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The N-terminal region of the 37-kDa translocated fragment of *Pseudomonas* exotoxin A aborts translocation by promoting its own export after microsomal membrane insertion

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ABSTRACT **The 37-kDa C-terminal fragment of *Pseudomonas* exotoxin A (PE; termed PE37 and composed of aa 280–613 of PE) translocates to the cell cytosol to cause cell death. PE37 requires a C-terminal endoplasmic reticulum retention sequence to be cytotoxic, indicating that the toxin may translocate to the cytosol from the endoplasmic reticulum. We show here that the N-terminal region of nascent PE37 can be inserted into the membrane of canine pancreatic microsomes by the preprocecropin signal sequence but then is exported or released from microsomes. The 34 N-terminal amino acids of the toxin fragment are sufficient to arrest translocation and prevent the microsomal accumulation of nascent chains that otherwise are sequestered into microsomes. These data support a role for the N-terminal region of PE37 in the translocation of the toxin from the endoplasmic reticulum to the cytosol in mammalian cells.**

Pseudomonas exotoxin A (PE) is a three-domain bacterial toxin of 66 kDa (1). Domain Ia (aa 1–252) mediates cell binding; domain II (aa 253–364) is responsible for translocation of domain III (aa 400–613) into the cell cytosol; domain III catalyzes the ADP-ribosylation of elongation factor 2, which arrests protein synthesis and causes cell death (2, 3). The function of domain Ib (aa 365–399) remains undefined, although aa 365–380 can be deleted without loss of cytotoxicity (4). Domain Ia of PE can be replaced with growth factors, antibody variable domains, or CD4 to produce recombinant toxins that are selectively targeted to cells (for review, see refs. 5 and 6).

After receptor-mediated endocytosis by the cell, PE is cleaved within domain II between Arg-279 and Gly-280 (7). Mutants of PE that cannot be cleaved by the intracellular protease are inactive (8). A 37-kDa fragment termed PE37 that is composed of aa 280–613 of PE ultimately reaches the cytosol to cause cytotoxicity (8). The 37-kDa C-terminal fragment can itself be targeted to cells expressing the epidermal growth factor receptor by inserting transforming growth factor α (TGF-α) near the C terminus to produce a molecule termed PE37–TGF-α (9). Deletion of 2, 4, or 7 aa from the N terminus of PE37–TGF-α substantially diminishes its cytotoxic activity (9), whereas the addition of the amino acid sequence MPQ does not (unpublished results). Because of this finding, we postulated that the N-terminal region of PE37 is active in translocating PE37 across a membrane from an extracytosolic compartment to the cytosol (9).

Proteolysis and disulfide bond reduction are necessary but not sufficient for toxin translocation. PE must also contain a proper C-terminal sequence to translocate into the cytosol (10). In PE, this sequence is REDLK, but it can be changed to REDL, KDEL, or other sequences that function to maintain or recycle proteins into the endoplasmic reticulum (ER) (10, 11). Based on this data, we have postulated that the ER is the compartment from which PE37 translocates to the cytosol and that PE37 may utilize elements of the preexisting protein transport apparatus to gain access to the cytosol (6).

Most mammalian secretory proteins are transported across the ER in a ribonucleoparticle-dependent manner as they are synthesized (for review, see refs. 12 and 13). These proteins contain a characteristic signal sequence at their N terminus that binds the signal recognition particle (14), which in turn binds to the signal recognition particle receptor on the ER (15, 16). After GTP-hydrolysis-dependent release of the signal recognition particle, the nascent protein translocates into the ER (17, 18). On the lumenal face of the ER, the signal sequence is cleaved by signal peptidase (19) and core glycosylation may occur. It is likely that translocation occurs through an aqueous channel or translocation pore (14, 20).

One way to examine the hypothesis that PE37 (aa 280–613 of PE) translocates from the ER into the cytosol is to target PE37 into microsomes and study its subsequent export. We attempted to do this by fusing the gene encoding PE37 to the preprocecropin signal sequence and producing nascent chains that were directed to microsomal membranes.

MATERIALS AND METHODS

Materials. The plasmid GEM-7Z and mRNA encoding β-lactamase were from Promega. The protein PE37–TGF-α contains a methionine residue, residues 281–607 of PE, and the sequence of TGF-α followed by aa 604–613 of PE (9).

Amplification. Oligonucleotides were purchased from Bioserve Biotech (College Park, MD). PCR was carried out using 10 ng of template and reagents by the manufacturer's instructions (Perkin–Elmer/Cetus) in the presence of 5% (vol/vol) formamide (Fluka) and 100 pmol of oligonucleotides as described (9).

Plasmid Construction. Plasmid CA37 encodes the SP6 promoter followed by the preprocecropin signal sequence and prolactin (PRL) (21). This plasmid served as an expression cassette for *in vitro* translation by utilizing the unique *Bsp*EI site that occurs immediately after the preprocecropin signal sequence and the unique *Pst* I site that lies downstream of the PRL termination codon. PE mutant plasmids were constructed by ligating *Bsp*EI/*Pst* I-digested PCR fragments into the *Bsp*EI and *Pst* I restriction sites found in plasmid CA37. After the preprocecropin signal sequence, these plasmids encode the amino acid sequence APQ followed by the relevant PE sequences (Fig. 1). All PE-encoding plasmids encode a serine at position 287 and the deletion of the

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Abbreviations: PE, *Pseudomonas* exotoxin A; PNGase F, peptide-*N*-glycosidase F; TGF-α, transforming growth factor α; ER, endoplasmic reticulum; PRL, prolactin.

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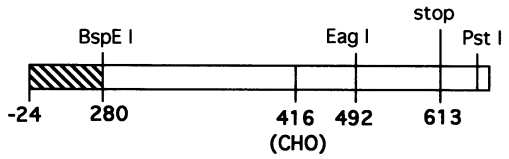

FIG. 1. Restriction sites in plasmid CT37 that encode the preprocecropin signal sequence followed by residues 280–613 of PE. Numbers indicate individual residues within the PE-encoding region. The potential N-glycosylation site is indicated by the letters CHO.

glutamate at position 553 and were confirmed by DNA sequencing.

***In Vitro* Translocation Assay.** *In vitro* transcription was carried out for 4 h at 37°C with either T7 or SP6 RNA polymerase in the presence of 5 mM ATP/5 mM CTP/5 mM UTP/4 mM 7-methylguanosine (5′)triphospho(5′)guanosine[m^7G(5′)ppp(5′)G] cap analog/1 mM GTP using a MEGAscript transcription kit (Ambion, Austin, TX). mRNA was purified by gel filtration through Sephadex G-50 Quick Spin columns (Boehringer Mannheim) and translated in a rabbit reticulocyte lysate with [^{3}H]leucine (165 Ci/mmol; 1 Ci = 37 GBq) or [^{35}S]methionine (1000 Ci/mmol) supplemented with canine pancreatic microsomes (where applicable) at 30°C for 1 h unless otherwise noted by the manufacturer's instructions (Boehringer Mannheim). Translation mixtures were diluted with 3 vol of 50 mM Tris·HCl, pH 7.4/100 mM NaCl and incubated at 22°C for 5 min with 2 mM tetracaine to stabilize microsomes (22). Proteinase K was added at 20 μg/ml with or without 1% Triton X-100. Digestion was carried out for 10 min at 22°C and terminated with 3 mM phenylmethylsulfonyl fluoride (PMSF). For deglycosylation, the proteinase K-treated samples containing microsomes and 3 mM PMSF were diluted with an equal volume of 0.5 M sodium phosphate, pH 7.4/20 mM EDTA/0.4% SDS/2% (vol/vol) β-mercaptoethanol and boiled for 5 min. Samples were cooled to 22°C, PMSF was added to 3 mM, peptide-*N*-glycosidase F (PNGase F) was added to 20 units/ml, and the mixture was incubated overnight at 37°C.

Sedimentation of Microsomes. Translation mixture (10 μl) was diluted with an equal volume of physiologic salt buffer (final concentration, 50 mM Tris acetate, pH 7.5/150 mM potassium acetate/2.5 mM magnesium acetate/1 mM dithiothreitol) and layered on a 160-μl 500 mM sucrose cushion containing physiologic salt buffer and sedimented at 20 psi (1 psi = 6.9 kPa) for 10 min using an Airfuge (Beckman) at 4°C. Proteins in the supernatant were precipitated in 10% (wt/vol) trichloroacetic acid prior to analysis by SDS/PAGE.

SDS/PAGE and Autoradiography. After SDS/PAGE (23), the gels were fixed for 1 h in 30% (vol/vol) methanol/10% (vol/vol) acetic acid and then treated with Entensify (New England Nuclear) for fluorography. Gels were dried under vacuum and exposed overnight to X-Omat AR film (Eastman Kodak) at −70°C.

RESULTS

PRL Is Sequestered in Microsomes After Cotranslational Targeting Using the Preprocecropin Signal Sequence. To demonstrate the efficacy of our translocation assay, we tested a fusion protein containing the preprocecropin signal sequence followed by PRL (termed ssPRL; Fig. 2). This protein undergoes signal sequence cleavage and is sequestered into microsomes in a cotranslational manner (21). *In vitro* translation of mRNA encoding ssPRL with [^{3}H]leucine yielded a single radiolabeled species of 24 kDa that was degraded by proteinase K (Fig. 3). When translation was done with microsomes, a smaller species of 22 kDa was produced, indicating that the signal sequence had been cleaved. The

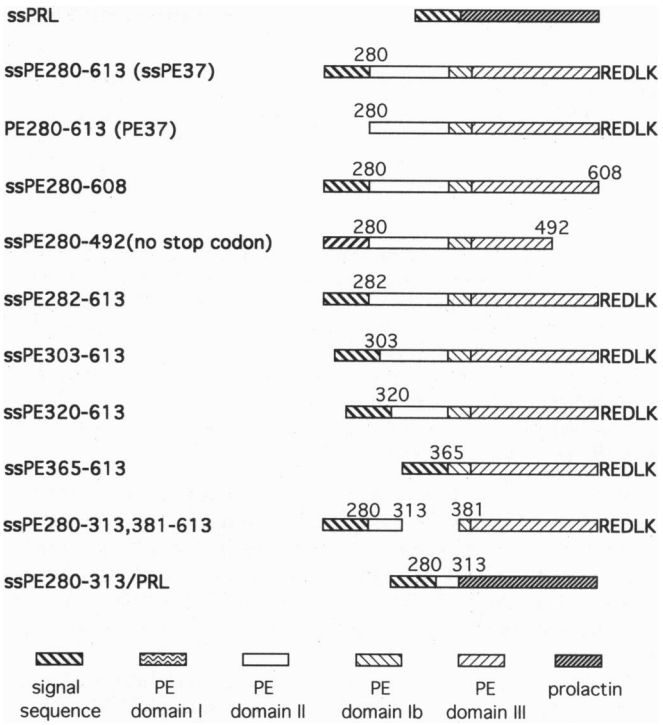

FIG. 2. Schematic representation of proteins used in the study. The positions of amino acids that span PE sequences are numbered. REDLK indicates the terminal 5 aa of PE. The protein name is to the left and its structure is to the right.

mature protein was sequestered within microsomes since degradation by proteinase K required solubilization of microsomes with Triton X-100.

PE280–613 Is Not Sequestered in Microsomes Despite Cotranslational Targeting Using the Preprocecropin Signal Sequence. We next constructed a DNA template (Fig. 1) that encodes the preprocecropin signal sequence followed by the aa 280–613 of PE (ssPE280–613; Fig. 2). The mature protein encoded by this template (PE280–613) is identical to that of the C-terminal fragment of PE that reaches the mammalian cell cytosol except for three mutations: Cys-287 → Ser that does not change the cytotoxicity of PE37–TGF-α (24); the Glu-553 deletion that eliminates the ADP-ribosyltransferase function of PE (25) and prevents ssPE280–613 from inactivating the reticulocyte lysate; and finally, after cleavage by signal peptidase, the amino acids APQ remaining at the N terminus of PE280–613. PE37–TGF-α containing the amino acids MPQ added to the N terminus is as active as PE37–TGF-α on A431 cells (unpublished results), indicating that the addition of 3 aa at the N terminus of the PE280–613 that results from signal sequence cleavage does not interfere with the function of the C-terminal PE fragment.

mRNA encoding ssPE280–613 was translated in the presence of [^{3}H]leucine and yielded a single radiolabeled species of 39 kDa (Fig. 4, lane 1) that was degraded by proteinase K

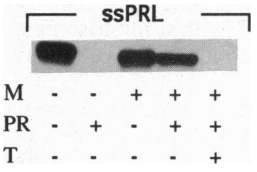

FIG. 3. PRL is sequestered into microsomes when targeted by the preprocecropin signal sequence. mRNA encoding ssPRL (Fig. 2) was translated using [^{3}H]leucine in the absence or presence of microsomes (M) and samples were treated with proteinase K (PR) and Triton X-100 (T) as indicated (+, treated; −, not treated).

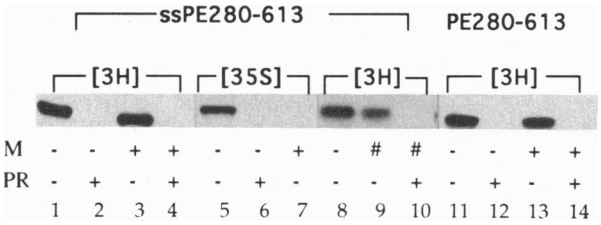

FIG. 4. Translocation of ssPE280–613 into microsomal membranes is ribonucleoparticle-dependent but does lead to sequestration of the processed protein. mRNA encoding ssPE280–613 or aa 280–613 of PE without a signal sequence (PE280–613; Fig. 2) was translated with either [^{3}H]leucine (3H) or [^{35}S]methionine (35S) for 45 min in the absence or presence of microsomes (M) as indicated. Microsomes were also added for 45 min after arrest of translation with 250 μM cycloheximide (#), and samples were treated with proteinase K (PR) as indicated (+, treated; −, not treated).

(Fig. 4, lane 2). Translation with microsomes produced a smaller species of 37 kDa (Fig. 4, lane 3) that would be expected if signal sequence cleavage had occurred. Strikingly, despite signal sequence cleavage, the processed product of ssPE280–613 was not sequestered into the lumen of microsomes since it remained sensitive to proteinase K (Fig. 4, lane 4).

To confirm that the signal sequence cleavage had occurred, translation was carried out in the presence of [^{35}S]methionine. ssPE280–613 lacks internal methionines so that a single methionine residue occurs at the N terminus. Translation of mRNA with [^{35}S]methionine yielded a single radiolabeled species of 39 kDa (Fig. 4, lane 5) that was degraded by proteinase K (Fig. 4, lane 6). Translation in the presence of microsomes, however, yielded no radiolabeled band (Fig. 4, lane 7), indicating that signal sequence cleavage had occurred. The lack of a radiolabeled species was not due to faulty translation since translation of β-lactamase mRNA in the presence of microsomes using the same reticulocyte lysate yielded a cleaved product of the expected size that was sequestered within microsomes (data not shown).

Signal peptidase is an integral ER membrane protein whose active site is localized to the lumenal face of microsomes (19, 26–29). The fact that ssPE280–613 undergoes signal peptide cleavage indicates that at least the N-terminal region of the processed form of the nascent polypeptides is inserted into the membrane to allow the signal sequence cleavage site access to the lumen of microsomes. If 20 aa are needed to span the lipid bilayer, then at least aa 280–299 of PE280–613 were within the membrane at one time prior to signal sequence cleavage.

When microsomes were added posttranslationally after termination of ssPE280–613 translation with cycloheximide, the 39-kDa radiolabeled species remained, indicating that signal sequence cleavage had not occurred (Fig. 4, lanes 8 and 9). Hence, ssPE280–613 is imported into microsomes only in a ribonucleoparticle-dependent fashion. When mRNA encoding PE280–613 lacking a signal sequence was translated, a 37-kDa species was produced whose size was not changed by microsomes (Fig. 4, lanes 11 and 13) and remained sensitive to proteinase K (Fig. 4, lane 14), indicating that it was not sequestered within microsomes. Thus, PE280–613 does not interact with microsomes unless it is preceded by a signal sequence.

To assess whether the processed product of ssPE280–613 remained associated with microsomes after signal sequence cleavage in a manner that allowed proteinase K digestion, microsomes were sedimented after translation. The processed product of ssPE280–613 did not associate with the microsomal pellet (Fig. 5). In contrast, when mRNA encoding ssPRL was translated with microsomes, the processed product of ssPRL was predominantly found in the microsomal pellet. Thus, after membrane insertion of the N-terminal region of ssPE280–613 as observed by signal sequence cleavage, the processed product of ssPE280–613 is released or exported from the microsomal membrane.

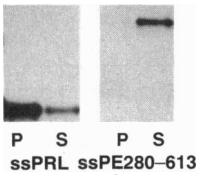

FIG. 5. Processed form of ssPE280–613 does not associate with sedimented microsomes. mRNA encoding ssPRL or ssPE280–613 (Fig. 2) was translated with [^{3}H]leucine in the presence of microsomes. Microsomes were then diluted with physiologic salt buffer and sedimented, and pellets (P) and supernatants (S) were analyzed.

The Ability of ssPE280–613 to Abort its Translocation Is Independent of the Need for a Proper C Terminus or Discharge from Ribosomes. To determine whether the REDLK sequence at the C terminus of ssPE280–613 was involved in the failure of PE37 to sequester in microsomes, mRNA encoding the preprocecropin signal sequence followed by aa 280–608 of PE (ssPE280–608; Fig. 2) was studied using the *in vitro* translocation system. Despite signal sequence cleavage, the processed product of ssPE280–608 was not sequestered within microsomes since it remained sensitive to proteinase K (Fig. 6). Thus REDLK at the C terminus is not necessary to abort the translocation of ssPE280–613 after membrane insertion.

We also linearized the DNA template encoding ssPE280–613 with the restriction enzyme *Eag* I (see Fig. 1) to produce a template that encodes the preprocecropin signal sequence followed by aa 280–492 of PE and lacks a stop codon [termed ssPE280–492(no stop codon); Fig. 2]. Translation of mRNA derived from this template produces nascent chains that are not released from ribosomes. Despite signal sequence cleavage, the processed product of ssPE280–492(no stop codon) was also not sequestered within microsomes since it remained sensitive to proteinase K (Fig. 6).

Deletion of Amino Acids in Domain II Allows ssPE280–613 To Be Sequestered into Microsomes. To assess whether amino acids of domain II aborted the translocation of ssPE280–613 after membrane insertion, we deleted residues from domain II of ssPE280–613 and studied the function of a protein containing the preprocecropin signal sequence followed by domains Ib and III (aa 365–613) of PE. When mRNA encoding ssPE365–613 (Fig. 2) was translated in the presence of microsomes, it yielded a single radiolabeled species of 30 kDa (Fig. 7) that was degraded by proteinase K. Translation in the presence of microsomes yielded a smaller species of 28 kDa and a larger species that were resistant to proteinase K. The larger species was converted by PNGase F to the 28-kDa species, indicating that core glycosylation and signal sequence cleavage had occurred. Since PNGase F specifically cleaves N-linked oligosaccharides (30), we conclude that

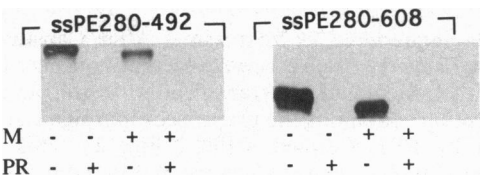

FIG. 6. Mutants of ssPE280–613 lacking the proper C terminus or a termination codon are also not sequestered into microsomes. mRNA encoding ssPE280–608 or ssPE280–492(no stop codon) (Fig. 2) was translated with [^{3}H]leucine in the absence or presence of microsomes (M) and samples were treated with proteinase K (PR) as indicated (+, treated; −, not treated).

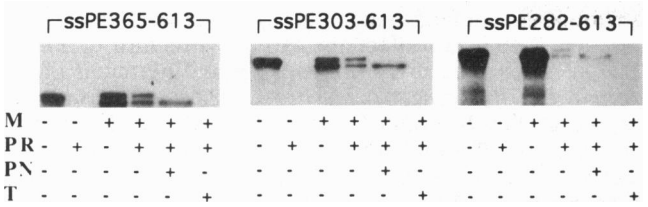

FIG. 7. Mutants of ssPE280–613 containing deletions of domain II residues are glycosylated and sequestered into microsomes. mRNA encoding ssPE365–613, ssPE303–613, or ssPE282–613 (Fig. 2) was translated with [^{3}H]leucine in the absence or presence of microsomes (M) and samples were treated with proteinase K (PR), PNGase F (PN), and Triton X-100 (T) as indicated (+, treated; −, untreated).

PE365–613 was glycosylated at the single potential N-linked glycosylation site located at residue 416 of PE (see Fig. 1). Since ssPE365–613 could be translocated, glycosylated, and sequestered in microsomes, there is no intrinsic "resistance" to translocation present within domains Ib and III of PE. Thus, sequences in domain II must abort the translocation of ssPE280–613 after membrane insertion.

Domain II of PE contains six α-helices (1). Helices B–F are contained within aa 280–364. To define which helices aborted the translocation of ssPE280–613 after membrane insertion, we constructed templates encoding the preprocecropin signal sequence followed by aa 303–613 or aa 320–613 of PE (see Fig. 2). These mutants are missing the B helix or the B and C helices contained in ssPE280–613, respectively. These mutants also were sequestered within microsomes after signal sequence cleavage and were glycosylated (Fig. 7 and data not shown). The degree of protease protection afforded PE303–613 and PE320–613 was similar to that afforded PE365–613. We conclude that aa 280–302 of PE are necessary to abort the translocation of ssPE280–613 from microsomes after membrane insertion.

To test further the importance of the N-terminal region, we deleted the first 2 aa of the processed product of ssPE280–613 to make ssPE282–613 (see Fig. 2). The processed product of this construct was sequestered within microsomes after signal sequence cleavage and was glycosylated. Although the overall degree of protease protection was poor for this mutant, it was superior to that seen in the case of ssPE280–613 (where neither protease protection nor glycosylation was detected).

PE aa 280–313 Are Sufficient to Prevent the Cotranslational Microsomal Accumulation of Nascent Polypeptides. ssPE280–313,381–613 contains a proper N terminus and a deletion of residues 314–380 from ssPE280–613. If aa 280–302 of PE37 are sufficient to prevent nascent polypeptide accumulation in microsomes, PE280–313,381–613, which contains a substitution of the 33 N-terminal residues from PE280–613 for the 16 N-terminal residues present in PE365–613, should not accumulate in microsomes. PE280–313,381–613 preceded by the preprocecropin signal sequence (ssPE280–313,381–613; Fig. 2) underwent signal sequence cleavage but was not sequestered into microsomes since it remained sensitive to proteinase K (Fig. 8). Thus replacement of the N terminus of PE365–613 with a peptide containing the N-terminal sequence of PE280–613 is sufficient to abort the translocation of this PE mutant.

To study the ability of the PE280–613 N-terminal region to prevent the microsomal accumulation of another polypeptide, we placed aa 280–313 of PE at the N terminus of PRL. ssPE280–313/PRL contains the preprocecropin signal sequence, followed by aa 280–313 of PE, which in turn are followed by PRL (Fig. 2). The chimeric protein underwent signal sequence cleavage but was not sequestered within microsomes since it remained sensitive to proteinase K (Fig. 8). We conclude that aa 280–313 of PE are sufficient to abort the translocation of a polypeptide unrelated to PE that, in the absence of aa 280–313 of PE, is sequestered in microsomes (Fig. 3).

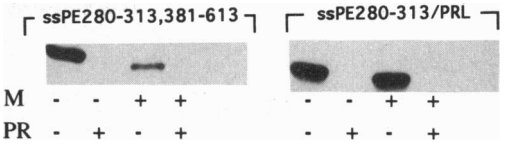

FIG. 8. PE aa 280–313 are sufficient to prevent the microsomal accumulation of nascent chains otherwise sequestered into microsomes. mRNA encoding ssPE280–313,381–613 or ssPE280–313/PRL (Fig. 2) was translated with [^{3}H]leucine in the absence or presence of microsomes (M) and samples were treated with proteinase K (PR) as indicated (+, treated; −, not treated).

DISCUSSION

We have postulated that the ER is the compartment from which PE37 translocates to the cytosol and that PE37 may utilize elements of the preexisting protein transport apparatus to gain access to the cytosol (6). To test this hypothesis, we fused the gene encoding PE37 to the preprocecropin signal sequence and produced nascent chains that were directed to microsomal membranes. We found that aa 280–313 of PE were sufficient to abort the translocation of downstream sequences into microsomes and appeared to do this by mediating the export or release of the N-terminal region of the nascent chain from microsomes after cotranslational membrane insertion.

Evidence that PE280–613 Is Inserted into Microsomal Membranes. We found that PE280–613 preceded by the preprocecropin signal sequence was proteolytically processed in a microsome- and signal sequence-dependent and cotranslational manner. This indicates that the targeting, signal-sequence cleavage, and translocation of ssPE280–613 were initiated in the usual ribonucleoparticle-dependent fashion (12, 13). If one assumes that 20 aa of PE280–613 were inserted into the lipid bilayer to allow signal-sequence cleavage, then at least aa 280–299 of PE280–613 were within the microsomal membrane at one time. At least this many amino acids must have been exported or released from the membrane since PE280–613 did not associate with sedimented microsomes.

PE280–613 did not undergo glycosylation in the presence of microsomes. This was not due to an intrinsic inaccessibility of the potential N-glycosylation site because PE282–613, PE303–613, PE320–613, and PE365–613 were glycosylated during translation in the presence of microsomes. Oligosaccharide transferase is a lumenal ER enzyme (31–33). It thus appears that the potential glycosylation site of ssPE280–613 did not reach the site within the lumen of microsomes where the transferase is located, was bound to an element of the ER that made the potential glycosylation site incapable of interacting with the transferase, or was imported and then subsequently rapidly exported. Since the N-glycosylation site present in ssPE280–613 was not utilized, there is no conclusive evidence that the entire 37-kDa fragment entered (or was exported from) microsomes. We can only conclude that the N-terminal region of PE280–613 was exported or released from microsomes after membrane insertion as shown by signal-sequence cleavage.

Model of Interaction of ssPE280–613 with Microsomes. To account for our data, we propose a model depicted in Fig. 9. After movement of the signal sequence through the translocation apparatus, the N terminus of the nascent peptide exists as a helical hairpin (13). The signal sequence is orientated so that its N terminus is directed toward the cytoplasmic face of the ER membrane. At this point the bias to protein import is mediated by binding of the signal sequence to a microsomal

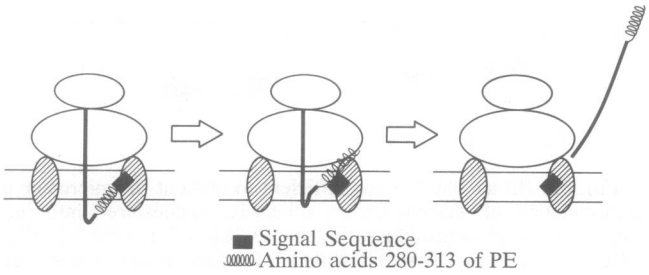

FIG. 9. Model depicting the N-terminal region of ssPE280–613 aborting translocation by promoting its own export after membrane insertion.

element. After signal-sequence cleavage, the bias to nascent chain import changes to one of protein export by interaction of the N-terminal region of PE280–613 with resident ER proteins and/or lipids. This model implies that amino acids after residue 313 act passively, a hypothesis that is consistent with the fact that two nascent chains that otherwise were sequestered into microsomes (PE365–613 and PRL) were prevented from accumulating in microsomes when preceded by aa 280–313 of PE37. The apparatus that mediates the export or release of the N-terminal region of PE280–613 is close in proximity or identical to the translocation apparatus since a ribosome-tethered ssPE280–492 construct also did not accumulate within microsomes. The model depicts the N-terminal region of PE280–613 moving through the same pore that mediated nascent chain import. The N-terminal region may, however, be exported or released from the microsomal membrane through a separate aqueous channel or through the lipid bilayer directly. In either case, it may interact with some proteins that are involved in ribonucleoparticle-dependent membrane translocation.

The ability of the N-terminal region of PE280–613 to abort translocation and prevent the microsomal accumulation of downstream sequences bears similarity to a function of the hepatitis B virus precore protein. The N terminus of the hepatitis B precore protein functions as an inefficient signal sequence (34). After signal sequence cleavage, however, translocation of the remaining nascent chains is partially aborted, with only 20–30% of the nascent chain becoming sequestered into microsomes. Interestingly, the N-terminal region of the processed product of the hepatitis B precoat protein contains tryptophans and negatively charged residues, a property shared by the N-terminal region of PE280–613.

Implications for Understanding the Intoxication of Mammalian Cells by PE. Our initial goal was to import the entire PE37 molecule into microsomes and then study its subsequent export. We were, however, unable to demonstrate complete import of the nascent chain because of the ability of the N-terminal region of PE280–613 to arrest translocation by exporting or releasing itself from microsomes after membrane insertion. Despite this limitation, the unique interaction of PE280–613 with microsomes is consistent with our understanding of the mechanism of intoxication of mammalian cells by PE.

The *in vitro* translocation assay indicates that sequences within domain II are essential to promote the microsomal export or release of the N-terminal region of PE280–613 after membrane insertion. This result is consistent with the hypothesis that domain II of PE mediates the translocation of PE280–613 through the ER during the intoxication of mammalian cells (3, 4, 22). In addition, we have shown previously that the deletion of 2 aa from PE37–TGF-α causes a 12-fold decrease in cytotoxic activity (9). The decrease in cytotoxic activity of this mutant was not the result of a decrease in ADP ribosyltransferase activity (domain III function) or a decreased affinity for the EGFR, indicating a defect in the ability of this mutant to reach the cell cytosol. Further, this deletion should not affect the tertiary structure of PE37–TGF-α, since it occurred at the exposed N terminus of the protein (1). By using the *in vitro* translocation assay, the export of the N-terminal region of a similar N-terminal mutant (ssPE282–613) after membrane insertion occurred less well than that of PE280–613. It is therefore possible that an interaction with the ER may be one rate-limiting step in the function of this mutant.

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