Identification of the Carboxyl-terminal Amino Acids Important for the ADP-Ribosylation Activity of Pseudomonas exotoxin A*

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The ADP-ribosylation domain of Pseudomonas exotoxin A (PE) has been identified to reside in structural domain III (residues 405–613) and a portion of domain Ib (residues 385–404) of the molecule (Hwang, J., FitzGerald, D. J., Adhya, S., and Pastan, I. (1987) Cell 48, 129–136). To further determine the carboxyl end region essential for ADP-ribosylation activity, we constructed sequential deletions at the carboxyl-terminal of PE. Our results show that a clone with a deletion of the carboxyl-terminal amino acid residues from Arg-609 to Lys-613 and replaced with Arg-Asn retained wild-type PE ADP-ribosylation activity. Deletion of the terminal amino acid residues from Ala-596 to Lys-613 and replaced with Val-Ile-Asn reduced ADP-ribosylation activity by 75%, while deletions of 36 or more amino acids from the carboxyl terminus completely lost their ADP-ribosylation activity. These modified PE fragments were also examined for their ability to block PE cytotoxicity. Our results showed that modified PE fragments which lost their ADP-ribosylation activity correspondingly lost their cytotoxicity. Furthermore, extracts containing PE fragments without ADP-ribosylation activity were able to block the cytotoxic activity of intact PE. Our results thus indicate that carboxyl-terminal amino acids in the Ser-595 region are crucial for ADP-ribosylation activity and, consequently, cytotoxicity of PE. The modified PE fragments which have lost their ADP-ribosylation activity may also be a route to new PE vaccines.

Pseudomonas exotoxin A (PE)* is the most toxic component of the extracellular products produced by Pseudomonas aeruginosa (1). This toxin is active on most eukaryotic cells and species, including humans (2). The intoxication process of PE is thought to proceed first by binding of PE to its specific receptor on the surface of toxin-sensitive cells. The PE receptor complex then enters the cell through receptor-mediated endocytosis. Finally, PE is translocated to the cytosol where it catalyzes the transfer of the ADP-ribose moiety of NAD* to elongation factor 2. This renders EF-2 inactive in protein synthesis and leads to the death of affected cells (3–5; reviewed in Refs. 6 and 7). Based on this proposed mechanism of action, PE should contain at least three functional domains. These are responsible for the binding to cells, translocation across a membrane, and ADP-ribosylation of EF-2.

Recent studies have correlated the structural domains of PE with specific biological functions. Based on the information obtained from the three-dimensional structure (8) and the study of PE using recombinant DNA methods (9–12), binding, translocation, and ADP-ribosylation domains have been identified to reside in domain Ia (residues 1–252), domain II (residues 253–364), and domain III (residues 405–613) with a portion of domain Ib (residues 385–404), respectively. Using photoaffinity labeling, Glu-553 was identified as a residue within the NAD*-binding site (13). This observation was further confirmed by oligonucleotide-directed mutagenesis experiments in which substitution of Glu-553 with aspartic acid drastically reduced PE cytotoxicity and ADP-ribosylation activity (11). In addition, analysis of a variant PE produced by P. aeruginosa, strain PAO1-PR1 (14), identified His-426 of PE to be essential for ADP-ribosylation of EF-2 (15). All of these studies provided further evidence that domain III is indeed the enzymatic domain of PE. Furthermore, crystallographic data show that domain III contains a distinct cleft into which substrates may become bound. Since the carboxyl-terminal amino acids were shown to constitute the flexible external arm of the cleft, this prompted us to investigate if the carboxyl-terminal amino acids are involved in the ADP-ribosylation activity. Therefore, we constructed sequential deletions at the carboxyl-terminal of PE and examined their ADP-ribosylation activity. Our results show that the carboxyl-terminal amino acids in the Ser-595 region are important for ADP-ribosylation activity.

**EXPERIMENTAL PROCEDURES**

Materials—Pseudomonas exotoxin A was obtained from ICN Biomedicals. Restriction endonucleases, T4 DNA ligase, and enzymes used in cloning DNA were obtained from New England Biolabs or Bethesda Research Laboratories and used under conditions recommended by the supplier. Synthetic deoxynucleotide linkers were prepared using Pharmacia Gene Assembler. Nicotinamide-[U-14C]Cladenine dinucleotide, ammonium salt ([U-14C]NAD*) was purchased from Amersham Corporation. The wheat germ extract enriched for elongation factor 2 was prepared as described by Legocki and Marcus (16) and Seal et al. (17). Reagents for gel electrophoresis were from Bio-Rad. All other chemicals were analytical reagent-grade. *Bacteria Strains—Escherichia coli strain HB101 (F*, recA 15, hisB, hisD, lacY1, supE44, ara, proA, mukK, xyl, mtl, rpsL), from Bethesda Research Laboratories, was used as host for most experiments (18). BL21(DE3), which has been lysogenized with a phage (DE3) carrying the T7 RNA polymerase gene under the control of a lacUV5 promoter (19), was used as host for recombinant PE expression.

*Expression of the Recombinant Toxins in BL21(DE3)—BL21(DE3) containing plasmid for the expression of PE or PE fragment, was cultured in LB broth with 50 µg/ml ampicillin at 37 °C. When absorbance at 650 nm reached 0.3, isopropyl-1-thio-β-D-galactopyranoside was added at a final concentration of 0.5 mM. Cells were

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‡‡ This abbreviation is used: PE, Pseudomonas exotoxin A; EF-2, elongation factor 2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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harvested 90 min later. PE clones with serial deletions at the carboxyl-terminal which expressed in BL21(DE3) were examined by SDS-PAGE, immunoblotting, ADP-ribosylation activity assay, and cell killing experiments.

**SDS-PAGE and Immunoblotting**—Samples containing different deletions of carboxyl-terminal of PE were dissolved in Laemmli buffer and kept for 5 min prior to application to a 0.1% SDS, 10% acrylamide slab gel, and stained by Coomassie Blue after electrophoresis as described by Laemmli (20). For immunoblotting, electrophoresed samples were transferred from gel to Millipore polyvinylidene difluoride paper, followed by reaction with antisera directed against intact PE. Intact PE and sequential deletions of the carboxyl-terminal of PE were visualized by treatment with biotinylated goat anti-rabbit IgG, followed by avidin horseradish peroxidase H and stained with oxidizable peroxidase substrate, 0.02% hydrogen peroxide, and 0.1% diaminobenzidine. Procedures were carried out under conditions recommended by the supplier (Vector Laboratories). The antisera to PE was generated in female New Zealand White rabbits as described previously (21).

**Assay of ADP-Ribosylation Activity**—For an assay of ADP-ribosylation activity, the general procedure of Collier and Kandel was followed (22). Wheat germ extracts enriched for EF-2 were used as a source of EF-2. Assays (500-μl total volume) contained about 10 pmol of EF-2, 37 pmol of [U-14C]NAD* (0.06 μCi), lysate of BL21(DE3) with different PE clones and buffer (40 mM dithiothreitol, 1 mM EDTA, and 50 mM Tris, pH 8.1). Cells were lysed with 2 mg/ml of lysozyme at room temperature in 50 mM Tris, pH 8.0, and 50 mM EDTA followed by centrifugation to separate supernatant and pellet. The pellet was then dissolved in 8 M urea. Activity in the supernatant and pellet was measured as picoimoles of ADP-ribose transferred to EF-2 in 30 min in separate reactions. After incubation for 30 min, 37°C, 0.6 ml of 12% trichloroacetic acid was added to each assay mixture. The assay mixtures were then set in an ice bath for 15 min, followed by centrifugation at 4°C, 3000 × g for 10 min. The pellet was washed with 1 ml of 6% trichloroacetic acid and centrifuged as above. The 14C content of the pellet was measured in a liquid scintillation counter as an index of the ADP-ribosylation activity. Purified PE at concentrations from 0.25 to 1.5 μg gives linear ADP-ribosylation activity. The ADP-ribosylation assays of expressed PE and PE deletions were, thus, performed within the same concentration range.

To quantitate the amount of PE fragment in each assay, a fraction of each transformant was run on SDS-PAGE and the protein band corresponding to the expressed PE fragment measured by densitometry. A standard using known concentrations of PE was established using SDS-PAGE and used to determine the amount of PE fragment in each aliquot. The control group was done in the presence of an equivalent amount of BL21(DE3) lysate. In addition, a 10-fold excess amount of each lysate, from BL21(DE3) transformed with pJJ3 to pJJ14, was assayed to further confirm that these PE deletions are negative in ADP-ribosylation activity.

**Cell Protection Assay**—Tests of the PE fragments on the protection of PE cytotoxicity were performed in Swiss 3T3 cells. Cells were seeded 24 h prior to the protection assay. Each well contained 2 × 105 cells. After incubation for 72 h in the presence of various concentrations of PE alone or with BL21(DE3) cell extracts containing PE fragments, then the monolayers were stained with methylene blue to detect the surviving cells. To estimate the amount of modified PE fragments added, we again relied upon densitometry readings on a fraction of the cells which were run on SDS-PAGE.

**RESULTS**

**Construction of Deletions at 3’ End of PE**—To express sequential deletions of the carboxyl-terminal of PE in the BL21(DE3) host, plasmids with serial deletions at the 3’ end of PE structural gene were constructed as shown in Fig. 1. Plasmid pJH4 which contains the entire coding sequence of mature PE with an additional methionine at the amino terminus was used for the deletion constructs (10). Using the sequenced data reported by Gray et al. (9), we noted that the PE structural gene conveniently contained multiple HhaI restriction enzyme cutting sites. Furthermore, pJH4 also had a unique EcoRI site just downstream of the PE gene. Therefore, we first partially digested pJH4 with HhaI. The linearized DNA of 5.9 kilobases was isolated and then completely digested with EcoRI. DNA fragments ranging from 4.4 to 5.9 kilobases were isolated. A synthetic oligonucleotide duplex with cohesive ends for HhaI and EcoRI was used to ligate the linearized fragments. The synthetic oligonucleotide duplex used to link the two noncomplementary ends was composed of a universal sequence for the termination of protein synthesis. It contained stop codons in all three reading frames.

**Identification of PE Deletion Clones**—Transformed HB101 cells were randomly picked up for deletion analysis. To iden-
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tify the clones containing deletions at the 3' end of PE, the plasmids were first double digested with EcoRI and ApaI, which cuts at 700 nucleotides upstream of the 3' end. Any clone having a restriction fragment smaller than the 800-base pair ApaI-EcoRI fragment of pJH4 were thus identified. To determine those constructs with deletions greater than 700 nucleotides, the plasmids were again double digested with EcoRI and KpnI, which cuts at 1530 nucleotides upstream of the 3' end. Out of 220 clones examined, 14 positive clones are shown in Fig. 2, A and B. The exact end point of each deletion can be calculated from the sequence data. Plasmid pJJ7, however, was first approximated to be a deletion of 470–476 nucleotides due to the clustering of four HhaI restriction sites. Plasmids pJJ1 to pJJ14 represent sequential deletions from 15 up to 1284 nucleotides at the 3' end of the PE coding sequence. Results are summarized in Fig. 2C.

The size of deletion in plasmid pJJ7 was resolved by restriction analysis and mapping by size. The clustering of four HhaI sites \((\text{GC}G^*\text{CG}^*\text{CG}^*\text{CG}^*\text{CG})\) also created the clustering of three restriction sites for BssHII (indicated by *) which recognizes the six base-pair sequence of \((\text{GC}G\text{CG}^*\text{CG}^*\text{CG}^*\text{CG})\). In plasmid pJJ7, none of these three BssHII sites remain intact; therefore, the site of deletion must be at the first or second HhaI restriction site. The difference between a deletion beginning at site 1 versus site 2 is the absence or presence of one HhaI site on a 150-base pair AatII to EcoRI fragment. After complete digestion with HhaI, the apparent size of the labeled fragment was decreased, suggesting that HhaI cut at site 2 in the cluster of four HhaI sites.

The exact junction point of four of the deletion clones was further confirmed by sequencing the 3' end of these PE clones (data not shown). A synthetic 20-nucleotide oligo(5'-AA-CAAATAGGGTTCCGCGC-3') was annealed to a complementary sequence 100 base pairs downstream of the 3' end of the cloned fragment and used to sequence upstream cloned PE gene. Our data revealed that the actual deletion end points of plasmid pJJ1, pJJ2, pJJ3, and pJJ7 correspond to the positions determined by restriction fragment analysis.

Expression of Cloned PE Fragments—Plasmids pJJ1 to pJJ14 were individually transferred to host BL21(DE3) by the method described in Ref. 23. Resulting transformants were immediately cultivated in LB with 50 µg/ml ampicillin and grown until absorbance at 650 nm reached 0.3. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to induce the synthesis of T7 polymerase located on the BL21(DE3) lysogenic phage. Induction of the T7 polymerase leads to a preferential and high level expression of the cloned PE gene under the control of the T7 promoter. Fifty million cells of each culture were dissolved in Laemmli buffer and analyzed by SDS-PAGE. The protein profile shows that the major product in each transformant corresponds to the expected size of the cloned PE fragment. The decrease in the

![Fig. 2. Identification and summary of PE deletion clones.](image-url)
molecular weight of the PE fragments correlates well to the size of the deletions on each plasmid. The expressed PE fragments are visible by Coomassie Blue staining (Fig. 3A). A duplicate SDS-PAGE gel containing the same samples but with a lesser number of cells (2 million cells/sample) was made and immunoblotted with antisera directed against PE. Our Western blot shows that the major protein band visualized by Coomassie Blue staining also reacts with the anti-PE antisera, but the control BL21(DE3) lysate is negative to the anti-PE antisera (Fig. 3B). There are some minor bands visible in lanes 3-17 which may be the degradative products of the cloned PE fragments. However, the major PE protein and the various PE deletion proteins appear to be intact and at the predicted molecular weight.

Assay of ADP-Ribosylation Activity in PE Fragments—PE fragments expressed in BL21(DE3) were further characterized by assaying their ADP-ribosylation activity. The transfer of ADP-ribose to EF-2 was monitored by the use of [14C]NAD+. Activity in the pellet and supernatant fractions were assayed separately and total activity pooled. The specific ADP-ribosylation activity of expressed PE and PE deletions was obtained by dividing the total activity by the estimated amount of enzyme present. The relative specific activity was obtained by comparing the specific ADP-ribosylation activity of PE deletions with that of full length PE expressed in BL21(DE3)/pJH4.

The PE fragment from plasmid pJJ1, which contains a deletion of the carboxyl-terminal amino acid residues from Arg-609 to Lys-613 and replaced with Arg-Asn, retained wild-type PE ADP-ribosylation activity. However, a deletion of the carboxyl-terminal amino acid residues from Ala-596 to Lys-613 and replaced with Val-Ile-Asn dramatically reduced the ADP-ribosylation activity by 75%. Deletions of 36 or more amino acids from the carboxyl terminus reduced all enzymatic activity to the level of BL21(DE3) host cells which were subjected to similar induction conditions (Fig. 4).

Protection against PE Cytotoxicity by Modified PE Fragments—Swiss 3T3 cells which are exquisitely sensitive to PE intoxication were used to assay the ability of modified PE fragments for protection against PE killing. Using the modified fragments produced by plasmids pJJ3 and pJJ4, which have completely lost their ADP-ribosylation activity, the survival of Swiss 3T3 cells was assayed. First, in Fig. 5A, we show that Swiss 3T3 cells are sensitive to less than 10 ng/ml of native PE. Our pJJ3 and pJJ4 deletion fragments which did not retain any ADP-ribosylating activity also appear to have lost their cytotoxicity. However, these deletion fragments retained their ability to competitively block the cytotoxicity of native PE on Swiss 3T3 cells (Fig. 5B). At an approximately 60-fold concentration excess of modified PE versus native PE, there was only partial protection. This suggests that all the receptor sites on the cells were not saturated and native PE could still enter. However, at higher concentrations, the modified fragments produced by plasmid pJJ3 and pJJ4 could effectively prevent intoxication by PE. Furthermore, this protection appears to be specific as cell lysates of untransformed host cells could not block PE cytotoxicity.

**DISCUSSION**

To establish the structure and function relationships of a molecule, it is of benefit to have the biophysical, biochemical, and genetic data. Pseudomonas exotoxin A is one of the best studied bacterial toxins. Recent studies have yielded new insights into the mechanism of PE action as well as a picture of the structure-function relationship of PE. The three-dimensional structure of PE has been determined by x-ray crystallography (8). It was found that PE was composed of three structurally distinct domains with a cleft formed by domain III. Further, biochemical and genetic analysis using site-directed mutagenesis identified Glu-553 which is located in the cleft to be important for NAD+ binding and ADP-ribosylation activity (11, 13). Deletion and immunoblotting
FIG. 4. ADP-ribosylation activity of recombinant PE and PE fragments. Aliquots (equivalent to 1 million cells) of each recombinant PE and PE fragments were incubated with [14C]NAD and EF-2. ADP-ribosylation assay was done in duplicate and ADP-ribosylation activity was measured as picomoles of NAD transferred to EF-2 in 30 min. Full length PE obtained from BL21(DE3)/pJH4 was used as positive control and stand for 100% of ADP-ribosylation activity. BL21(DE3)/- was used as negative control. The relative ADP-ribosylation activity of all other PE fragments obtained from BL21(DE3)/pJJ1 to BL21(DE3)/pJJ14 was measured. The specific ADP-ribosylation activity of PE deletions were compared with that of full length PE obtained from BL21(DE3)/pJH4.

FIG. 5. Cytotoxicity and cell protection assay. Swiss 3T3 cells were seeded in 24-well microtiter plates 24 h prior to assay. A, for cytotoxic assay, cells were challenged with 0, 1, 3, 10, 30, 100 ng/ml of PE, or cell lysates from BL21(DE3)/pJJ3 and BL21(DE3)/pJJ4 equivalent to 0, 0.2, 0.6, 2, 6, 20 μg/ml of toxin fragment. Equivalent amounts of cell lysate from BL21(DE3) were used as negative control for cytotoxic assay. B, for protection assay, the same amount of cell lysate was added to cells as in panel A. After 30 min at 37 °C, cells, which had been preincubated with cell lysates from BL21(DE3)/pJJ3, BL21(DE3)/pJJ4, and BL21(DE3), were challenged with 30 ng/ml of native PE.

studies by Hwang et al. (10, 21) localized the receptor binding to domain Ia and the entry site of elongation factor 2 to the interface of domain I and III, respectively. A naturally existing CRM form of PE was recently isolated (15). The analysis of elongation factor 2 recognition. Since His-426 is located near this mutant PE shows that residue His-426 is essential for the interface of domain I and III, this fact provides further support that the interface of domain I and III is involved in the recognition of elongation factor 2 and that the cleft is
Indeed, the catalytic center for ADP-ribosylation activity of PE.

Analyzing the components of the cleft prompted us to investigate the role of the carboxyl-terminal amino acids due to its unique position in forming part of the cleft. By creating sequential deletions from the carboxyl-terminal end of PE, we found that deletion of the carboxyl-terminal amino acid residues from Arg-609 to Lys-613 and replaced with Arg-Asn retained wild-type PE ADP-ribosylation activity. Deletion of the terminal amino acids from Ala-596 to Lys-613 and replaced with Val-Ile-Asn reduced ADP-ribosylation activity by 75%, while deletions of 36 or more amino acids from the carboxyl terminus completely abolished all enzymatic activity. The use of the oligo linker added three amino acids, Val-Ile-Asn, to the pJJ2 deletion. The extra amino acids Val, Ile, and Asn in pJJ2 replaced the corresponding amino acids, Ala (596), Leu (597), Pro (598), and Asp (599) in PE. Due to the similar characteristics shared by branched chain amino acids, the 75% decrease in activity is more likely to result from a deletion of the 15-terminal amino acids rather than from the added amino acids. We conclude that the formation of a structurally intact cleft is important for ADP-ribosylation activity. It is clear that we should further pursue our structure-function study of the PE deletion fragments by using x-ray crystallography and confirm our biochemical results. We postulate that the elongation factor 2-binding region is near residue His 426, and the binding region of NAD+ near residue Glu-553 while the carboxyl-terminal amino acids in the region of Ser-595 may be involved in the catalytic transfer activity (Fig. 6).

In addition to further clarifying the structure-function relationships of PE, we also intended to investigate the possibility of modifying the PE molecule and developing a better vaccine. It has been previously shown that injecting domain Ia can illicit a protective antibody response against challenge with native PE (21). However, the efficacy of the antibody raised against domain Ia is only one-tenth as effective as the antibody response to native PE. Therefore, a safer and better antigen would be a nontoxic fragment of PE which includes domains I, II, and as much as possible, the enzymatic domain, domain III.

We have done preliminary studies to show that pJJ3, the largest enzymatically inactive fragment of PE, can protect Swiss 3T3 cells from PE cytotoxicity. It is feasible that deletions at the carboxyl-terminal portion of PE would allow the remaining portion to retain all the required epitopes for antigenicity. Furthermore, reversion of the deletion-containing PE to a catalytically active form would be extremely rare. Since our previous study has demonstrated that domain Ia could be used for vaccination against PE (21), we expect that the modified PE deleted for its carboxyl-terminal 36 amino acids would be a safe and improved antigen. Nevertheless, it is clear that further work needs to be done especially at the animal level.

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