when the C2I\textsuperscript{1-225} adaptor was positioned in the middle of a fusion protein. Amino acid residues 1-87 of C2I were sufficient for interaction with C2IIa and for translocation of C2I fusion toxins into HeLa cells. Residues 1-87 were the minimal part of C2I to bind to C2IIa on the cell surface as detected by fluorescence-activated cytometry. An excess of C2I\textsuperscript{1-87} (but not of further truncated C2I fragments) competed with Alexa488-labeled C2I for binding to C2IIa. Also the fragment C2I\textsuperscript{30-431} and the fusion toxin C2I\textsuperscript{30-225}-C3, respectively, competed with C2I-Alexa488 for binding to C2IIa. C2I\textsuperscript{30-225}-C3 did not induce cytotoxic effects on cells when applied together with C2IIa, indicating that amino acid residues 1-29 are involved in translocation of C2I but are not absolutely essential for binding to C2IIa.
INTRODUCTION

*Clostridium botulinum* C2 toxin belongs to the family of binary actin-ADP-ribosylating toxins. Further members of this toxin family are iota toxin from *C. perfringens* (1), the *C. sputriforme* toxin (2), the *C. difficile* ADP-ribosyltransferase (3) and the recently crystallized vegetative insecticidal protein (VIP) from *Bacillus cereus* (4). These toxins are composed of two separated proteins, a binding/translocation component and an enzyme component which must assemble on the surface of target cells to exhibit cytotoxicity. This mechanism of toxin uptake is also shared by anthrax toxin, which binding and translocation component protective antigen (PA) is related to the binding components of the actin-ADP-ribosylating toxins (5).

C2 toxin consists of the ADP-ribosylating enzyme component C2I (Mr ~50,000) and the binding component C2II (Mr ~80,000). For cellular uptake of C2 toxin, trypsin-activated C2IIa forms a heptamer (6) which interacts with the C2I enzyme component and binds to the cellular receptor, a hybrid and/or complex carbohydrate structure (7). Both C2IIa and C2I are internalized via receptor-mediated endocytosis. In an acidic endosomal compartment, the C2IIa oligomers insert in the membrane and form pores (6). Most likely, C2I is translocated through this pore across the endosomal membrane into the cytosol to ADP-ribosylate G-actin

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1 *Abbreviations* - C., Clostridium; C2I, enzyme component of *C. botulinum* C2 toxin; C2II, binding component of *C. botulinum* C2 toxin; C2IIa, trypsin-activated binding component of *C. botulinum* C2 toxin; C3, *C. limosum* C3-like exoenzyme; HBSS, Hank’s balanced salt solution; MEM, minimal essential medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
at Arg-177 (8). As a consequence, actin polymerization is blocked and actin filaments disassemble. Site-directed mutagenesis of C2I (9) and of the related ADP-ribosyltransferase iota α from \textit{C. perfringens} (10) indicates that the catalytic site of these enzymes is located in their C-terminal part. This is in line with the recently reported crystal structure and the suggested catalytic mechanism of the ADP-ribosyltransferase VIP2 (4).

The N-terminal domain of C2I (amino acid residues 1-225) is enzymatically inactive but interacts with the C2IIa oligomer and mediates cellular uptake of C2I (11). This allows C2I\textsuperscript{1-225} to act as an adaptor for C2IIa-mediated delivery of fusion proteins into cells. In the C2I\textsuperscript{1-225}-C3 fusion toxin, C2I\textsuperscript{1-225} was used to deliver C3 transferase from \textit{C. limosum} (Mr ∼23,000) (11) which inactivates Rho GTPase by ADP-ribosylation at Asn-41 (12). C3 transferase by its own does not enter cells efficiently because it lacks any specific binding and translocation unit (13). We characterized the C2I/C2IIa contact site in more detail by the use of various truncations of C2I and their fusions with C3 transferase. Here, we report that amino acid residues 1-87 of C2I are sufficient for C2IIa-mediated delivery of C2I fusion toxins into cells.
EXPERIMENTAL PROCEDURES

Materials - Cell culture medium was from Biochrom (Berlin, Germany), fetal calf serum from PAN Systems (Aidenbach, Germany) and cell culture materials from Falcon (Heidelberg, Germany). The C2II binding component from C. botulinum C2 toxin was purified as recombinant GST-fusion protein in E. coli and activated with trypsin as described (6). Antiserum against C2I and antiserum against C3 transferase was raised in rabbits. Donkey anti-rabbit antibody coupled to peroxidase and the enhanced chemiluminescence detection kit were purchased from Amersham (Braunschweig, Germany). The nitrocellulose blotting membrane was from Schleicher and Schuell (Dassel, Germany). Molecular weight protein marker was obtained from BIO RAD (Hercules, USA). Oligonucleotides were obtained from MWG (Ebersberg, Germany), the pGEX2T vector (included in the GST Gene Fusion System) and glutathione Sepharose 4B was from Pharmacia Biotech (Uppsala, Sweden), DNA molecular weight marker (Lambda Hind III) and restriction enzymes were from Boehringer Mannheim (Mannheim, Germany) and T4 ligase and competent E. coli cells from Stratagene (Heidelberg, Germany). PCR was performed in the Gene Amp PCR System 2400 from Perkin-Elmer (Langen, Germany) and DNA sequencing was done with the Cycle Sequencing Ready Reaction Kit (ABI PRISM ) from Perkin-Elmer. Thrombin was from Sigma (Deisenhofen, Germany). [32P]-NAD (30 Ci/mmol) was from DuPont NEN (Bad Homburg, Germany). Alexa488 was purchased from Molecular Probes (Eugene, USA).

Cell culture and cytotoxicity assay - HeLa cells and African green monkey kidney (Vero) cells were cultivated in tissue culture flasks at 37 °C and 5% CO2 in Dulbeccos MEM, containing 5% heat-inactivated (30 min, 56 °C) fetal calf serum, 2 mM L-glutamate, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were routinely trypsinized and reseeded
twice a week. For cytotoxicity assays, cells were grown as subconfluent monolayers in 12-well plates and treated with trypsin-activated C2IIa together with the respective fusion toxin in serum free medium or in Hanks' balanced salt solution (HBSS). HBSS contained in g/l: 0.185 CaCl₂, 0.098 MgSO₄, 0.4 KCl, 0.06 KH₂PO₄, 8 NaCl, 0.048 Na₂HPO₄, and 1 glucose, to which 10 mM HEPES (pH 7.4) was added. At the indicated times, pictures were taken and the total number of cells as well as the number of rounded cells per field were counted and the percentage of rounded cells was calculated. All experiments were performed at least three times.

**Fluorescence-activated cytometry** – A cysteine residue was introduced in C2I protein at amino acid position 16 instead of a serine residue by site-directed mutagenesis of the pGEX-C2I plasmid with the Quick Change Kit from Stratagene (Heidelberg, Germany) and the 5’-primer: CAA TAA ACC TGA ATG CGA AGC AAA AAA ATG G and the 3’-primer: CCA TTT TTT TGC TTC GCA TTC AGG TTT ATT G (Stiles et al., submitted). A cysteine residue was introduced in C2I30-431 at amino acid residue 408 by site-directed mutagenesis of the pGEX-C2I plasmid with the Quick Change Kit and the 5’-primer: GAA TAA CTC CAA TTT GCA TAA TTA ATA GAG and the 3’-primer: CTC TAT TAA TTA TGC AAG TTA TTG GAG TTA TTC. A cysteine residue was introduced in the C2I30-225-C3 fusion toxin at amino acid residue 187 of C3 instead of a serine residue by site directed mutagenesis of the pGEX-C2I30-225-C3 plasmid with the Quick Change Kit and the 5’-primer: GCA CCT ACA CTA TAT GTG ATA TGC AAA TAG C and the 3’-primer: GCT ATT TGC ATA TCA CAT ATA GTG TAG GTG C. The mutations were confirmed by cycle sequencing. Labeling of C2IS16C, C2I30-431S408C and C2I30-225-C3S187C proteins, respectively, with Alexa488 was done as described in the manufacturer’s protocol (Molecular Probes, Eugene, USA). Excess label was removed from the protein mixture by washing with ice cold
phosphate buffered saline (PBS), pH 7.4, in a Micro Bio-Spin 6 chromatography column (Biorad, Hercules, USA). Binding studies by cytometry were done with African green monkey kidney (Vero) cells. Cells were detached from flasks and about 1 x 10^6 cells per sample were used. For direct binding of C2I1-225-C3-A488 and C2I30-225-C3-A488, respectively, cells were incubated for 15 min at 4 °C with CIIa together with C2I1-225-C3-A488 and C2I30-225-C3-A488, respectively in Hanks balanced salts solution (HBSS) containing 0.2% bovine serum albumin (BSA). Cells were washed four times with HBSS + BSA (for some controls, cells were not washed) and cell-associated fluorescence detected by a FACSCalibur fluorescence-activated cell sorter (Becton Dickinson, Heidelberg, Germany). For competition assays, cells were incubated for 30 min with C2IIa and C2I-A488 (with or without C2I-C3 fusion toxins as competitors) at 4 °C in HBSS + BSA. Cells were washed twice with HBSS + BSA and cell-associated fluorescence was detected by flow cytometry.

*Construction of the C2I fusion toxins* - C2I-truncations were obtained by PCR with the pGEX2T-C2I plasmid (1296 bp from *Clostridium botulinum* strain 92-13; Genbank Acc. No. AJ224480) (9) as a template with the respective primers. 3' primers: C2I1-27: GAA TCC GGA TCC TTT TTC TTC TTC TTC TTT ACC, C2I1-47: GAA TCC GGA TCC CTT ATT TTT AAG TTG ATT, C2I1-67: GAA TCC GGA TCC TGC AGT TAA TGA AGA AAA, C2I1-87: GGA TCC TTC CCT AAT TCT TTC AAC ATC AAA, C2I1-137: GGA TCC TAT TAT TTG CTG TCT AAC TTT ATC, C2I1-187: GGA TCC TAA TAA TAA TGA TAC TGT TTC TTT. To generate these constructs, the following 5' primer was used: AGA TCT ATG CCA ATA ATA AAA GAA CCC. For amplification of C2I10-225 and C2I110-225, respectively, the following 3' primers containing a *BgIII*-site were used: C2I10-225: AGA TCT TTC ATC AAT AAA CCT GAA TCT GAA, C2I110-225: AGA TCT GTA ATT ATT TTT TCT ATA AGA GAT. The 5' primer for these constructs contained both an *EcoR*I- and a *BamH*I-site: GAA TCC GGA
TCC TCC TTT ATT ATA GAA ATC. The following PCR programm was used for amplification: 2 min at 94° C, 10 sec at 94° C, 30 sec at 56° C, 2 min at 68° C (20 cycles) and 10 min at 68° C. These various C2I-truncations were used for cloning of C2I-C3 fusion toxins as described for C2I1-225-C3 (11). C2I30-431 and C2I30-225-C3 were obtained by PCR with the pGEX2T-C2I (9) and the pGEX2T-C2I1-225-C3 (11) plasmid, respectively, as templates and the 5’-oligonucleotide primer: GGA GAT CTT TTA CGA AAT TAA ATA ATC TTG AAG AAG TAG C and the 3’-primer CCA TTG CTG CAG GCA TC. This primer bound to a sequence in pGEX 2T. The fragments were cloned into the pCR2.1 plasmid (Invitrogen, NV Leek, The Netherlands). From the pCR2.1 plasmid, the C2I30-431 fragment (2145 bp) was excised with BgII/PstI and ligated into BamHI/PstI-digested pGEX 2T plasmid. C2I30-225-C3 (1300 bp) was excised from pCR2.1 plasmid with BgII/EcoRI and ligated into BamHI/EcoRI-digested pGEX 2T plasmid. The constructs were sequenced using the sequencing primers 5’ pGEX2T-58 and 3’ pGEX2T-43. The cycle sequencing reaction was performed according to the manufacturer's instructions.

Expression and purification of recombinant proteins - All proteins were expressed as recombinant GST-fusion proteins in E. coli, harboring the respective DNA fragment in the plasmid pGEX-2T and cleaved with thrombin as described (11).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting - SDS-PAGE was performed according to the methods of Laemmli (14). Immunoblot analysis of the various proteins was performed as described (11).
ADP-ribosylation assay - The in vitro ADP-ribosylation assay for G-actin and for Rho was performed as described previously (8,15,16). Radioactively labeled proteins were detected by phosphorimaging.
RESULTS

Cytotoxicity of GST-C2I\(^{1-225}\)-C3 – The fusion toxin C2I\(^{1-225}\)-C3 is efficiently delivered into the cytoplasm of various cell lines via the C2IIa transport component (11). To study whether it is essential that the C2I\(^{1-225}\) adaptor is located at the N-terminal end of fusion toxins, we tested whether GST-C2I\(^{1-225}\)-C3 (\(~\) 75 kDa) was delivered into HeLa cells by C2IIa. Therefore, 200 ng/ml and 1 µg/ml of GST-C2I\(^{1-225}\)-C3, respectively, were added to cells with or without C2IIa (200 ng/ml). Whereas GST-C2I\(^{1-225}\)-C3 without C2IIa had no effect on cells (Fig. 1 A and C), cells showed "C3-morphology" (e. g., contraction of the cell body, formation of thin cell processes and rounding up) when incubated for 3 h with GST-C2I\(^{1-225}\)-C3 plus C2IIa (Fig. 1 B and D). This finding indicates that i) the N-terminus of C2I has not to be located necessarily at the very N-terminus of the fusion proteins but can be located at the inner part of a fusion toxin, and ii) C2IIa delivers molecules into the cytoplasm which have a higher molecular mass than C2I (49.4 kDa).

Cloning, expression and characterization of N-terminally truncated C2I proteins and their fusion toxins with C. limosum C3 exoenzyme - To investigate the role of the N-terminal part of C2I in interaction with C2IIa and in the enzyme activity of C2I, we cloned N-terminally truncated C2I\(^{30-431}\) and expressed the GST-fusion protein. Following cleavage with thrombin, the protein was analyzed for enzyme activity. ADP-ribosylation assay revealed that the C2I\(^{30-431}\) protein possessed no enzyme activity (not shown). The reason for the loss of enzyme activity is not clear but we suggest that the amino acid residues 1-29 have an important role in folding of C2I. In a tryptic digest of C2I and C2I\(^{30-431}\) the truncated C2I\(^{30-431}\) protein yielded in increased degradation products (not shown). While the catalytic domain of C2I (amino acids 226-431) appears to need the N-terminal domain for expression of enzyme...
activity, C2I\(^{1-225}\) was functional with respect to binding to C2IIa and delivery of peptides into the cell. Therefore, we constructed various C2I deletions and made fusion toxins to study which part of the N-terminal domain of C2I interacts with C2IIa. The resulting fusion toxins were: C2I\(^{1-187}\)-C3, C2I\(^{1-137}\)-C3, C2I\(^{1-87}\)-C3, C2I\(^{1-67}\)-C3, C2I\(^{1-47}\)-C3, C2I\(^{1-27}\)-C3 and C2I\(^{110-225}\)-C3, C2I\(^{30-225}\)-C3 and C2I\(^{10-225}\)-C3. The various C2I-C3 fusion toxins were expressed as described above and analyzed by SDS-PAGE. Each protein run with the molecular mass expected (Fig. 2 A). All fusion toxins were recognized in an immunoblot analysis with anti-C2I antiserum (Fig. 2 B) and by anti-C3 antiserum (not shown). To analyze the various C2I-C3 fusion toxins for their enzyme activity and substrate specificity, \textit{in vitro} ADP-ribosylation of cell lysates was performed. The autoradiography shown in Fig. 2 C confirmed that exclusively Rho was ADP-ribosylated by C2I-C3 fusion toxins.

\textit{Cytotoxicity of C2I-C3 fusion toxins} – Cytotoxic activity of chimeric C2I-C3 toxins was tested on HeLa cells. Therefore, subconfluently growing monolayer cells were incubated in HBSS with C2IIa and C2I-C3 fusion toxins. For control, cells were incubated with C2IIa plus C3 or without any toxin. The finding that C2I\(^{1-87}\)-C3 intoxicated cells (Fig. 3), strongly suggests that amino acid residues 1-87 of C2I are sufficient for delivery of fusion toxins into cells. We compared the action of C2I\(^{1-87}\)-C3 with C2I\(^{1-225}\)-C3 on HeLa cells (Fig. 3). The single components C2IIa (not shown), C2I\(^{1-225}\)-C3 (Fig. 3 A) or C2I\(^{1-87}\)-C3 (Fig. 3 B) did not exhibit cytopathic effects but cells exhibited "C3-morphology" when treated with C2I\(^{1-225}\)-C3 plus C2IIa (Fig. 3 C) or with C2I\(^{1-87}\)-C3 plus C2IIa (Fig. 3 D) for 3 h. A more quantitative analysis is given in Fig. 4. At concentrations of 500 ng/ml of C2I\(^{1-225}\)-C3 or C2I\(^{1-87}\)-C3, respectively, the fusion toxins caused similar cytotoxic effects when added to HeLa cells in the presence of C2IIa (Fig. 4 B). When 200 ng/ml of the respective fusion toxin were applied with C2IIa, the cytotoxic action of C2I\(^{1-87}\)-C3 was delayed compared to C2I\(^{1-225}\)-C3, but after
3 hours, no significant difference in the action of both toxins was observed (Fig. 4 A). To confirm that the concentration of 200 ng/ml C2IIa was not a limiting factor in these experiments, we incubated HeLa cells with various concentrations of C2IIa (200, 500 and 1000 ng/ml) together with a constant concentration of 200 ng/ml of the C2I1-225-C3 fusion toxin. These studies showed that after 2, 4 and 6 h of incubation there was no detectable difference in the amount of intoxicated cells indicating that the binding component was not limiting (data not shown).

Further truncations of C2I were made to identify the minimal length of C2I to transport C3 into cells. No cytotoxic effects were observed when C2I1-67-C3, C2I1-47-C3 and C2I1-27-C3, respectively, were applied to HeLa cells together with C2IIa (not shown). These findings indicate that residues 1-87 of C2I were necessary to mediate binding to C2IIa and/or translocation of C2I into the cytosol.

Next, we tested whether the N-terminal amino acid residues 1-9 and 1-109, respectively, were necessary for C3 delivery. HeLa cells were treated with C2I10-225-C3 and C2I110-225-C3, respectively. C2I110-225-C3 plus C2IIa showed only poor cytotoxic effects but C2I10-225-C3 plus C2IIa was cytotoxic when applied together with C2IIa (Fig. 5).

_Competition of truncated C2I proteins with C2I1-225-C3 for C2IIa binding_ – To study the minimal part of C2I which interacts with C2IIa, those fusion toxins which did not intoxicate cells were tested for competition for the C2IIa binding site with C2I1-225-C3. Therefore C2I1-67-C3, C2I1-47-C3 and C2I1-27-C3, respectively (40 µg/ml medium) were added together with C2IIa (200 ng/ml medium) to HeLa cells for 30 min at 4 °C to allow binding but not endocytosis. For controls, cells were incubated without any protein or with C2IIa alone. Cells were washed to remove free protein, C2I1-225-C3 (200 ng/ml) was added and the cells were incubated at 37 °C. After 2, 4, and 6 h, pictures were taken and the intoxicated cells
were determined. No inhibitory effect on the intoxication of HeLa cells was observed when cells were pretreated with the fusion toxins C2I\(^{1-67}\)-C3, C2I\(^{1-47}\)-C3 and C2I\(^{1-27}\)-C3, respectively (data not shown). To make sure that the binding of C2I\(^{1-67}\)-C3, C2I\(^{1-47}\)-C3 and C2I\(^{1-27}\)-C3, respectively to C2IIa on the cells was not removed by the washing step, the same experiment was done without washing the cells after incubation with the proteins on ice. When C2I\(^{1-225}\)-C3 was added directly to cells preincubated with C2IIa together with C2I\(^{1-67}\)-C3, C2I\(^{1-47}\)-C3 and C2I\(^{1-27}\)-C3, respectively on ice, no delay in intoxication with C2I\(^{1-225}\)-C3 was observed after shifting the cells to 37 °C.

Next, we tested whether residues 1-87 of C2I show any competition with C2I\(^{1-225}\)-C3 for C2IIa binding sites. Therefore, the C2I\(^{1-87}\) peptide was used because the C2I\(^{1-87}\)-C3 fusion toxin was taken up via C2IIa and changed cellular morphology. HeLa cells were pretreated for 30 min at 4 °C with C2IIa (200 ng/ml) together with C2I\(^{1-87}\) (40 µg/ml). For control, cells were treated without any protein or with C2IIa alone. Cells were washed and C2I\(^{1-225}\)-C3 (200 ng/ml) was added. The cells were incubated at 37 °C and after 1, 2, 3, and 4 h, pictures were taken. Fig. 6 shows the intoxication of cells. After 1 h of incubation, cells showed no alteration in morphology. After 2 h, those cells which were incubated with C2IIa plus C2I\(^{1-225}\)-C3 showed “C3 morphology” (Fig. 6 B). Less cells showed “C3 morphology” after pretreatment with C2I\(^{1-87}\) plus C2IIa (Fig. 6 F) compared with cells pretreated only with C2IIa (Fig. 6 B). After 3 (Fig. 6 C and G) and 4 h (Fig. 6 D and H) this protective effect was no longer detectable. Fig. 6 I gives the percentage of intoxicated cells after the indicated incubation periods. To make sure that the delay in C2I\(^{1-225}\)-C3 action was due to a specific competition of this fusion toxin with C2I\(^{1-87}\), we tested the effect of C3 exoenzyme from \textit{C. limosum} (40 µg/ml) in the competition assay with C2I\(^{1-225}\)-C3 on HeLa cells. C3 is not able to enter cells and does not specifically interact with C2IIa and, therefore, can be used as a control. C3 had no influence on the intoxication of cells with C2I\(^{1-225}\)-C3 together with C2IIa.
The delay in intoxication of cells with C2I^{1-225}-C3 after pretreatment of cells with C2I^{1-87} shows that C2I^{1-87} is not only the minimal part of C2I which mediates translocation of fusion toxins into the cytoplasm, but also represents the minimal essential part of C2I for which binding to C2IIa was detected.

This finding was confirmed by binding experiments of C2 toxin on Vero cells done by fluorescence-activated cytometry. Vero cells were incubated at 4 °C in solution with C2IIa together with the C2I-C3 fusion toxins C2I^{1-225}-C3, C2I^{1-87}-C3, C2I^{1-67}-C3, C2I^{1-47}-C3 and C2I^{1-27}-C3, respectively, to allow binding of the proteins to the cells. After 15 min, C2I labeled with the fluorescence dye Alexa488 was added and cells were further incubated in the cold for 15 min. Thereafter, cell-associated fluorescence was analyzed by flow cytometry. The cell binding studies revealed that pretreatment of cells with C2I^{1-225}-C3 and C2I^{1-87}-C3, respectively, in the presence of C2IIa decreased the fluorescence signal from C2IA488 on the cell surface compared to cells, which were treated with C2IIa and C2IA488 (Fig. 7). This indicates that C2I^{1-225}-C3 and C2I^{1-87}-C3 proteins act as competitors for binding of C2IA488 to C2IIa on the cells, when added in a 200-fold excess to C2IA488. The other fusion toxins C2I^{1-67}-C3, C2I^{1-47}-C3 and C2I^{1-27}-C3, respectively, showed no competition of binding of C2IA488 to C2IIa (Fig. 7).

To study the role of the N-terminal 30 amino acid residues of C2I, we constructed the fusion toxin C2I^{30-225}-C3 (Fig. 8 A). C2I^{30-225}-C3 was active in the ADP-ribosylation assay (Fig. 8 B) but was not cytotoxic when applied to HeLa cells together with C2IIa for 3 h (Fig. 8 D). By contrast, C2I^{1-225}-C3 plus C2IIa showed a cytotoxic effect after a 3 h incubation period (Fig. 8 C). Even at a concentration of 1 µg/ml, the C2I^{30-225}-C3 fusion toxin induced no cytotoxic effect (not shown). Furthermore, in the cytotoxicity assay, C2I^{30-225}-C3 was not able to compete with C2I^{1-225}-C3 (not shown).
Truncated C2I\(^{30-431}\), which was enzymatically inactive, was not able to bind together with C2IIa on Vero cells as tested by fluorescence activated cytometry with Alexa488-labeled protein (not shown). We used fluorescence-activated cytometry to test whether C2I\(^{30-431}\) and C2I\(^{30-225}\)-C3 proteins, respectively, compete with C2IA488 for binding to C2IIa on the surface of Vero cells (Fig. 9 A). For controls, full length C2I and the C2I\(^{1-225}\)-C3 fusion toxin were also tested for competition in this assay. As shown in Fig. 9 A, all proteins decreased binding of C2IA488 to cells. C2I reduced the fluorescence signal to control level and the truncated C2I\(^{30-431}\) and C2I\(^{30-225}\)-C3 proteins, respectively, to about 50 % of the signal obtained without competitors. All competitors were used in a 200 fold excess to C2IA488.

Because C2I\(^{30-225}\)-C3 decreased the binding of C2IA488 to cells, we tested whether the C2I\(^{30-225}\)-C3 fusion toxin shows binding to C2IIa in a direct assay. Therefore, we introduced a cystein residue into the C3 part of C2I\(^{1-225}\)-C3 and C2I\(^{30-225}\)-C3, respectively and studied binding of the Alexa488-labeled proteins to cells. The recombinant proteins C2I\(^{1-225}\)-C3(S187C) and C2I\(^{30-225}\)-C3(S187C) both were able to catalyze ADP-ribosylation of Rho protein \textit{in vitro} (not shown). Fig. 9 B shows the relative fluorescence after binding of C2I\(^{1-225}\)-C3(S187C)-A488 + C2IIa (column 2) and of C2I\(^{30-225}\)-C3(S187C)-A488 + C2IIa (column 3) to Vero cells. For control, cells were treated with C2IIa only (column 1). When cells were washed four times after incubation with the proteins and analyzed by flow cytometry, C2I\(^{30-225}\)-C3(S187C)-A488 showed some binding to C2IIa. However, the binding of C2I\(^{30-225}\)-C3(S187C)-A488 + C2IIa to cells was decreased compared to that of C2I\(^{1-225}\)-C3(S187C)-A488 + C2IIa (Fig. 9 B).

Taken together, our studies show that amino acid residues 1-87 of C2I are sufficient for C2IIa-mediated delivery of C3 fusion toxins into cells. Further C-terminal truncations of this adaptor were not able to transport C3 transferase into cells. Amino acid residues 10-29 are not
absolutely essential for binding of C2I to C2IIa but they are necessary for translocation of C2I-C3 fusion toxins.
DISCUSSION

For cellular uptake of the binary *C. botulinum* C2 toxin, the activated binding component C2IIa forms heptamers, which bind to the cellular receptor, and eventually interacts with the enzyme component C2I. Both components are internalized by receptor-mediated endocytosis. Most likely, in an acidic early endosomal compartment, the C2IIa heptamers insert in the membrane and form pores (6). We assume that C2I translocates through the pores across the endosomal membrane into the cytosol. To this end, at least a partial unfolding of the C2I ADP-ribosyltransferase is necessary, because the inner diameter of the C2IIa pore appears to be ~2 nm (6). After the translocation process, a proper refolding of C2I is a prerequisite to recover its enzymatic activity in the cytoplasm. Most likely, a specific adaptor region of the C2I protein is required for binding to C2II on the cell surface and/or for initiation of the translocation process in the acidic endosomal compartment. However, little is known about the interaction of the enzymatic component with the binding component C2II. We reported earlier that the N-terminal domain of C2I (residues 1-225) is sufficient to mediate the cellular uptake of *C. limosum* C3 transferase by means of the C2I1-225-C3 fusion toxin and the activated C2IIa binding component (11). C3 by itself appears to enter cells via non-specific pinocytosis when large amounts of C3 are applied because it lacks a receptor binding domain (13). The N-terminal 225 amino acids of C2I (C2I1-225) represent an adaptor region which binds to C2IIa and coordinates the translocation of C2I and of the C2I1-225-C3 fusion toxin, respectively, through the pore formed by C2IIa. The surprisingly efficient transport of C2I1-225-C3 fusion toxin by C2IIa may be explained by at least two reasons. First, C3 transferase shares structural features with the C-terminal part of C2I transferase (4) and second C2I1-225-C3 (~50 kDa) has nearly the same molecular mass as C2I (~50 kDa) (11). We tested whether the C2I1-225 adaptor molecule must be located at the N-terminus or C-terminus
of the fusion toxins for efficient transport. Here we report that GST-C2I\(^{1-225}\)-C3 was taken up by cells. We also constructed fusion toxins containing C2I\(^{1-225}\) and the catalytic domain of C. difficile toxin B. In one of these proteins the C2I\(^{1-225}\) adaptor was located at the N-terminus and in the other protein at the C-terminus. Both fusion toxins were delivered into HeLa cells by C2IIa (manuscript in preparation). This finding suggests that the part of C2I that functions as an adaptor to interact with C2IIa can be located at different parts of the fusion toxin. Obviously, it is not essential for interaction with C2IIa that the C2I\(^{1-225}\) part is located at the very N-terminus of the respective fusion toxin. Furthermore, using these fusion toxins in cytotoxicity assays, we observed that molecules of a higher molecular mass than that of C2I were also translocated into the cytosol. The GST-C2I\(^{1-225}\)-C3 fusion toxin (~ 75 kDa) exhibits a higher mass than C2I (~ 50 kDa).

Recently, the first member of the family of binary actin ADP-ribosylating toxins, VIP2, the enzymatic component of the VIP toxin (vegetative insecticidal protein) from Bacillus cereus, was crystallized (4). In analogy to the two components C2I and C2II of C2 toxin, VIP toxin consists of VIP1 and VIP2. VIP1 is the membrane binding component (100 kDa) which mediates cellular uptake of the enzymatically active component VIP2 (52 kDa) into insect cells. According to recent crystal structure analysis (4), VIP2 consists of two domains which are separated by a central cleft (see Fig. 10). The N-terminal domain consists of amino acid residues 60-265 and the C-terminal catalytic domain of residues 266-461. VIP2 shares significant sequence homology with the enzymatic components of other binary actin ADP-ribosylating toxins including C2 toxin from C. botulinum (see Fig. 10 A) and iota toxin from C. perfringens (4). Sequence alignment between the N-terminal domains of VIP2 and C2I suggest that C2I\(^{1-225}\) is most likely also composed of 5 \(\alpha\)-helices and 8 \(\beta\)-sheets (Fig. 10 A). The shortest part of C2I which allowed the transport of C3 transferase into cells, C2I\(^{1-87}\), contains the region covering helices \(\alpha1\)-4 in the VIP2 structure (see Fig. 10): helix \(\alpha1\) (amo
acid residues 15-29 in C2I), helix α2 (residues 34-45 in C2I), helix α3 (residues 52-61 in C2I) and helix α4 (residues 69-86 in C2I). Pretreatment of cells with C2IIa plus an excess of the C2I\(^{1-87}\) peptide delayed intoxication of cells with C2I\(^{1-225}\)-C3 toxin for about 2 h. Furthermore, C2I\(^{1-225}\)-C3 and C2I\(^{1-87}\)-C3, respectively, competed with C2I for binding to C2IIa on the cell surface as demonstrated by fluorescence-activated cytometry. These observations indicate that C2I\(^{1-87}\) competes with C2I\(^{1-225}\)-C3 for the C2IIa binding sites. However, our findings show that binding of C2I\(^{1-87}\) to C2IIa is less stable as interaction of C2I\(^{1-225}\) with C2IIa. C2I\(^{1-87}\) was the shortest of the described C2I fragments which delayed intoxication of cells with C2I\(^{1-225}\)-C3. The further truncations of C2I (e.g., C2I\(^{1-67}\)) were not able to deliver C3 into the cytoplasm of cells and did not compete with C2I\(^{1-225}\)-C3 for C2IIa on the cell surface. This suggests that helices α1-4 are essential at least for translocation of the protein into the cytoplasm and most likely also for interaction with the C2IIa binding component. To study the role of helix α1 (residues 15-29), amino acid residues 1-29 were deleted from C2I\(^{1-225}\) and C2I\(^{30-225}\) was fused to C3. This protein was not delivered into cells by C2IIa. When binding of C2I and truncated C2I\(^{30-431}\) to cell bound C2IIa was analyzed by fluorescence-activated cytometry with Alexa-labeled proteins, no significant binding of C2I\(^{30-431}\) was measured. Furthermore, the C2I\(^{30-225}\)-C3 fusion toxin did not inhibit or delay intoxication of HeLa cells with C2I\(^{1-225}\)-C3 when the protein was tested for competition in cytotoxicity assays. In competition studies with fluorescence-activated cytometry with Vero cells, however, C2I\(^{30-431}\) and C2I\(^{30-225}\)-C3, respectively, competed with full length C2I for the C2IIa binding to cells. One possible explanation for the different findings in the binding (FACS analysis) and the cytotoxicity assays is a weak interaction of C2I\(^{30-225}\)-C3 with C2IIa. This weak interaction is detectable in FACS analysis but not in the cytotoxicity assay, because the assay procedure requires an additional washing step. Direct binding of Alexa-labeled C2I\(^{30-225}\)-C3 protein
(together with C2IIa) to Vero cells, revealed that this fusion toxin showed some binding to C2IIa but with apparent lower affinity than C2I1-225-C3.

Therefore we suggest that the amino acid residues 1-29 of C2I are not absolutely necessary for interaction with C2IIa but most likely stabilize the interaction between C2I and C2IIa. Because the toxins, lacking residues 1-29, were not taken up via C2IIa, these amino acids seem to be involved in translocation of the enzyme component into the cytoplasm.

The recombinant C2I30-431 protein was enzymatically inactive with respect to ADP-ribosylation of actin. This suggests that residues 1-29 of C2I are essential for the expression of a functional C-domain. Because the tryptic digestion of C2I30-431 was increased compared to the full length C2I protein, amino acids 10-29 are most likely essential for proper folding of C2I.

The binary lethal toxin from *Bacillus anthracis* represents the combination of the transport component protective antigen (PA) and lethal factor (LF) or edema factor (EF), respectively. LF (776 amino acids) interacts with PA to enter cells (5). Because PA shares homology with C2II, interaction of the binding components and the enzymatic components from anthrax toxin and C2 toxin may be similar. Arora and Leppla reported earlier that amino acids 1-254 from LF are necessary for binding to PA and for translocation of LF and LF-fusion toxins, respectively, into the cytosol (17). Fusion proteins containing amino acids 1-198 of LF were inactive, suggesting that the PA-binding domain of LF is located within residues 1-254. When amino acids 1-40 of LF were deleted, the resulting protein was not toxic because it did not bind to PA (17). Therefore, the C2II/C2I system and the PA/LF system share the importance of the N-terminus of the enzyme component to interact with the binding component.

Recently, Kumar et al. reported that residues Tyr137, Tyr138, Ile140 and Lys142 are required for binding of edema factor (767 amino acids) to the PA heptamer (18). Obviously, the minimum length of C2I (C2I1-87) to function as an adaptor for interaction with C2IIa is shorter...
than that of LF to interact with PA. So far it is not clear whether LF\(^{1-254}\) and C2I\(^{1-87}\) share structural similarities which are responsible for the interaction with the binding components. However, it is important to note that the complete N-terminal domain e.g., amino acids 1 to 225, was still more efficient as an adaptor for binding and transport of fusion toxins than the short C2I\(^{1-87}\) adaptor. Supported by the structure analysis of the related VIP2 (Fig. 10), we suggest that the complete N-terminal half of C2I (amino acids 1-225) forms a functional unit which is optimal for interaction with C2II. Interaction occurs mainly through the N-terminal 4 α-helices, which can be fused to variable parts of a "cargo" protein. Further studies are necessary to get more insight into the translocation process of C2I across the C2IIa pore and to understand the molecular interactions involved in binding, defolding, translocation and refolding of cargo proteins.
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LEGENDS TO FIGURES

Fig. 1. Cytotoxic effects of GST-C2I1-225-C3 fusion toxin. Subconfluent HeLa cells were incubated with the respective toxin at 37° C and pictures were taken after 3 h. (A) GST-C2I1-225-C3 (200 ng/ml), (B) GST-C2I1-225-C3 (200 ng/ml) with C2IIa (200 ng/ml) (C) GST-C2I1-225-C3 (1 µg/ml) (D) GST-C2I1-225-C3 (1 µg/ml) with C2IIa (200 ng/ml).

Fig. 2. Characterization of C2I-C3 fusion toxins. Proteins were expressed as GST-fusion proteins in *E. coli* and cleaved with thrombin from glutathione Sepharose beads. Proteins were run in a 12.5 % SDS-PAGE and subsequently stained with Coomassie (A) or analyzed by anti-C2I immunoblot analysis (B). Lane 1, C3; lane 2, C2I1-27-C3; lane 3, C2I1-47-C3; lane 4, C2I1-67-C3; lane 5, C2I1-87-C3; lane 6, C2I1-137-C3; lane 7, C2I1-187-C3; lane 8, C2I10-225-C3; lane 9, C2I110-225-C3. For control C2I was used. (C) Autoradiography of [32P]ADP-ribosylated actin and [32P]ADP-ribosylated Rho after *in vitro* incubation of cell lysates with C3 and the respective C2I-C3 fusion toxins. For control, C2I was used for ADP-ribosylation of actin. The lanes represent the same proteins as in (B).

Fig. 3. Cytotoxic effects of C2I1-225-C3 and C2I1-87-C3. Subconfluent HeLa cells were incubated at 37 °C with C2I1-225-C3 (200 ng/ml) (A), with C2I1-87-C3 (200 ng/ml) (B), with C2I1-225-C3 (200 ng/ml) plus C2IIa (200 ng/ml) (C) and with C2I1-87-C3 (200 ng/ml) plus C2IIa (200 ng/ml) (D). After 3 h, pictures were taken.

Fig. 4. Time course of cytotoxic effects of C2I1-225-C3 and C2I1-87-C3 fusion toxins. Subconfluent HeLa cells were incubated with C2IIa (200 ng/ml) with 200 ng/ml (A) and 500 ng/ml (B) of C2I1-225-C3 (★) and C2I1-87-C3 (▼), respectively at 37 °C. For control, cells
were incubated with the single components C2IIa (●), C2I\(^{1-225}\)-C3 (♦) or C2I\(^{1-87}\)-C3 (■). After the indicated time points, pictures were taken and the percentage of cells showing "C3 morphology" was determined. Values are given as mean ± S. D. (n = 3).

Fig. 5. Time course of cytotoxic effects of C2I\(^{10-225}\) -C3 and C2I\(^{110-225}\) -C3. Subconfluent HeLa cells were incubated with C2IIa (200 ng/ml) + 500 ng/ml of C2I\(^{10-225}\) -C3 (♦) and C2I\(^{110-225}\) -C3 (●), respectively at 37 °C. For control, cells were incubated with C2IIa (200 ng/ml) with C3 (500 ng/ml) (▲) or without any toxin (■). Immediately and after 1, 2 and 3 h, pictures were taken and the percentage of cells showing "C3 morphology" was determined. Values are given as mean ± S. D. (n = 3).

Fig. 6. Competition of C2I\(^{1-87}\) with C2I\(^{1-225}\) -C3 for C2IIa binding. HeLa cells were incubated for 30 min at 4 °C with C2IIa (200 ng/ml) together with 40 µg/ml of C2I\(^{1-87}\) in serum free medium (E - G). For control, cells were incubated with C2IIa (200 ng/ml) alone (A - D). Cells were washed one time with serum free medium and fresh prewarmed medium (37 °C) containing fetal calf serum and 200 ng/ml C2I\(^{1-225}\) -C3 was added. Cells were further incubated at 37 °C and after 1 (A, E), 2 (B, D), 3 (C, G) and 4 h (D, H) pictures were taken. I. The percentage of intoxicated cells showing "C3 morphology" was determined by cell counting from 3 selected fields from the pictures (each field contained between 60 and 130 cells). Black columns, cells were preincubated with C2IIa (200 ng/ml) for 30 min at 4 °C and subsequently with C2I\(^{1-225}\) -C3 for 1, 2, 3 and 4 h at 37 °C. Grey columns, cells were preincubated with C2IIa (200 ng/ml) together with C2I\(^{1-87}\) (40 µg/ml) and subsequently with C2I\(^{1-225}\) -C3 for 1, 2, 3 and 4 h at 37 °C. Values are given as mean ± S. D. (n = 3).
Fig. 7. Competition of various C2I-C3 fusion toxins with C2I-Alexa488 for C2IIa binding on Vero cells by fluorescence-activated cytometry. Vero cells in suspension were incubated for 15 min at 4 °C in HBSS + 0.2% BSA together with C2IIa (10 µg/ml) and the respective C2I-C3 fusion toxin (100 µg/ml each) as a competitor. Thereafter, C2I-Alexa488 (0.5 µg/ml) was added and cells were incubated for further 15 min at 4 °C. Cells were washed twice with HBSS + 0.2% BSA, analyzed by fluorescence-activated cytometry and mean fluorescence was recorded. Values are given as mean ± S. D. (n = 3) and significance was tested by a Students t-test (* = P < 0.05; ** = P < 0.01). 1, control cells; 2, C2IIa only; 3, C2IIa + C2I-Alexa488; 4, C2IIa + C2I1-225-C3 + C2I-Alexa488; 5, C2IIa + C2I1-47-C3 + C2I-Alexa488; 6, C2IIa + C2I1-67-C3 + C2I-Alexa488; 7, C2IIa + C2I1-87-C3 + C2I-Alexa488; 8, C2IIa + C2I1-225-C3 + C2I-Alexa488.

Fig. 8. Characterization of C2I130-225-C3 fusion toxin. A. SDS-PAGE of C2I1-225-C3 and C2I30-225-C3 fusion toxins. Proteins were expressed as GST-fusion proteins in E. coli, cleaved with thrombin and run in 12.5% SDS-PAGE (1 µg of each). Coomassie staining of proteins is shown. B. In vitro ADP-ribosylation of Rho by C2I1-225-C3 and C2I30-225-C3 fusion toxins. Human platelet cytosol was incubated in the presence of [32P]-NAD for 15 min at 37 °C with C2I1-225-C3 and C2I30-225-C3, respectively. Proteins were run in 12.5% SDS-PAGE and detected by subsequent phosphorimaging. C, D. Cytotoxic effects of C2I1-225-C3 and C2I30-225-C3 fusion toxins on HeLa cells. Cells were incubated with 200 ng/ml C2IIa together with either 200 ng/ml C2I1-225-C3 (C) or with 200 ng/ml C2IIa together with 200 ng/ml C2I30-225-C3 (D) and after 3 h pictures were taken.

Fig. 9. A. Competition of C2I30-431 and C2I30-225-C3 fusion toxin with C2I-Alexa488 for C2IIa binding on Vero cells by fluorescence-activated cytometry. Vero cells in suspension were
incubated for 15 min at 4 °C in HBSS + 0.2% BSA together with C2IIa (10 µg/ml) and C2I\textsuperscript{30-431}, C2I\textsuperscript{30-225}-C3, C2I, C2I\textsuperscript{1-225}-C3 fusion toxin, respectively (100 µg/ml each) as a competitor. Thereafter, C2I-Alexa488 (0.5 µg/ml) was added and cells were incubated for further 15 min at 4 °C. Cells were washed twice with HBSS + 0.2% BSA, analyzed by fluorescence-activated cytometry and mean fluorescence was recorded. Values are given as mean ± S. D. (n = 3) and significance was tested by a Students t-test (* = P < 0.05). 1, control cells; 2, C2IIa + C2I-Alexa488; 3, C2IIa + C2I\textsuperscript{1-225}-C3 + C2I-Alexa488; 4, C2IIa + C2I\textsuperscript{30-225}-C3 + C2I-Alexa488; 5, C2IIa + C2I + C2I-Alexa488; 6, C2IIa + C2I\textsuperscript{30-431} + C2I-Alexa488. B. Analysis of the binding of C2I\textsuperscript{1-225}-C3(S187C)-Alexa488 + C2IIa and C2I\textsuperscript{30-225}-C3(S187C)-Alexa488 + C2IIa, respectively to Vero cells. Vero cells in suspension were incubated for 15 min at 4 °C in HBSS + 0.2% BSA together with C2IIa (10 µg/ml) and C2I\textsuperscript{1-225}-C3(S187C)-Alexa488 and C2I\textsuperscript{30-431}-C3(S187C)-Alexa488 fusion toxin, respectively (0.5 µg/ml each). Thereafter, cells were washed for four times and analyzed by fluorescence-activated cytometry. The mean fluorescence was recorded and values are given as mean ± S. D. (n = 3). 1, C2IIa; 2, C2I\textsuperscript{1-225}-C3(S187C)-Alexa488 + C2IIa; 3, C2I\textsuperscript{30-225}-C3(S187C)-Alexa488 + C2IIa.

Fig. 10. A. Sequence alignment of the N-terminal domains of \textit{Bacillus cereus} VIP2 (sequence see (4)) and C2I from \textit{C. botulinum} strain 92-13 (Genbank Acc. No. AJ224480). The alignment was done with NCBI Blast. The locations of secondary structure elements are indicated over the aligned sequences. B. Schematic structure of \textit{Bacillus cereus} VIP2 protein modified according Han et al. (4). VIP2 consists of two similar parts, which share the same protein folding. The N-terminal half of VIP2 (shown) functions most likely as the adaptor to interact with the binding component, and the C-terminal part (indicated as \textit{C terminus}) harbors the ADP-ribosyltransferase. The α4 helix of VIP2 ends at Phe133, which corresponds to Arg86 of C2I. C2I\textsuperscript{1-87} was the minimal region to function as an adaptor. The most efficient
adaptor region of C2I was C2I\textsuperscript{1-225}. Gly225 of C2I corresponds to Glu275 of VIP2. VIP2\textsuperscript{60-265} covers the complete N-terminal domain of VIP.
Barth et al., Fig. 3

A

B

C

D
Barth et al., Fig. 4

A

B

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http://www.jbc.org/Downloaded from
Barth et al., Fig. 6

A C B D E F G H

I

intoxicated cells [%]

0 1 2 3 4

intoxicated cells [%]

0 20 40 60 80 100 120

120

110

100

90

80

70

60

50

40

30

20

10

0

0 1 2 3 4

time [h]
Barth et al., Fig. 8
Barth et al., Fig. 9

A

B

fluorescence

**

*
Barth et al., Fig. 10

A

VIP2  60 ... TDKVEDFREDKERAKREWGKEREKER--KLTAEGKMNNFLDN--KNDKTYKAPFT 113
+ ++ ++ ++ +AE+WGK+EK W KL E+ +N + K I + I F
C2I  1 MPIKEP1DPINKPESKAEKREWGKEERERWFKLNLEEVARVQLKNKREYTKIDNEFSTDILF 62

VIP2  114 SMAGSFED-----DEIKLDKEIDKMFED---KTNLSNSITYKUVFPTTTIGNKSLTE---GNTI 165
S + E E +L +++ + K L I Y N P +G N S+ + I
C2I  63 SLLAIEIMKEDNNFLFDVERIREALIAKTDLRDAIGTYNTPKELGINSIRDEVNLRSI 124

VIP2  166 NSDMAQFKEQPLDRDSDKFQYLTDAQVFSSKERVILKVTQSVGKSTTPKAGVILNN
+ + + ++Q ++++ + K L I Y N P +G N S+ + I
C2I  125 SDETLDKVRQIINQEQYTKFSISLGLNDNSINESVPVIVKTRV---TFDYGVLNDK 180

VIP2  228 SEYKMLIDNGYMHVHSVKVKVGVECDLQIEGTLVKKSNDKNDINAE ... 276
+L++ G+ + + KG + + IEG+L + LDF N +
C2I  181 ETIVSLLLQGFSIIPEASAITITTKGDYILIEGSLQELDF----YNKGS ... 226

B

ADP-ribosyltransferase domain similar folding as N terminus

VIP2 Glu275  C2I Gly225

β2  β7  β8

β4  β3  β2  β3  β1

VIP2  Phe133  C2I  Arg86

N
The binary clostridium botulinum C2 toxin as a protein delivery system - identification of the minimal protein region necessary for interaction of toxin components

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