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Translocation Mediated by Domain II of *Pseudomonas* Exotoxin A: Transport of Barnase into the Cytosol

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ABSTRACT: *Pseudomonas* exotoxin A (PE) is a protein toxin composed of three structural domains. Functional analysis of PE has revealed that domain I is the cell-binding domain and that domain III functions in ADP ribosylation. Domain II was originally designated as the translocation domain, mediating the transfer of domain III to the cytosol, because mutations in this domain result in toxin molecules with normal cell-binding and ADP-ribosylation activities but which are not cytotoxic. However, the results do not rule out the possibility that regions of PE outside of domain II also participate in the translocation process. To investigate this problem, we have now constructed a toxin in which domain III of PE is replaced with barnase, the extracellular ribonuclease of *Bacillus amyloliquefaciens*. This chimeric toxin, termed PE^{1-412}-Bar, is cytotoxic to a murine fibroblast cell line and to a murine hybridoma resistant to the ADP-ribosylation activity of PE. A mutant form of PE^{1-412}-Bar with an inactivating mutation in domain II at position 276 was significantly less toxic. Because the cytotoxic effect of PE^{1-412}-Bar was due to the ribonuclease activity of barnase molecules which had been translocated to the cytosol, we conclude that domain II of PE is not only essential but also probably sufficient to carry out the translocation process.

Pseudomonas exotoxin A (PE) is a 66 000-dalton (Da) Leppla, 1976) protein toxin that kills eukaryotic cells bearing specific receptors for the toxin (FitzGerald et al., 1983; Pastan et al., 1986). The toxin enters the cell via receptor-mediated endocytosis (FitzGerald et al., 1980), and a 37 000-Da carboxyl-terminal fragment is translocated into the cytosol (Ogata et al., 1990), where it catalyzes the ADP ribosylation of elongation factor 2 (EF-2) (Iglewski & Kabat, 1975), which leads to cell death. The three-dimensional structure of PE has been solved (Allured et al., 1986) and function assigned to each of the three domains (Hwang et al., 1987). Domain Ia (amino acids 1–252) is responsible for cell recognition and binding, and domain III (amino acids 405–613), for the ADP-ribosylation activity. No role for domain Ib (amino acids 365–404) has been determined (Siegall et al., 1989). Recently, the carboxyl terminus of PE was shown to be essential for cytotoxicity (Chaudhary et al., 1990). Mutational analysis of this region revealed that the last few amino acids of PE (...REDLK) are critical for cytotoxic activity, but have a role independent of ADP-ribosylation activity. Further investigations showed that the sequence REDLK could be replaced with KDEL, which is the endoplasmic retention signal sequence (Monro & Pelham, 1987). Toxins with this modified carboxyl end were more cytotoxic than their native counterparts (Seetharam et al., 1991). These results suggest that the carboxyl end of PE has a role in the translocation process, possibly via interaction with a cellular component involved in translocation of the toxin to the cytosol.

It was originally proposed that domain II (amino acids 253–364) was responsible for translocation across the cell membrane (Hwang et al., 1987) since a construct in which the first half of this domain was deleted had both cell binding and ADP-ribosylation activity but was not cytotoxic. Since these initial experiments, several studies have served to define further the regions of domain II that are necessary for cytotoxicity. The amino terminus appears to be necessary since deletion of amino acids 254–263 inactivates PE. Further deletion analysis has shown that amino acids 346–364 can be deleted without loss of toxin activity (Siegall et al., 1989, 1991); further deletion of amino acids 344 and 345 resulted in complete inhibition of cytotoxic action (Siegall et al., 1991). In addition, arginine residues at positions 276, 279, 330, and

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337 are important for full toxicity (Jinno et al., 1989). Residues Arg-276 and Arg-279 are situated on a loop on the surface of domain II which is the site of intracellular processing (Ogata et al., 1990). This processing generates a 37-kDa fragment generated from the carboxyl end of the toxin consisting of a portion of domain II and all of domain III. This fragment is translocated to the cytosol. A role for domain Ia in the translocation process can be discounted, since this domain can be replaced with many other targeting moieties of diverse structure (FitzGerald & Pastan, 1989; Pastan & FitzGerald, 1989). However, we cannot discount the possibility that portions of domain III, other than the REDLK sequence at the carboxyl terminus, are needed for translocation of PE into the cytosol.

Barnase is an extracellular ribonuclease produced by *Bacillus amyloliquefaciens* [for review see Hartley (1989)]. The protein is synthesized as a proenzyme (Paddon & Hartley, 1986), and the secreted, native form is small (110 amino acids), thus facilitating physical studies, including determination of its three-dimensional structure to 2 Å (Mauguen et al., 1982). Since some toxins, for example, ricin, exert their cytotoxic effects by the hydrolysis of ribosomal RNA (Endo et al., 1987), we were interested in whether the ribonuclease activity of barnase could be introduced into PE, thus creating a chimeric toxin with both ribonuclease and ADP ribosylation activities. We have recently described such a molecule (Prior et al., 1991). The results indicated that it was possible to construct chimeric toxins with two or more enzymatic activities. Furthermore, we were able to demonstrate that PE could deliver barnase to the cytosol of target cells.

Having shown that barnase could be translocated to the cytosol when inserted just before the carboxyl end of domain III, we next wished to determine if sequences in domain III were necessary for translocation of barnase, or if the presence of domain II is sufficient. In this paper, we describe the construction, expression, purification, and activities of chimeric fusion proteins composed of barnase and domains Ia, II, and Ib of PE. The chimeric toxin is cytotoxic to a murine fibroblast cell line even in the absence of almost all (amino acids 413–606) of domain III. This data indicates that domain II of PE is sufficient to promote the translocation of proteins inserted in place of domain III.

Materials and Methods

Reagents. Chemicals and enzymes were purchased from standard suppliers. [^{3}H]Thymidine (6.7 Ci/mmol) and [^{3}H]leucine (125 Ci/mmol) were purchased from NEN/Du Pont (Boston, MA). Purified barnase was a gift from Dr. R. W. Hartley, National Institutes of Health.

Plasmids, Bacterial Strains, and Cell Lines. Plasmid pTP32 f(+)T carries the DNA encoding the chimeric toxin PE-Bar (Prior et al., 1991). This plasmid was designed such that the DNA encoding barnase (amino acids 1–110) is preceded by amino acids 1–607 and followed by amino acids 604–613 of PE. The DNA encoding the barnase inhibitor, barstar, was inserted between the stop codon of the chimeric toxin and the transcription terminator. Plasmid pTP36 f(+)T encodes a mutant form of PE-Bar lacking amino acids 604–613 of PE (Prior et al., 1991). Plasmid pJY4A2Q-2 (Jinno et al., 1989) encodes a mutant form of PE in which the arginine residue at position 276, which is necessary for efficient processing of PE (Ogata et al., 1990; Jinno et al., 1989), has been changed to glycine. *Escherichia coli* strain BL21 (λDE3) has been described previously (Studier & Moffat, 1986). L929 is a murine fibroblast cell line. Cell line 103-PE^R is a PE-resistant subclone of the murine hybridoma OVB3 (Willingham et al., 1987), which has a mutation in EF-2 such that it can no longer be ADP ribosylated by PE (E. Lovelace, M. Gallo, and I. Pastan, unpublished data).

Plasmid Construction. Plasmid DNA was prepared using QIAGEN columns (QIAGEN, Inc., Studio City, CA). Plasmid pTP32 f(+)T contains a *Stu*I recognition site immediately prior to the DNA encoding barnase. This plasmid also has a *Sac*II recognition site close to the junction between domain Ib and domain III of PE. Plasmid pTP32 f(+)T was digested with *Stu*I and *Sac*II, and the 3′-protruding end of the *Sac*II site was filled in using T4 DNA polymerase. After purification on 1% (w/v) low melting point agarose (Sea Plaque GTG agarose, FMC Bioproducts, Rockland, ME), the 4995 bp fragment was self-ligated and the recombinants were screened by restriction digestion analysis. Several positive clones were checked for protein expression. Plasmid pTP50 f(+)T was identified as carrying the gene for the chimeric toxin PE$^{1-412}$-Bar.

Plasmid pTP70 f(+)T, which encodes PE$^{1-412,Gly276}$-Bar, was constructed as follows. Plasmid pTP50 f(+)T and plasmid pJY4A2Q-2 (Jinno et al., 1989), which encodes PE^{Gly276}, both have an *Nco*I recognition site in domain I of PE and an *Apa*I recognition site in domain Ib; these sites span domain II, which contains amino acid 276. After digestion with *Nco*I and *Apa*I, the 3992 bp fragment from plasmid pTP50 f(+)T and the 1003 bp fragment from plasmid pJY4A2Q-2 were purified and ligated and the resulting recombinants analyzed as discussed earlier.

Plasmid pTP72 f(+)T was constructed from plasmid pTP50 f(+)T and plasmid pTP36 f(+)T (Prior et al., 1991), which encodes a mutant form of PE-Bar lacking amino acids 604–613 of PE. Plasmid pTP50 f(+)T contains two *Xma*I recognition sites; one is within the DNA encoding barnase, and the other is within the region coding for the carboxyl end of PE$^{1-412}$-Bar (amino acids 604–613 of PE). Since plasmid pTP36 f(+)T lacks the carboxyl terminus of PE, it only has the *Xma*I recognition site within barnase. Both plasmids have a unique *Eco*RI recognition site immediately following the stop codon of barstar. After digestion with *Xma*I and *Eco*RI, the 4514 bp fragment from plasmid pTP50 f(+)T and the 445 bp fragment from plasmid pTP36 f(+)T were purified and ligated. Plasmid pTP72 f(+)T, encoding PE$^{1-412}$-Bar-COOH, was identified as described above.

Protein Expression and Purification. The chimeric toxins were prepared using *E. coli* strain BL21 (λDE3). All of the constructions used in this study contained the OmpA signal peptide; consequently, the expressed proteins were secreted into the periplasm. After induction with isopropyl β-D-galactopyranoside (IPTG), the chimeric toxins were purified essentially as described earlier (Prior et al., 1991). The proteins were purified using Q-Sepharose Fast-Flow and MonoQ (Pharmacia/LKB Biotechnology Inc., Piscataway, PA) anion-exchange chromatography and gel filtration chromatography (TSK G3000SW column), with removal of the tightly bound barstar from the barnase chimeras accomplished by denaturation in 7 M guanidine hydrochloride, 5 mM EDTA, and 100 mM Tris-HCl (pH 8.0) and chromatography over a TSK G3000SW column equilibrated with this same solution. The larger PE$^{1-412}$-Bar was separated from the smaller barstar by this procedure. The chimeric toxin, in approximately 2 mL, was renatured by dialysis versus 500 mL of 20 mM Tris-HCl (pH 7.4) and 1 mM EDTA with one change.

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli (1970).

DNA Synthesis Inhibition Assay. The cytotoxicity of the chimeric toxins on L929 cells was measured using a [^{3}H]-thymidine incorporation assay as described (Prior et al., 1991). Briefly, the cells were placed in 96-well tissue culture dishes at 5×10^3 cells per well for 24 h before assaying. The toxins were diluted in phosphate-buffered saline containing 0.2% (w/v) human serum albumin and added at various concentrations to the cells. After incubation for 48 h at 37 °C in 5% CO_2, the cells were cultured with [^{3}H]thymidine (0.2 μCi) for 3 h. The cells were harvested by filtration onto glass fiber membranes (TomTec 96-well harvester), and the amount of [^{3}H]thymidine incorporation was measured (Beta-Plate Scintillation Counter, Pharmacia/Wallac Ltd.).

Protein Synthesis Inhibition Assay. Since cell line 103 is a thymidine uptake-negative strain, cytotoxicity was evaluated using [^{3}H]leucine. This assay was performed exactly as above except that [^{3}H]leucine (1.0 μCi) was added to the cells.

Ribonuclease Assay. The ribonuclease activity of the barnase-containing chimeric toxins was estimated using the qualitative detection assay described in Hartley and Smeaton (1973). Appropriately diluted enzyme preparations were spotted on 2% (w/v) agar plates containing 0.5% (w/v) yeast RNA (Boehringer-Mannheim, Indianapolis, IN) and 0.1 M ammonium acetate (pH 8.2). After 15-min incubation at 37 °C, the plates were developed by treating the plates with 5 mL of 2 N H_2SO_4. Precipitated RNA turns the agar cloudy, and clear spots indicate the presence of ribonuclease: activity was estimated by comparison to the spots obtained with known amounts of barnase.

Results

Construction of Expression Plasmid Encoding PE$^{1-412}$-Bar and Derivatives. The plasmid pTP50 f(+)T encoding PE$^{1-412}$-Bar was constructed as described in Materials and Methods. The chimeric toxin is composed of the OmpA signal peptide, amino acids 1–412 of PE fused to amino acids 1–110 of mature barnase, and finally, amino acids 604–613 of PE. There is a single amino acid residue, proline, in between amino acid 412 of PE and the first amino acid of barnase, which was introduced through the cloning procedure and corresponds to Pro-607 of PE. In a similar fashion, the genes encoding the chimeric proteins PE$^{1-412,Gly276}$-Bar, which has a mutation in position 276 of PE (Arg to Gly) and therefore has poor translocation activity (Jinno et al., 1989), and PE$^{1-412}$-Bar-COOH, which lacks amino acids 604–613 of PE required for translocation of PE into the cytosol (Chaudhary et al., 1990), were prepared. A schematic representation of the proteins encoded by these genes is shown in Figure 1.

Expression and Purification of the Chimeric Toxins. The chimeric toxins were expressed by cultures of *E. coli* strain BL21 (λDE3). Since the genes contained the OmpA signal sequence, induction with IPTG resulted in the appearance of the expressed protein in the periplasm. The chimeric toxins were purified from this source. The initial purification steps included chromatography over Q-Sepharose Fast-Flow and MonoQ anion-exchange columns and gel filtration over a TSK G3000SW sizing column. The proteins eluted from the anion-exchange columns at salt concentrations (130 mM NaCl) significantly lower than expected for PE and PE-Bar (220 mM NaCl) (Prior et al., 1991) (data not shown). Since barstar binds tightly to, and copurifies with, barnase (Hartley, 1989) and PE-Bar chimeras (Prior et al., 1991), it is necessary to chaotropically separate the two proteins. Thus, the purified material was treated with guanidine hydrochloride and loaded onto a TSK G3000SW column equilibrated with buffer containing guanidine hydrochloride. The first peak, corresponding to PE$^{1-412}$-Bar, was renatured by removing the guanidine hydrochloride by dialysis. The recovered protein was greater than 95% pure, as determined by SDS–PAGE (Figure 2).

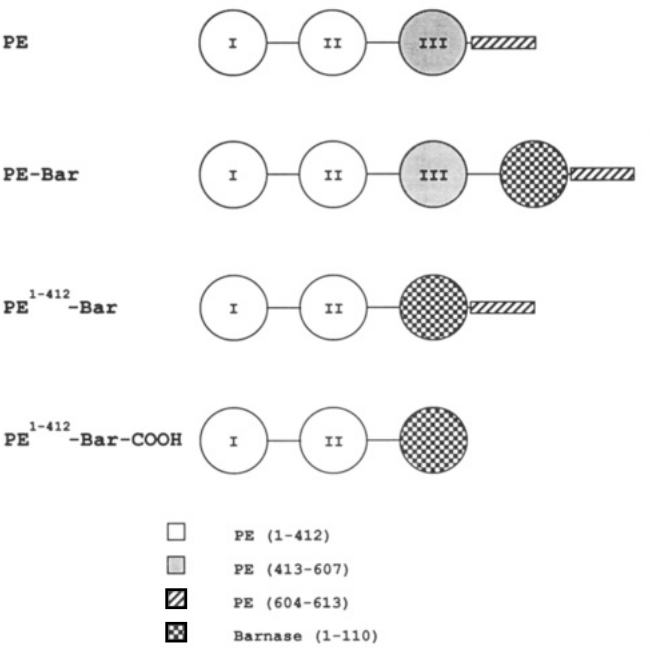

FIGURE 1: Schematic representation of the domain structure of PE and the barnase toxins. Roman numerals refer to the domain of PE.

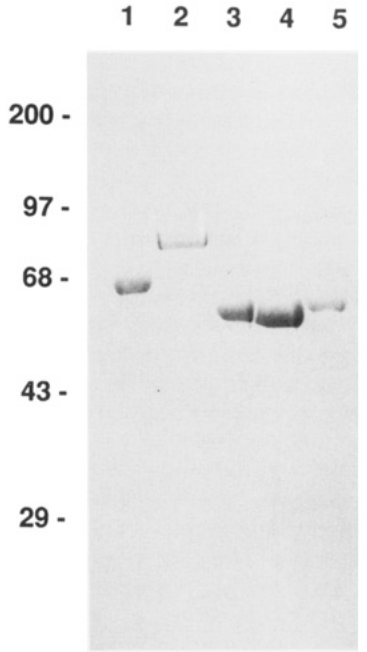

FIGURE 2: SDS–PAGE of PE and the barnase toxins. Purified proteins were analyzed using 10% (w/v) SDS–PAGE and stained with Coomassie blue. Lane 1, PE; lane 2, PE-Bar; lane 3, PE$^{1-412}$-Bar; lane 4, PE$^{1-412,Gly276}$-Bar; lane 5, PE$^{1-412}$-Bar-COOH.

Enzymatic Activity of the Chimeric Toxins. The ribonuclease activity of the recombinant toxins was determined (Table I). Corrected for the difference in the molecular mass between barnase (12.5 kDa) and the chimeric toxins (78.5 kDa for PE-Bar; 57.0 kDa for PE$^{1-412}$-Bar and its derivatives), the proteins PE-Bar, PE$^{1-412}$-Bar, PE$^{1-412,Gly276}$-Bar, and PE$^{1-412}$-Bar-COOH had at least 80% of the ribonuclease activity of barnase. PE had no detectable ribonuclease activity (Prior et al., 1991).

Cytotoxic Activity of the Chimeric Toxins. The cytotoxic activity of the various PE$^{1-412}$-Bar constructions was evaluated

Table I: Ribonuclease Activity of Chimeric Toxins

protein	relative activity (% of control)[a]	protein	relative activity (% of control)[a]
barnase	100	$PE^{1-412,Gly276}$-Bar	85
PE-Bar	80	PE^{1-412}-Bar-COOH	80
PE^{1-412}-Bar	90		

[a] Ribonuclease activity was determined using the RNA precipitation assay described in Materials and Methods. Results are expressed as a percentage of the activity observed with barnase.

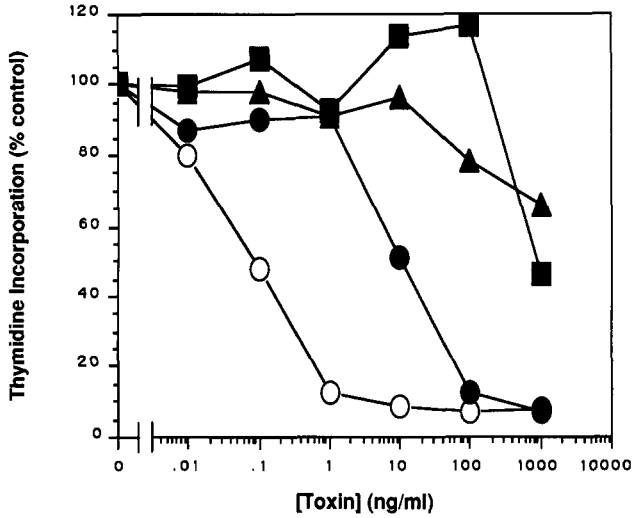

FIGURE 3: DNA synthesis inhibition by PE and barnase toxins. L929 cells (5×10^3 cells in 0.2 mL) were incubated overnight. Various amounts of PE (open circles), PE^{1-412}-Bar (closed circles), $PE^{1-412,Gly276}$-Bar (closed squares), or PE^{1-412}-Bar-COOH (closed triangles) were added to the cultures. After 48 h of incubation, DNA synthesis was measured. Results are expressed as the percentage of DNA synthesis activity of cells incubated without toxin.

on the murine fibroblast cell line L929. The cells were incubated for 48 h with varying amounts of toxin, and the rate of DNA synthesis was measured to assess the cytotoxic activity of the proteins. As shown in Figure 3, PE and the chimeric toxins PE^{1-412}-Bar inhibited DNA synthesis on L929 cells. The concentration of toxin giving a 50% inhibition of DNA synthesis (ID_{50}) was 0.1 ng/mL for PE and 10 ng/mL for PE^{1-412}-Bar. $PE^{1-412,Gly276}$-Bar, which has a mutation at residue 276 of PE (Arg to Gly), necessary for efficient processing and translocation of PE (Ogata et al., 1990; Jinno et al., 1989), was significantly less cytotoxic (ID_{50} = 900 ng/mL). PE^{1-412}-Bar-COOH, which lacks the carboxyl terminus of PE necessary for cytotoxicity (Chaudhary et al., 1990), had no detectable effect on DNA synthesis (ID_{50} > 1000 ng/mL). Thus, the chimeric toxin PE^{1-412}-Bar is cytotoxic even in the absence of almost all (96%) of domain III.

Effect of Chimeric Toxins on a PE-Resistant Cell Line. Cell line 103-PE^R is a PE-resistant hybridoma that contains a mutant EF-2 that cannot be ADP ribosylated by PE (E. Lovelace, M. Gallo, and I. Pastan, unpublished observations). Since cell line 103-PE^R is a thymidine uptake-negative strain, cytotoxicity was evaluated using [^{3}H]leucine. As shown in Figure 4, cell line 103, the PE-sensitive parent of 103-PE^R, was sensitive to both the toxins PE and PE^{1-412}-Bar. Conversely, cell line 103-PE^R was sensitive only to PE^{1-412}-Bar. PE was not cytotoxic to cell line 103-PE^R (ID_{50} > 10000 ng/mL). This result supports the conclusion that the chimeric toxin PE^{1-412}-Bar is cytotoxic to target cells because the barnase moiety is translocated to the cytosol in the absence of domain III.

Discussion

In this paper, we describe the replacement of almost all of domain III of PE with a foreign protein, barnase, and present evidence that the barnase moiety is translocated to the cytosol. Previously, there was no direct biochemical evidence to indicate that domain II could translocate protein into the cytosol independently of domain III. We have recently described the properties of a new chimeric toxin, called PE-Bar, which was constructed by inserting the DNA encoding *B. amyloliquefaciens* barnase to a gene encoding PE (Prior et al., 1991). The cytotoxic action of PE-Bar was due to its ribonuclease activity, which had been translocated into the cytosol. We have now constructed a series of chimeric toxins in which domain III of PE was deleted from PE-Bar. These hybrid toxins were produced by *E. coli* and purified from this source. The fusion protein, termed PE^{1-412}-Bar, which contains the first 412 amino acids of PE, barnase, and the last 10 amino acids of PE, was cytotoxic to the murine fibroblast cell line L929 and to a murine hybridoma resistant to PE. Several lines of evidence indicate that the cytotoxic action of PE^{1-412}-Bar is due to its ribonuclease activity, which is transferred to the cytosol by

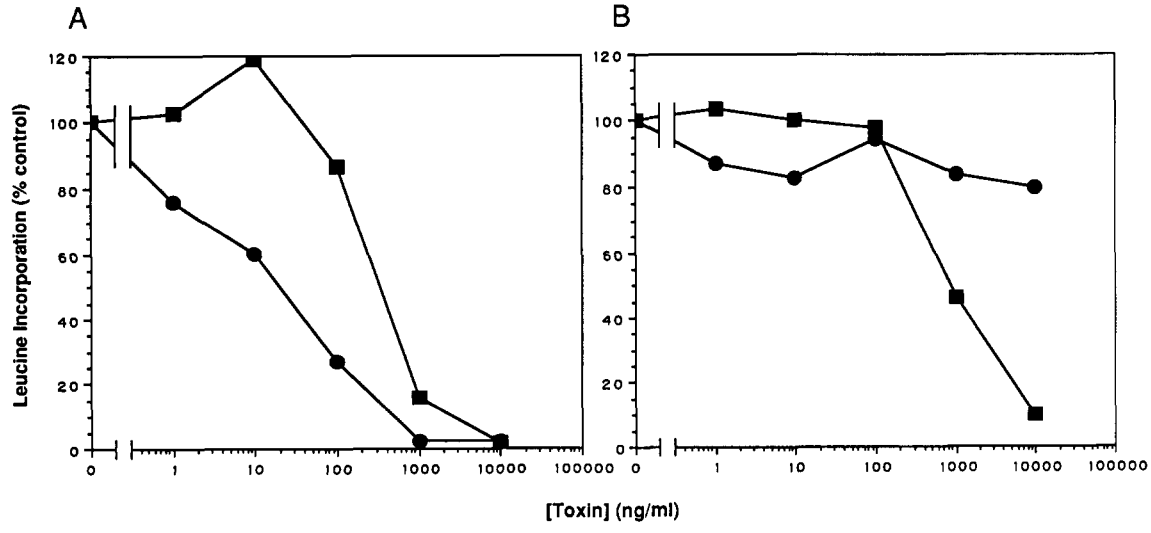

FIGURE 4: Treatment of cell lines 103 and 103-PE^R with barnase toxin. 103 (A) and 103-PE^R (B) cells were prepared as described in the legend to Figure 3. Various amounts of PE (closed circles) or PE^{1-412}-Bar (closed squares) were added to the cultures. After 48 h of incubation, protein synthesis was measured. Results are expressed as the percentage of protein synthesis activity of cells incubated without toxin.

the action of domain II of PE. First, PE^{1-412}-Bar was cytotoxic to cells despite lacking the ADP ribosylation domain. Second, a derivative of PE^{1-412}-Bar ($PE^{1-412,Gly276}$-Bar), in which the arginine residue at position 276 necessary for cytotoxicity (Jinno et al., 1989) and cellular processing of PE (Ogata et al., 1990) was changed to glycine, was significantly less toxic. Third, the chimeric toxin PE^{1-412}-Bar-COOH, which lacks the carboxyl amino acids of PE (604–613) necessary for translocation of the 37-kDa fragment of PE into the cytosol (Chaudhary et al., 1990), was not active. Further, PE^{1-412}-Bar was toxic to a PE-resistant cell line with a mutant EF-2 that cannot be ADP ribosylated and inactivated by PE.

Although the chimeric toxin PE-Bar (Prior et al., 1991) has both ADP-ribosylation and ribonuclease activities, it was significantly less cytotoxic (2 ng/mL) to cells than PE (0.1 ng/mL). This decrease in cytotoxicity was hypothesized to be due to inhibition of the translocation process by the presence of the additional 12.5 kDa of protein (barnase) that must be translocated across the cell membrane. This conclusion is supported by the observation that PE^{1-412}-Bar was more cytotoxic (10 ng/mL) than the chimeric toxin $PE^{\Delta 553}$-Bar (90 ng/mL; Prior et al., 1991); this latter construction is a full-length derivative of PE-Bar in which the ADP ribosylation activity had been abolished by mutation. We have recently found that the carboxyl region of domain II and almost all of domain Ib (amino acids 346–404) can be deleted without affecting cytotoxicity (Siegall et al., 1991). Therefore, there are additional sequences which can be removed from this construction and still maintain efficient translocation across the cell membrane.

The results presented here show that domain II of PE can transport a soluble enzyme into the cytosol essentially independent of the other domains of PE, although the carboxyl terminus of PE is also necessary for interaction with cellular components of the translocation machinery (Seetharam et al., 1991). This is the first direct evidence that domain II is the translocation domain of PE, although this was suggested by other studies (Hwang et al., 1987; Jinno et al., 1989; Siegall et al., 1989, 1991; Ogata et al., 1990). Since domain Ia of PE, which binds to all cells as a targeting domain, can be replaced with growth factors, lymphokines, or single-chain antibodies for targeting to specific cells in vitro or in animals (FitzGerald & Pastan, 1989; Pastan & FitzGerald, 1989), the results suggest a novel approach of drug delivery. For example, polypeptides of diagnostic or therapeutic interest can be targeted to a cell and delivered by domain II into the cell in an active form.

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