Evidence that Arg-295, Glu-378, and Glu-380 are active-site residues of the ADP-ribosyltransferase activity of iota toxin

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Received 16 July 1996; revised version received 2 September 1996

Abstract **The active site of the enzymatic component (Ia) of the *Clostridium perfringens* iota toxin has been studied by site-directed mutagenesis. Sequence alignment showed that Ia and C3 enzymes display a segment in their C-terminal part which is homologous to that forming the active domain of pertussis toxin, cholera toxin, and *Escherichia coli* thermolabile toxins. This structure consists of a β-strand and an α-helix which forms the NAD-binding cavity and which is flanked by two catalytic spatially conserved residues involved in catalysis [Domenighini et al. (1994) Mol. Microbiol. 14, 41–50]. Substitutions (Arg-295-Lys, Glu-378-Ala, Glu-380-Asp, and Glu-380-Ala) induced a drastic decrease in ADP-ribosylation and cytotoxic activities, while substitution of the adjacent Arg (Arg-296-Lys) only partially affected the enzymatic activity and cytotoxicity. These results indicate that Arg-295, Glu-378 and Glu-380 of Ia are involved in the ADP-ribosylation activity which is essential for the morphological changes of cells treated with iota toxin.**

Key words: Iota toxin; *Clostridium perfringens*; C3 enzyme; ADP-ribosylating toxin; *C. spiroforme* toxin; *C. difficile* ADP-ribosyltransferase

1. Introduction

ADP-ribosylation is a mechanism widely used by toxins to kill or alter the metabolism of eukaryotic cells. This enzymatic reaction consists in modification of a target protein by transfer of the ADP-ribose (ADPR) moiety from NAD to a specific amino acid. The ADP-ribosylating toxins are composed of two functionally different domains, an enzymatic domain (A) and a binding domain (B) which recognizes a cell surface receptor and allows the internalization of subunit A into the cytosol. They can be divided into 4 major groups according to the nature of the target protein. The first group encompasses diphtheria toxin (DT) and *Pseudomonas* exotoxin A (PAETA) that ADP-ribosylates elongation factor 2. A second group includes cholera toxin (CT), *Escherichia coli* heat-labile enterotoxins (LT1 and LT2), pertussis toxin (PT), and *Pseudomonas* exoenzyme S (exoS) that ADP-ribosylate membrane-associated G proteins [1]. The third group includes the C3 enzymes from *Clostridium botulinum*, *C. limosum*, *Bacillus cereus* and epidermal cell differentiation inhibitor (EDIN) from *Staphylococcus aureus* which ADP-ribosylate the low molecular weight GTP-binding protein Rho [2–4]. The target protein of toxins from group 4 is actin, an ATP-binding protein. These toxins include *C. botulinum* C2 toxin, iota toxin from *C. perfringens*, *C. spiroforme* toxin and *C. difficile* ADP-ribosyltransferase (CDT) [5–8].

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Significant homologies have been found in the amino acid sequences of toxins from groups 1 and 2. However, even though no sequence homology was detected between the two groups of proteins, the secondary and tertiary structures of the enzymatic domain are conserved [1]. The NAD-binding site is composed of a cavity formed by an α-helix bent over a β-strand. Two amino acids (Glu and His or Arg) that are essential for catalysis are located on two β-strands flanking the NAD-binding cavity in ADP-ribosylating toxins from groups 1 and 2 [1,9]. In the C3 enzyme from *C. botulinum* C-003-9 and *C. limosum*, Glu-173 and Glu-174, respectively, have been found to be essential for the ADP-ribosyltransferase activity [10,11].

Iota toxin is a binary toxin consisting of two independent protein chains called iota a (Ia) (M_r 47 500) and iota b (Ib) (M_r 71 500), which are not associated by either covalent or non-covalent bonds [12,13]. Ia catalyzes the ADP-ribosylation of globular skeletal muscle and non-muscle actin at Arg-177 [8,14]. The Ia structural gene encodes a 413 amino acid mature protein, and the Ib gene encodes a 836 amino acid precursor protein [15,16].

In this study, we report first that the enzymatic site is located in the C-terminal moiety of Ia, and then that sequence alignment of Ia with other ADP-ribosyltransferases indicates that Arg-295 and Glu-380 could be two important catalytic residues. The functional role of Arg-295 and Glu-380 of Ia was confirmed by site-directed mutagenesis.

2. Material and methods

2.1. Constructions of Ia mutants

Manipulations of DNA were performed by using standard methods [17]. The *Pst*I-*Hin*cII DNA fragment from pMRP67 containing the Ia gene and its own region promoter [15], was subcloned into the pBluescript SK(−) vector (Stratagene, Montigny-le-Bretonneux, France) to yield the pMRP189 recombinant plasmid (Fig. 1). Site-directed mutagenesis was performed by using the Chameleon kit (Stratagene) with pMRP189 as template, the *Kpn*I → *Srf*I substitution primer (Stratagene), and the appropriate oligonucleotides. The resultant plasmids were subjected to DNA sequencing to confirm the presence of the desired mutation.

2.2. Expression of Ia mutants

E. coli TG1 strain carrying the recombinant plasmids were grown overnight at 37°C with shaking in LB medium [17] containing 100 μg of ampicillin per ml. 1 ml of the cell suspension was pelleted in a microcentrifuge, resuspended in 100 μl of 10 mM HEPES (pH 7.5), and sonicated. The protein contents of the lysates were assayed using the BioRad reagent (BioRad, Paris, France). 10–20 μg proteins of bacterial lysates were electrophoresed in a 0.1% SDS-12% polyacrylamide gel (PAGE). Immunoblotting was performed as previously described [15] by using a 1:400 dilution of immunopurified Ia rabbit antibodies [7,18]. Bound antibodies were detected with peroxidase-labeled protein A and the chemiluminescence kit provided by Amersham.

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PII S0014-5793(96)01035-6

Purification of recombinant Ia and Ib was performed as described previously [12,15].

2.3. ADP-ribosylation assays

2.3.1. Gel assay. In vitro ADP-ribosylation assays were performed with G actin isolated from *Xenopus laevis* oocytes as described previously [15]. The mixture for the PAGE assay (total volume, 20 μl) contained 7 μg of oocyte actin, 100 mM HEPES, 5 mM ATP, 2.5 mM ADP-ribose, and 5×10^5 cpm of [^{32}P]NAD (spec. act. 30 Ci/mmol; Dupont, NEN Research Products, Boston, MA). Between 1 and 10 μl of a fraction to be tested for enzymatic activity was added and the preparation was fractionated by SDS-PAGE and processed by autoradiography.

2.3.2. Filter assay. The ADP-ribosylation reaction was performed as described above, and terminated by the addition of 0.5 ml of trichloracetic acid (100%, w/v). The precipitated protein was collected on a Whatman GF/C glass filter (diameter 2.5 cm). The filter was washed with 10 ml of trichloracetic acid (10%, w/v) and counted for radioactivity.

2.4. Culture cell cytotoxicity assay

Cells were cultivated in Dulbecco's modified Eagles medium (DMEM) supplemented with 5% fetal calf bovine serum. Vero (African green monkey kidney) cells were used for cytotoxic assays. Vero cells plated into a 96-well Falcon tissue culture plate (Becton Dickinson LabWare, Oxnard, CA) and grown for 24 h to form monolayers. Purified Ib (3.3 μg/ml) was combined with purified Ia (1.7 μg/ml) or bacterial lysates containing wild-type or mutated Ia protein (1.7 μg/ml). Serial 2-fold dilutions in 100 μl final volume were added to the monolayers. The cells were observed at 18 h after incubation for morphological changes.

3. Results and discussion

3.1. The enzymatic domain is localized on the C-terminal part of Ia

In the first report of the molecular characterization of the iota toxin, the amino acid sequence alignment of Ia with DT, PAETA, PT and C3 suggested that Glu-14 and Trp-19 could be part of the active site of Ia [15]. Glu-14 was replaced with Ala, and Trp-19 with Ser. The mutants showed similar ADP-ribosylation activity to the wild type (Fig. 1), indicating that these two residues are not involved in the enzymatic site of iota toxin.

A deletion in the 3′ terminal part of Ia gene was constructed by using the *Hin*dIII restriction site from pMRP67 plasmid. The natural mature protein comprises 413 amino acids [16], whereas the truncated Ia protein encoded by pMRP117 comprises 205 C-terminal residues. pMRP117 showed an immunolabeled protein of 24 kDa corresponding to the expected size (24 kDa) plus 27 additional amino acids encoded by the vector, which reacted with anti Ia antibodies (Fig. 2). The Ia truncated protein did not show any significant ADP-ribosylation activity, as tested by gel assay or filter assay (Figs. 1 and 3), suggesting that the enzymatic domain is localized on the C-terminal moiety of Ia.

3.2. Sequence homology between Ia and PT, LT, CT and C3

The amino acid sequence of Ia displays low overall homology with other ADP-ribosylating toxins. Ia is more related to C3 enzymes (22 to 25% identity) than to the S1 subunit of PT, and subunits A of LT and CT (15 to 19% identity, respectively). Pileup alignment of these sequences with the corrected iota toxin sequence [16] shows that segments of Ia (residues 376–394), C3 (residues 171–187) and EDIN (residues 146–162) can be aligned with sequences forming the β-strand and α-helix structure of PT, LT and CT that constitute the NAD-binding cavity (Fig. 4). The Ia and C3 segments are also predicted to form β-strand and α-helix structures. It is noteworthy that the amino acids recognized as forming the β-strand of the cavity binding the nicotinamide rings of NAD in PT, CT and LT [1] are highly conserved in Ia and C3 while those involved in the α-helix are less conserved. The two amino acids (Arg and Glu) flanking the NAD-binding cavity, which have been found to be essential for catalysis in PT, CT and LT can be aligned with Arg-295 and Glu-380 of Ia and Arg-188 and Glu-173 or Glu-174 of C3 sequences, respectively (Fig. 4). This suggests that the spatial arrangement of the enzymatic site is conserved among these toxins and that

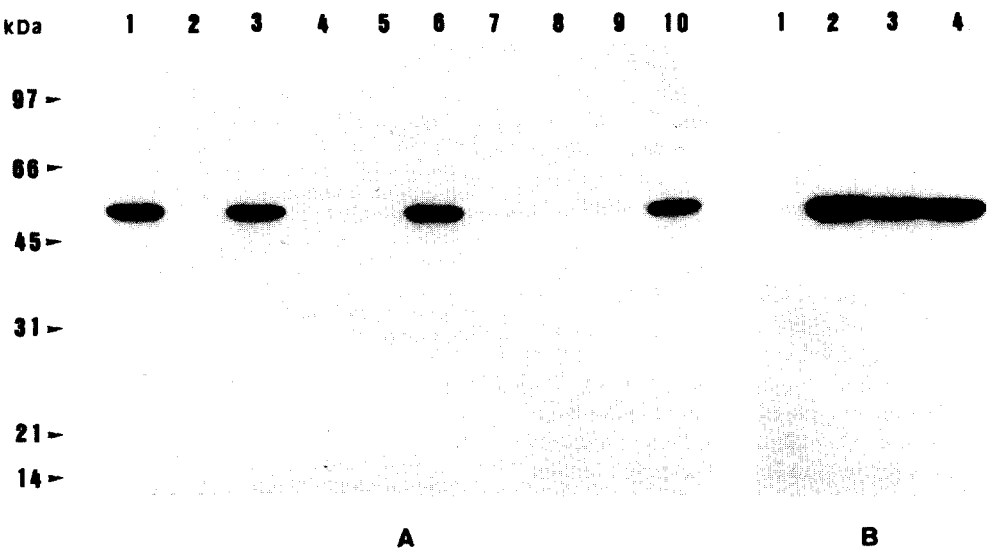

Fig. 1. Autoradiogram of actin ADP-ribosylation in gel assay by wild-type and mutated Ia proteins (4 ng by reaction): (A) purified Ia (lane 1), cell extract from *E. coli* harboring pUC19 (negative control) (lane 2), pMRP67 (lane 3), and pMRP189 (lane 6) (wild-type Ia), and pMRP117 (C-terminal truncated protein) (lane 4), pMRP297 (E378A) (lane 5), pMRP251 (E380D) (lane 7), pMRP252 (E380A) (lane 8), pMRP260 (R295K) (lane 9), and pMRP261 (R296K) (lane 10); (B) pBluescript SK (negative control) (lane 1), pMRP189 (wild-type Ia) (lane 2), pMRP207 (W19S) (lane 3), and pMRP208 (E14A) (lane 4).

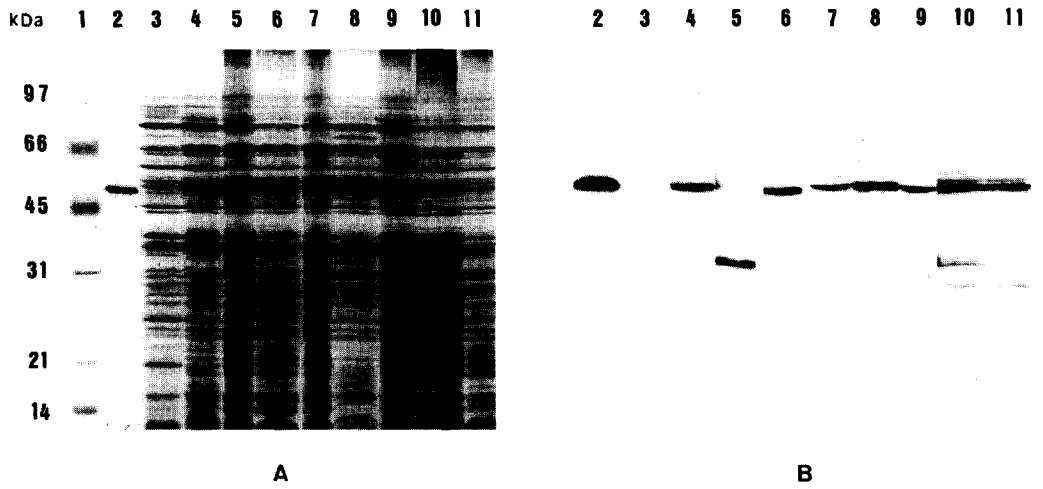

Fig. 2. Recombinant Ia proteins from *E. coli* sonic extracts and purified Ia: (A) silver staining SDS-PAGE of purified Ia (0.5 μg) (lane 2), and 45 μg of total protein from bacterial extract of *E. coli* carrying pUC19 (negative control) (lane 3), pMRP67 (lane 4), and pMRP189 (lane 6) (wild-type Ia), and pMRP117 (C-terminal truncated protein) (lane 5), pMRP297 (E378A) (lane 7), pMRP251 (E380D) (lane 8), pMRP252 (E380A) (lane 9), pMRP260 (R295K) (lane 10), and pMRP261 (R296K) (lane 11); molecular mass standards are shown in lane 1; (B) Western blotting of the same samples with anti-Ia antibodies.

Glu-380 and Arg-295 of Ia should play a key role in the ADP-ribosyltransferase reaction. We have investigated this possibility.

3.3. *Glu-378, Glu-380 and Arg-295 are involved in the Ia ADP-ribosylation site*

Glu-380 was replaced by the conservative residue Asp (Glu-380-Asp) and by the hydrophobic residue Ala (Glu-380-Ala). Wild-type Ia and mutated proteins were produced in *E. coli*, and their yields were determined by silver staining SDS-PAGE and immunoblotting (Fig. 2). Equivalent amounts of enzymes (4 ng) were used in the gel assay for ADP-ribosylation. Conservative and non-conservative substitutions of Glu-380 induced a pronounced decrease in enzymatic activity (Fig. 1). The filter assay for ADP-ribosylation performed with 4 ng of enzymes indicated that both mutants were over 50-times less active than the wild-type toxin. These results indicate that Glu-380 is essential for the enzymatic activity of Ia.

Arg-295 was substituted by the analogous amino acid Lys. Since Arg-295 is adjacent to another Arg (Arg-296), the latter was also independently replaced by Lys. The Arg-295-Lys substitution caused a significant decrease of ADP-ribosylation activity, while that of Arg-296-Lys caused only a slight diminution of activity (Figs. 1 and 3). In the filter assay, the proteins with Arg-295-Lys and Arg-296-Lys showed a 96 and 26% reduction of activity, respectively, compared to the wild-type Ia. Theses results indicate clearly that the conserved Arg-295 is the functional residue and that the adjacent Arg-296 is not involved in the catalytic activity of Ia.

It has recently been shown that NAD can be cross-linked by UV irradiation to Glu-378 of Ia [19]. The substitution Glu-378-Ala induced a drastic decrease in ADP-ribosylation (Figs. 1 and 3). Taken together, these results indicate that Glu-378 is essential for the NAD-binding and the enzymatic activity. The sequence Glu-x-Glu is conserved in a region corresponding to that of iota in other ADP-ribosyltransferases such as CT, LT, *B. sphoericus* mosquitocidal toxin, and mammalian ADP-ribosyltransferases [19]. The replacement of either Glu-110, or Glu-112 of LT, and either Glu-238 or Glu-240 of the rabbit muscle ADP-ribosyltransferase (rMT) with Asp was accompanied by a severe reduction of the enzymatic activity [20,21]. The biglutamic motif of the iota toxin and related ADP-ribosyltransferase probably participates in NAD binding and in the catalytic reaction.

Iota toxin causes susceptible cells to be roundish by disorganization of the actin cytoskeleton. Actin filaments (F-actin) are polar structures and are in dynamic equilibrium with monomeric actin (G-actin). F-Actin polymerizes at one end and depolymerizes at the other end. Ib component mediates the internalization of Ia which ADP-ribosylates G-actin into the cytosol. Modified G-actin is incorporated at the growing end of F-actin and blocks further polymerization by capping, while depolymerization occurs at the other end of F-actin

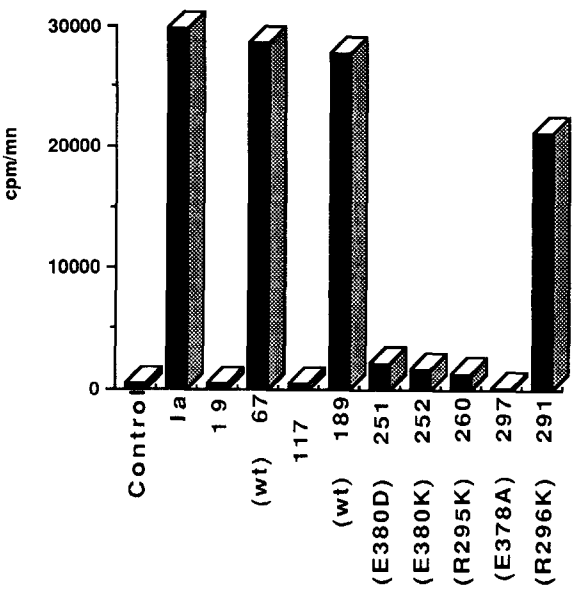

Fig. 3. Quantitative ADP-ribosylation in filter assay of wild-type and mutant Ia proteins. Ia indicates purified wild-type (wt) Ia, numbers denoting the recombinant plasmids (see legend to Fig. 2). Control is the ADP-ribosylation assay without Ia protein.

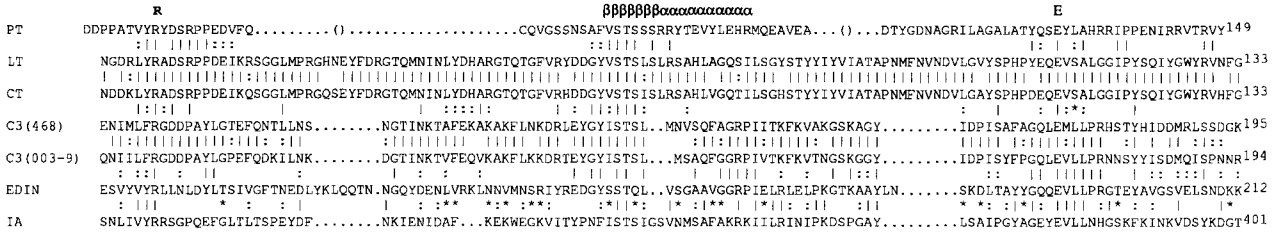

Fig. 4. Sequence alignment of Ia with EDIN, C3 from *C. botulinum* strains C-468 and C-003-9, CT, LT, and PT. β (beta strand) and α (alpha helix) indicate the conserved structural organization of the NAD binding site. The conserved catalytic residues (E, R) are shown. A vertical bar indicates identity between amino acids, ':' denotes homology, and '*' indicates identity with at least another amino acid among the sequence of the group.

leading to the destruction of microfilament network [22,23]. In order to determine whether the morphological changes of cells treated with iota toxin are due to the ADP-ribosylation activity, we examined the effects of the Ia variants in cultured cells. Vero cells were treated with purified Ib (3.3 μg/ml) and wild-type or mutated Ia (1.7 μg/ml). The truncated Ia encoded by pMPR117, and the Glu-378-Ala, Glu-380-Asp, and Glu-380-Ala variants showed an above 97% decrease in cytotoxicity of the wild-type Ia from pMRP67 and pMRP189. The reduction in cytotoxicity level as a result of the substitution of Arg-295 with Lys and Arg-296 with Lys was 94 and 25%, respectively. The Glu-14-Ala and Trp-19-Ala variants displayed cytotoxic activity similar to that of the wild type. These results provide further evidence that Vero cell cytotoxicity induced by iota toxin is strongly correlated with the actin ADP-ribosylation activity.

In conclusion, we have shown that Arg-295, Glu-378, and Glu-380 of Ia are essential for the ADP-ribosylation activity of iota toxin. The sequence alignment suggests that the NAD-binding and catalytic site which has been elucidated in other ADP-ribosylating toxins [1] is conserved in Ia. Arg-295 and Glu-380 probably correspond to the two catalytic residues flanking a β-strand and α-helix structure which forms the NAD-binding cavity. Glu is conserved in all ADP-ribosylating toxins, while the second catalytic residue is an Arg in PT, CT, LT, C3 enzymes, and presumably in Ia, and a His in DT and PAETA [1]. A second Glu residue is also involved in the enzymatic activity in Ia and other ADP-ribosyltransferases such as CT, LT and rMT.

Acknowledgements: We thank Dr. K. Aktories and I. Just for fruitful discussions on the structure of iota toxin.

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