Analysis of Sequences in Domain II of *Pseudomonas* Exotoxin A Which Mediate Translocation

Clay B. Siegall,‡ Masato Ogata, Ira Pastan, and David J. FitzGerald*

Laboratory of Molecular Biology, Division of Cancer Biology Diagnosis and Centers, National Cancer Institute, National Institutes of Health, Building 37, Room 4E16, Bethesda, Maryland 20892

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ABSTRACT: *Pseudomonas* exotoxin (PE) contains 613 amino acids that are arranged into 3 structural domains. PE exerts its cell-killing effects in a series of steps initiated by binding to the cell surface and internalization into endocytic vesicles. The toxin is then cleaved within domain II near arginine-279, generating a C-terminal 37-kDa fragment that is translocated into the cytosol where it ADP-ribosylates elongation factor 2 and arrests protein synthesis. In this study, we have focused on the functions of PE which are encoded by domain II. We have used the chimeric toxin TGFα-PE40 to deliver the toxin's ADP-ribosylating activity to the cell cytosol. Deletion analysis revealed that sequences from 253 to 345 were essential for toxicity but sequences from 346 to 364 were dispensable. Additional point mutants were constructed which identified amino acids 339 and 343 as important residues while amino acids 344 and 345 could be altered without loss of cytotoxic activity. Our data support the idea that domain II functions by first allowing PE to be processed to a 37-kDa fragment and then key sequences such as those identified in this study mediate the translocation of ADP-ribosylation activity to the cytosol.

Protein toxins are found in many organisms and function as potent cytotoxic agents. Many of these toxins kill cells by attacking the cellular machinery which is used to synthesize proteins (Olsnes & Sandvig, 1988). Directing the action of toxins to specific cells can be accomplished by replacing the native cell recognition region of the toxin with a ligand that specifically binds only to certain cells. There are many examples of targeted cell-killing using protein toxins such as *Pseudomonas* exotoxin, diphtheria toxin, and ricin (Pastan & FitzGerald, 1989; Pastan et al., 1986; Vitetta et al., 1987; Estworthy & Neville, 1984; Greenfield et al., 1987; FitzGerald & Pastan, 1989). We have been employing *Pseudomonas* exotoxin A (PE) to kill a variety of cell types by attaching PE or modified forms of PE to different ligands (Chaudhary et al., 1987, 1988; Siegall et al., 1988, 1989b; Lorberboum-Galski et al., 1988; Ogata et al., 1989; Batra et al., 1989). Along with their utility in killing target cells, chimeric toxins can be used to identify important sequences of toxin function (Johnson & Youle, 1989; Siegall et al., 1989a; Williams et al., 1990; Chaudhary et al., 1990).

PE is composed of three structural domains as determined by crystallographic analysis (Allured et al., 1986). Domain I is made up of two subunits, domain Ia (amino acids 1–252) and domain Ib (amino acids 365–404), which are closely associated in the crystal structure but are separated by domain II in the amino acid sequence (Gray et al., 1984). The amino acids of domain Ia encode the PE cell recognition function (Hwang et al., 1987), while the function of domain Ib is not yet known (Siegall et al., 1989a). Mutational analysis of the lysine residues in domain Ia indicated that Lys-57 is essential for cell binding (Jinno et al., 1988). Further, mutational analysis revealed that amino acids 246, 247, and 249 are also important for killing by the native toxin.

Domain II (amino acids (253–364), which contains six consecutive α-helices, has been demonstrated to be responsible for translocation of the toxin across membranes (Hwang et al., 1987; Siegall et al., 1989a; Jinno et al., 1989; Edwards et al., 1989). Previous studies have determined that there are several distinct regions within domain II which are essential for toxin activity. Processing of PE to its active form requires the amino end of domain II, with the actual cleavage occurring around amino acid 279 (Ogata et al., 1990). Deletion of the amino end of domain II, amino acids 254–263, inactivates the cytotoxicity of PE, but deletion of the carboxyl end (361–364) and part of domain Ib (amino acids 361–380) does not affect the cytotoxicity of PE (Siegall et al., 1989a). When amino acids 337–380 were deleted, PE cytotoxicity was lost, indicating that there was an essential region between aa 337 and 360 (Siegall et al., 1989a). We have also determined the importance of several arginine residues in domain II (Jinno et al., 1989). The results indicate that the arginines at aa 276, 279, 330, and 337 are important for full PE toxicity. In particular, mutations at Arg-276 or Arg-279, which lie in a loop on the surface of domain II, completely inactivated the toxin. We have recently determined that cellular proteolytic processing of PE required for translocation of a 37-kDa fragment of PE occurs within this loop (Ogata et al., 1990).

Domain III, which functions as the enzymatic unit of the toxin contains the ADP-ribosylation activity. PE kills cells by ADP-ribosylating elongation factor 2 and halting protein synthesis. The structural boundary of domain III as defined by crystallography structure is 405–613 (Allured et al., 1986). The functional NH_2-terminal boundary of domain III is amino acid 400, and the carboxyl boundary is 601 (Siegall et al., 1989a; Chaudhary et al., 1990). Previous studies of domain III have been shown that His-426, Tyr-481, Glu-553, and Trp-558 are essential for ADP-ribosylation (Carroll & Collier, 1987; Wozniak et al., 1988; Brandhuber et al., 1988; Lukas & Collier, 1988) and the carboxyl five amino acids (609–613) are important for translocation of PE into the cytosol (Chaudhary et al., 1990).

In the current study, we have focused on the role of amino acids at the carboxyl end of domain II containing the E helix (aa 333–350) and F helix (aa 361–364) in the cytotoxic action

* To whom correspondence should be addressed.
‡ Present address: Pharmaceutical Research Institute, Bristol-Myers Squibb, Wallingford, CT.

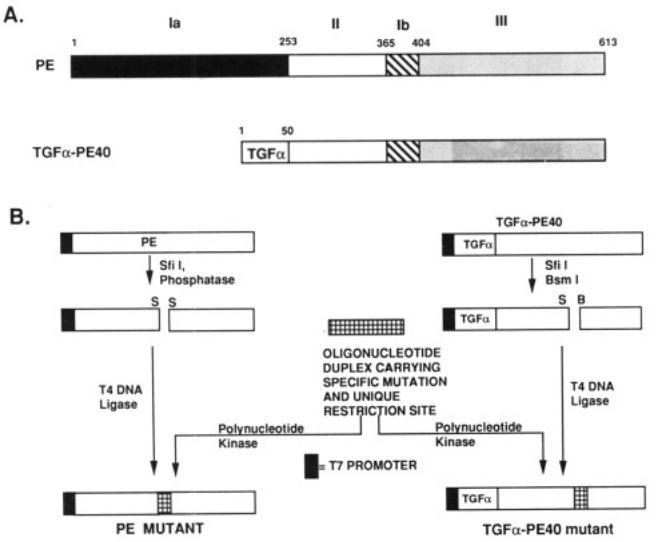

FIGURE 1: (A) Schematic diagram of PE and TGFα-PE40. (B) Scheme for construction of mutant plasmids encoding either PE or TGFα-PE40. S, *Sfi*I; B, *Bsm*I.

of PE. We have found that all of the F helix (aa 361–364) and part of the E helix (aa 346–350) can be deleted but residues at positions 339–345 are required.

Materials and Methods

Bacterial Strains and Plasmids. HB101 (Bethesda Research Laboratories, Gaithersburg, MD) was used for propagation of the plasmids. BL21 (λDE3), which carries an inducible T7 RNA polymerase gene on a λ prophage, was used to express the recombinant proteins (Studier & Moffatt, 1986). The plasmids pVC 387 (encoding TGFα-PE40) and pVC 45f(+)T (encoding native PE) were used as templates in all the constructions (Siegall et al., 1989b; Jinno et al., 1989).

The TGFα-PE40 mutants (pVC387 derivatives) were produced by the insertion of oligonucleotide duplexes into the *Sfi*I–*Bsm*I restriction sites found in the PE gene (Figure 1). The PE mutants [pVC 45f(+) T derivatives] were made by insertion of oligonucleotide duplexes containing the appropriate mutation into the gap formed by a complete *Sfi*I digestion (Figure 1). Several plasmids of each mutant were identified by the presence of a unique restriction site in the new sequence. Additionally, many of the mutants were DNA-sequenced to confirm the presence of the nucleotide substitution.

Expression and Analysis of Recombinant Proteins. Several plasmids from each mutant were expressed into protein by using the inducible T7 promoter system in BL21 (λDE3), and proteins either were used in nonpurified form for certain analysis or were purified from inclusion bodies (TGFα-PE40 derivatives) or from the periplasm fraction (PE and derivatives) (Siegall et al., 1989a,b). Proteins were visualized by electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) using the Laemmli method. Proteins were also tested for authenticity by immunoblot analysis using rabbit antibodies to *Pseudomonas* exotoxin or goat antibodies to TGFα (Biotope, Seattle, WA) and detection using vector stain kits (Vector Laboratories, Burlingame, CA). Proteins were quantitated by using Bradford analysis. ADP-Ribosylation activities of mutant proteins were measured according to Collier and Kandel (1971).

Protein Synthesis Inhibition Analysis. The cytotoxic activities of mutant proteins were compared to that of the native protein using A431 cells (TGFα-PE40) or Swiss-3T3 cells (PE). Protein synthesis was assessed by determining the incorporation of [^{3}H]leucine into cellular protein (Siegall et al., 1989b; Jinno et al., 1989).

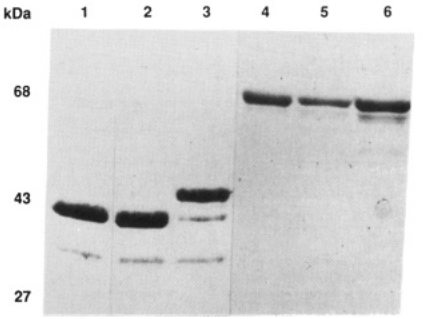

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of TGFα-PE40, PE, and mutant derivatives of both. Samples were applied to 10% SDS–polyacrylamide gels and stained with Coomassie Blue R-250. Lane 1, TGFα-PE40 Δ346–380; lane 2, TGFα-PE40 Δ344–380; lane 3, TGFα-PE40; lane 4, PE; lane 5, PE^{Gln343}; lane 6 $PE^{Gln344,345}$.

Binding Displacement and Cell-Associated Processing Analysis. ^{125}I-PE binding assays were performed by using Swiss-3T3 cells plated at 1×10^5 cells/mL. Radiolabeled PE (5 ng) was added to cells in the presence of potential competitors at concentrations of 8, 40, and 200 μg as previously described (Jinno et al., 1989). For the processing assay, PE or mutated forms of PE were metabolically radiolabeled with [^{3}H]leucine as described (Ogata et al., 1990). Swiss 3T3 cells (confluent in 150-mm tissue culture dishes) were incubated with labeled toxins at 37 °C for 3 h, washed 5 times with cold PBS, and lysed in 1 mL of RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% deoxycholic acid, and 0.1% SDS) containing 1 mM PMSF. PE or PE-processed fragments were immunoprecipitated by rabbit anti-PE serum as described (Ogata et al., 1990). Samples were analyzed on 12.5% SDS–PAGE and exposed to X-ray film for 5 days.

Results

Precipitation of TGFα-PE40, PE, and Mutant Proteins. To express TGFα-PE40, PE, and mutant proteins, we transformed *Escherichia coli* BL21 (λDE3) with the appropriate plasmid, cultured the cells, and induced expression of protein with isopropyl β-D-thiogalactoside. TGFα-PE40 and derived proteins were isolated from sucrose-washed spheroplasts and quantitated by comparison of serial dilutions with purified TGFα-PE40 on immunoblots using an antibody to *Pseudomonas* exotoxin. PE and related mutant proteins were expressed from a plasmid containing the OmpA signal sequence and secreted as soluble proteins into the periplasmic space. All proteins were purified by anion-exchange and gel filtration chromatography. Crude and purified proteins were analyzed by SDS gel electrophoresis and by immunoblotting using antibodies to PE (Figure 2).

Cytotoxic Activity of TGFα-PE40 and Related Mutant Proteins. To determine how much of the carboxyl end of domain II was necessary for cell-killing activity, we made deletions of increasing size in domain II of TGFα-PE40. We began making deletion mutants using information from a previous study in which various portions of domains Ib and II were deleted from TGFα-PE40 (Siegall et al., 1989a). We focused on amino acids 337–360 which were demonstrated to contain important residues for cell-killing activity. The various deletion mutants we constructed and a summary of the cytotoxicity of the mutant molecules on A431 cells are shown in Figure 3. Cytotoxicity was assessed by measuring inhibition of protein synthesis. To do this, various dilutions of the mutant

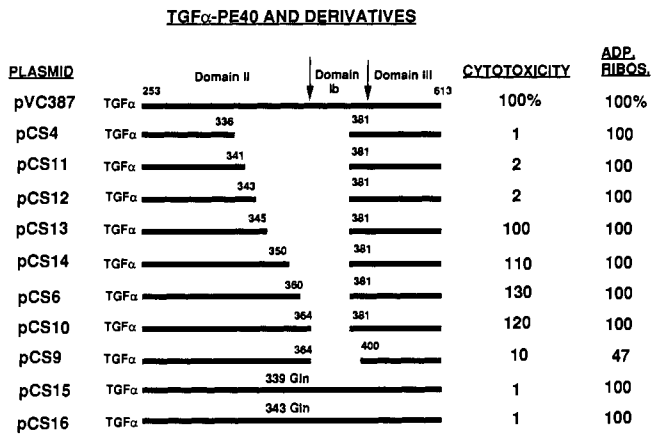

FIGURE 3: Representations of TGFα-PE40 and the various mutant derivatives. Deletion and substitution mutants are shown, and numbers indicate amino acids present or mutated in PE40. The new amino acid in the substitution mutants is indicated next to the residue number. The cDNA for TGFα encodes amino acids 1–50 in all the constructs. Cytotoxicity was determined by comparing the ID_{50} of unmodified TGFα-PE40 to the various mutant forms. ID_{50} is the concentration of toxin which is required to inhibit protein synthesis by 50% as measured by [^{3}H]leucine incorporation after 20-h incubation of A431 cells (1×10^5 cells/mL) with the various toxins. ADP-ribosylation was determined as previously described (Collier & Kandel, 1971).

TGFα-toxins were added to A431 cells and cultured for 20 h followed by a 1-h pulse with [^{3}H]leucine as detailed under Materials and Methods. In each case, we compared the activity of the mutants with unmodified TGFα-PE40. We also measured the ADP-ribosylation activity of each mutant protein (Collier & Kandel, 1971). All the TGFα-toxin mutants had ADP-ribosylation levels identical with that of TGFα-PE40 except for pCS9, described in Hwang et al. (1987) and Figure 3. The results indicate that deletion of amino acids (aa) 346–380 did not reduce the cytotoxicity of TGFα-PE40. However, deletion of two additional residues, aa 344–380, resulted in a molecule which retained only 2% of the cytotoxicity of TGFα-PE40. Deletion mutants extending to amino acids 342 and 337 had almost no cytotoxic activity against A431 cells (Figure 3).

Cytotoxicity of TGFα-PE40 and PE Point Mutants. To define further the important residues in this portion of the toxin molecule, we constructed two point mutations in TGFα-PE40 (Figure 3). The results show that mutations at amino acids 339 and 343, which change alanine to glutamine, eliminated cell-killing activity (Figure 3).

Since we wanted to perform additional analysis in this region of the molecule and also study the intracellular processing of mutant proteins, we made similar mutations in native PE (Figure 4). By generating mutations in native PE, we were able to study the cell-associated processing of the molecules as recently described by Ogata et al. (1990). The processing of chimeric toxins such as TGFα-PE40 has not yet been studied. As with TGFα-PE40, the mutations at amino acids 339 and 343, which changed alanine to glutamine, inactivated the cytotoxic activity of native PE (Figure 4). However, the toxicity of PE was not changed when Ala-343 was changed to leucine or valine (data not shown). Additional point mutations were made in this cluster of amino acids. Surprisingly, when we changed the alanines at amino acids 344 and 345 to glutamine, singularly or together, PE retained full toxicity. Also, when amino acid 341 was mutated from threonine to phenylalanine, the toxin retained activity. The cytotoxicity of the PE point mutations on Swiss 3T3 cells is summarized in Figure 4, and representative dose response curves are shown in Figure 5.

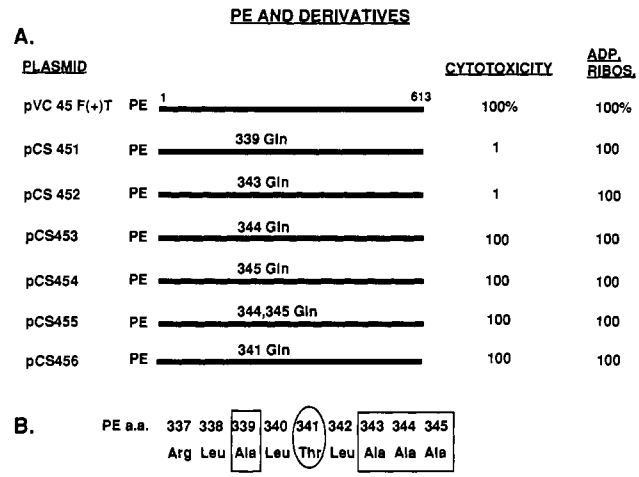

FIGURE 4: (A) Representations of PE and the various mutant forms. Substitution mutants are shown with the new amino acid (Gln in all cases) listed next to the position which was mutated. Cytotoxicity and ADP-ribosylation were done as in Figure 3. (B) The amino acid sequence of PE between residues 338 and 345. The boxes indicated alanine residues which were mutated to glutamine. The circled amino acid indicated a threonine which was mutated.

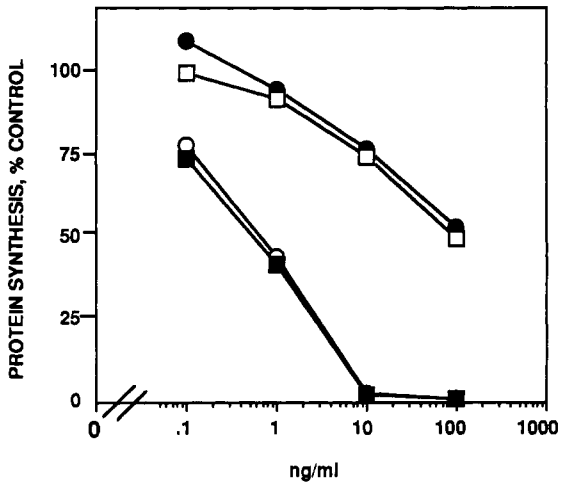

FIGURE 5: Toxic activity of PE and various mutant proteins on Swiss 3T3 cells. PE and derivatives were prepared from the periplasm of *E. coli*. The concentration of the various mutant toxins was estimated by Bradford assay. [^{3}H]Leucine incorporation was measured, and the results are expressed as percent of control cells not treated with PE. PE (○), PE^{Gln343} (●), PE^{Gln339} (□), $PE^{Gln344,345}$ (■).

Binding of PE Mutants. The decrease in cytotoxicity of the PE mutants could be a result of diminished binding to the PE receptor through some indirect affect of domain II on domain Ia. To determine whether the PE mutants had the same affinity as PE for the PE receptor, we compared their displacement of ^{125}I-PE to Swiss 3T3 cells (Figure 6). We added various amounts of competitor (8–200 μg/mL) to 200 ng/mL ^{125}I-PE and incubated for 1 h at 37 °C followed by washing the cells with phosphate-buffered saline and harvesting of the cells for counting. We compared PE and two mutants (PE^{Gln343}; $PE^{Gln344,345}$) which were representative of the mutants generated. A similar degree of displacement of ^{125}I-PE was obtained with PE, PE^{Gln343} and $PE^{Gln344,345}$. Thus, PE and two mutant forms of PE, one, PE^{Gln343}, which inactivates the toxin and a second, $PE^{Gln344,345}$, which does not alter PE cytotoxicity, bind in an identical manner to PE.

Detection of Cell-Associated Processing. PE is proteolytically cleaved after endocytosis, and a 37-kDa C-terminal fragment that contains the ADP-ribosylation activity is translocated into the cytosol (Ogata et al., 1990). To test

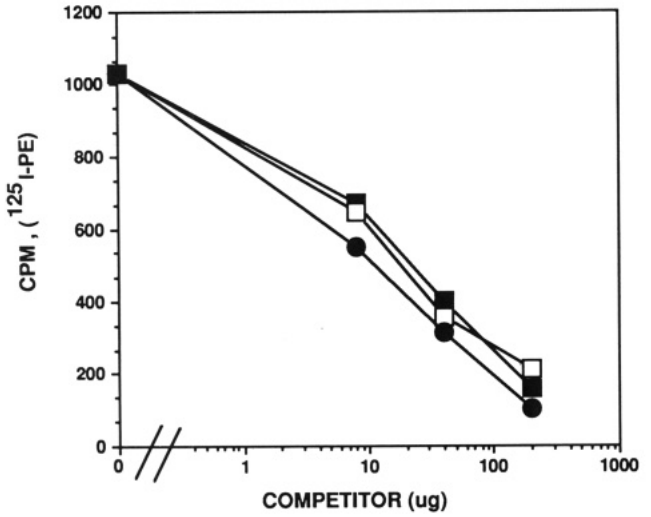

FIGURE 6: PE and PE mutant displacement assay. Binding of PE and two mutant forms was assessed by displacement of trace amounts of ^{125}I-PE. ^{125}I-PE bound to Swiss 3T3 cells was measured as counts per minute. PE (■), $PE^{Gln344,355}$ (□), PE^{Gln343} (●).

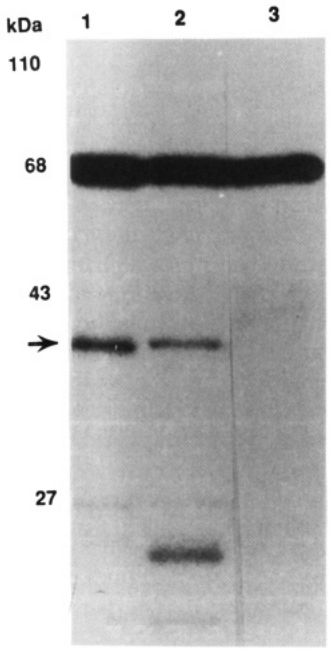

FIGURE 7: Cell-associated processing of radioactive PE and mutants in Swiss 3T3 cells. PE and derivatives were metabolically labeled with [^{3}H]leucine. The radiolabeled proteins were incubated with Swiss 3T3 cells for 3 h, lysed, and immunoprecipitated by using rabbit anti-PE antibodies. Lane 1, PE; lane 2, PE (Gln-343); lane 3, PE (Gly-276). SDS–PAGE was done by using 12.5% gels and reducing conditions. Previously, we showed that PE is first cleaved to produce two fragments, one of 28 kDa, derived from the N-terminus, and one of 37 kDa, derived from the C-terminus. The arrow shows the position of the 37-kDa fragment (which, if the toxin is active, is translocated to the cytosol). The 28-kDa fragment, derived from domain Ia, is further degraded to produce fragments of 25 and 18 kDa (Ogata et al., 1990).

whether the mutant forms of PE which are not cytotoxic to Swiss 3T3 cells are processed in a similar fashion to native PE, we examined the processing of several different PE molecules by Swiss 3T3 cells. We used the PE^{Gln343} mutant and compared its processing to that of PE and PE^{Gly276}, a nontoxic mutant that is not processed appropriately (Figure 7). In this experiment, PE or mutant forms of PE were metabolically radiolabeled with [^{3}H]leucine, purified, and incubated with Swiss 3T3 cells at 37 °C for 3 h. Then the cells were washed, lysed, and immunoprecipitated by rabbit anti-PE serum. The results (Figure 7) indicate that PE and PE^{Gln343} are both processed to a 37-kDa fragment while the mutant PE^{Gly276} is not (Ogata et al., 1990). Therefore, the cytotoxic-deficient mutant PE^{Gln343} can be processed to a 37-kDa fragment. In the PE^{Gln343} mutant, an additional low molecular weight fragment was observed which was derived from domain Ia (Ogata et al., 1990).

Table I: Bioactivities of PE and Mutant Molecules

	binding	processing	ADP-ribosylation	cytotoxicity
PE	+	+	+	+
PE^{Gln343}	+	+	+	–
PE^{Gly276}	+	–	+	–
$PE^{Gln344,345}$	+	ND[a]	+	+

[a] ND, not done.

Discussion

In this study, we have defined by deletion analysis how much of the carboxyl end of PE domain II is necessary for cytotoxic activity. Previously, we showed that part of domain Ib, amino acids 365–380, could be deleted without loss of activity. We now show that removal of up to 19 aa (Δ346–364) did not result in loss of activity. However, the removal of two additional aa (Δ344–364) did result in a dramatic loss of activity. The α-carbon backbone structure of PE is shown in Figure 8A, and that of domain II is shown in Figure 8B. These deletions remove all of the F helix (aa 360–363) and part of the E helix (aa 333–350). These amino acids, which lie grouped together on the surface of the PE molecule, appear to have no particular role in cytotoxicity. However, they could have a function not detected by our assays.

To gain further information about the E helix, we made point mutations (substitutions) in the essential region that could not be deleted at positions 339, 341, 343, 344, and 345 (Figure 8B). This is a hydrophobic region rich in alanine residues. The mutations changed this small hydrophobic residue to a bulky hydrophilic residue. Two of these mutants (Ala-339 and Ala-343 to Gln) inactivated PE cytotoxicity, while two others (Ala-344 and -345 to Gln) did not inactivate PE (Figures 3 and 4). To determine if these mutations affected other PE functions, we measured ADP-ribosylation and cell binding and found that there were no changes in the mutant molecules as compared to PE (Figures 3–5).

Domain II is important for translocation in a process in which the molecule is first cleaved by a cell-associated protease near residue 279, generating a 37-kDa fragment which is then translocated to the cytosol. Therefore, we examined the processing of the mutant PE^{Gln343} which had lost activity. We found that the mutant molecule was processed to a 37-kDa fragment as was native PE and also retained normal ADP-ribosylating activity (Figure 4). We interpret this result to mean that the mutant molecule (Ala → Gln) generated the same 37-kDa fragment (aa 280–613), as did native PE, but the fragment did not reach the cytosol so that protein synthesis was not arrested. We assume the same result would be obtained with the other inactivating mutations but have not yet examined them. This class of mutants differs from others in domain II such as the change of arginine to glycine at position 276 or 279 which prevents the molecule from being processed normally (Ogata et al., 1990). Currently, their are only a limited number of assays for toxin function, and the results obtained for several mutant proteins are summarized in Table I. Unfortunately, a direct assay for toxin translocation does not yet exist. However, if and when such an assay is developed, we can then examine the role of key amino acids in the E helix.

Table II: Sequence Comparison between Clathrin Light Chain and PE[a]

clathrin	30	31	32	33	34	35	36	37	38	39
light chain	Ala	Ala	Phe	Leu	Ala	Gln	Gln	Glu	Ser	Glu
Pseudomonas	339	340	341	342	343	344	345	346	347	348
exotoxin A	Ala	Leu	Thr	Leu	Ala	Ala	Ala	Glu	Ser	Glu
	↓		↓		↓	↓	↓			
	Gln		Phe		Gln	Gln	Gln			
PE cytotoxicity	−		+		−	+	+			

[a] Amino acids with identity are underlined. Mutation in PE and the cytotoxic activity of the mutant protein are indicated.

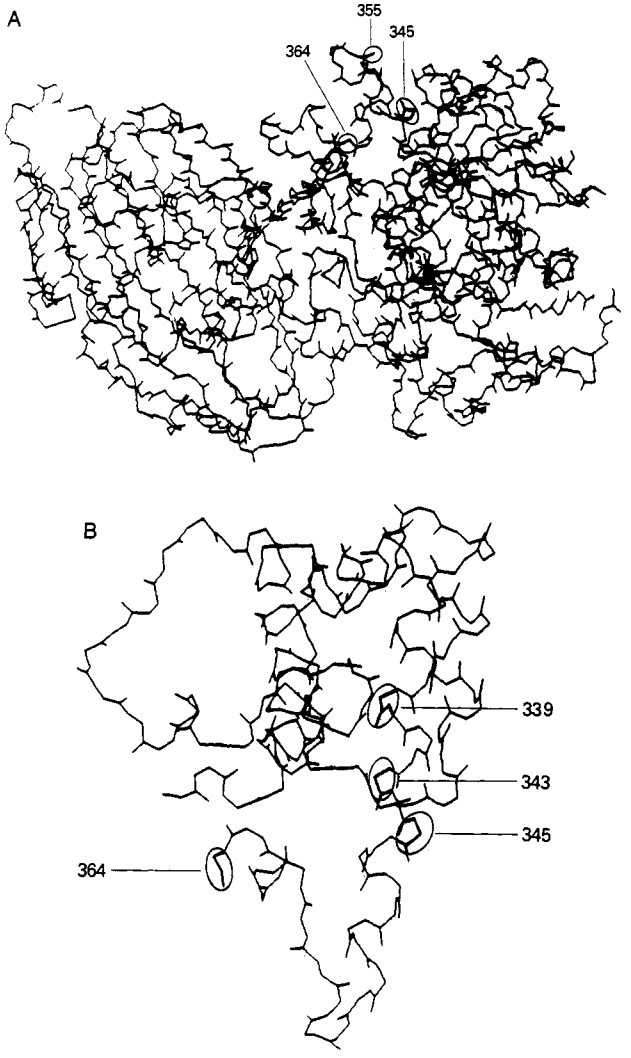

FIGURE 8: Structural representations of *Pseudomonas* exotoxin showing regions where mutations and deletions were made. (A) The entire PE molecule is depicted, and the regions of domains II and Ib which could be deleted are shown (aa 346–380). (B) Structure of PE domain II showing the region from residue 346 to residue 364 which could be removed without affecting cytotoxicity. Amino acids 339 and 343 (both alanines) which are essential for PE cytotoxicity are indicated by their residue number. These two amino acids are on the inside of the helix facing the interior of the PE molecule. Perhaps the inactivating mutations in which glutamine is substituted for alanine at positions 339 and 343 disrupt its structure due to its side chains. Alternatively, the alanines at residues 344 and 345 which can be mutated to glutamine are not on the interior of the E helix. The structures were made by using a Silicon Graphics Iris computer and a GEMM software program developed by Dr. B. K. Lee, NIH.

The region of PE examined in this study, aa 339–345, is rich in hydrophobic residues (Ala, Leu, Thr, Leu, Ala, Ala, Ala). This prompted us to examine whether domain II of PE is related to other proteins that contain hydrophobic stretches of aa residues. The best fit of any protein examined was to clathrin light chain, which had 28.6% identify in a 77 amino acid overlap. When one focuses only on the region of interest in domain II (amino acids 339–348), there is a striking resemblance between the two proteins (Table II). Analysis of the mutations produced (Figures 3 and 4) demonstrates that when we altered an amino acid that was conserved between PE and clathrin light chain, the toxicity of PE was lost. Alternatively, when we mutated an amino acid of PE to an amino acid residue that is found at the corresponding site in clathrin light chain, cytotoxicity was retained (Table II).

It is intriguing that clathrin light chain has significant homology to domain II of PE, since clathrin is found on the cytoplasmic face of coated pits. Clathrin is thought to be involved in facilitating the internalization of cell-surface receptors and to facilitate receptor transport in a process that targets proteins to intracellular compartments (Brodsky, 1988). The region of PE that we have mutated aligns with a region of clathrin light chain that is conserved between all species tested (Jackson & Parham, 1988). The function of this region (aa 23–44 in clathrin light chain) is not known. The central region of clathrin light chain (residues 93–157) is thought to be involved in clathrin heavy-chain binding (Brodsky, 1987). Clathrin light chains also interact with proteins found in the cytoplasm and in vesicles (Brodsky, 1988). It is possible that PE interacts with the same element (perhaps a protein) as clathrin light chains during the translocation process. It is also possible that, coincidentally, this portion of the clathrin light chain has a helical structure and the relationship to PE is more one of common structure than common function.

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Average Membrane Penetration Depth of Tryptophan Residues of the Nicotinic Acetylcholine Receptor by the Parallax Method†

Amitabha Chattopadhyay‡ and Mark G. McNamee*
Department of Biochemistry and Biophysics, University of California, Davis, California 95616
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ABSTRACT: The membrane penetration depths of tryptophan residues in the nicotinic acetylcholine receptor from *Torpedo californica* have been analyzed in reconstituted membranes containing purified receptor and defined lipids. Dioleoylphosphatidylcholine and three spin-labeled phosphatidylcholines with the nitroxide group at three different positions on the fatty acyl chain were used for reconstitution of the receptor. The spin-labeled phospholipids serve as quenchers of tryptophan fluorescence. Differential quenching of the intrinsic fluorescence of the acetylcholine receptor by the spin-labeled phospholipids has been utilized to analyze the average membrane penetration depth of tryptophans by the parallax method [Chattopadhyay, A., & London, E. (1987) *Biochemistry 26*, 39–45]. Analyses of the quenching data indicate that the tryptophan residues on the average are at a shallow location (10.1 Å from the center of the bilayer) in the membrane. In addition, the generally low levels of quenching imply that the majority of tryptophan residues are located in the putative extramembranous region of the receptor. These results are consistent with several proposed models for the tertiary structure of the acetylcholine receptor and are relevant to ongoing analyses of the overall conformation and orientation of the acetylcholine receptor in the membrane.

Due to the inherent difficulty of crystallizing membrane proteins, most analyses of membrane protein structure have utilized indirect biophysical techniques with an emphasis on spectroscopic methods. One such analysis involves determination of membrane penetration depth, the location of a molecule or a specific site within a molecule in relation to the membrane surface. Knowledge of the precise depth of a membrane-embedded group or molecule helps define the conformation and topology of membrane proteins and probes. Fluorescence has been one of the most widely used techniques to determine depth. Both long-range dipole–dipole (Forster)

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* Address correspondence to this author.
‡ Present address: Centre for Cellular and Molecular Biology, Uppal Rd., Hyderabad 500 007, India.

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