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Pseudomonas exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity

(endocytosis/toxin/translocation/endoplasmic reticulum/signal sequence)

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ABSTRACT **_Pseudomonas_ exotoxin (PE), a single-chain polypeptide toxin of 613 amino acids, consists of three functional domains: an amino-terminal receptor-binding domain, a middle translocation domain, and a carboxyl-terminal ADP-ribosylation domain. Deletion of as few as 2 or as many as 11 amino acids from the carboxyl terminus of PE does not affect ADP-ribosylation activity but produces noncytotoxic molecules. Deletions and substitutions between positions 602 and 611 of PE show that the last 5 amino acids of PE are very important for its cytotoxic action. The carboxyl-terminal sequence of PE is Arg-Glu-Asp-Leu-Lys. Mutational analysis indicates that a basic amino acid at 609, acidic amino acids at 610 and 611, and a leucine at 612 are required for full cytotoxic activity. Lysine at 613 can be deleted or replaced with arginine but not with several other amino acids. Mutant toxins are able to bind normally to target Swiss mouse 3T3 cells and are internalized by endocytosis, but apparently they do not penetrate into the cytosol. A PE molecule that ends with Lys-Asp-Glu-Leu, which is a well defined endoplasmic reticulum retention sequence [Munro, S. and Pelham, R. B. (1987) *Cell* 48, 899–907], is fully cytotoxic, suggesting that a common factor may be involved in intoxication of cells by PE and retention of proteins in the lumen of the endoplasmic reticulum. Sequences similar to those at the carboxyl end of PE are also found at the end of *Cholera* toxin A chain and *Escherichia coli* heat-labile toxin A chain.**

The mechanism by which protein toxins kill cells is quite complex. Many toxins bind to receptors on the surface of mammalian cells, are internalized by endocytosis, translocate to the cytosol, and there exert an enzymatic activity that kills the target cell (for review, see ref. 1). Accordingly, these toxins have separate domains for cell binding, translocation, and an enzymatic activity that inactivates an essential cellular function. *Pseudomonas* exotoxin A (PE) is a single polypeptide chain of 613 amino acids (2). X-ray crystallographic studies and mutational analysis of the PE molecule (3, 4) have shown that PE consists of three domains; an amino-terminal cell receptor-binding domain (domain I), a middle translocation domain (domain II), and a carboxyl-terminal activity domain (domain III). Domain III catalyzes the ADP-ribosylation and inactivation of elongation factor 2, which inhibits protein synthesis and leads to cell death. Mutational analysis of domain I has revealed that Lys-57 plays a major role in receptor binding (5). Similarly Glu-553, Tyr-481, and His-426 have been shown to be important for ADP-ribosylation activity (6–8). Recently, mutational analysis of domain II has shown that certain portions of this domain are absolutely required for the cytotoxicity of PE as well as a chimeric toxin made up of transforming growth factor α (TGFα) and a portion of PE (9, 10). In particular, the arginines at positions 276 and 279 are required for the cytotoxicity of PE (9).

While constructing various chimeric toxins in which growth factors were fused to a form of PE (PE40) that was devoid of domain I, we observed that the recombinant fusion proteins made by attaching TGFα, interleukin 2 (IL2), or interleukin 4 (IL4) at the carboxyl end of PE40 had poor cytotoxic activity. PE40-TGFα was about 40-fold less active than TGFα-PE40 (11, 12), and PE40-IL2 and PE40-IL4 were inactive in killing target cells (unpublished results). Furthermore, immunoconjugates made from PE40 were less cytotoxic than anticipated, probably due to conjugation of the antibody with one of the three lysine residues present near the carboxyl end of domain III (13). These observations led us to examine the possibility that the carboxyl terminus of the activity domain of PE, domain III, may have an additional role in the cytotoxic action of PE. Here we present evidence that the carboxyl-terminal 5 amino acids of PE (Arg-Glu-Asp-Leu-Lys, REDLK) have important information for the cytotoxicity of PE that is unrelated to ADP-ribosylation. Similar sequences are also found at the carboxyl end of *Escherichia coli* heat-labile toxin (14) and *Cholera* toxin A chain (15). The sequence REDLK may be a recognition sequence required for entry of the ADP-ribosylation domain of PE into the cytosol.

MATERIALS AND METHODS

Materials. Sources of most reagents have been described (11, 16). The polymerase chain reaction (PCR) was performed using a GeneAmp kit (Perkin–Elmer/Cetus).

Mutants and Plasmid Constructions. Mutants were created by oligonucleotide-directed mutagenesis using plasmid pVC45f+T as described (5, 9) or using PCR as described below. pVC45f+T carries a PE gene under control of a T7 promoter and also contains a T7 transcriptional terminator and an f1 phage origin (9, 16). The PE gene in this plasmid contains an OmpA signal sequence that is cleaved upon secretion of PE into the periplasm, leaving a 3-amino acid (Ala-Asn-Leu) extension at the amino terminus (17). For PCR mutagenesis, two oligonucleotides and a 1.0-kilobase-pair (kbp) *Sal* I–*Eco*RI fragment of pVC45f+T were employed. One oligonucleotide was the same as nucleotides 2216–2236 of the PE gene (2). Other oligonucleotides were complementary to the 3′ end of the PE coding sequence, contained desired mutations, and created an *Eco*RI site after the stop codon. Other unique restriction sites were created without changing amino acids, to identify the mutants. A 30-cycle PCR was performed with denaturation at 94°C for 1 min, annealing at 55°C for 90 sec, and polymerization at 72°C for 2 min with a 10-sec extension per cycle, using a thermal cycler (Perkin–Elmer/Cetus). The PCR-amplified fragment was cut with *Eco*RI and *Bam*HI and purified by electropho-

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Abbreviations: PE, *Pseudomonas* exotoxin; TGFα, transforming growth factor α.

resis in low-melting-point agarose. PCR fragments were ligated with a 4.5-kbp dephosphorylated *Eco*RI–*Bam*HI fragment of pVC45f+T. Mutants were identified by the unique restriction sites created during mutagenesis and were finally confirmed by dideoxy sequencing using Sequenase (United States Biochemical).

pVC4915f+T. This plasmid contains two mutations; Codons 608 (CCG) and 609 (CGC) were changed to CCC and GGG, respectively. This mutation results in glycine at 609 in place of arginine and creates a *Sma* I site between codons 608 and 609. This plasmid was used to clone various carboxyl-terminal fragments of PE.

pVC4975f+T. A 1-kbp *Bam*HI–*Pst* I fragment of pVC8 (8) was cleaved with *Nar* I, treated with phage T4 DNA polymerase to make blunt ends, and then cut with EcoRI, and a 286-bp fragment was ligated to a 4.9-kbp dephosphorylated *Sma* I–*Eco*RI fragment of pVC4915f+T.

pVC4985f+T. A 1-kbp *Bam*HI–*Pst* I fragment of pVC8 was cleaved with *Hin*fI and treated with T4 DNA polymerase followed by *Eco*RI, and a 237-bp fragment was ligated to the 4.9-kbp *Sma* I–*Eco*RI fragment of pVC45f+T.

pVC4995f+T. A synthetic oligodeoxynucleotide duplex, VK192/193 (not shown), containing codons 598–613 of PE with a stop codon and an *Eco*RI-compatible 3′ end, was ligated to the 4.9-kbp *Sma* I–*Eco*RI fragment of pVC-4915f+T.

pVC4715f+T. This plasmid was created by PCR mutagenesis. It contains restriction sites for *Stu* I, *Nde* I, *Sma* I, *Eco*RV, and *Eco*RI within the 3′ end of the PE gene and encodes amino acids RPHMPGDILK in place of PREDLK at 608–613. These unique restriction sites were later used to make insertions and to attach various DNA segments encoding carboxyl-terminal portions of PE.

pVC47195f+T. This was created by ligating oligonucleotide duplex VK192/193 to a 4.9-kbp *Eco*RV–*Eco*RI fragment of pVC4715f+T. The carboxyl terminus of this PE mutant contains RPHMPGDPDYASQPGKPPREDLK in place of amino acids 608–613 (PREDLK) of PE.

Protein Expression and Purification. Cultures of *E. coli* strain BL21(λDE3) containing various plasmids were grown to OD_{650} of 0.6–0.8 and induced with 1 mM isopropyl β-D-thiogalactoside for 90 min at 37°C. Periplasmic fractions were prepared as described (17). By virtue of having an OmpA signal sequence, >90% of each of the expressed toxin proteins was secreted into the periplasm. These proteins have a residual Ala-Asn-Leu sequence at the amino end left behind after the processing of the OmpA signal sequence. Periplasmic fractions were assayed for ADP-ribosylation activity and cytotoxicity. Later, PE mutants were purified using a Mono Q anion-exchange column (HR5/5) attached to a Pharmacia FPLC system. PE and mutant proteins were eluted at NaCl concentrations at 0.22–0.26 M. Upon SDS/PAGE the toxins were >90% pure. Protein concentration was measured by the Coomassie blue G-250 binding assay (Bio-Rad Protein Assay) with bovine serum albumin as a standard.

ADP-ribosylation and Cytotoxicity Assays. ADP-ribosylation activity was assayed after the activation of PE and mutant proteins with 4 M urea and 50 mM dithiothreitol (18) unless otherwise stated. Cytotoxicity of PE mutants was determined by adding various dilutions of periplasmic proteins or purified proteins to 10^5 Swiss mouse 3T3 cells in 24-well plates, as described (5, 9). ADP-ribosylation and cytotoxic activities of recombinant PE and native PE (from Swiss Serum and Vaccine Institute, Berne, Switzerland) were indistinguishable

Toxin Binding and Internalization. The ability of various mutant PE proteins to compete with ^{3}H-labeled PE for binding to Swiss mouse 3T3 cells was assessed as described (9). Internalization of various mutant PE derivatives was studied by immunofluorescence as described (9).

Table 1. Deletion analysis of the carboxyl terminus of PE

Mutant protein			
Name*	Amino acids present	Cytotoxicity, % of PE	ADP-ribosylation, % of PE
PEΔ590–613	1–589	<0.1	0
PEΔ600–613	1–599	<0.1	20
PEΔ603–613	1–602	<0.1	100
PEΔ606–613	1–605	<0.1	100
PEΔ607–613	1–606	<0.1	100
PEΔ611–613	1–610	<0.1	100
PEΔ612,613	1–611	<0.1	100
PEΔ613	1–612	100	100

Mutant PE proteins were expressed in *E. coli* using a T7 promoter-based vector (16) and purified from the periplasm. All proteins contain a 3-amino acid (Ala-Asn-Leu) amino-terminal extension remaining after the processing of the OmpA signal sequence. These amino acids were not considered when assigning residue numbers to the mutant proteins. Cytotoxicity was determined by assaying inhibition of protein synthesis on Swiss mouse 3T3 cells. All results are expressed as percent of the activity obtained with recombinant full-length PE (amino acids 1–613). All the assays were done in duplicate and at least two separate clones were tested.
*Δ means deletion of the indicated amino acid(s).

RESULTS

To investigate the role of amino acid residues at the carboxyl end of PE, a series of terminal deletion mutants were constructed that removed 1, 2, 3, 7, 8, 11, 14, and 24 amino acids. Removal of 2 or more amino acids eliminated cytotoxicity without affecting ADP-ribosylation activity (Table 1, Fig. 1). In fact, even 11 amino acids (Δ603–613) could be removed without any loss of ADP-ribosylation activity. However, removal of 14 amino acids (Δ600–613) resulted in a protein with low but measurable ADP-ribosylation activity, and removal of 24 amino acids (Δ590–613) produced an enzymatically inactive protein. These results indicate that residues at the carboxyl end of PE have a role in toxin action and that these residues are not involved in the ADP-ribosylation reaction.

The role of the carboxyl-terminal residues in toxin action was further defined by creating a series of internal deletions and substitutions (Table 2). These mutations began at amino acid 602, so that ADP-ribosylating activity would not be

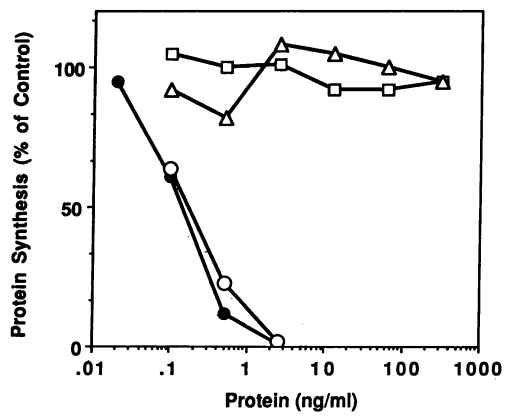

FIG. 1. Cytotoxicity of PE and PE mutants on Swiss mouse 3T3 cells. Various dilutions of PE proteins in phosphate-buffered saline containing 0.2% human serum albumin were added to Swiss 3T3 cells (10^5 cells per well) in 24-well plates. Sixteen hours later the cells were metabolically labeled with [^{3}H]leucine for 90 min, and trichloroacetic acid-precipitable cell-associated radioactivity was determined as a measure of protein synthesis. The results are expressed as percent of control where no toxin was added. ●, PE; ○, PEΔ613; □, PEΔ612,613; △, PEΔ611–613. All the assays were done in duplicate and repeated twice.

Table 2. Internal deletions and substitutions within the carboxyl terminus of PE

pVC plasmid	Carboxyl-terminal sequence* (601 A S Q P G K P P R E D L K 613)	Cytotoxicity, % of PE
45	A S Q P G K P P R E D L K	100
49215	A L K	<0.1
49235	A G K P P R E D L K	100
49245	A S Q P G R E D L K	100
49255	A S Q P G E D L K	0.3
4955	A S Q P G P K P R E D L K	100
4935	A S G S H L A A R E D L K	100
4955	A S E G K S S G R E D L K	100
49315	A S Q P G M M M R E D L K	100

Mutant PE proteins were expressed in *E. coli* and purified from the periplasm. ADP-ribosylation activities of all the mutants were indistinguishable from that of the full-length PE.

*Amino acids within the carboxyl end of PE (residues 601–613) and PE mutants are shown as single-letter code. The substitutions have been underlined. Blanks indicate deletions.

affected, and extended to position 611. We found that several small deletions encompassing amino acids 602–604 and 606–608 did not reduce cytotoxicity. Furthermore, two substitutions that altered amino acids 603–608 as well as two other substitutions within amino acids 606–608 of PE did not reduce cytotoxicity. Therefore, the sequence of amino acids in positions 602–608 did not appear to be important for cytotoxicity. However, deletions that removed Arg-609 (pVC49215 and pVC49255) greatly reduced the cytotoxic action of PE. These results, together with the experiments in Table 1 showing that deletion of amino acids 612 and 613 abolishes cytotoxicity, focused our attention on amino acids 609–613, which are situated at the carboxyl terminus of PE.

The role of Arg-609 was studied by either deleting it or replacing it with several different amino acids. Replacement with another basic amino acid, lysine, did not reduce the cytotoxic activity of PE (Table 3). However, deleting Arg-609 (pVC49115) or replacing it with glycine, glutamic acid, or leucine reduced cytotoxicity by a factor of 6–10. Thus, a basic amino acid appears to be important at position 609.

To study the sequence specificity of the last 5 amino acids of PE, several other mutant molecules were then constructed. In two of these, the order of the acidic amino acids at positions 610 and 611 was reversed and Lys-613 was deleted (Table 4, pVC49415 and pVC49425). These molecules were fully active whether residue 609 was a lysine or an arginine. We also created a molecule with a leucine at position 609 and an arginine at 612 (pVC49435) that was inactive.

Although deletion of the terminal amino acid, Lys-613, did not affect cytotoxicity, we suspected that other mutations in this position might affect cytotoxicity because of the low activity of various chimeric toxins in which the ligand was placed in peptide linkage at the carboxyl terminus of PE (ref. 11 and unpublished data). Therefore, Lys-613 was converted to glutamine, asparagine, or aspartic acid. Each of these mutations produced a less cytotoxic molecule (Table 5).

Table 3. Mutations at position 609 of PE

pVC plasmid	Mutant protein	Cytotoxicity, % of PE
49115	PEΔ609	12
49125	[Lys^{609}]PE	100
4915	[Gly^{609}]PE	10
49135	[Glu^{609}]PE	16
49155	[Leu^{609}]PE	15

Mutant PE proteins were expressed in *E. coli* and purified from the periplasm.

Table 4. Sequence specificity of last 5 amino acids of PE

pVC plasmid	Carboxyl-terminal sequence (609 R E D L K 613)	Cytotoxicity, % of PE
45	R E D L K	100
49125	K E D L K	100
4215	R E D L	100
49415	K D E L	100
49425	R D E L	100
49435	L D E R	<0.03

For details see legends to Tables 1 and 2.

Addition of 6 or 11 amino acids to the carboxyl terminus of PE also produced a less cytotoxic molecule (data not shown). However, replacement of Lys-613 with the basic amino acid arginine did not decrease cytotoxicity. Thus, positions 609 and 613 both require a basic amino acid for full cytotoxic activity. There are two other lysine residues within the carboxyl end of PE; these are situated at positions 590 and 606. Both of these lysines could be converted to the uncharged amino acid glutamine without a decrease in cytotoxicity, showing that a positively charged amino acid was not required at position 590 or 606 (Table 5).

Having shown the importance of the amino acids at the carboxyl terminus of PE, we wished to determine whether the carboxyl-terminal 5 amino acids could be separated from the ADP-ribosylation domain to regenerate an active toxin. As shown in Table 6, a fully active cytotoxic molecule could be generated from PEΔ609–613 (which is not cytotoxic) by the addition of amino acids 551–613, 567–613, or 598–613 of an intact PE to the carboxyl terminus of PEΔ609–613. Thus, the distance between the ADP-ribosylation domain, which ends around amino acid 600, and the essential amino acids at positions 609–613 was not critical and could be substantially

Table 5. Mutations of lysines at positions 590, 606, and 613 in the carboxyl-terminal domain of PE

Mutant protein	Cytotoxicity, % of PE	ADP-ribosylation, % of PE
PEΔ613	100	100
[Arg^{613}]PE	100	100
[Gln^{613}]PE	1	100
[Glu^{613}]PE	1	100
[Asn^{613}]PE	1	100
[Gln^{606}]PE	100	100
[Gln^{590}]PE	100	100
[Gln^{590,606,613}]PE	1	100
[Gln^{590,606},Arg^{613}]PE	100	100

Analyses were as described in legends to Tables 1 and 2.

Table 6. Addition of various portions of the PE carboxyl terminus to PEΔ609–613

pVC plasmid	Mutant protein	Cytotoxicity, % of PE
4905	PEΔ609–613	<0.1
4975	PEΔ609–613+(551–613)	100
4985	PEΔ609–613+(567–613)	100
4995	PEΔ609–613+(598–613)	100
4715	PEΔ609–613-RPHMPGDILK	<0.1
47195	PEΔ608–613-RPHMPGD+(598–613)	50

A plasmid (pVC4915) with a *Sma* I site between codons 608 and 609 of PE was created and various portions of the carboxyl-terminal coding sequence were attached after codon 608. pVC4995 was constructed by using synthetic oligonucleotides. The last 16 amino acids (residues 598–613) of PE are PDYASQPGKPPREDLK (also see Tables 1 and 2). All the mutants had full ADP-ribosylation activity (100% that of PE).

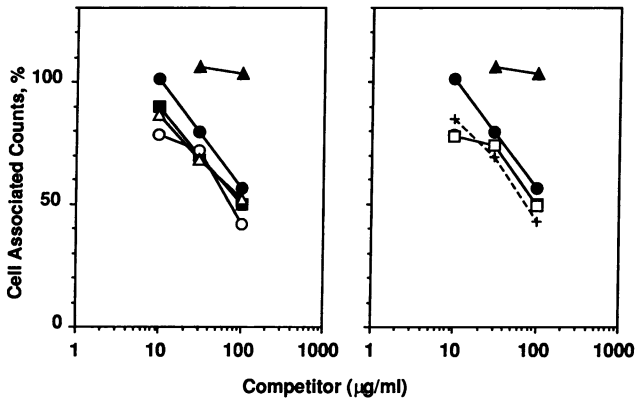

FIG. 2. Competition for cellular uptake of recombinant PE. Swiss mouse 3T3 cells were incubated with 400 ng of ^{3}H-labeled PE (specific activity, 3.5×10^5 dpm/μg) and various concentrations of purified mutant proteins for 1 hr at 37°C. Cell monolayers were washed and cell-associated radioactivity was determined. ●, PE; ▲, [Glu^{57}]PE; △, PEΔ612,613; ○, PEΔ613; ■, [Gly^{276}]PE; □, PEΔ609–613; +, PEΔ609–613+(598–613).

increased without a decrease in cytotoxicity. Also shown in Table 6 is a PE molecule with RPHMPGDILK (pVC4715) at the carboxyl terminus in place of PREDLK. This molecule, in which Arg-609 and Asp-611 were altered, was not cytotoxic. But attaching the last 16 amino acids of an intact PE molecule to give a carboxyl terminus of RPHMPGDPDYASQPGKPPREDLK (pVC47195) restored cytotoxicity to this molecule. The data clearly show that cytotoxic activity can be restored by attaching an intact carboxyl end to a PE molecule that is inactive due to a deletion or modification within the carboxyl end. It is now possible to create active chimeric molecules by inserting a binding ligand such as TGFα at 608 within the carboxyl end of PE, thus retaining the last 5 amino acids as REDLK.

Although it was previously demonstrated that domain I of PE is the domain responsible for cell binding, we felt it was important to show that the mutations at the carboxyl end of PE that decreased cytotoxicity did not also somehow decrease cell binding. To test this, the ability of various mutant forms of PE to compete for the uptake of ^{3}H-labeled PE was evaluated. As shown in Fig. 2, several PE mutants that had decreased cytotoxicity due to mutations at the carboxyl terminus were just as able to compete for the uptake of ^{3}H-labeled PE as authentic wild-type PE. In this competition assay, PE40, which lacks domain I, and [Glu^{57}]PE, with a point mutation in domain I, were inactive, as reported previously (9).

These uptake results were confirmed by a fluorescence assay that measured the internalization of PE and various mutant PE molecules (Fig. 3). In this assay, cells are incubated with various toxins for 30 min to allow binding and internalization into endocytic vesicles. [Glu^{57}]PE and PE40 were not internalized. In contrast, all the other PE molecules,

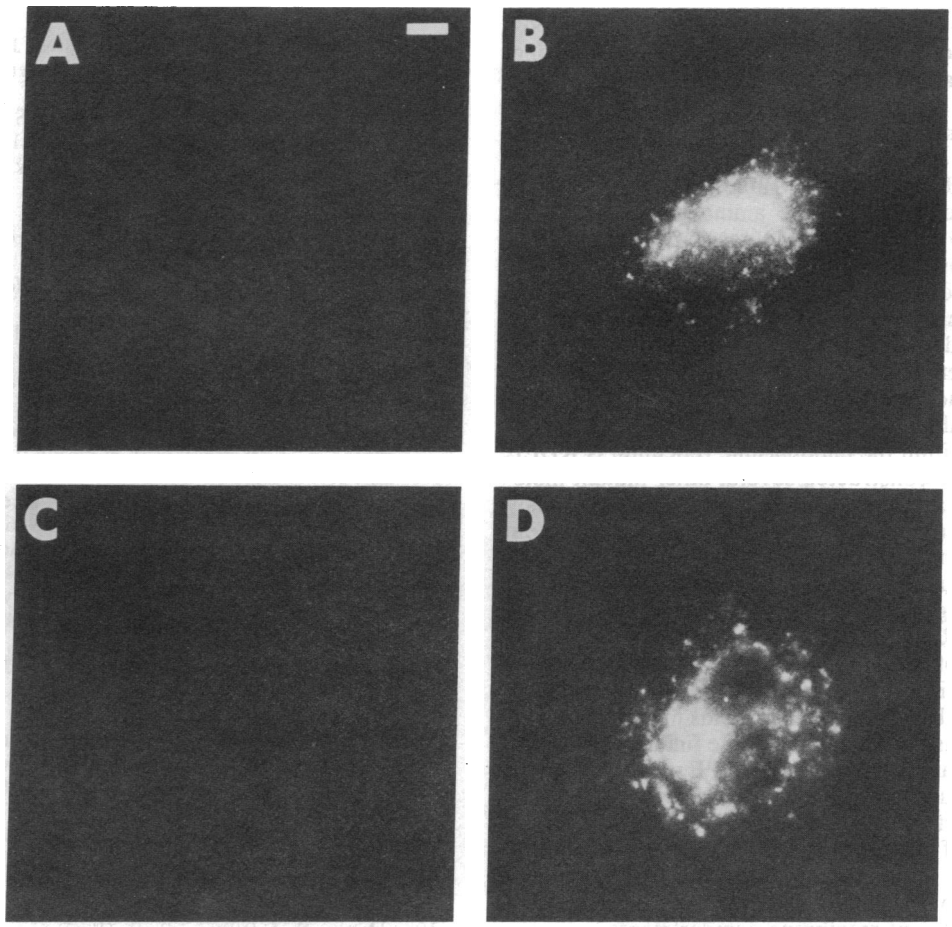

FIG. 3. Immunofluorescence detection of binding and internalization of PE and its recombinant variants in Swiss 3T3 cells. Cells were incubated at 37°C for 30 min in the absence of toxin (*A*) or in the presence (10 μg/ml) of native PE (*B*), recombinant [Gly^{57}]PE (*C*), or recombinant PEΔ612,613 (*D*). Following this incubation, the cells were fixed in formaldehyde and further incubated in the continuous presence of saponin. The cells were incubated with mouse monoclonal anti-PE (M40-1, 10 μg/ml), followed by affinity-purified rhodamine-labeled goat anti-mouse IgG (25 μg/ml). (Bar = 10 μm.)

whether or not they contained mutations at the carboxyl end of domain III, were found to have bound and been internalized into endocytic vesicles and other elements in the trans-Golgi system in the perinuclear area of the cells (Fig. 3 *B* and *D*). These results clearly show that decreased cytotoxicity of carboxyl-terminal mutants is not due to decreased receptor binding or cellular uptake of PE molecules.

DISCUSSION

We have shown that mutations at the carboxyl end of PE and particularly in the last 5 amino acids of PE result in molecules with full ADP-ribosylation activity but greatly reduced cytotoxicity. Depending on the type of mutation introduced, cytotoxicity was often undetectable and at a minimum was decreased 6-fold. PE must undergo several steps in order to intoxicate a cell. These include (*i*) binding to a cell surface receptor, (*ii*) internalization via coated pits into endocytic vesicles and perhaps other endocytic compartments, (*iii*) translocation of a portion of PE into the cytosol, which probably requires proteolytic processing of PE, and finally (*iv*) ADP-ribosylation of elongation factor 2. Here we show that carboxyl-terminal mutants of PE have retained their capacity to bind to 3T3 cells (Fig. 2) and to be taken up into endocytic vesicles (Fig. 3). Furthermore, after 30 min of uptake there was no obvious difference in the pattern of the vesicles that contained PE or that contained several different carboxyl-terminal mutants of PE. This result indicates that the mutant toxins were located in the same intracellular compartment as native PE. Also, these mutants did not have any defect in ADP-ribosylation activity. Thus, the mutations at the carboxyl end of PE must affect some step occurring after internalization and before ADP-ribosylation. The most likely step affected by these mutations is the one in which PE is processed and translocated into the cytosol.

The amino acid sequence at the carboxyl end of PE is Arg-Glu-Asp-Leu-Lys (REDLK, Table 2). Arg-609 can be replaced by lysine, but nonbasic amino acids cannot be tolerated (Table 3). Lys-613 is not essential and can be deleted without loss of cytotoxic activity (Table 1), but it cannot be replaced with a nonbasic amino acid (Table 5). Thus, having either REDL or KEDLK at the carboxyl terminus produced a fully cytotoxic molecule (Table 4). A search of the literature for similar sequences that were present in other molecules and performed a specific biological function revealed that the sequence which retains newly formed proteins within the endoplasmic reticulum is KDEL (19). Therefore, we constructed several other mutant molecules, one of which contained the exact sequence previously described as being responsible for the retention of the protein in the lumen of the endoplasmic reticulum (Table 4). We found that a molecule ending with KDEL was fully cytotoxic. A molecule ending with RDEL was fully active, but one ending with LDER was not. These findings suggest that the successful entry of PE into the cytosol from an endocytic compartment requires interaction with a cellular component similar to the one that helps retain proteins made by the cells within the endoplasmic reticulum. These findings also imply that the sequence at the carboxyl end of PE acts as some type of recognition sequence to assist translocation of PE from an endocytic compartment into the cytosol. The nature and cellular location of the molecule that might interact with this portion of PE are not known. The cellular component that assists translocation could be found either in the cytosol or in one of several different membrane compartments.

Other mutations that decrease the cytotoxic effect of PE and do not affect binding to the surface of the cell include those located in domain II. In particular, arginines at positions 276 and 279 are required for full cytotoxicity and these cannot be changed, even to lysines, without a dramatic decrease in activity (8). A proteolytic clip occurs within domain II near Arg-276 of native PE (M. Ogata, V.K.C., I.P., and D.F., unpublished data). It is likely that a fragment consisting of a part of domain II as well as all or most of domain III is translocated into the cytosol. This implies that at least two specific recognition events occur within the cells, one in the center of domain II that leads to processing and one at the carboxyl end of domain III. Diphtheria toxin also undergoes processing in order to reach the cytosol (1). However, diphtheria toxin does not contain a sequence that closely resembles REDLK, indicating that it probably has a different mechanism of cellular entry. A search of a protein data base (National Biomedical Research Foundation, August 1989) for the presence of similar sequences showed that the A chains of cholera toxin and *E. coli* heat-labile toxin contain KDEL and RDEL as their last 4 amino acids, respectively. It is possible that other toxins may have similar sequences at the carboxyl terminus and use similar mechanisms to translocate into the cytosol.

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