Domain II of *Pseudomonas* Exotoxin A Arrests the Transfer of Translocating Nascent Chains into Mammalian Microsomes

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ABSTRACT: The translocation of PE from the extracytosolic compartment to the cytosol during the intoxication of mammalian cells is mediated by domain II of the toxin. We have shown previously that within domain II amino acids 280–313 of PE promote their own export from mammalian microsomes following signal sequence-directed membrane insertion. In this study, we attempted to target full-length PE into mammalian microsomes using the preprocecropin signal sequence, but found that translocation was arrested to generate a transmembrane protein. "Stop transfer" required the presence of amino acids 280–313 of PE, and the first 313 amino acids of PE were sufficient to generate a transmembrane protein (N-terminus-in/C-terminus-out). The mechanism of stop transfer appears to be different from that described previously because amino acids 280–313 of PE are not highly hydrophobic and do contain many charged residues. In addition, the transmembrane segment appeared to be influenced by the cytoplasmic domain of the transmembrane proteins.

Pseudomonas exotoxin A (PE)[1] is a three-domain bacterial toxin of 66 kDa (Allured et al., 1986). During the intoxication of mammalian cells, domain Ia (amino acids 1–252) mediates cell binding; domain II (amino acids 253–364) is responsible for translocation of domain III (amino acids 400–613) into the cell cytosol; domain III catalyzes the ADP-ribosylation of elongation factor 2, which arrests protein synthesis and causes cell death (Hwang et al., 1987; Iglewski & Kabat, 1975). The function of domain Ib (amino acids 365–399) remains undefined, although amino acids 365–380 can be deleted without loss of cytotoxicity (Siegall et al., 1989). Domain Ia of PE can be replaced with growth factors, antibody variable domains, or CD4 to produce recombinant toxins that can be selectively targeted [reviewed in Pastan and FitzGerald (1991) and Pastan et al. (1992)].

Following receptor-mediated endocytosis by the cell, PE is cleaved within domain II between Arg-279 and Gly-280 (Ogata et al., 1992). Mutants of PE that cannot be cleaved by the intracellular protease are inactive (Ogata et al., 1990). A 37-kDa fragment termed PE37, that is composed of amino acids 280–613 of PE, ultimately reaches the cytosol to cause cytotoxicity (Ogata et al., 1990). The 37-kDa C-terminal fragment can itself be targeted to cells expressing the epidermal growth factor receptor (EGFR) by inserting transforming growth factor α (TGFα) near the C-terminus to produce a molecule termed PE37/TGFα, which begins at residue 280 of PE (Theuer et al., 1992). Deletion of two, four, or seven amino acids from the N-terminus of PE37/TGFα substantially diminishes its cytotoxic activity (Theuer et al., 1992). These findings led us to postulate that the N-terminal region of PE37 is active in translocation across a membrane from an extracytosolic compartment to the cytosol.

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 (Chaudhary et al., 1990). 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) (Chaudhary et al., 1990; Seetharam et al., 1991). On the basis of these 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 (Pastan et al., 1992).

Most mammalian secretory and transmembrane proteins are transported across the ER in a ribonucleoparticle-dependent manner as they are synthesized [reviewed in Rapoport (1992) and Wiech et al. (1991)]. These proteins contain a characteristic signal sequence at their N-terminus that binds the signal recognition particle (SRP; Blobel & Dobberstein, 1975) which, in turn, binds to the signal recognition particle receptor on the ER (Gilmore et al., 1982a, 1992b). Following GTP hydrolysis-dependent release of the SRP, the nascent protein translocates into the ER (Connolly & Gilmore, 1986; Connolly et al., 1991). On the lumenal face of the ER, the signal sequence is cleaved by signal peptidase (Jackson & Blobel, 1977), and core glycosylation may occur. It is likely that translocation occurs through an aqueous channel, or translocation pore (Blobel & Dobberstein, 1975; Simon & Blobel, 1991).

Recently, using an *in vitro* transcription/translation system, we reported that PE37 can be targeted into mammalian microsomes by the preprocecropin signal sequence. Following initial membrane insertion, however, the N-terminal region of PE37 arrests translocation by promoting its own export or release from the membrane (Theuer et al., 1993). The release of the N-terminal region occurs even when the nascent chain

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[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PE, *Pseudomonas* exotoxin A; EGFR, epidermal growth factor receptor; TGFα, transforming growth factor α; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; PNGase, peptide-*N*-glycosidase F; ER, endoplasmic reticulum.

Table 1: DNA Primers Used in This Study[a]

CT8	5′-TCC CTT TCG GGC TTT GTC TGC AGC CGA ATT CAT ATT CAA TT-3′
CT23	5′-GTA GCG CAG GCC GCT CCG GAA GCC GAA GAA GCT TTC GAC CTC T-3′
CT31	5′-GCT GCC GGG GCT GGC CAG GGC GTT GCG CGG CTG GCG ATG ACG GGT GAA-3′
CT252	5′-GCG GTC AGC GCG GCC AGC TGC AGT TAG CCC TCG GGA AAG TGC A-3′
CT279	5′-GCA CTG CTC CAG TTG CTG CAG TTA GCG CGG CTG GCG ATG AC-3′
CT313	5′-CCG CTG CCG GGG CTG GCC TGC AGT TAG CGG ATC ACC TGG TCG A-3′
CT335	5′-GCG GCC AGG GTC AGG GCC TGC AGT TAC TGC TCC GGC TGC TCG CGG AT-3′
CT346	5′-TGC CCT GCC GGA CGA ACT GCA GTT ACT CGG CGG CGG CCA GGG T-3′
CT364	5′-GCA GGT CAG GCT CAC CTG CAG TTA GTT GGA CGC GCC GGC CTC GT-3′
CT400	5′-AAG CTG ACG TCG CCG CCC TGC AGT TAG AAC TCC GCG CCA GTG GGA T-3′
CT101	5′-AAT GGT CAA TGC GGC TCCGGACTGT-3′
CT102	5′-ATG GAA TGG ATC CGC AGT TGT TGT TGT AGA TGA TTC T-3′
CT103	5′-ATC GCC AGG GAT CCG GCT GGG AAC AAC TGG A-3′
CT104	5′-CCG CTG CCG GGG AGC TCC TGC AGT TAG CGG ATC ACC TGG TCG A-3′
CT103N	5′-TGC ACT TTG GAT CCG GCG GCA GCC TGG CCG CGC T-3′

[a] Oligonucleotides were used as discussed under Experimental Procedures. Underlined sequences represent unique restriction enzyme sites for *Pst*I (CT8, CT252, CT279, CT313, CT335, CT346, CT364, CT400, and CT101), *Bsp*EI (CT23 and CT101), or *Bam*HI (CT102, CT103, and CT103N).

remains tethered to ribosomes, indicating that it is mediated by microsomal elements close in proximity to the translocation apparatus (Theuer et al., 1993).

Protein translocation across microsomes can be interrupted by amino acids sequences referred to as "stop transfer" sequences that are distinct from signal sequences (Yost et al., 1983). These sequences typically contain a stretch of hydrophobic amino acids that form a transmembrane segment followed by a stretch rich in basic amino acids that form the cytoplasmic segment [reviewed in Blobel (1980), Sabatini et al. (1982), Singer et al. (1987), and von Heijne and Gavel (1988)]. Since the N-terminal region of PE37 (amino acids 280–313) promotes its own export from microsomes, we wanted to know how this sequence would behave when preceded by the large N-terminal sequence of PE. Our results indicate that a portion of domain II of PE functions to promote "stop transfer" of the translocating nascent chain.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes, T4 DNA ligase, polynucleotide kinase, and calf intestinal alkaline phosphatase were from New England BioLabs (Beverly, MA). *Escherichia coli* strain DH5α from GIBCO/BRL (Gaithersburg, MD) was used for the propagation of plasmids. AmpliTaq DNA polymerase was from Perkin-Elmer/Cetus (Norwalk, CT). Phenylmethanesulfonyl fluoride (PMSF), canine pancreatic microsomes, and rabbit reticulocyte lysate were from Boehringer Mannheim (Indianapolis, IN). Tetracaine hydrochloride, cycloheximide, and proteinase K were from Sigma Chemical Co. (St. Louis, MO). The Sequenase DNA sequencing kit and DNase-free RNase were from United States Biochemical (Cleveland, OH). [^{3}H]Leucine (165 Ci/mmol) and protein molecular mass standards (rainbow standards) were from Amersham Corp. (Arlington Heights, IL). PVDF membranes were from Novex (San Diego, CA). PNGase was from Oxford Glycosystems (Rosedale, NY).

Amplification. Oligonucleotides are detailed in Table 1 and were purchased from Bioserve Biotechnologies (College Park, MD). Polymerase chain reactions were carried out using 10 ng of template and reagents as per the manufacturer's instructions (Perkin-Elmer/Cetus Instruments) in the presence of 5% formamide (Fluka Chemika-Biochemika, Ronkonkoma, NY) and 100 pmol of oligonucleotides. Each polymerase chain reaction totaled 25 cycles consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 90 s, and polymerization at 72 °C for 2 min with a 10-s extension in each cycle. Amplified fragments were purified from unused oligonucleotides by ultrafiltration using a Centricon 100 microconcentrator (Amicon, Beverly, MA).

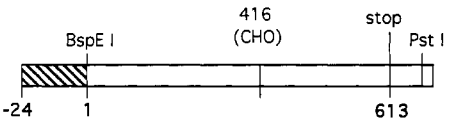

FIGURE 1: Restriction sites in pCT45 which encodes the preprocecropin signal sequence followed by the sequence of *Pseudomonas* exotoxin A, including a deletion of residue 553. Numbers indicate individual residues within the *Pseudomonas* exotoxin A encoding region. The potential N-glycosylation site is indicated by the letters CHO.

Plasmid Construction. Plasmid DNA was prepared using QIAGEN columns (QIAGEN Incorporated, Studio City, CA) according to the manufacturer's instructions. pVC45D (f+T) encodes amino acids 1–613 of PE, with a deletion of the glutamate residue at position 553 (Prior et al., 1991).

pCA37 encodes the SP6 promoter followed by the preprocecropin signal sequence and prolactin (Schlenstedt et al., 1992). 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 prolactin termination codon (Figure 1). PE-derived plasmids were constructed by ligating *Bsp*EI–*Pst*I-digested PCR fragments into the *Bsp*EI–*Pst*I restriction sites in pCA37. pCT37 was made using a PCR fragment amplified with oligonucleotides CT23 and CT8 (Table 1) using pVC45D as template and encodes the preprocecropin signal sequence, amino acids APQ, and residues 1–613 of PE, except for deletion of the glutamate residue at position 553. pCT47 was made using oligonucleotide-directed mutagenesis by a modification of the method of Kunkel (1985). Single-stranded DNA from pVC45 (f+T) was prepared from first-cycle phage in uridine-containing medium, annealed with the phosphorylated oligonucleotide CT31, treated with T4 DNA polymerase and T4 DNA ligase, and transformed into *E. coli* strain DH5α. DNA from individual colonies was screened for the loss of a unique *Sal*I site, and pCT47 was verified by DNA sequencing to encode amino acids 1 through 279 and 314 through 613 of PE. pCT48 was made by ligating a *Bsp*EI–*Pst*I-digested PCR fragment made with oligonucleotides CT23 and CT8 (Table 1) using pCT47 as template into the *Bsp*EI–*Pst*I sites found in pCA37. To make pCT49, which encodes the preprocecropin signal sequence, amino acids APQ, and amino acids 1 through 279 and 314 through 613 of PE, as well as deletion of the glutamate residue at position 553, the 383 base pair *Eag*I fragment of pVC45D was ligated into the *Eag*I sites of pCT48. pCT252 was made using a PCR fragment amplified with oligonucleotides CT23 and CT252 (Table 1) using pVC45D as template and encodes the preprocecropin signal sequence, amino acids

APQ, and residues 1–252 of native PE. pCT279 was made using a PCR fragment amplified with oligonucleotides CT23 and CT279 (Table 1) using pVC45D as template and encodes the preprocecropin signal sequence, amino acids APQ, and residues 1–279 of PE. pCT313 was made using a PCR fragment amplified with oligonucleotides CT23 and CT313 (Table 1) using pVC45D as template and encodes the preprocecropin signal sequence, amino acids APQ, and residues 1–313 of PE. pCT335 was made using the PCR fragment amplified with oligonucleotides CT23 and CT335 (Table 1) using pVC45D as template and encodes the preprocecropin signal sequence, amino acids APQ, and residues 1–335 of PE. pCT346 was made using the PCR fragment amplified with oligonucleotides CT23 and CT346 (Table 1) using pVC45D as template and encodes the preprocecropin signal sequence, amino acids APQ, and residues 1–346 of PE. pCT364 was made using the PCR fragment amplified with oligonucleotides CT23 and CT364 (Table 1) using pVC45D as template and encodes the preprocecropin signal sequence, amino acids APQ, and residues 1–364 of PE. pCT400 was made using the PCR fragment amplified with oligonucleotides CT23 and CT400 (Table 1) using pVC45D as template and encodes the preprocecropin signal sequence, amino acids APQ, and residues 1–400 of PE. Plasmids were confirmed by DNA sequencing or restriction analysis (Sanger et al., 1977).

In Vitro Transcription and Translocation Assay. In vitro transcription was carried out for 4 h at 37 °C with SP6 RNA polymerase in the presence of 5 mM ATP, 5 mM CTP, 5 mM UTP, 4 mM $m^7G(5')ppp(5')G$ cap analog, and 1 mM GTP using a MEGAscript transcription kit (Ambion Incorporated, Austin, TX). mRNA was purified by gel filtration through Sephadex G-50 Quick Spin columns (Boehringer Mannheim) and translated in rabbit reticulocyte lysate in the presence of [^{3}H]leucine supplemented with canine pancreatic microsomes (where applicable) at 30 °C for 1 h unless otherwise noted according to the manufacturer's instructions (Boehringer Mannheim). Translation mixtures were diluted with 3 volumes of 50 mM Tris-HCl (pH 7.4)/100 mM NaCl and incubated at room temperature for 5 min in the presence of 2 mM tetracaine to stabilize microsomal membranes (Scheele, 1983). Proteinase K was added to a final concentration of 20 μg/mL in the presence or absence of 1% Triton X-100, and digestion was carried out for 10 min at room temperature. The reaction was terminated by the addition of PMSF to a final concentration of 3 mM. One-third volume of 4 × SDS sample buffer (Laemmli, 1970) was added, and samples were heated at 85–90 °C for 3–5 min prior to analysis by SDS–PAGE.

For deglycosylation, PMSF was added to a final concentration of 10 mM following proteinase K treatment of translation mixtures containing microsomes. Mixtures were diluted with an equal volume of 0.5 M sodium phosphate (pH 7.4), 20 mM EDTA, 0.4% SDS, and 2% β-mercaptoethanol and boiled for 5 min. Samples were allowed to cool to room temperature, PMSF was added to a final concentration of 3 mM, and PNGase F was added to a final concentration of 20 units/mL. The mixture was incubated overnight at 37 °C. For analysis by SDS–PAGE, one-third volume of 4 × SDS sample buffer was added, and the sample was heated at 85–90 °C for 3–5 min.

SDS–PAGE and Autoradiography. SDS–PAGE was carried out using the method of Laemmli (1970). Precast gels of 12% were purchased from Novex. After electrophoresis, the gels were fixed for 1 h in 30% methanol/10% acetic acid, and then fluorography was done with Entensify (New England Nuclear, Boston, MA) according to the manufacturer's instructions. Gels were dried under vacuum and exposed overnight to X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at −70 °C.

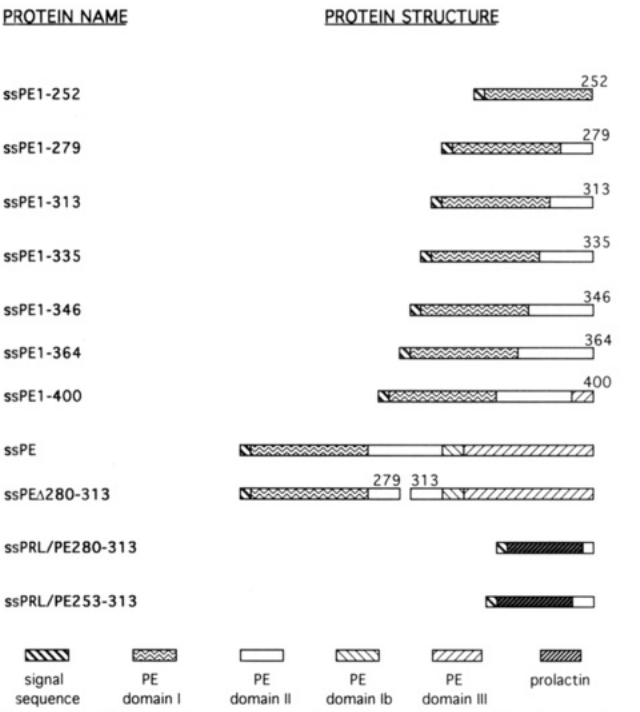

FIGURE 2: Schematic representation of proteins used in the study. The positions of amino acids that span *Pseudomonas* exotoxin A sequences are numbered.

Protein Sequencing. Following *in vitro* translation in the presence of [^{3}H]leucine and microsomes and treatment with proteinase K, samples were prepared in sample buffer as described above and subjected to SDS–PAGE. Proteins were blotted onto 0.45-μm PVDF membranes in 10 mM CAPS and 10% methanol for 45 min at 300 mA. The PVDF membranes were allowed to dry, and the radioactive bands representing the protease-resistant species were cut out and subjected to amino acid sequencing (Coligan et al., 1983; Sheer et al., 1991). Aliquots from each round of Edman degradation were counted for the presence of [^{3}H]leucine.

RESULTS

PE Is Prevented from Fully Translocating into Microsomes When Targeted Using the Preprocecropin Signal Sequence. The gene encoding PE was cloned downstream of DNA encoding the preprocecropin signal sequence in a plasmid containing an SP6 promoter (see Experimental Procedures). mRNA generated from the plasmid was tested using an *in vitro* translation assay that has been described (Theuer et al., 1993; see Experimental Procedures). The protein, termed ssPE, is shown schematically in Figure 2. The toxin portion of the protein contains a deletion of the glutamate residue at position 553 of PE to prevent domain III from ADP-ribosylating elongation factor 2 and possibly inactivating the reticulocyte lysate (Lukac et al., 1988). This mutation does not prevent the translocation function of PE (Prior et al., 1991) or affect its processing in mammalian cells (Ogata et al., 1990). *In vitro* translation of mRNA encoding ssPE in the presence of [^{3}H]leucine yielded a single radiolabeled species of 69 kDa that was degraded by proteinase K (Figure 3, lanes 1 and 2). When translation was done in the presence of microsomes, a smaller radiolabeled species of 67 kDa was produced, indicating that signal sequence cleavage had occurred (Figure 3, lane 3). Following translation in the presence of microsomes, only a 33-kDa fragment of PE was

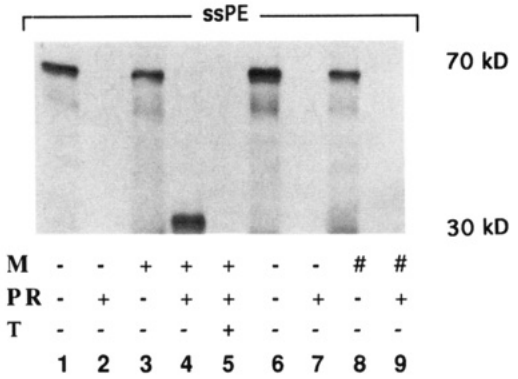

FIGURE 3: PE is partially sequestered into microsomes in a cotranslational manner when targeted by the preprocecropin signal sequence. Plasmid CT37 encoding the preprocecropin signal sequence followed by PE was linearized with *Pst*I (a site 3′ to the termination codon) and transcribed with SP6 RNA polymerase. Translation using [^{3}H]leucine was carried out in the absence (–) or presence (+) of canine pancreatic microsomes (M), or microsomes were added following the arrest of translation (#). Treatment with proteinase K (PR) and Triton X-100 (T) and analysis of radiolabeled species on SDS–PAGE were carried out as described under Experimental Procedures.

protected from degradation by proteinase K. The 33-kDa fragment did not represent a protease-resistant domain because full-length PE was completely degraded by proteinase K (Figure 3, lane 2). When mRNA encoding mature prolactin fused to the preprocecropin signal sequence was translated in the presence of microsomes, it was completely sequestered within microsomes in a cotranslational manner (Theuer et al., 1993). The 33-kDa protease-resistant PE fragment was thus not the result of "leaky microsomes" or a unique function of the preprocecropin signal sequence. The 33-kDa fragment was indeed sequestered within microsomes since degradation by proteinase K required solubilization of microsomes with Triton X-100 (Figure 3, lane 5).

When microsomes were added posttranslationally following termination of ssPE translation with cycloheximide, the 69-kDa radiolabeled species remained, indicating that signal sequence cleavage had not occurred (Figure 3, compare lanes 6 and 8). Hence, ssPE is imported into microsomes only in a ribonucleoparticle-dependent fashion (Wiech et al., 1991). Furthermore, treatment of the 69-kDa fragment by proteinase K following the posttranslational addition of microsomes did not yield a resistant 33-kDa fragment (Figure 3, lane 9). This indicates that the microsomes did not interact with PE in a posttranslational fashion to protect a portion of the protein from proteinase K. We conclude that a portion of PE is translocated into microsomes in a cotranslational manner and that sequences of PE subsequently arrest translocation to generate a transmembrane protein.

Amino Acids 280–313 of PE Are Required To Prevent the Full Translocation of PE Preceded by the Preprocecropin Signal Sequence. Amino acids 280–313 of PE are sufficient to abort the translocation of downstream sequences into microsomes following initial membrane insertion using the preprocecropin signal sequence (Theuer et al., 1993). To test whether these amino acids were also required to arrest the translocation of PE preceded by the preprocecropin signal sequence, these 34 residues were deleted to produce a protein termed ssPEΔ280–313 (Figure 2). Translation of mRNA encoding ssPEΔ280–313 yielded a single 66-kDa radiolabeled species that was degraded by proteinase K (Figure 4). Translation in the presence of microsomes yielded a smaller species of 64 kDa and one of increased size. Both species were resistant to proteinase K, indicating that they were

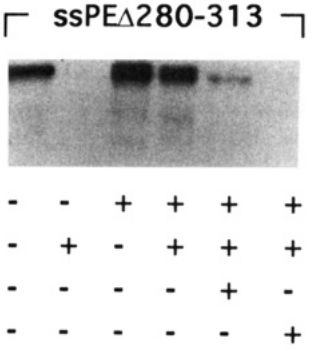

FIGURE 4: Amino acids 280–313 of PE are necessary to prevent the full translocation of PE into microsomes. pCT49, encoding the preprocecropin signal sequence followed by amino acids 1–279 and 314–613 of PE (ssPEΔ280–313), was linearized with *Pst*I and transcribed with SP6 RNA polymerase. Translation with [^{3}H]leucine was carried out in the absence or presence of microsomes (M) as indicated. Treatment with proteinase K (PR), PNGase F (PN), and Triton X-100 (T) and analysis of radiolabeled species on SDS–PAGE were carried out as described under Experimental Procedures.

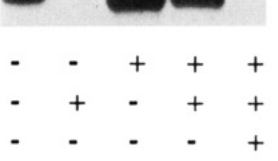

FIGURE 5: Domain I of PE is fully sequestered into microsomes when targeted using the preprocecropin signal sequence. pCT252 encoding the preprocecropin signal sequence followed by amino acids 1–252 of PE was linearized with *Pst*I and transcribed with SP6 polymerase. Translation with [^{3}H]leucine was carried out in the presence of microsomes (M) as indicated. Treatment with proteinase K (PR) and Triton X-100 (T) and analysis of radiolabeled species on SDS–PAGE were carried out as described under Experimental Procedures.

completely sequestered inside of microsomes. The species of increased molecular mass was eliminated by PNGase treatment, indicating that core glycosylation as well as signal sequence cleavage had occurred (Figure 4). Since PNGase F specifically cleaves N-linked oligosaccharides (Plummer et al., 1984), we conclude that ssPEΔ280–313 was glycosylated at the single potential N-linked glycosylation site located at residue 416 of PE (see Figure 1). Since the mature form of ssPEΔ280–313 could be fully translocated, glycosylated, and sequestered into microsomes, amino acids 280–313 of PE are required to arrest the translocation of PE into microsomes.

The Mature Form of ssPE That Is Translocated into Microsomes Includes the N-Terminal Portion and Contains Approximately Amino Acids 1–313. We reasoned that the simple explanation for the failure of PE to be fully translocated into microsomes was that a portion of the PE sequence functioned as a stop transfer sequence, producing a type I transmembrane protein (N-terminus-in/C-terminus-out). To investigate this point, we constructed a series of ssPE mutants containing progressive deletions of C-terminal portions of PE (see Figure 2). If only the N-terminal portion of PE was sequestered into microsomes, then some mutants would be fully sequestered into microsomes, while larger mutants (those including a functional stop transfer sequence) would be only partially translocated.

In vitro translation of mRNA encoding the preprocecropin signal sequence followed by amino acids 1–252 (domain I) of PE (termed ssPE1–252; Figure 2) is shown in Figure 5.

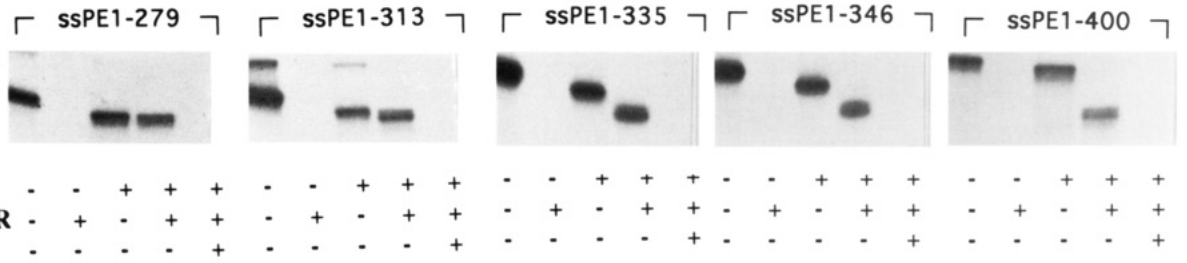

FIGURE 6: Mutants of ssPE containing deletions of the C-terminal portion are partially sequestered into microsomes. pCT279 encoding the preprocecropin signal sequence followed by amino acids 1–279 of PE (ssPE1–279), CT313 encoding the preprocecropin signal sequence followed by amino acids 1–313 of PE (ssPE1–313), CT335 encoding the preprocecropin signal sequence followed by amino acids 1–346 of PE (ssPE1–346), and pCT400 encoding the preprocecropin signal sequence followed by amino acids 1–400 of PE were linearized with *Pst*I and transcribed with SP6 RNA polymerase. Translation with [^{3}H]leucine was carried out in the absence or presence of microsomes (M) as indicated. Treatment with proteinase K (PR) and Triton X-100 (T) and analysis of radiolabeled species on SDS–PAGE were carried out as described under Experimental Procedures.

Translation in the absence of microsomes yielded a single radiolabeled species of 27 kDa that was degraded by proteinase K. Translation in the presence of microsomes yielded a smaller species indicating that signal sequence cleavage had occurred. The mature protein was fully sequestered into microsomes since its size was not affected by treatment with proteinase K unless microsomes were solubilized with Triton X-100. Amino acids 1–279 of PE were also targeted to microsomes by the preprocecropin signal sequence (ssPE1–279; Figure 2). Following signal sequence cleavage, the mature product also completely resisted degradation by proteinase K unless microsomes were solubilized with Triton X-100, indicating that the mature product was fully sequestered into microsomes (Figure 6). These data indicate that the N-terminal portion of PE (amino acids 1–279) does not, by itself, interact with microsomes to interrupt translocation. This result is consistent with data showing that the sequences of toxin that interact with mammalian membranes start at amino acid 280 (Theuer et al., 1992).

Amino acids 1–313, 1–335, 1–346, 1–364, and 1–400 of PE were also targeted to microsomes by the preprocecropin signal sequence (to produce the proteins ssPE1–313, ssPE1–335, ssPE1–346, ssPE1–364, and ssPE1–400; Figure 2). In each case, translation of these five longer mutants in the presence of microsomes yielded species of slightly smaller size in comparison to the translation product produced without microsomes, indicating that signal sequence cleavage had occurred (Figure 6 and data not shown). However, the mature products were not fully sequestered into microsomes since treatment with proteinase K in the presence of microsomes in each case produced products of lower molecular mass (Figure 6 and data not shown). To allow a direct comparison of the sizes of the proteinase K-resistant fragments of these mutants as well as that produced from ssPE, mRNA encoding each protein was translated in the presence of microsomes, an aliquot of each translation mixture was then treated with proteinase K, and results were analyzed by SDS–PAGE and autoradiography. The mature product of ssPE1–279 was used as a size standard for comparison (Figure 7). In most cases, the fully translocated products that remained resistant to proteinase K occurred as a wide band, indicating two poorly resolved species. One had a molecular mass (30 kDa) slightly higher than that of PE1–279. The second had a molecular mass (32 kDa) close to that of PE1–313. Interestingly, the fully translocated product of PE occurred as a single species with a molecular mass (33 kDa) slightly higher than that of PE1–313.

These data are consistent with the hypothesis that the N-terminal portion of PE was translocated into microsomes

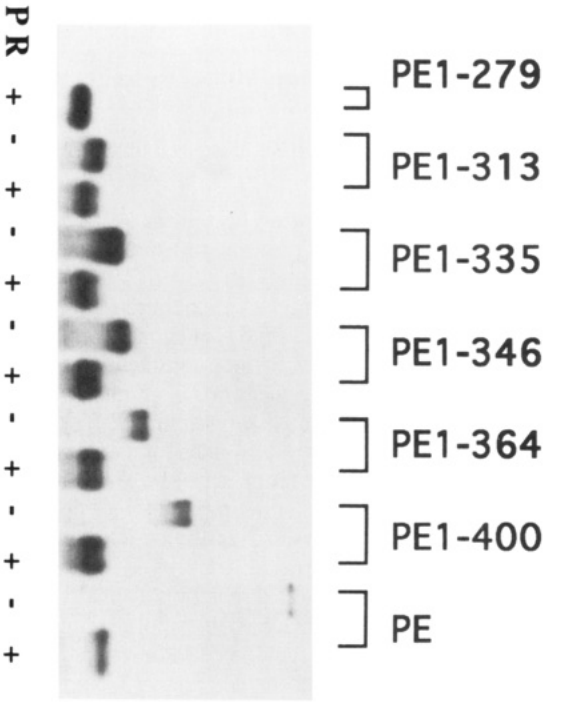

FIGURE 7: Comparison of the relative sizes of translocated fragments of PE and PE mutants. Plasmids were linearized with *Pst*I and transcribed with SP6 RNA polymerase. Translation with [^{3}H]leucine was carried out in the presence of microsomes (M). Treatment with proteinase K (PR) and analysis of radiolabeled species on SDS–PAGE were carried out as described under Experimental Procedures. Five microliters of sample was loaded in lanes treated without proteinase K, and 10 μL of sample was loaded in lanes treated with proteinase K in order to see individual bands more clearly.

and that translocation was arrested by a specific PE sequence leaving the C-terminal portion outside microsomes and sensitive to proteinase K. To substantiate this hypothesis, [^{3}H]leucine-labeled proteinase K-generated doublets from ssPE1–313, ssPE1–335, ssPE1–364, and ssPE1–400 were subjected to sequential rounds of Edman degradation. Following signal sequence cleavage, the initial N-terminal leucine residue of each of these mutants, as well as PE, occurs at the 10th position. If the proteinase K-generated fragments occurred as a result of cleavage at the C-terminal region, we expected nearly identical sequence patterns for the N-terminus of each PE mutant fragment. In contrast, if the proteinase K-generated fragments occurred as a result of cleavage at the N-terminal region of the mature proteins, we expected a unique sequence pattern for each PE mutant fragment. In each case, the radioactive peak of [^{3}H]leucine was located primarily in the 10th cycle (Figure 8). These data indicate that the

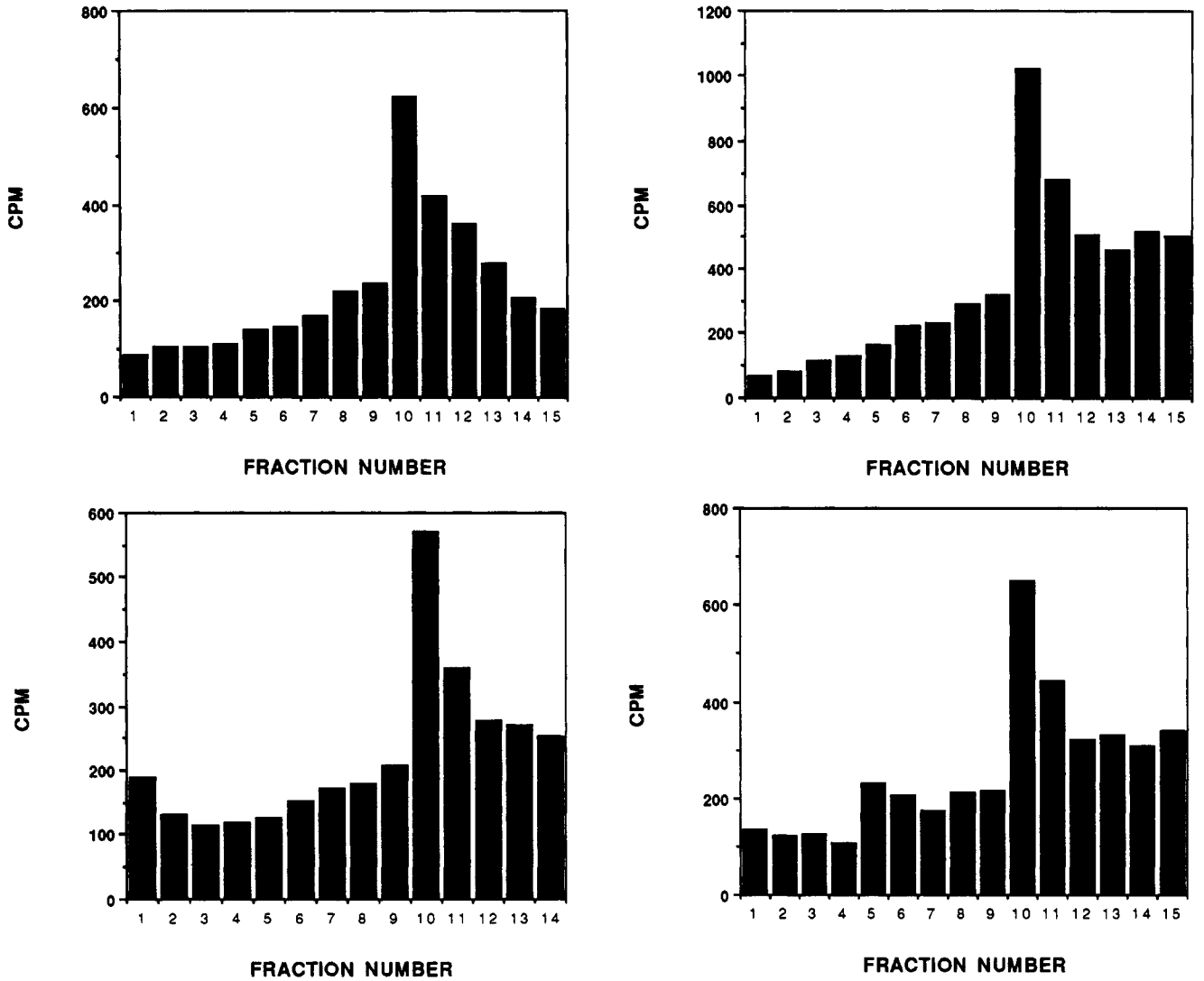

FIGURE 8: Analysis of sequential Edman degradations of proteinase K-resistant mature fragments of C-terminal deletion mutants of PE. mRNA encoding ssPE1–313 (upper left panel), ssPE1–335 (upper right panel), ssPE1–364 (lower left panel), and ssPE1–400 (lower right panel) was translated in the presence of microsomes and treated with proteinase K as described under Experimental Procedures. Following SDS–PAGE, bands were transferred to PVDF membranes, and doublets representing the proteinase K-resistant fragments of each mutant protein were cut out and subjected to Edman degradation. Fractions were analyzed for the presence of [^{3}H]leucine.

N-terminal portion of the mature proteins PE1–313, PE1–335, PE1–364, and PE1–400 remained intact following translocation in the presence of microsomes and treatment with proteinase K. The site of cleavage of these mutants occurred at the C-terminal portion of each molecule. Furthermore, since the proteinase K-generated mutant PE fragments were of nearly identical size in each case, we conclude that the two proteinase K-resistant fragments were produced by cleavage at nearly the same two sites in each mutant. These data indicate that a specific PE sequence arrested translocation. Translocation arrest was only detected with mutants containing the domain II sequence. These data are consistent with the result of Figure 4 which indicates that amino acids 280–313 of PE are required to arrest the translocation of PE.

DISCUSSION

In the current study, residues within domain II of PE were targeted to cross the microsomal membrane in the opposite direction from that which occurs during the intoxication of mammalian cells (Pastan et al., 1992). We found that amino acids 280–313 of PE prevented transfer of PE into microsomes, and thereby functioned as a "stop transfer" sequence. The resulting transmembrane protein contained an N-terminus sequestered within the microsomal lumen and a C-terminus that remained outside of microsomes.

Stop transfer sequences typically consist of a transmembrane segment of at least 20 nonpolar amino acids that are frequently followed by a segment rich in basic residues (Sabatini et al., 1982). While a lumenally disposed charged domain is also required for stop transfer of the prion protein (Lopez et al., 1990; Yost et al., 1990), this is not a universal requirement (von Heijne & Gavel, 1988). The sequence of domain II of PE necessary for translocation arrest is markedly distinct from the typical "stop transfer" sequence. Amino acids 280–313 of PE are contained within a long α helix (Allured et al., 1986). The helix contains numerous nonpolar amino acids, and the longest stretch of uncharged amino acids is Leu-Val-Ala-Leu-Tyr-Leu-Ala-Ala. Kuroiwa et al. (1991) have shown that nine leucine residues followed by two lysine residues are sufficient to arrest the translocation of interleukin-2. However, a much greater number of less hydrophobic residues (19 alanines) was required to achieve the same effect. It is unlikely that the eight-residue stretch of amino acids 294–301 of PE, which are not entirely hydrophobic, can function as a classic transmembrane segment.

Mechanism of Stop Transfer. When the microsome-associated mutants PE1–335, PE1–346, PE1–364, and PE1–400 were treated with proteinase K, two protease-resistant species were observed, and these had been cleaved at two nearly indentical sites within each protein. These data suggest that the transmembrane segments of each of these proteins existed as two distinct stable structures. In contrast, a single proteinase K-resistant fragment was generated in the case of the transmembrane form of PE. While the N-termini of PE and its C-terminal mutants are identical, PE is unique in that it contains a large C-terminal domain (domain III) capable of forming a stable tertiary structure. It is possible that the two transmembrane forms of the C-terminal PE deletion mutants may be a consequence of alternative protein folding pathways of the truncated C-termini.

We previously showed that when amino acids 280–313 are present at the amio end of a protein preceded by a signal sequence, they promote their own export after the signal sequence is removed within the lumen of the microsome. We suggested that this export is catalyzed by some interaction of the 280–313 sequence with a component of the protein import complex. We believe that this same interaction is responsible for the arrest of translocation that occurs when PE is targeted to the lumen of microsomes. Presumably, amino acids 280–313 are capable of promoting their own export, but are unable to do so because domain I exists within the microsome in a folded state and this folded structure cannot be exported through the pore.

In summary, we have shown that a sequence of amino acids in domain II of PE functions to stop the transfer of the protein into microsomes. The mechanism of stop transfer appears to be unique from that described for other proteins.

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