Alteration of a protease-sensitive region of *Pseudomonas* exotoxin prolongs its survival in the circulation of mice

(immunotoxin/cancer/protein engineering/trypsin/plasmin)

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ABSTRACT *Pseudomonas* exotoxin A (PE) is a single-chain 66-kDa polypeptide that kills eukaryotic cells by ADP-ribosylation of translational elongation factor 2. PE is composed of three major structural domains whose functions are binding of cells (I), translocation (II), and ADP-ribosylation (III). Here we describe a protease cleavage target that is located near arginine-490 on the surface of domain III. We made several different types of mutations near arginine-490. Deletion of arginine-490 or replacement of arginine-490 and -492 with serine and lysine or with two lysines resulted in protease-resistant molecules that were fully cytotoxic and had normal ADP-ribosylation activity. However, the half-life in mouse blood of the PEΔ490 mutant was 24 min whereas that of PE was 13 min. Furthermore, two PE mutants that were protease-hypersensitive, PEGlu246,247,249 and PEGlu57,246,247,249 (in which glutamate residues replace basic residues at the indicated positions), had very short half-lives. These data indicate that protease sensitivity is an important determinant in the half-life of PE in the circulation and suggest that the half-life of other proteins may be prolonged by removal of protease sites. Deletion of arginine-492 or the replacement of amino acids 486–491 with three glycines markedly diminished ADP-ribosylation activity and cytotoxicity, indicating that this region of domain III is also important for catalytic activity.

Pseudomonas exotoxin A (PE) (1, 2) is a single-chain protein that is arranged into three major structural domains and is able to kill eukaryotic cells by ADP-ribosylation and inactivation of elongation factor 2 (EF-2) (3). Domain I of PE is responsible for cell binding, domain III contains the ADP-ribosylating activity, and domain II has an important role in translocating the ADP-ribosylating region of the toxin into the cytosol (4–7). To kill cells, PE must undergo a series of steps that involves conformational changes and complex processing in order for the ADP-ribosylating activity present in domain III to reach the cytosol of cells, where EF-2 is located (6, 7). Intoxication begins with internalization of the toxin into endocytic vesicles; then proteolytic cleavage occurs near arginine-279, generating a 37-kDa fragment that is translocated to the cytosol (7). In addition, PE has other protease-sensitive sites, but these are not believed to be essential for the activation of the toxin. Of special interest is the finding that limited digestion with trypsin at pH 7.4 generates a 55-kDa fragment due to a cleavage in domain III (7, 8).

Toxins such as PE, ricin, and diphtheria toxin have been coupled to antibodies to create immunotoxins and are being developed as therapies to treat cancer and other diseases (9–11). Toxins have been genetically altered and fused to growth factors or other targeting ligands to make chimeric toxins. For example, PE40, a modified form of PE in which the binding domain has been deleted, has been fused to transforming growth factor α; interleukins 2, 4, and 6; insulin-like growth factor 1, CD4, or single-chain antibodies to kill target cells with receptors for these ligands on their surfaces (10, 12).

Because immunotoxins and chimeric toxins are given intravenously for therapeutic applications, understanding the mechanisms that influence the lifetime of these proteins in animals is of special interest. If the factors that affect the lifetime were known, it should be possible to create altered molecules with a more desirable lifetime. Several factors are thought to control the lifetime of proteins in the circulation, including binding to tissues, filtration in the kidney, and inactivation or degradation of proteins by proteolysis. Our hypothesis is that molecules that are sensitive to proteases can be rapidly proteolyzed within the vascular system and display a short lifetime, whereas molecules that are protease-resistant should have a longer survival in the circulation. Recent studies with mutated tumor necrosis factor α molecules are in accord with this hypothesis (G. Keilhauer, personal communication).

In this study, we have treated PE with trypsin, plasmin, and thrombin to determine whether the native molecule contains any regions that are particularly sensitive to these proteases. We find that all three proteases cleave the molecule at a site close to the active site for ADP-ribosylation. Proteolytic susceptibility appears to be a factor influencing the lifetime of PE in mice, because mutations that change the proteolytic sensitivity of the molecule can alter its lifetime.

MATERIALS AND METHODS

Plasmids were propagated, site-directed mutagenesis was performed, and proteins were expressed as described (6, 13).

PE was harvested from the periplasm by osmotic shock and purified by Mono Q ion-exchange chromatography (5). To obtain radiolabeled PE, cells were grown as described (7) with 200 μCi (7.4 MBq) of [^{3}H]leucine. The proteins in the periplasm were bound on QMA cartridges (Waters) and washed with 5 ml of 20 mM Tris, pH 7.4, followed by 5 ml of 100 mM NaCl/20 mM Tris, pH 7.4. PE was eluted with 250 mM NaCl/20 mM Tris, pH 7.4, and further purified on a TSK 250 column. A single band (66 kDa) was obtained after SDS/PAGE and radiography. To preserve activity, human serum albumin (HSA, 0.2% final concentration) was added.

ADP-ribosylation assays using wheat germ extracts were carried out as described (14). Unless noted otherwise, the samples were activated with 4 M urea and 40 mM dithiothreitol.

Protein-synthesis inhibition assays were performed on L929 mouse fibroblasts (5, 15).

Abbreviations: PE, *Pseudomonas* exotoxin; EF-2, elongation factor 2; HSA, human serum albumin.
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To determine protease sensitivity, 10 μg of toxin was added to 50 μl of phosphate-buffered saline (PBS: 150 mM NaCl/1.7 mM KH_2PO_4/5 mM Na_2HPO_4, pH 7.4) containing 1 mM EDTA and 0.5 unit of trypsin (Sigma), plasmin, or thrombin (Boehringer Mannheim). After 1 hr at 20°C, for trypsin and thrombin, or 2 hr, for plasmin, equal amounts of protein were analyzed by SDS/PAGE (16).

Female BALB/c mice, 17–18 g, were used to determine animal toxicity (LD_{50}) and plasma lifetime. To determine LD_{50}, PE molecules diluted in PBS/0.2% HSA were injected intraperitoneally into 6-week-old mice. To analyze the pharmacokinetics, groups ($n = 8$–12 mice), of 10- to 12-week-old mice were used, with 10 μg of toxin in 200 μl of PBS/0.2% HSA injected into the tail vein. Two minutes later the first blood samples (about 70 μl) were collected from the retroorbital plexus. In these samples, toxin was always between 6 and 7 μg/ml. Each mouse was bled no more than three times in the first 40 min and no more than four times in total. The samples were allowed to clot on ice and were centrifuged to obtain the serum. Toxin concentrations in sera were determined by (i) a cytotoxicity assay, in which various dilutions of the serum were incubated with L929 cells and the toxin concentration was determined by comparison with a standard curve of toxin in normal mouse serum; (ii) injecting ^{3}H-labeled toxins and determining the radioactivity in 15 μl of serum dissolved in 10 ml of scintillation fluid; and (iii) ELISA in which 96-well plates were coated with a 1:2000 dilution of goat serum containing anti-PE antibodies and then were blocked with 3% gelatin in PBS. Dilutions of samples and standard proteins in PBS and mouse serum were added, followed by incubation with polyclonal rabbit anti-PE antibodies and goat anti-rabbit IgG coupled to peroxidase. In between the incubation steps (1 hr each, 37°C), the plates were washed three times with PBS/0.05% Tween 20 and, before the peroxidase reaction, three extra times with PBS. A solution containing 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) and H_2O_2 was then added. After 5 min, the reaction was stopped with 100 μl of 10% SDS, and the absorbance at 405 nm was read. The concentration of PE in the samples was quantitated by comparison of sample dilutions to a standard curve of absorbance vs. concentration of PE.

RESULTS

Because toxins are being used for treatment of cancer and other diseases, it is important to understand what controls the half-life of these agents in the circulation. To investigate the possibility that proteases contribute to the rapid clearance of PE from the circulation, we determined the sensitivity of PE to trypsin, plasmin, and thrombin. All three proteases yielded a 55-kDa fragment. However, thrombin, because it cuts at additional sites, generated only a small amount of the 55-kDa fragment, along with many smaller fragments (Fig. 1). The fragment generated by trypsin digestion was isolated, subjected to N-terminal sequencing, and shown to possess the same N-terminal sequence as untreated PE (2).

Since plasmin and thrombin cleave proteins preferentially at arginine residues, we examined PE for the presence of arginine residues at or near residue 500. If the average amino acid in PE has a molecular mass of 110 Da, an arginine near position 500 might serve as the cleavage site that would produce the 55-kDa N-terminal fragment. There are arginines at positions 500, 505, and 513, but from the x-ray crystallography data these are not prominently exposed on the surface of PE and should not be easily accessible to proteases (1). There is also a cluster of arginines at positions 490, 492, and 494. Arginine-494 is partially buried within the PE molecule, but the two other arginines are in a group of amino acids, 485–492, whose location was not determined in the x-ray crystallographic model of PE (1). Because the adjacent amino
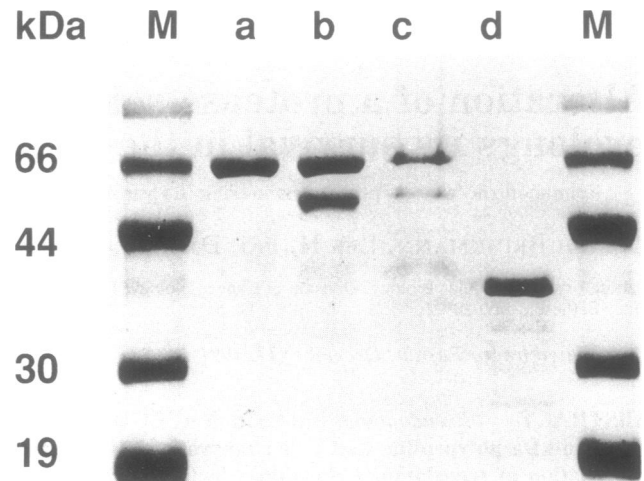
FIG. 1. Reducing SDS/12.5% PAGE analysis of PE samples before and after incubation with proteases at pH 7.4. Lanes: M, molecular size markers; a, untreated PE (66 kDa); b, after trypsin digestion (66 kDa and 55 kDa fragment); c, plasmin digestion; d, thrombin digestion.

acids at positions 484 and 493 lie on the surface of the PE molecule, we assume that these residues were difficult to resolve into a structure, because they are arranged in a flexible conformation or loop on the surface of the PE molecule (Fig. 2).

It seemed possible that this exposed structure might contain a predominant protease target. However, we could not detect a discrete 11- to 12-kDa fragment as the corresponding part to the 55-kDa fragment and obtain its N-terminal sequence to identify the cleavage site. Therefore we took an alternative genetic approach and made mutations that either deleted or replaced arginines 490 and 492. We also removed the entire loop region and replaced it with three glycine residues (Table 1).

PE Mutants with Altered Protease Sensitivity. Each mutant molecule was produced in *Escherichia coli* with expression levels similar to that of native PE, and all molecules were secreted into the periplasm where they were present in an unaggregated state. Each molecule was purified to homogeneity and treated with different proteases. When the molecule in which the loop was deleted and replaced with three glycines (PEΔ485–492/3G) was treated with trypsin or plasmin, very little of the 55-kDa fragment was generated (Fig. 3; Table 1). The trypsin resistance of the molecules (PEΔ490, PES490K492, and PEK490K492) with arginine residues de-
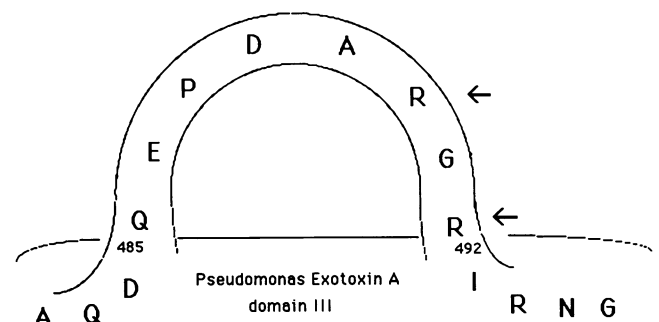
FIG. 2. An exposed structure on the surface of the enzymatic active domain III of PE. The proposed "loop" includes the amino acids from glutamine-485 to arginine-492. The positions of aspartate-484 and isoleucine-494 are defined; the backbone at these positions is facing outward from the molecule. Arrows indicate potential exposed targets for proteases. Arginine-494 is also on the surface but is not prominently exposed.

Table 1. Characterization of PE mutant molecules

| Protein | Plasmid | Sequence | 55-kDa fragment | | ID$_{50}$, ng (% PE) | Mouse LD$_{50}$, μg |
			Trypsin	Plasmin		
PE	pVC45F+T	$\overset{485}{D}$QEPDAR$\overset{492}{G}$RIR	++	+	0.3 (100)	0.2
PEΔ490	pPE45Δ490	DQEPDA–GRIR	–	–	0.3 (100)	0.2
PES490K492	pPE45SK490/2	DQEPDASGKIR	–	–	0.3 (100)	0.2
PEK490K492	pPE45KK490/2	DQEPDAKGKIR	–	(+)	0.3 (100)	ND
PEK490	pPE45K490	DQEPDAKGRIR	(+)	++	0.3 (100)	ND
PEΔ492	pPE45Δ492	DQEPDARG–IR	++++	–	3.0 (10)	1.5
PEΔ485–492/3G	pPE45Δ485/92G3	D——GGGIR	–	–	70 (0.4)	10

Amino acid substitutions in the proposed loop region in domain III are underlined. The proteolysis results are from Figs. 1 and 3, and the data from protein-synthesis inhibition assays (ID$_{50}$) are from Fig. 4. ND, not done.

leted or replaced by lysine enabled us to identify arginine-490 as the trypsin target. A plasmin cleavage site is located at arginine-492, since PEΔ492 was completely resistant to plasmin cleavage but still very sensitive to cleavage by trypsin. PES490K492 was also resistant to plasmin cleavage. Cleavage by plasmin was dependent on adjacent sequences: PEΔ490, which still has arginine-492, was not hydrolyzed by plasmin. In summary, these studies indicate that the 55-kDa fragment of PE is generated by cleavage at position 490 preferentially with trypsin and at position 492 preferentially with plasmin.

Also shown in Fig. 3 is the protease digestion result with PEGlu57,246,247,249, a mutant form of PE that has low animal and cellular toxicity (17). This molecule, as well as PEGlu246,247,249 (data not shown), was very sensitive to protease digestion, with very little undigested PE or 55-kDa fragment remaining after even short incubation periods.

Toxic Activity of PE Mutants. Most of the mutated PE molecules did not show any unusual properties in the purification procedure or in their ability to kill cells. This was so despite the fact that alterations were made in the ADP-

ribosylation domain of PE (4). However, we also produced two mutant molecules that had diminished or undetectable ADP-ribosylation activity and, therefore, reduced cellular toxicity. ADP-ribosylation activity and cytotoxicity was reduced by 90% in PEΔ492, and in PEΔ485–492/3G there was no ADP-ribosylation activity detected and cytotoxicity was reduced by >99% (Figs. 4and 5; Table 1). In animal studies, PEΔ492 showed an ≈8-fold reduction in toxicity and PEΔ485–492/3G about 50-fold (Table 1). We also noticed a slight reduction in the ADP-ribosylation activity of PEΔ490 and PES490K492, although these mutants had full cytotoxic activity (Table 1).

Effect of Mutations on the Survival of PE in the Circulation of Mice. To analyze whether the removal of protease recognition sites influences the lifetime of PE in animals, blood levels of PE were determined at various times after intravenous injection of either 10 μg of PE or 10 μg of several of the mutant toxins. The concentration of PE molecules in the circulation was determined by three different methods; a cytotoxicity assay, ELISA (when mutant molecules with reduced cytotoxic activity were used), or by the injection of PE molecules labeled uniformly with [^{3}H]leucine.

PE was found to have a half-life in mice ($t_{1/2}$) of 13–20 min depending on which assay was used to quantify the toxin (Fig. 6 and Table 2). The use of ^{3}H-labeled toxin gave the most reproducible results, probably because there was less sample processing in this assay. This method was chosen for routine use with toxins that could be easily metabolically labeled and purified from the periplasm. For the plasma decay studies, we chose to analyze PEΔ490, which is resist-
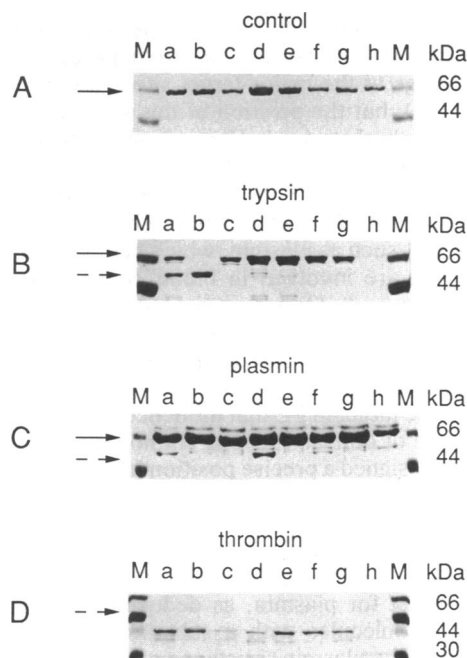

control

FIG. 3. Cleavage of PE and PE mutants by serine proteases. PE (lanes a), and mutants PEΔ492 (lanes b), PEΔ485–492/3G (lanes c), PEK490 (lanes d), PEΔ490 (lanes e), PES490K492 (lanes f), PEK490K492 (lanes g), and PEGlu57,246,247,249 (lanes h) were incubated with trypsin (*B*), plasmin (*C*), or thrombin (*D*) or left untreated (*A*) and were analyzed by reducing SDS/PAGE in 12.5% (*A–C*) or 18% (*D*) gels. Lanes M, molecular size standards. Arrows mark the 66-kDa PE protein and the 55-kDa fragment (broken arrows).
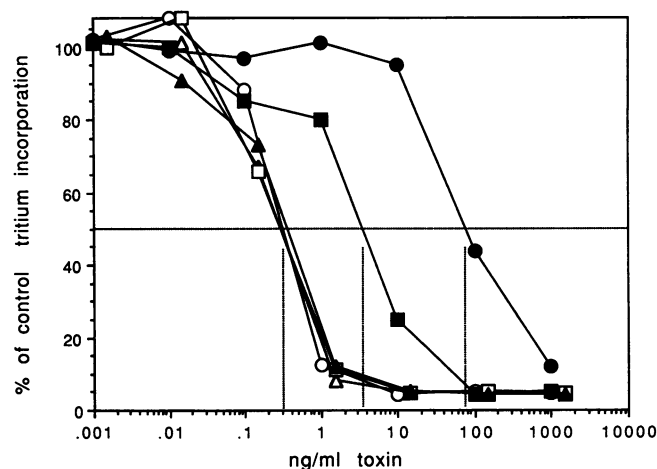

FIG. 4. Inhibition of protein synthesis by PE and mutant molecules. Inhibition of protein synthesis was assayed in L929 mouse fibroblasts. ID$_{50}$ is the concentration of toxin that reduces the ^{3}H incorporation to 50% of the control and was determined in at least two experiments, using three samples for each point. ○, PE; △, PEΔ490; □, PES490K492; ▲, PEK490K492; ■, PEΔ492; ●, PEΔ485–492/3G.
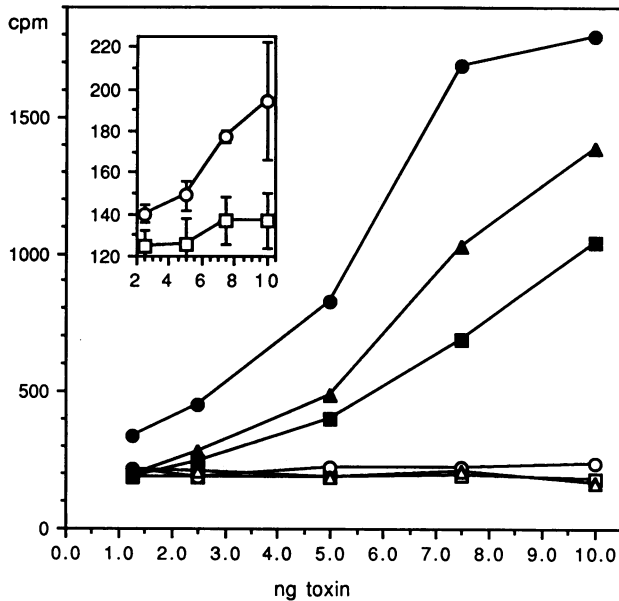
FIG. 5. ADP-ribosylation of eukaryotic EF-2 by PE and derivatives. All assays were performed as described (14) in duplicate. (*Inset*) Enlargement of the range below 250 cpm of samples PEΔ553 and PEΔ492 to show the remaining activity of PEΔ492. □, PEΔ553; △, PEΔ485–492/3G; ○, PEΔ492; ■, PES490K492; ▲, PEΔ490; ●, PE.

ant to both trypsin and plasmin yet is fully cytotoxic. PEΔ490 has a significantly longer survival in the circulation of mice than PE, with a $t_{1/2}$ of 24 min compared with a $t_{1/2}$ of 13 min for PE (Fig. 6). The *P* values calculated by Student's *t* test from this data are shown in the legend to Fig. 6. At every point the differences were highly significant.

Half-Life of Other PE Mutants in the Circulation of Mice. We previously described other mutant forms of PE that have diminished cellular cytotoxicity. One of these is PEGlu246,247,249. In this molecule, three basic amino acids in domain I are replaced by glutamates interrupting hydrogen bonds that connect domains Ia and II (17). Another molecule is PEGlu57,246,247,249, in which lysine-57, which is required for cell binding is also replaced by a glutamate. When these molecules were injected into mice, both molecules were
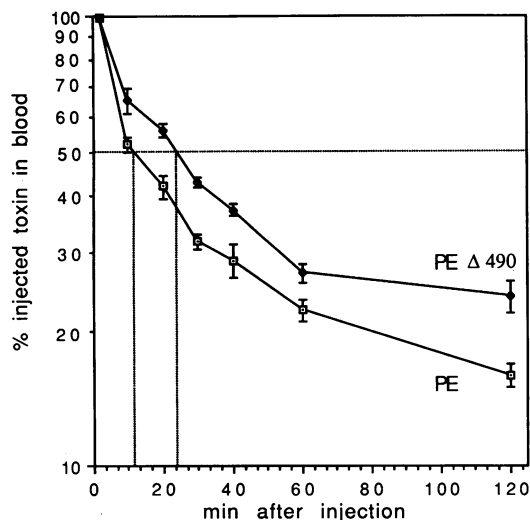

FIG. 6. Lifetime of PE and mutant PEΔ490 in mice. The amount of toxin at each time point represents the blood level of six mice; bars describe the standard error of the samples. The *P* values at 10, 20, 30, 40, 60, and 120 min were 0.017, 0.0017, 0.00045, 0.01, 0.026, and 0.0042, respectively.

Table 2. Half-lives in BALB/c mice

Molecule	$t_{1/2}$, min		
	^{3}H	ELISA	Cytotoxicity
PE	13	20	18
PEΔ490	24	ND	33
PES490K492	ND	ND	35
PEGlu246,247,249	ND	7	6
PEGlu57,246,247,249	ND	8	ND

Half-lives were determined by the three assays described in *Materials and Methods*. ND, not done.

found to have shorter half-lives in the circulation than native PE (Table 2). With the ELISA, the $t_{1/2}$ of PE was 20 min whereas the $t_{1/2}$ of PEGlu246,247,249 was 7 min, and the $t_{1/2}$ of PEGlu57,246,247,249 was 8 min. Thus, these mutations, which increased the sensitivity of PE to proteolysis (Fig. 3), shortened the half-life of PE in the circulation of mice.

DISCUSSION

We have found that mutations which eliminate a protease recognition site (region) in domain III of PE prolong the survival of PE in the circulation of mice. Although this region of PE is important for the ADP-ribosylation activity, we were able to mutate certain residues at the cleavage site without losing cytotoxic and ADP-ribosylation activities and thereby creating mutant biologically active molecules that were more stable to proteolytic attack than native PE. Although those molecules had a significantly longer $t_{1/2}$ than PE, they were not more toxic to mice. Since molecules that are present in the blood for a longer period of time should have an increased chance to intoxicate cells, this finding suggests that elimination of the protease recognition site might also diminish interactions that contribute to animal toxicity.

Treatment of PE with trypsin at neutral pH produces several proteolytic products. A minor cleavage site is close to arginine-279, a residue essential for toxin processing by cells (6, 7). Cleavage at the major target site generates a 55-kDa fragment (7, 8), but the position of this site and its function had not been characterized. Here we show that this major protease target is in domain III, which contains the ADP-ribosylating activity of PE. We observed that this target was recognized not only by trypsin but also by proteases that are found in blood, such as plasmin, which belongs to a family of proteases that are involved in blood coagulation and clot lysis. Other enzymes of this group include tissue plasminogen activator (tPA), thrombin, and other coagulation factors. They act at similar target sites as trypsin and might influence the lifetime of PE in the circulation of animals.

The protease target in PE that influenced half-life is located on the surface of domain III. The amino acids in this region could not be assigned a precise position in the structure of PE (1). We suggest they might be arranged in a flexible loop on the surface of PE and therefore be very susceptible to proteolysis. There are two protease recognition sites in this loop; R490 is the major target for trypsin and R492 is probably the major target for plasmin, as deduced from the trypsin resistance of molecules with a substitution or deletion of R490 and from the plasmin resistance of molecules that were mutated at R492 (Table 1). We believe that these mutant molecules still have the native conformation of PE except at the position of the mutation, because they are secreted into the periplasm of *E. coli* like PE, behave in an identical manner to PE in the purification procedure, and cannot be distinguished from PE in cytotoxicity assays, which are very sensitive indicators of changes of the PE structure (17). The context of the plasmin target, R492, shows homology to proteolytic processing sites of serum proteases which are

involved in the clot lysis cascade. In PE, the site is GR/IRNG, which is similar to the plasmin-processing site GR/VVGG (18) or the tPA-processing site FR/IKGG (19). Thus, in addition to exposure in the loop structure, the amino acid context of the protease target in PE is probably important for recognition and cleavage. This is consistent with the observation that changes in the amino acids around the PE protease target site not involving the cleavage site itself can enhance or reduce proteolysis (see Fig. 3, PEΔ492 cleaved by plasmin or PEK490 cleaved by trypsin).

The region of the PE molecule harboring the protease target was also found to be important for the toxicity of PE. Some mutations not only affected the protease susceptibility but also reduced the ability of the PE molecule to transfer the ADP from NAD to EF-2. The three-dimensional structure of PE (1) indicates that the described protease site is on the same side of domain III as the proposed active center. However, the distance of this structure from the active center (glutamate at 553; ref. 20) makes it unlikely that amino acids in this region interfere directly with the catalysis of ADP transfer. This region more probably participates in the binding of either NAD or EF-2 to the PE molecule.

The determination of the half-life of the protease-resistant molecules PEΔ490 and PES490K492, of native PE, and of the two protease-hypersensitive molecules PEGlu57,246,247,249 and PEGlu246,247,249 showed an inverse correlation of protease sensitivity with the lifetime in the blood of animals. The mutants PEGlu246,247,249 and PEGlu57,246,247,249 have a shorter lifetime in mice than native PE. They are hypersensitive to proteolysis (see Fig. 3), probably because they are more unfolded than PE as indicated by the observation that these molecules do not need activation with urea and dithiothreitol to express their ADP-ribosylation activity. In these molecules, extra protease targets must be exposed. On the other hand, PE molecules that are more protease-resistant than PE, such as PEΔ490 and PES490K492, have a prolonged half-life. The correlation between protease sensitivity and serum lifetime implicates proteolytic susceptibility as one important factor in controlling the lifetime of PE in animals. Our experimental results also indicate that the method of proteolytic digestion with serum proteases *in vitro* may be useful to predict the behavior (stability or clearance) of genetically engineered molecules in animals.

The protease-sensitive site near arginine-490 in domain III may also contribute to the toxicity of PE in animals. Because plasmin and thrombin are associated with the surfaces of liver cells and endothelial cells (21, 22), PE might become bound by their protease recognition sites to those cells. This would explain its rapid clearance from the blood, because the first contact of intravenously injected proteins is with endothelial cells and liver cells because the liver, unlike most tissues, has openings between the endothelial cells, giving access to hepatocytes. This might also explain the accelerated clearance of mutant molecules with extra exposed protease sites and the slower clearance of toxins in which the site has been removed. In addition, molecules could be bound by their protease recognition sites but might not necessarily be im-

mediately inactivated by cleavage and may even be proteolytically activated for example at the arginine-279 activation site. Changing lysine-57 of PE to glutamate decreased the binding and toxicity of PE toward cultured cells by 100-fold, yet the mutant molecule was only 5-fold less toxic to the liver of mice (5). This suggests that other regions of the PE molecule can bind to liver cells, and the current data suggest that exposed protease recognition sites have a role in this process.

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