Mechanism of Action of Pseudomonas Exotoxin

IDENTIFICATION OF A RATE-LIMITING STEP*

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Pseudomonas exotoxin (PE), which is cytotoxic for mammalian cells, enters cells by receptor-mediated endocytosis, and requires proteolytic processing before it can reach the cytosol and inhibit protein synthesis (1). While several steps in the toxin pathway have been delineated, the fate of the toxin after processing is poorly understood.

PE is composed of three structural domains (2). Specific amino acids in domain I (3, 4) mediate binding and entry via the α-granulokrin receptor (5, 6). This step brings the toxin to the endosomal compartment where it is processed by an unidentified membrane-associated protease (1, 7). Processing cleaves a specific peptide bond in an arginine-rich loop at the beginning of domain II. Recently, it was shown that the site of cleavage is between Arg279 and Gly280 (8). To understand how the 37-kDa fragment of PE acts, we focused on the role of specific amino acids located near its NH₂ terminus. We found that there was a 4–250-fold loss in toxic activity when tryptophan 281, leucine 284, or tyrosine 289 was changed to other residues. Mutations at these three positions did not interfere with the receptor binding, cell-mediated proteolytic cleavage, or ADP-ribosylating activity.

To determine the role of these amino acids, a competition assay was devised in which the addition of excess PEΔ53, a mutant form of PE that lacks ADP-ribosylating activity, competed efficiently for the toxicity of PE. Excess PE with mutations near the NH₂ terminus of the 37-kDa fragment competed poorly. This competition occurred after proteolysis since PEΔ53, a mutant form of PE that is not cleaved, did not compete. We conclude that specific amino acids at the NH₂ terminus of the 37-kDa fragment interact in a saturable manner with an unknown intracellular component.

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1 The abbreviations used are: PE, Pseudomonas exotoxin; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-galactopyranoside.

2 M. Chiron and D. J. Fitzgerald, unpublished results.

outcome of processing is the generation of an NH₂-terminal fragment of 28 kDa which contains the toxin's binding domain and a COOH-terminal fragment of 37 kDa (composed of domains II and III) which translocates to the cytosol and ADP-ribosylates elongation factor 2. Mutant toxins that cannot be processed appropriately are nontoxic for cells (1, 7).

Existing evidence suggests that cleavage occurs in the endosomal compartment. Kinetic data showed that at 37 °C toxin fragments were generated within 10 min of incubating toxin with cells (1); cleavage of the toxin by crude cellular membranes, detergent-solubilized membranes (7), or by a pepsin-released soluble protease was optimal at pH 5.5 and, finally, Percoll gradients of disrupted cells revealed that toxin cleavage was mediated by a protease that migrated with light cellular fractions such as the plasma membrane, endosome, or Golgi and not with heavy fractions such as lysosomes (7).

After toxin cleavage, the 37-kDa fragment is separated from domain I by reduction of the disulfide bond linking cysteines 265 and 287. Then, to inhibit protein synthesis, the 37-kDa fragment translocates to the cytosol. While the mechanism for translocation is currently unknown, residues at the COOH terminus of the fragment, resembling an endoplasmic reticulum (ER) retention sequence, are essential for toxicity (9, 10). Evidence suggests that the toxin must reach the ER before it can translocate to the cytosol (1, 9).

Three recent studies have suggested that following the cleavage step sequences at the NH₂ terminus of the 37-kDa fragment are also important for toxicity. When residues 280–282 were changed to methionine, only the change at tryptophan 281 produced a toxin molecule with substantially reduced cytotoxic activity (8). When alanine scanning mutagenesis was performed on surface residues near the NH₂ terminus of the 37-kDa fragment, amino acids 280–282, 285–286, and 290 were changed to alanine and assayed for cytotoxic activity. Only the tryptophan 281 to alanine change reduced toxicity by as much as 100-fold (11). And finally when a chimeric toxin composed of the 37-kDa fragment fused with transforming growth factor α was tested for cytotoxic activity it had maximum activity when the protein started at residue 280. Activity was reduced 12-fold when amino acids 281–282 were deleted, 20-fold when amino acids 281–284 were absent, and 200-fold when amino acids 281–288 were removed (12). Thus one or more residues at the NH₂ terminus of the 37-kDa fragment were found to be important for toxicity.

To understand the requirement for particular amino acid side chains at position 281, additional mutations were generated at this residue. And because the side chains of amino acids 284 and 289 are clustered near the side chain of tryptophan 281, the importance of these amino acids was also investigated. Because all the mutations were located close to...
the Arg-Gly bond where PE is cleaved, mutant toxins were
assayed for changes in susceptibility to proteolysis and in the
patterns of fragments generated. And finally, a competition
assay was devised to show that these 3 specific residues
contribute to a saturable interaction with a cellular compo-
nent.

MATERIALS AND METHODS

Plasmids and Cells—Plasmids were propagated in Escherichia coli
DH5a (Bethesda Research Laboratories). Recombinant strain
cell C1236 was used to generate single-stranded DNA for mutagenesis (see below). For
expression of PE or mutant forms of PE, plasmids were transformed into
E. coli BL21 (DE3) (13). Plasmid pMOA12VK352 f T (8), which
encodes native PE with a unique XhoI site in domain II (at amino
acid 281, 264–285), was used as the template for mutagenesis. Plasmid
pVC45D f T which lacks the codon for glutamic acid 553
was used as to subclone the 553 mutation into various constructions.

Restriction Enzymes—XhoI, NarI, BamHI, EcoX, Ral, Apal, and
EcoRI were from Boehringer Mannheim; BspHI and CfrIOL were from
New England Biolabs. The MutageneT4 polymerase Refill Pack (Bio-Rad) was used for mutagenesis.

Tissue Culture—Murine L-929 cells and human HT-29 cells were
obtained from ATCC and maintained in Dulbecco's modified Eagle's
medium, 5% fetal bovine serum with penicillin and streptomycin.

Purification of Recombinantly Expressed Proteins—At Ab% of 0.5–
0.6 M, DNA was induced with 1 mM isopropyl-1-
thio-β-D-galactoside (IPTG). Cells were harvested 90 min later. PE
and mutant forms were obtained from the periplasm of E. coli
by osmotic lysis. Proteins were further purified using successive ion-
exchange columns. Routinely, PE proteins were purified to 80%
holo(1) or better (Fig. 4).

Production of Intrinsically Radiolabeled Proteins—Radiolabeling of
PE and PE mutants was performed as described (1, 8). Briefly, E.
coli was transformed as usual but the resulting colonies were grown
in 25 ml of minimal medium. IPTG was added when the culture
reached an absorbance of 0.3. Immediately after the addition of IPTG, 1.0
mCi of [3H]leucine was added; and thereafter, 1.0 mCi of [3H]
leucine was added every 15 min until a total of 5 mCi had been added.
At the end of the induction period, cells were harvested and periplasm
prepared by osmotic lysis. Toxin-enriched periplasm was filtered
using a rabbit anti-PE antibody and protein A-Sepharose (Pharmacia
LKB Biotechnology Inc.). Sequencing was performed as described previously (8).

Site-directed Mutagenesis—Oligonucleotide-directed mutagenesis
was carried out using a modification of the method of Kunkel (14). Oligonucleotides
used for mutagenesis are listed in Table I. Mutations were
identified by restriction enzyme analysis and confirmed by
sequencing using the Sanger dideoxy method. Sequencing was per-
formed using the Sequenase Kit from U. S. Biochemical Corp. The
primer used for sequencing was 5'-AGGGCGTTGCGGATCACC-3'.

Construction of Enzymatically Inactive Mutants—Enzymatically
inactive form PE mutants were constructed by subcloning the Apal-
EcoRI fragment obtained from pVC45D f T into each plasmid.

Cytotoxicity Assay—To measure toxicity, PE or mutant forms were
added to L-929 or HT-29 cells. Cells at 1 x 10⁶ per well were seeded
1 day prior to testing and then a range of toxin concentrations was
added for 20 h at 37°C. At the end of this period, [3H]-leucine was
added to a final specific activity of 2 μCi/ml for a further 1 h. To
assay the receptor onto the membrane. Following the addition
of bovine serum albumin to block nonspecific binding, PE or mutant
toxins were added to the receptor-coated membranes. Evidence
of toxin binding was obtained by adding peroxidase-labeled (Jackson
Laboratories) affinity-purified rabbit anti-PE.

ADP-ribosylation Assay—The toxin-catalyzed transfer of
ADP-ribose from elongation factor-2 was determined using raw wheat germ
as the source of elongation factor-2 and performed according to
the method of Collier and Kandel (16).

In Vitro Proteolysis of PE and PE mutants—PE or PE mutants
(approximately 6 μg) were mixed with a protease from beef liver that
was highly enriched for an activity that cleaved PE at the Arg-Gly
(279–280) bond. The assay was at room temperature in 200 mM
sodium acetate, 1 mM CaCl₂, pH 5.5, conditions already established
to be optimal for the cleavage of PE.³ Incubation times ranged from
1 h to an overnight incubation. The degree of cleavage was determined
by SDS-PAGE analysis.

Competition Assay—PE at 300 ng/ml in the presence or absence
of potential competitors was added to L929 cells for 4 h at 37°C.
During the last hour of the 4-h incubation, [3H]leucine was added to
the culture to a final concentration of 2 μCi/ml. To determine the
extent of inhibition of protein synthesis, the monolayers were
processed as described above.

RESULTS

Replacement of Tryptophan 281 with Various Amino Acids—Results by Ogata et al. (8) and Kasturi et al. (11)
indicated that tryptophan 281 is important for the toxicity of PE. When this amino acid was replaced by either methionine
or alanine there was a 100-fold decrease in toxicity. Likewise, when tryptophan was deleted from the NH₂ terminus of a
chimeric toxin composed of the COOH-terminal 37-kDa fragment
of PE fused with transforming growth factor α, there was a profound reduction in activity as progressively more
amino acids were removed (12). Taken together, the three
studies indicated an important role for tryptophan 281 and,
that reduction in activity was not due to a unique
interaction with L929 cells.

To understand the role of tryptophan 281, it was systematically
replaced by other amino acids exhibiting a variety of side chains (see Table I for construction of mutants). PE or
mutant forms were added to L929 cells to determine their
cytotoxic activity. To compare activities, the IC₅₀ of each
toxin was determined and then compared with native PE
representative experiments are shown in Table II and Fig.
1). Replacement of tryptophan with basic amino acids was the
least detrimental to activity. With lysine at 281, toxicity
was reduced 4-fold; with either arginine or histidine at 281,
toxicity was reduced by 10-fold. However, the replacement
with either acidic or neutral amino acids reduced toxicity by
as much as 100-fold and when glutamic acid was present, by
as much as 250-fold. There was no apparent requirement for
an aromatic side chain since the introduction of phenylalanine
caus ed a 30-fold reduction in toxicity (Table II and Fig. 1).

L929 cells are very sensitive to PE. To determine if the requirement for tryptophan 281 extended to other cells and
species, some experiments were repeated by adding PE or
selected mutants to the human colon cancer line HT-29. The
same relative reduction in activity was seen (Table III). This
confirmed that tryptophan is important for PE-mediated
toxicity and that reduction in activity was not due to a unique
interaction with L929 cells.

In addition to testing the effects of simple amino acid
substitutions, the positional role of tryptophan relative to
the other amino acids was determined. Tryptophan at 281
was exchanged with glutamic acid at position 282. This exchange
resulted in the generation of a toxin molecule with 30-fold

³ M. Chiron, C. M. Fryling, and D. J. Fitzgerald, manuscript in preparation.
Rate-limiting Step for Toxicity of Pseudomonas Exotoxin

TABLE I
Scheme for site-directed mutagenesis of native PE

The WT sequence of PE is from plasmid pMOA129VK929, T (8) and is slightly different in nucleotide composition from the sequence originally reported by Gray et al. (22). The underlined sequences reveal the restriction sites used for screening. Several clones with changes at PELeu284 were screened for the loss of the XhoI site (in these cases no sequences are underlined).

### Table I

**Oligonucleotides**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5'-ggatcc gca tct cca cga ggg ctc ggt cgg -3'</td>
</tr>
<tr>
<td>Ala283A</td>
<td>5'-ggatcc gca tct cca cga ggg cac gtc ggt cgg -3'</td>
</tr>
<tr>
<td>Phe281A</td>
<td>5'-ggatcc gca tct cca cga ggg cag gtc ggt cgg -3'</td>
</tr>
<tr>
<td>Ser283A</td>
<td>5'-ggatcc gca tct cca cga ggg cgg gtc ggt cgg -3'</td>
</tr>
<tr>
<td>Arg281A</td>
<td>5'-ggatcc gca tct cca cga ggg ccc gtc ggt cgg -3'</td>
</tr>
<tr>
<td>Lys281A</td>
<td>5'-ggatcc gca tct cca cga ggg cct gtc ggt cgg -3'</td>
</tr>
<tr>
<td>His283A</td>
<td>5'-ggatcc gca tct cca cga ggg caa gtc ggt cgg -3'</td>
</tr>
<tr>
<td>Glu281A</td>
<td>5'-ggatcc gca tct cca cga ggg cta gtc ggt cgg -3'</td>
</tr>
<tr>
<td>Met283A</td>
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</tr>
<tr>
<td>Gln281A</td>
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</tr>
<tr>
<td>Trp283A</td>
<td>5'-ggatcc gca tct cca cga ggg caa gtc ggt cgg -3'</td>
</tr>
<tr>
<td>Ala283A</td>
<td>5'-ggatcc gca tct cca cga ggg cgg gtc ggt cgg -3'</td>
</tr>
<tr>
<td>Arg281A</td>
<td>5'-ggatcc gca tct cca cga ggg ccc gtc ggt cgg -3'</td>
</tr>
<tr>
<td>Phe283A</td>
<td>5'-ggatcc gca tct cca cga ggg cct gtc ggt cgg -3'</td>
</tr>
</tbody>
</table>

* Restriction enzymes, XhoI.
* NarI.
* BamHI.
* BspHI.
* EclXI.
* CfrloI.
* RsaI.

**TABLE II**

Toxicity of PE and PE mutants on L-929 cells

The results of individual experiments are shown in each column. The data from each first column is also represented as a figure.

<table>
<thead>
<tr>
<th>Residue 281</th>
<th>Residue 284</th>
<th>IDm/ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan (wt)</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>20</td>
<td>3.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>12</td>
<td>3.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>30</td>
<td>3.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>80</td>
<td>3.0</td>
</tr>
<tr>
<td>Serine</td>
<td>100</td>
<td>3.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>30</td>
<td>3.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>250</td>
<td>3.0</td>
</tr>
<tr>
<td>283/282 switch</td>
<td>35</td>
<td>3.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>5.0</td>
<td>0.45</td>
</tr>
<tr>
<td>Glutamine</td>
<td>15.0</td>
<td>0.35</td>
</tr>
<tr>
<td>Serine</td>
<td>20.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>25</td>
<td>3.5</td>
</tr>
<tr>
<td>284/283 switch</td>
<td>40</td>
<td>1.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Residue 289, Tyrosine (wt)</th>
<th>Residue 289, Tyrosine (wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>35</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>50</td>
</tr>
</tbody>
</table>

Reduced activity (Fig. 1 and Table II), indicating that the position of tryptophan relative to the other amino acids is important.

**Site-directed Substitutions at Leucine 284 and Tyrosine 289**—Tryptophan 281 is at the beginning of an α-helical structure (2) and the side chains of leucine at position 284 and tyrosine at position 289 are clustered close to it. To determine if these amino acids are also important for toxicity, various amino acid substitutions were made. When leucine was replaced by other amino acids there was a small but reproducible loss in toxic activity (Fig. 2A and Table II). A maximum of a 10-fold reduction was noted when leucine was replaced by either tryptophan or serine. There was a 2-fold loss in activity when leucine was replaced by either methionine or glutamine. In addition, when leucine at 284 was switched with glutamine 283 there was a 10-fold loss in activity.

The substitutions at position 289 caused greater reductions in activity (Fig. 2B and Table II). When tyrosine was replaced with either alanine or glutamic acid there was a 30-100-fold loss in activity. A 10-fold loss was seen with phenylalanine and about a 2-fold loss in activity was seen with arginine.

Taken together, these results indicate that tryptophan 281 and tyrosine 289 and to a lesser extent leucine 284 are important for the activity of PE. These specific amino acids appeared to be needed for a particular function or structure since not all substitutions in this part of the toxin molecule reduced toxicity. Previous methionine and alanine replacement studies indicated that glycine 280, glutamic acid 282, glutamic acid 285, and proline 290 could be changed without loss of activity (8, 11). The change of glutamine 286 to alanine reduced (by less than 10-fold) activity on L929 cells but enhanced activity on MCF-7 cells (11).

**Proteolysis of PE Mutants**—Because tryptophan 281, leucine 284, and tyrosine 289 are physically close to the Arg-Gly cleavage site, changes in amino acid composition at these locations could influence the toxin's susceptibility to proteolysis. Three types of assays were performed. Initially, PE and two of the PE281 mutants were intrinsically labeled with [3H]leucine and then added to cells to determine the extent of intracellular proteolysis. To nullify any influence that toxin-mediated inhibition of protein synthesis might have on the outcome of the experiment, enzymatically inactive forms termed PEAla553, PEArg281A553, and PEAla281A553 were used.
A second series of experiments tested unlabeled mutant toxins (including mutants at 281, 284, and 289) as in vitro substrates for a protease preparation enriched in the putative cellular protease responsible for the in vivo cleavage. And finally, to determine if mutations altered the site of cleavage, the 37-kDa fragment derived from one of the least toxic mutants (PEAlaZ8') was subjected to NH₂-terminal sequence analysis.

Cell-mediated cleavage of PE and the two mutant toxins was assessed by adding 3H-toxins to L929 cells for 6 h. PE-related protein was recovered by immunoprecipitation and analyzed by SDS-PAGE and fluorography (Fig. 3). The first involved the sequencing of the 37-kDa fragment generated from native PE. This indicated that leucine was the fifth amino acid after residue 281. The second involved the sequencing of the 37-kDa fragment generated from native PEAlaZ8', which was cleaved the best. In each case, fragments of 37- and 28-kDa were generated. Since previously it was shown that the site of cleavage occurred at the same Arg-Gly bond, this indicated that the cleavage occurred at the same Arg279-Gly280 bond. The same result had been obtained previously with the PEArg281 mutant.

A second series of experiments tested unlabeled mutant toxins (including mutants at 281, 284, and 289) as in vitro substrates for a protease preparation enriched in the putative cellular protease responsible for the in vivo cleavage. And finally, to determine if mutations altered the site of cleavage, the 37-kDa fragment derived from one of the least toxic mutants (PEAlaZ8') was subjected to NH₂-terminal sequence analysis.

Cell-mediated cleavage of PE and the two mutant toxins was assessed by adding 3H-toxins to L929 cells for 6 h. PE-related protein was recovered by immunoprecipitation and analyzed by SDS-PAGE and fluorography (Fig. 3). These experiments indicated that native PEAla553 was cleaved least well within cells, PEArg281Δ553 was intermediate, and PEAlaZ8'Δ553 was cleaved the best. In each case, fragments of 37- and 28-kDa were generated. Since previously it was shown that the generation of the 37-kDa fragment by cell-mediated cleavage was necessary for toxicity, this result seemed to conflict with the relative toxicities of these proteins (see below).

To investigate whether the increased production of 28- and 37-kDa fragments from the PEArg281 and PEAlaZ8' mutants was due to changes in the nature of these proteins as substrates or some other parameter such as toxin routing within cells, mutant forms were tested as substrates in a biochemical assay. Purified toxins were mixed with a partially purified protease (isolated from beef liver) that cleaves PE at the Arg279-Gly280 bond. PE or PE mutant proteins (approximately 0.1 nmol of toxin) were mixed with the protease. Samples were incubated for either 1 or 18 h under conditions determined to be optimal for cleaving PE, i.e. pH 5.5, 0.2 M sodium acetate, 1 mM CaCl₂ at room temperature. The production of fragments was assessed by SDS-PAGE (Fig. 4, A and B). After the 1-h incubation, substantial amounts of the 28- and 37-kDa fragments had been produced from PEAlaZ8', small amounts from PEArg281, and none from PE, PEArg280, PEAlaZ8', PEMet284, or PETrp284. With the longer incubation, the cleavage of PEAlaZ8' went to completion, the cleavage of PEArg281 was substantial, and small but detectable amounts of fragments were produced from PE and the other mutants. Results indicated that the mutations introduced at positions 281 apparently changed the structure of PE to make it a better substrate for cleavage but alterations at positions 284 and 289 did not affect the extent of cleavage relative to native toxin (Fig. 4, A and B).

To investigate the possibility that the loss in toxic activity was due to a change in the site of cleavage, two sequencing experiments were performed using PEAlaZ8' as the substrate. The first involved the sequencing of the 37-kDa fragment produced after the addition of [3H]PEAla281A553 to cells. The radioactive 37-kDa fragment was retrieved from cells by immunoprecipitation, located by SDS-PAGE and fluorography, transferred to polyvinylidene difluoride membranes, and then subjected to repeated rounds of Edman degradation. As shown in Fig. 5, there was a peak of radioactivity released after the fifth cycle. Since the toxin had been labeled with [3H]leucine, this indicates that leucine was the fifth amino acid after cleavage. The same result had been obtained previously with the PEAlaZ8' protein was incubated with the partially purified protease and the NH₂ terminus of the 37-kDa fragment sequenced by automated techniques. Again, the site of cleavage was the Arg-Gly bond at residues 279 and 280.

**Table III**

<table>
<thead>
<tr>
<th>Residue 281</th>
<th>Trytophan (wt)</th>
<th>ID₅₀/μg/ml</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 1. Inhibition of protein synthesis by PE mutants with substitutions at position 281.** Purified mutant proteins were added to L929 cells for an overnight incubation at 37 °C. At the end of the incubation period the level of protein synthesis was determined by measuring the incorporation of [³H]leucine into cellular protein. One representative experiment is shown. The results of several similar experiments are presented in Table 1.

**Binding and ADP-ribosylating Activity**—Although no mutations were introduced in either domain Ia, the binding domain, or in domain III, the ADP-ribosylating domain, selected mutants were analyzed for these activities. Since PE
FIG. 2. Inhibition of protein synthesis by PE mutants with substitutions at positions 284 and 289. A, purified PE284 mutants were added to L929 cells for an overnight incubation at 37 °C. At the end of the incubation period the level of protein synthesis was determined by measuring the incorporation of [3H]leucine into cellular protein. B, purified PE289 mutants were added to L929 cells for an overnight incubation at 37 °C. At the end of the incubation period the level of protein synthesis was determined by measuring the incorporation of [3H]leucine into cellular protein.

binds to the low density lipoprotein receptor-related protein/ α₂-macroglobulin receptor (5), analysis of binding activity was carried out by performing ligand blots on various amounts of immobilized receptor. Receptor-bound toxin was detected using an affinity purified peroxidase-labeled anti-PE rabbit antibody. Results indicated that all the mutants with changes in domain I bound to the receptor in a comparable fashion to native PE or at least had no more than a 2-fold reduction (Fig. 6). Retention of receptor binding activity by these mutants was important to establish since competition experiments with excess toxin were undertaken to demonstrate the existence of a saturable intracellular site in the toxin pathway (see below). PEGlu°, which does not bind the receptor, was used as the negative control.

Likewise, when selected mutants were assayed for ADP-ribosylating activity, all appeared fully active (data not shown).

Competition Assay Reveals Role of Key Amino Acids in Defining Rate-limiting Step—To understand the possible role of these 3 amino acids at the NH₂ terminus of the 37-kDa fragment, a competition assay was devised that could discriminate between native PE and the mutant forms. The addition of excess PEΔ553 competed for the toxicity of native PE (Fig. 7A). In a 3-h assay, PE at 300 ng/ml caused an 80–90% reduction in protein synthesis. However, the addition of excess PEΔ553 inhibited PE-mediated toxicity in a dose-dependent manner. In the presence of 14 μg/ml PEΔ553 there was only a 40% reduction in protein synthesis. Initially, we considered the observed competition was due to the occupation of surface receptors by excess PEΔ553. However, in separate experiments it was demonstrated that when excess PEΔ553 was added to intact cells, it reduced the uptake of radiolabeled PE very poorly (9). Thus it was reasoned that the competition was for an intracellular rate-limiting step. To
determine if the various mutant toxins could compete for PE toxicity to the same extent as PEΔ553, additional experiments were performed. Again, to avoid the complication of adding mutant proteins that exhibited different levels of cytotoxic activity, the Δ553 versions of each mutant toxin was used.

When excess PEArg^{281}Δ553 or PEAla^{281}Δ553 was added to cells, there was less competition compared to the same concentrations of PEΔ553 (Fig. 7B). PEAla^{281}Δ553 exhibited minimal competition while PEArg^{281}Δ553 showed intermediate competition. Likewise the other mutants were added as potential competitors (Fig. 7, B and C). It was noted that PEMet^{281}Δ553 and the PEArg^{289}Δ553 mutants competed at an intermediate level while PETrp^{284}Δ553 and PEAla^{280}Δ553 competed minimally. Generally, the level of competition reflected the level of toxicity exhibited by each mutant.

To understand other features of the competition assay, additional PE mutants were analyzed. When PEGly^{770}, which cannot be cleaved by cells (1, 7), was tested, there was little or no competition for PE activity (Fig. 7B). This indicated that the competition step required proteolytic processing and the production of the 37-kDa fragment. As mentioned in the Introduction, for PE to be toxic it also requires the presence of specific sequences, resembling the KDEL ER-retention sequence at its COOH terminus. When these are missing or jumbled there is no toxicity, presumably because there is no translocation to the cytosol. When PE lacking the last 15 amino acids, PEΔ599-613, was added to cells there was excellent competition. This class of mutant is cleaved well by cells but ultimately cannot kill cells because it does not get retained in the ER (1). This suggested that the competition step occurred after proteolysis and before translocation from the ER.

![Graphical representation of cell-mediated proteolysis](image)

**Fig. 3. Cell-mediated Proteolysis of radiolabeled PE proteins.** [^{3H}]PEΔ553, [^{3H}]PEArg^{281}Δ553, or [^{3H}]PEAla^{281}Δ553 were added to cells for 6 h at 37 °C. At the end of this period, PE-related protein was recovered by immunoprecipitation. Lane 1 shows the extent of cell mediated cleavage of PEΔ553; lane 2 of PEArg^{281}Δ553; and lane 3 PEAla^{281}Δ553. Approximately equal number of counts were recovered from each dish of cells.

![Graphical representation of proteolysis by cellular protease](image)

**Fig. 4. Proteolysis of PE and PE mutants by a partially purified cellular protease.** Proteins were incubated at room temperature in the presence (+) or absence (−) of protease. A, SDS-PAGE analysis of toxin cleavage after 1 h. B, SDS-PAGE analysis of toxin cleavage after 18 h.
DISCUSSION

PE is internalized by receptor-mediated endocytosis and is then cleaved by a cellular protease. The resulting 37-kDa COOH-terminal fragment eventually reaches the ER where it translocates to the cytosol. Here we show that specific amino acids near the NH₂ terminus of the 37-kDa fragment are important for a rate-limiting step in the toxin pathway that occurs after the cleavage step and before translocation.

Tryptophan 281—We focused first and most extensively on the role of tryptophan 281 since it has been shown by several recent studies that it was necessary for toxicity. When tryptophan was changed to either methionine or alanine there was at least a 100-fold loss in toxicity (1, 11). One puzzle was why this amino acid was so important when glycine 280 and glutamic acid 282 could be changed to other amino acids without any loss in toxin activity (1, 11). Since it was possible that such a profound change in the chemical nature of the side chain, from tryptophan to either methionine or alanine, was sufficient to greatly reduce activity, additional amino acid substitutions were made. The replacement of tryptophan with basic amino acids caused the least disruption in toxicity. Replacement with neutral or negatively charged amino acids resulted in a loss of activity of at least 100-fold.

The first indication that mutations at residue 281 caused PE to interact with cells differently from the native toxin came from early experiments that examined the cellular processing of radioactive PE proteins. It appeared that the processing of [³H]PEMet₅₅₃ generated greater amounts of the 37- and 28-kDa fragments than was seen with native toxin (1). To clarify the situation we generated enzymatically inactive forms of some of the mutants at residue 281. Since NAD binds to glutamic acid at position 553, the deletion of this amino acid (Δ553) renders the toxin unable to catalyze the ADP-ribosylation of elongation factor 2 (17–19).

³H-Labeled PEA₅₅₃, PEArg₅₅₃, and PEAla₅₅₃ were produced, added to cells, and the degree of cell-mediated cleavage was determined for each protein. Results showed that PEA₅₅₃ was cleaved most efficiently, then PEArg₅₅₃ and PEAla₅₅₃ was cleaved least well. However, having shown that there was a clear difference in cell-mediated cleavage, two additional issues needed to be settled. One was to determine the reason for the increased proteolysis. Was it because the mutants were better substrates or was it because mutant toxins were routed differently within cells? In addition, even though cell-mediated cleavage of PEA₅₅₃, PEArg₅₅₃, and PEAla₅₅₃ produced fragments of 28 and 37 kDa, it was necessary to determine the exact site of cleavage since it was possible that the mutations altered toxin structure to favor another cleavage site. Results from biochemical assays suggested that the 281 mutants were struc-

![Graph](image_url)

FIG. 5. Determination of NH₂-terminal sequence of the 37-kDa fragment generated by L929 cells. [³H]PEAla⁵⁵₃, PEAla⁵₅₃, PEAla⁵₅₃, PEAla⁵₅₃, PEAla⁵₅₃ were added to cells, the 37-kDa fragment was recovered by immunoprecipitation. The correct band was identified by fluorography. The fragment was then subjected to repeated cycles of Edman degradation. The counts from each cycle are shown.

![Graph](image_url)

FIG. 6. Toxin binding to immobilized receptor. Approximately 2 µg of affinity-purified α₂-macroglobulin receptor, and sequential 2-fold dilutions were immobilized on nitrocellulose. PE or various PE mutants were added to determine binding activity.
petition for the toxicity of 300 ng/ml native PE. The toxins were added to L929 cells for a total of 4 h. The results are expressed as the percentage of control protein synthesis.

Fig. 7. Competition assay using PE mutants as potential competitors. The ability of excess amounts of enzymatically inactive (PEGP92 and PEGS92) and PE589 are approximately 1000-fold less cytotoxic for mammalian cells than native PE. For this reason it was not necessary to introduce the Δ553 mutation) PE (PEGΔ553) and PE mutants to compete for the toxicity of native PE was assessed. (The Δ553 mutation is sometimes represented on the graphs by a upper case “D”). A, increasing concentrations of PEGΔ553 were added as a competitor for the toxicity of 300 ng/ml native PE. The toxins were added to L929 cells for a total of 4 h. Results are expressed as the percentage of control protein synthesis. B, various concentrations of PE mutants were mixed with 300 ng/ml native PE and added to L929 cells for a total of 4 h. The results are expressed as cpm/well of cells. C, various concentrations of PE mutants were mixed with 300 ng/ml native PE and added to L929 cells for a total of 4 h. The results are expressed as the percentage of control protein synthesis.

turally different from native PE since they were cleaved more readily. This discounted the possibility that the mutants were routed differently within cells or spent more time associated with a protease-rich organelle. Furthermore, NH₂-terminal sequence analysis of the 37-kDa fragment derived from the PEAla281 mutant, produced either within cells or in biochemical assays, revealed that the cleavage site had not been altered. To understand why the production of greater amounts of the correctly cleaved fragment produced less toxicity other assays had to be devised (see below).

Leucine 284 and Tyrosine 289—Domain II is composed of 6 α-helices (2). An arginine-rich loop is located between the A helix and the B helix. In native PE, tryptophan 281 is at the beginning of the B helix. The aromatic side chain of tryptophan is close to the side chains for leucine 284 and tyrosine 289. Neither of these amino acids had been analyzed previously in the alanine scanning study since neither was prominently exposed on the surface of the toxin. To understand the possible role of these 2 amino acids, single amino acid substitutions were undertaken.

Changing leucine 284 to other amino acids caused a maximum of a 10-fold reduction in activity. The largest reduction in activity was caused by substituting either serine or tryptophan while the introduction of either methionine or glutamic acid had almost no effect on toxicity.

Residue 289 was more sensitive to change than residue 284. A 30–100-fold loss in activity was noted when tyrosine 289 was replaced by either alanine or glutamic acid, a 10-fold loss when phenylalanine was introduced, and almost no change in activity when arginine was substituted.

Competition Assay Reveals Intracellular Rate-limiting Step—Previous attempts to saturate the surface binding of PE have been difficult (9, 20). We now know that PE binds to the α2-macroglobulin receptor, a receptor with many internal repeat units (21). While the PE binding site on this receptor has not been determined, if the toxin interacted with one of these repeat units, it might be very difficult to saturate surface binding. Previously, we showed that the addition of as much as 100 μg/ml PE reduced the uptake of radioactive PE by only 50% (Fig. 2 of Ref. 9) but reduced toxicity by as much as 100-fold. Together these results suggest that competition for toxicity is not at the level of surface binding but for some other step in the toxin pathway. This rate-limiting step has been identified using the 281, 284, and 289 mutants. While the nature of the toxin interaction with this component is not understood, we note that the two most important residues, 281 and 289, tolerated changes from aromatic side chains to basic ones without much loss in activity but changes to acidic residues produced large decreases in toxicity. Our speculation as to the identity of the cellular component that interacts with the NH₂ terminus of the 37-kDa fragment includes: an unfolding protein such as a chaperone, a transport protein that could bring the toxin to the Golgi or the ER, or a component of a translocation complex. And clearly, the primary structure and not just the composition of the NH₂ terminus is important to effect this interaction. This is best illustrated with the result that showed that the exchange of tryptophan 281 with glutamic acid 282 produced a mutant toxin with very little cytotoxic activity.

Based on the change in susceptibility to protease cleavage, the replacement of tryptophan with other side chains at position 281 clearly produced changes in PE structure. However, these structural changes were not necessary for loss in activity since the 289 mutants had reduced toxic activity, reduced competitive activity intracellularly but no change in protease susceptibility. The mutations at position 281 deserve a special comment. The addition of PEAla281 to cells produced much more of the 37-kDa fragment that the addition of native PE. And yet these additional amounts of fragment still competed very poorly for the intracellular site. Thus on a molar
Rate-limiting Step for Toxicity of Pseudomonas Exotoxin

basis the alterations at the 281 position caused the greatest disruption of normal toxin function.

Conclusion—Tryptophan at position 281 is critical for the toxicity of native PE despite the fact that toxin cleavage is not very efficient. Together, tryptophan 281, leucine 284, and tyrosine 289 are needed for a rate-limiting step in the pathway that occurs after the cleavage step.

REFERENCES
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