An Early Step in Pseudomonas Exotoxin Action Is Removal of the Terminal Lysine Residue, Which Allows Binding to the KDEL Receptor

Jill L. Hessler and Robert J. Kreitman*

Laboratory of Molecular Biology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, 37/4E16, 37 Convent Drive, MSC 4255, Bethesda, Maryland 20892

Received June 17, 1997; Revised Manuscript Received September 5, 1997[⊗]

ABSTRACT: During the intoxication process, Pseudomonas exotoxin (PE) and immunotoxins containing PE internalize into the target cell and become processed into two fragments, and the carboxyl fragment translocates into the cytosol where it inactivates elongation factor 2. We have proposed that after internalization into cells the carboxyl-terminal fragment of PE (amino acids 280−613), which ends in REDLK, binds to the KDEL receptor (ERD2) which carries it to the endoplasmic reticulum, from which the PE fragment translocates to the cytosol. Earlier experiments showing that REDL but not REDLK binds to the KDEL receptor suggested that the terminal lysine is removed sometime during the intoxication process. To determine if and where this occurs, we exposed a peptide ending in REDLK to malignant cells in culture and found that binding to the KDEL receptor was restored. Restoration of receptor binding also occurred if a peptide or toxin ending in REDLK at its carboxyl terminus was incubated with plasma, indicating that the terminal lysine is removed prior to entry of the toxin into the cell. We conclude that plasma carboxypeptidase(s) cleave(s) the lysine residue from the carboxyl terminus of PE and PE-containing immunotoxins as an early and essential step in their cellular intoxication pathway.

Pseudomonas exotoxin (PE) is a 613 amino acid protein produced by *Pseudomonas aeruginosa* which kills cells by inhibition of protein synthesis. The major domains of PE elucidated by its crystallographic structure (Allured et al., 1986) include domain Ia (amino acids 1−252), which binds to the α_2-macroglobulin receptor present on animal cells (Kounnas et al., 1992), domain II (amino acids 253−364), which contains a proteolytic site for furin cleavage located between amino acids 279 and 280 (Chiron et al., 1994; Ogata et al., 1992) and a protein translocating sequence (amino acids 280−313) (Theuer et al., 1993a, 1994), and domain III (amino acids 400−613), which contains the ADP-ribosylating enzyme within amino acids 400−602 (Chaudhary et al., 1990; Hwang et al., 1987). Replacement of the last five amino acids of PE, REDLK, with the endoplasmic reticulum (ER) retention sequence KDEL improves cytotoxicity (Kreitman et al., 1993; Seetharam et al., 1991). The current model describing how PE kills cells includes the following steps: The toxin binds to its receptor; it is internalized into endosomes and at low pH unfolds and is cleaved by the protease furin between amino acids 279 and 280. The disulfide bond connecting Cys265 and Cys287 is reduced, and the carboxyl terminus of the toxin binds to the KDEL receptor and is shuttled from the trans-Golgi (Miesnbock & Rothman, 1995) to the ER where sequences in domain II induce translocation of the protein to the cytoplasm, possibly through preexisting ER pores. In the cytosol, the toxin catalytically ADP-ribosylates elongation factor 2 (EF2), inhibits protein synthesis, and induces programmed cell death (Brinkmann et al., 1995).

It has been shown that introduction of only a few molecules of PE into the cytoplasm of a cell by microinjection is sufficient to kill the cell (Willingham and Pastan, unpublished data). PE-based recombinant toxins containing various binding domains have been made to target cancer cells. These immunotoxins can kill cells that have only a few hundred sites per cell (Kreitman et al., 1992), even though only a small percentage of toxin molecules that bind to a cell actually reach the cytoplasm (Ogata et al., 1990). If the delivery of the toxin to the cytosol is inefficient, the target cell will escape intoxication. Thus, elucidating and refining the steps necessary for cellular intoxication by PE has clinical importance.

We have found that immunotoxins and peptides ending in KDEL or REDL bind to the KDEL receptor, but those ending in REDLK, the native sequence of PE, do not (Kreitman & Pastan, 1995). These data suggested that the terminal lysine residue of PE is removed prior to the binding of the toxin to the intracellular KDEL receptor. Our goals in the present study were to test the validity of this hypothesis, and in addition to determine if lysine removal occurs before or after binding of the toxin to the cell surface. We show here that the terminal lysine residue of PE is removed during incubation with target cells, allowing peptide or protein ending in REDLK to bind to the KDEL receptor. We also show that removal of this lysine can occur outside the cell in the plasma.

MATERIALS AND METHODS

Plasmid Construction. Plasmid pCT11 encodes PE35, composed of methionine followed by amino acids 281−364 and 381−613 of PE (Theuer et al., 1993b). To make pRK35K, encoding PE35 ending in KDEL, the 0.36 kb *Bam*HI−*Eco*RI fragment of pRK749K (Kreitman et al., 1994) was ligated to the 3.6 kb *Bam*HI−*Eco*RI fragment of

* To whom correspondence should be addressed. Phone: (301) 496-4797. Fax: (301) 402-1344.

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1997.

pCT11. To make pRK35RED, encoding PE35 ending in REDL, the 0.36 kb *Bam*HI−*Eco*RI fragment of pRK79RED (Kreitman & Pastan, 1995) was ligated to the 3.6 kb fragment of pRK35K. To make pRK35KK, encoding PE35 ending in KDELK, pRK35K was amplified by PCR using the primers VK116 (5′-TGG-CGC-GGT-TTC-TAT-ATC-GCC-3′) and BK200 (5′-TTG-TTA-GCA-GCG-AAT-TCA-CTT-AAG-CTC-GTC-TTT-CGG-CGG-3′). The 0.37 kb *Bam*HI−*Eco*RI fragment of the amplification product was ligated to the 3.6 kb *Bam*HI−*Eco*RI fragment of pRK35K. The PCR amplification contained 30 cycles and required the presence of 5% formamide. Cycles contained a 1 min denaturation at 94 °C, a 2 min annealing at 55 °C, and a 3 min polymerization at 72 °C with a 10 s extension per cycle. The correct sequence was confirmed by automated sequencing using a Dyedeoxy terminator cycle sequencing kit from Applied Biosystems (Foster City, CA).

Purification of Recombinant Proteins. Production of PE35, PE35REDL, PE35KDELK, and PE35KDEL from their respective plasmids pCT11, pRK35RED, pRK35KK, and pRK35K required a modified protocol, since these proteins were secreted into the periplasm of *E. coli* and each contains a free cysteine residue. BL21/lDE3 was transformed with plasmid and grown on LB-ampicillin plates overnight at 37 °C. The bacteria were fermented in 2 L flasks, each containing 500 mL of superbroth media containing 100 μg/mL ampicillin, 1.6 mM $MgSO_4$, and 12.5 mL of 20% dextrose. At an OD_{650} of 2−3, isopropyl β-D-thiogalactopyranoside (IPTG; United States Biochemicals, Cleveland, OH) was added to a final concentration of 1 mM. After 90−120 min, the cell paste was obtained and resuspended for 10−20 min in cold sucrose solution (20% sucrose, 30 mM Tris, pH 7.4, 2 mM EDTA), and then the centrifuged cells were resuspended in cold water to obtain periplasm. The periplasmic protein was clarified by centrifugation at 13 000 rpm in a GSA rotor (Sorvall, Wilmington, DE) and filtration through a 0.45 μm filter. The protein was then treated with mercaptoethanol (ETSH) to a final concentration of 1 mM and in this concentration of ETSH purified by Q Sepharose and MonoQ anion exchange chromatography (Pharmacia, Piscataway, NJ). Monomeric protein was >80−90% pure by reducing SDS−PAGE gels (data not shown). ETSH was removed from the protein using PD-10 columns (Pharmacia).

KDEL Receptor Binding Assays. The protocol for measuring relative binding to the KDEL receptor was reported previously (Kreitman & Pastan, 1995; Wilson et al., 1993). Golgi membranes were purified from rat liver as described (Tabas & Kornfeld, 1979). [^{125}I]-YTSEKDEL, radioiodinated using chloramine T, was incubated in 1.8 mL microfuge tubes (Robbins Scientific, Sunnyvale, CA) in aliquots of 25 μL of 1 ng (18−150 μCi/μg) in the presence or absence of different concentrations of unlabeled peptides or proteins. The binding buffer consisted of 50 mM calcium cacodylate, 20 mM NaCl, 100 mg/mL BSA, and 0.02% Triton X-100, adjusted to a pH of 5.0 using acetic acid. This was the optimal pH to study the binding of both peptides and PE-related proteins. After ∼15 min of incubation at 4 °C, the Golgi membranes were resuspended with a pipet tip and centrifuged at 12 000−14 000 rpm for 5−7 min at 4 °C. The supernatants were aspirated and the tubes counted.

Incubation with Cell Lines and Plasma. The medium for cell culture was RPMI containing 10% FBS. The adherent colon lines LS174T or SW403 (available from ATCC, Rockville, MD) were grown in 96 well plates overnight at 15 000 cells/well. The adult T-cell leukemia (ATL) HUT-102 and MT-1 cells, which grew in suspension, were obtained from Dr. T. Waldmann and were grown at 40 000 cells/well. Assays to measure activation of the carboxy terminus of PE in the presence of cells were performed by incubating the colon lines with 2.3 mM and HUT-102 cells with 3 mM of the peptides YTSEREDL and YTSEREDLK. After 24 h at 37 °C, the medium was incubated in the Golgi assay to determine displacement of [^{125}I]-YTSEKDEL from the KDEL receptor. Plasma was obtained by collecting blood in citrate-containing tubes and was stored at −80 °C until use.

Carboxy Terminal Sequencing. Plasma was diluted 10-fold with PE35 to a final PE35 concentration of 95 μM (3.3 mg/mL) and a final albumin concentration of ∼4 mg/mL. After incubating at 37 °C for 6 h, the PE35 (35 kDa) was separated from the albumin (68 kDa) on a 10% nonreducing SDS−PAGE gel and electroeluted to a Teflon membrane which was presoaked in methanol followed by electroelution buffer. The electroelution buffer consisted of 2 mg/mL CAPS and 10% methanol. The Teflon membrane was stained with 0.1% Coomassie blue in methanol and destained in 50% methanol containing 10% acetic acid. The appropriate bands were cut out, and the protein was sequenced by the formation and hydrolysis of carboxy-terminal peptidyl-thiohydantoins (Bailey & Shively, 1990). Carboxyl-terminal sequencing was performed by the City of Hope Mass Spectrometry and Micro Sequencing Core Facility at the Beckman Research Institute of the City of Hope (Duarte, CA).

RESULTS

We had previously found that peptides ending in the native PE sequence REDLK, which is found at the carboxyl terminus of native PE, do not bind to the KDEL receptor, but those ending in REDL do (Kreitman & Pastan, 1995). In the present study, we determined whether the terminal lysine residue of PE is removed during cellular intoxication, since removal would allow the toxin to bind to the KDEL receptor and enable the toxin to be transported to the ER where it could be translocated to the cytosol.

Effect of the Terminal Lysine of PE in KDEL Receptor Binding and Cytotoxicity. To determine the effect of the terminal lysine residue in the REDLK sequence of PE on binding to the KDEL receptor, Golgi membranes isolated from rat liver were incubated with the peptide [^{125}I]-YTSEKDEL in the presence and absence of different concentrations of recombinant immunotoxins ending in REDLK or REDL, and the centrifuged Golgi pellets were counted. [^{125}I]-YTSEKDEL was chosen for displacement studies since it contains the native binding sequence for the KDEL receptor. The immunotoxins tested were anti-Tac-(Fv)-PE38, which ends in REDLK, and anti-Tac(Fv)-PE38REDL. These each contain the single-chain Fv form of the anti-Tac monoclonal antibody replacing domain Ia at the amino terminus of PE (Figure 1). They also have a deletion of amino acids 365−380 of PE. Anti-Tac(Fv)-PE38 is referred to here as anti-Tac(Fv)-PE38REDLK to emphasize the presence of REDLK at the carboxyl terminus of PE. Anti-Tac(Fv)-PE38REDL is identical to this molecule except it is missing the carboxyl-terminal lysine residue.

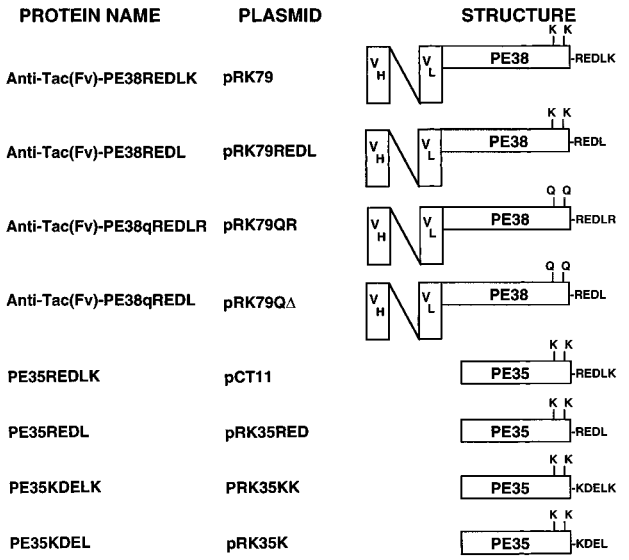

FIGURE 1: Schematic diagram of recombinant proteins. Anti-Tac(Fv)-PE38REDLK, also referred to previously as anti-Tac(Fv)-PE38, is composed of the anti-Tac(Fv) ligand fused to amino acids 253−364 and 381−613 of PE. For each protein, the carboxyl-terminal residues following amino acid 608 of PE are shown. PE38q in the protein name indicates K590Q and K606Q mutations in PE, which do not affect biological activity (Debinski & Pastan, 1992). PE35REDLK, referred to previously as PE35, is composed of methionine followed by amino acids 281−364 and 381−613 of PE.

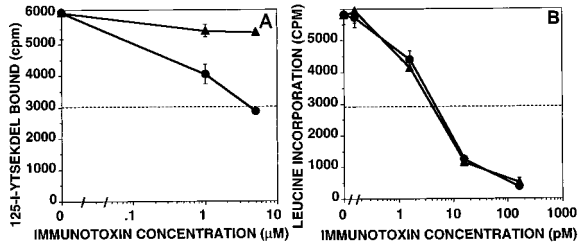

FIGURE 2: Effect of the terminal lysine of PE on binding and cytotoxicity. In (A), Golgi membranes isolated from rat liver were incubated with the peptide [^{125}I]-YTSEKDEL (0.04 μM) in the presence and absence of different concentrations of anti-Tac(Fv)-PE38REDLK (▲) or anti-Tac(Fv)-PE38REDL (●), and the centrifuged Golgi membranes were counted. Each point represents the net cpm bound after subtracting background, which is the cpm associated with the Golgi pellet in the presence of a maximal concentration of anti-Tac(Fv)-PE38KDEL. In (B), anti-Tac(Fv)-PE38REDLK (▲) or anti-Tac(Fv)-PE38REDL (●) was incubated with MT-1 cells (40 000/well in 100 μL of DMEM + 10% FBS) for 24 h, and incorporation of [^{3}H]leucine was determined. Error bars indicate standard deviations from the means of duplicate or triplicate experiments.

As shown in Figure 2A, anti-Tac(Fv)-PE38REDL but not anti-Tac(Fv)-PE38REDLK was able to displace [^{125}I]-YTSEKDEL from the KDEL receptor of rat Golgi membranes. The concentration of immunotoxin required for 50% displacement of [^{125}I]-YTSEKDEL (EC_{50}) was previously reported to be >5 μM for both anti-Tac(Fv)-PE38REDL and anti-Tac(Fv)-PE38REDLK. Concentrations exceeding 5 μM could not reliably be tested due to toxin aggregation. In the present assay (Figure 2A), the EC_{50} for anti-Tac(Fv)-PE38REDL was ~5 μM, and it displaced [^{125}I]-YTSEKDEL much more than anti-Tac(Fv)-PE38REDLK. In contrast, anti-Tac(Fv)-PE38REDL and anti-Tac(Fv)-PE38REDLK have been reported to have the same cytotoxic activity (Kreitman & Pastan, 1995), and as shown in Figure 2B display similar

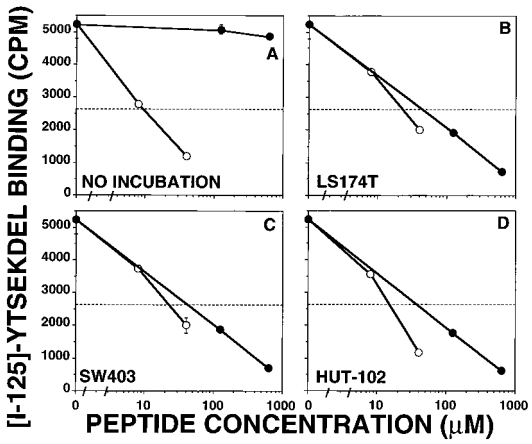

FIGURE 3: Activation of REDLK during exposure to cells to a sequence which binds to the KDEL receptor. The peptides YTSEREDLK (●) and YTSEREDL (○) were tested for displacement of [^{125}I]-YTSEKDEL as in Figure 2 after incubation overnight with LS174T cells (B), SW403 cells (C), or HUT-102 cells (D) at a peptide concentration of 2−3 mM. In (A), the peptides were incubated without exposure to cells.

inhibition of protein synthesis when incubated with MT-1 target cells. This indicates that if KDEL receptor binding is necessary for the cytotoxic activity of PE, the terminal lysine residue of PE must be removed prior to this step.

The Terminal Lysine of the REDLK Sequence Is Removed during Intoxication. To determine if the terminal lysine of PE is removed during cellular intoxication, the two colon carcinoma lines LS174T and SW403 and the adult T-cell leukemia line HUT-102 were each incubated 20 h with 2−3 mM of either YTSEREDLK or YTSEREDL. Peptide-containing medium was then diluted with KDEL receptor binding buffer and exposed to Golgi membranes at concentrations of 8 and 40 μM for YTSEREDL or 125 and 625 μM for YTSEREDLK. As shown in Figure 3A, without incubation YTSEREDL binds with an EC_{50} of 9.4 μM, compared to >625 μM for YTSEREDLK. However, as shown in Figure 3B−D, when medium which contains YTSEREDLK is incubated with target cells, the peptide can now bind to the KDEL receptor (EC_{50} < 125 μM), indicating that the terminal lysine was removed. Incubation of the peptide YTSEREDL in cell culture did not significantly affect its binding, indicating that the improvement in the binding of YTSEREDLK present in cell culture was not due to metabolism of the peptide at a site other than the terminal lysine residue. These experiments are consistent with the hypothesis that the terminal lysine residue is removed during the intoxication process. Not addressed by these experiments, however, is whether the lysine residue is removed prior to endocytosis, at the cell surface, or somewhere in the endocytic pathway.

Activation of the Carboxyl Terminus of PE in Human Plasma. To determine whether the terminal lysine of PE can be removed prior to binding or internalization by a target cell, YTSEREDLK was incubated with human plasma and then tested for binding to the KDEL receptor. The peptides YTSEREDLK and YTSEREDL, each dissolved in 0.01 M sodium phosphate, pH 6.0, were diluted 1:1 with human plasma and incubated at 37 °C. Dilutions of the plasma peptide mixture were then tested for displacement of [^{125}I]-YTSEKDEL from the KDEL receptor. Figure 4A shows that at the beginning of incubation YTSEREDLK had no

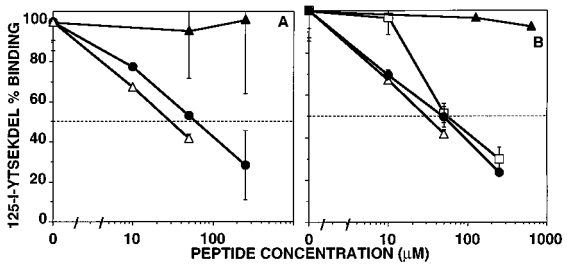

FIGURE 4: Activation of the REDLK sequence in human plasma. In (A), the peptides YTSEREDLK and YTSEREDL, each dissolved in 0.01 M sodium phosphate, pH 6.0, were diluted 1:1 with human plasma and incubated at 37 °C. Dilutions of the plasma−YTSEREDLK mixture after 0 (▲) or 15 min (△) or of the plasma−YTSEREDL mixture after 15 min (●) were tested for displacement of [^{125}I]-YTSEKDEL from the KDEL receptor. In (B), the plasma−YTSEREDLK mixture was tested for displacement of [^{125}I]-YTSEKDEL from the KDEL receptor before (▲) or after 15 min (△), 4 h (□), or 24 h (●) of incubation.

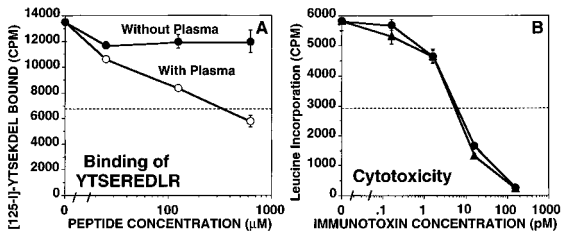

FIGURE 5: Activation by plasma when the REDL sequence is blocked by arginine. In (A), the peptide YTSEREDLR was used without plasma (●) or after incubation at 37 °C for 5 h with human plasma (○) to test for displacement of [^{125}I]-YTSEKDEL from the KDEL receptor. In (B), MT-1 cells (40 000/well) were incubated for 24 h with anti-Tac(Fv)-PE38qREDL (▲) or anti-Tac(Fv)-PE38qREDLR (●) and then tested for [^{3}H]leucine incorporation.

significant binding to the KDEL receptor. After 15 min, the EC_{50} was 30−60 μM for both YTSEREDLK and YTSEREDL, indicating that the terminal lysine was removed by the plasma. As shown in Figure 2B, a time course experiment showed no significant difference in activation of the carboxyl terminus when YTSEREDLK was incubated 0.25, 4, and 24 h. Thus, when the carboxyl terminus of PE is exposed to human plasma at 37 °C, the terminal lysine is rapidly removed, even before its exposure to cells.

Activation of the Carboxyl Terminus of PE in Plasma. It was previously reported that replacement of lysine with arginine at the carboxyl terminus of PE does not affect cytotoxic activity (Chaudhary et al., 1990). The truncated PE mutant PE38q (Figure 1), containing arginine at the carboxyl terminus and glutamine substituting for lysine at positions 590 and 606, has been useful for chemically conjugating to antibodies, because new residues at those positions could not be chemically derivatized (Debinski & Pastan, 1992). We hypothesized that an arginine residue at the carboxyl terminus of PE would also prevent binding to the KDEL receptor but that the arginine residue would be removed by plasma. To determine if this is the case, we tested the peptide YTSEREDLR for its displacement of [^{125}I]-YTSEKDEL from the KDEL receptor. As shown in Figure 5A, YTSEREDLR showed no displacement even at 625 μM, indicating that arginine like lysine blocks the binding of the REDL sequence to the KDEL receptor. To determine the effect of a carboxy-terminal arginine residue on the cytotoxic activity of PE toward MT-1 cells, the cytotoxic activities of anti-Tac(Fv)-PE38qREDLR and anti-Tac(Fv)-PE38qREDL

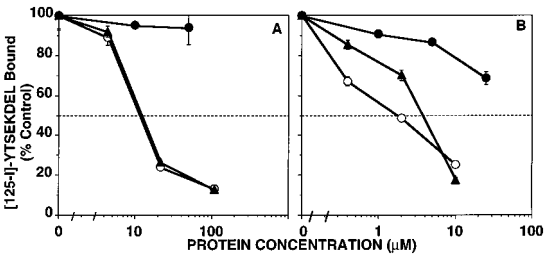

FIGURE 6: Binding of PE35 derivatives to the KDEL receptor. In (A), [^{125}I]-YTSEKDEL displacement is shown for PE35 before (●) or after (○) incubation with plasma for 1 h at 37 °C, and is also shown for PE35REDL (▲). In (B), [^{125}I]-YTSEKDEL displacement is shown for PE35KDELK before (●) or after (○) incubation with plasma for 1 h at 37 °C, and is also shown for PE35KDEL (▲).

were compared on MT-1 cells. Anti-Tac(Fv)-PE38qREDLR and anti-Tac(Fv)-PE38qREDL differ from each other only in the presence or absence of an arginine residue following the REDL sequence (see Figure 1 and Materials and Methods). MT-1 cells were incubated with the recombinant immunotoxins for 24 h, and then [^{3}H]leucine incorporation was measured. As shown in Figure 5B, the terminal arginine residue did not affect cytotoxicity, suggesting that it, like lysine, is removed from the carboxyl terminus of PE. To confirm that this was the case, the peptide YTSEREDLR was incubated with human plasma and then assessed for binding to the KDEL receptor. As shown in Figure 5A, incubation with plasma restored binding of YTSEREDLR to the KDEL receptor. Thus, a terminal arginine residue, like the native terminal lysine residue, is removed from the carboxyl-terminal sequence of PE.

Plasma Enables Truncated PE Protein To Bind to the KDEL Receptor. To measure the binding of proteins to the KDEL receptor, it is preferable to use peptides, because peptides can be tested in much higher molar concentrations and they are stable at the low pH required in the binding assay. Moreover, the background in the assay is higher for protein compared to peptide, due to nonspecific interactions (Kreitman & Pastan, 1995). To determine whether plasma could remove the carboxyl-terminal lysine residue of a smaller and more soluble form of PE, we chose to study a 35 kDa truncated form of PE, termed PE35 (Theuer et al., 1993b). PE35 (Figure 1), referred to here as PE35REDLK, contains methionine followed by amino acids 281−364 and 381−613 of PE, and is identical to the fragment of anti-Tac(Fv)-PE38 that undergoes translocation after proteolytic processing between Arg279 and Gly280 of PE (Ogata et al., 1992), except that PE35 begins with methionine instead of glycine at position 280. PE35 is an appropriate protein to study binding to the KDEL receptor, since the binding step very likely occurs after proteolytic processing. To determine whether the terminal lysine residue is removed from PE35 prior to interaction with target cells, plasma was diluted 6-fold with 0.01 M sodium phosphate, pH 6.0, and PE35 was added to a final concentration of 187 μM. After incubating for 1 h at 37 °C, the protein was tested for binding to the KDEL receptor. As shown in Figure 6A, PE35, which ends in REDLK, does not bind to the KDEL receptor before incubation with plasma, but after incubation PE35 is able to bind with the same relative affinity as PE35REDL. This result, like those shown above, indicates that the carboxy-terminal lysine residue is removed in plasma.

Activation of a Blocked KDEL Sequence. To determine if the terminal lysine could be removed from other ER retention sequences by incubation in plasma, we tested a protein ending in KDELK. Plasma was diluted 6-fold and PE35KDELK added to a final protein concentration of 15.6 μM; after incubation at 37 °C for 4 h, the mixture was tested for KDEL receptor binding activity. As shown in Figure 6B, before incubation in plasma PE35KDELK showed slight or no significant binding to the KDEL receptor, but after incubation with plasma bound with an affinity similar to that of PE35KDEL. Thus, the carboxy-terminal lysine residue was also removed from toxin sequences ending in KDEL.

Direct Evidence for Amino Acid Removal from the Carboxy Terminus of PE. To determine directly whether the carboxyl-terminal lysine residue is removed from PE by plasma, carboxy-terminal sequencing was performed on PE35REDLK before and after exposure to human plasma. Plasma was diluted 10-fold and PE35REDLK added to a final concentration of 95 μM (3.3 mg/mL) and a final albumin concentration of ~4 mg/mL. After incubating at 37 °C for 6 h, the PE35REDLK (35 kDa) was separated from the albumin (68 kDa) on a 10% nonreducing SDS−PAGE gel and electroeluted to a Teflon membrane. The 35 kDa protein (~1000 pmol/30 μg) was then sequenced by the formation and hydrolysis of carboxy-terminal peptidylthiohydantoins (Bailey & Shively, 1990). Equal amounts of untreated PE35REDLK and PE35REDL were sequenced as negative and positive controls, respectively. The position of the leucine residues in the control proteins could not be assessed due to technical reasons, but it could be determined that lysine was the last amino acid and aspartate was the third to last amino acid in PE35REDLK, and that aspartate was the second to last amino acid in PE35REDL. In the PE35REDLK sample exposed to plasma, lysine was no longer present at the carboxyl terminus, and aspartate was the second to last amino acid. Thus, the carboxylterminal sequencing of truncated PE is consistent with the hypothesis that plasma cleaves the carboxyl-terminal lysine of PE, leaving the carboxyl terminus REDL which can bind intracellularly to the KDEL receptor.

Competition of the Removal of Carboxy-Terminal Lysine with Protease Inhibitors. To characterize the protease or proteases in human plasma responsible for removing the carboxyl-terminal lysine residue, the peptide YTSEREDLK was incubated at a concentration of 4.4 mM in 50% human plasma in the presence or absence of protease inhibitors. After a 4 h incubation at 37 °C, the mixture was tested for Golgi membrane binding activity by displacement of [^{125}I]-YTSEKDEL as described in the legend to Figure 4. The percent of peptide molecules converted to YTSEREDL was calculated by comparing the Golgi binding activity of each plasma−YTSEREDLK−protease inhibitor mixture with that of an identical mixture where YTSEREDL was substituted for YTSEREDLK. As shown in Table 1, in the absence of protease inhibitors, the cleavage of the carboxyl-terminal lysine from the REDLK sequence increased from <0.8% to 31 ± 2% after incubation with plasma. Addition of the protease inhibitors PMSF, aprotinin, or leupeptin each resulted in a partial decrease in removal of the terminal lysine to 12−19% of peptide molecules. However, addition of 10 mM EDTA completely blocked removal of the carboxyl-terminal lysine residue. The lack of Golgi binding activity in the plasma and EDTA-tested YTSEREDLK sample was not due to the interference of EDTA in the Golgi binding assay, since EDTA did not decrease the Golgi binding activity when YTSEREDL was incubated in plasma. Thus, EDTA is the best inhibitor of lysine removal from the REDLK sequence in human plasma.

Table 1: Effect of Protease Inhibitors on the Activation of the REDLK Sequence by Human Plasma[a]

incubation time at 37 °C (h)	protease inhibitor	relative Golgi binding activity (%)
0	0	<0.8
4	0	31 ± 2
4	PMSF, 1 mM	19 ± 1
4	aprotinin, 10 μM	12 ± 2
4	leupeptin, 100 μM	15 ± 2
4	EDTA, 10 mM	<0.8

[a] The peptide YTSEREDLK (8.8 mM) in 0.01 M sodium phosphate, pH 6.0, was diluted 1:1 with human plasma containing PMSF (2 mM), aprotinin (20 μM), leupeptin (200 μM), EDTA (20 mM), or no inhibitor and incubated at 37 °C for 4 h. To determine the percent Golgi binding activity, the displacement of [^{125}I]-YTSEKDEL binding (as in Figure 4) of each YTSEREDLK−plasma mixture was compared to that of the exact same mixture where YTSEREDL was substituted for YTSEREDLK.

DISCUSSION

We found that the terminal lysine residue of PE is removed when it is incubated with cells in medium containing serum or incubated in plasma without cells. Removal of this lysine residue is essential for binding of PE to the KDEL receptor which functions to transport the toxin to the ER. This indicates that removal of the terminal lysine of PE is an early step which is essential for the cellular intoxication by PE.

Removal of a Carboxy-Terminal Basic Residue by Plasma. Human plasma is known to contain a variety of different carboxypeptidases, many of which may be responsible for cleaving the carboxy-terminal lysine residue from PE (Skidgel, 1988). Human carboxypeptidase N is a 280 kDa plasma glycoprotein which removes the lysine or arginine from the carboxyl terminus of peptides such as bradykinin and kallidin. It has also been purified from the conditioned medium of carcinoma cells (Grimwood & Plummer, 1988). Carboxypeptidase H, which also removes basic carboxy-terminal residues, was reported to be secreted from Hep G2 hepatoma cells at levels 2−3-fold greater than that of carboxypeptidase N (Grimwood et al., 1989). Another carboxypeptidase has been identified in blood during clotting or inflammation which also removes basic residues (Campbell & Okada, 1989). Carboxypeptidase M is a membrane-bound carboxypeptidase B-like protease which cleaves carboxy-terminal basic amino acids (Skidgel, 1988). The inhibition of lysine removal in plasma by EDTA is consistent with any of these carboxypeptidases being responsible for removing the lysine, since they each require cobalt for activity (Grimwood et al., 1989; Skidgel, 1988). We have found that antibodies to carboxypeptidase N do not prevent the removal of lysine from the amino terminus of YTSEREDLK (data not shown). It is likely that PE can be cleaved in the plasma by more than one peptidase. It is also possible that removal of the terminal lysine residue can take place on the cell surface or within the cell if it does not take place outside the cell. Interestingly, we found that the peptides YTSEREDLN and YTSEREDLE also showed significant improvement in their KDEL receptor binding after

incubation with plasma, indicating that the terminal residue could be removed by other peptidases as well (data not shown). However, these residues were not removed as fast as basic ones, which probably protects recombinant toxin and other proteins from complete degradation. In fact, analysis of [^{125}I]-anti-Tac(Fv)-PE38REDLK by SDS−PAGE after 2 h of incubation with the same plasma used in the above experiments to cleave the carboxy-terminal lysine residue showed no evidence of significant proteolytic degradation (data not shown).

Naturally occurring substrates for basic carboxypeptidases include proteins such as bradykinin, anaphylatoxins, enkephalins, fibrinopeptides, protamine, calmodulin, complement proteins, renin, enolase, creatine kinase, and erythropoietin (Skidgel, 1988). PE is a nonhuman protein toxin which should be added to this list. One may speculate that the terminal lysine residue may enhance the secretion or decrease the degradation of PE while it is being produced by *Pseudomonas aeruginosa*.

Could removal of the terminal lysine residue be a limiting factor in the cellular intoxication by PE? Due to the rapidity of removal of the terminal lysine in plasma, it is unlikely that this step is rate-limiting in the intoxication of cells by PE, at least when administered or secreted by *Pseudomonas aeruginosa in vivo*. However, the fact that REDL blocked by amino acids does not bind to the KDEL receptor suggests that construction of PE derivatives which are blocked at the carboxyl terminus with amino acids which are selectively removed in cancer or other target cells may be useful for enhanced selectivity.

High Concentrations of PE Are Required for Binding to the KDEL Receptor. While 5 μM anti-Tac(Fv)-PE38REDL was necessary to displace the binding of [^{125}I]-YTSEKDEL by 50% (Figure 2), concentrations 10^6-10^7 lower are sufficient for >50% cytotoxicity or protein synthesis inhibition when the immunotoxin is incubated with cells. Only 2 logs of this difference can be accounted for by the fact that REDL binds with ~1% of the affinity of KDEL (Kreitman & Pastan, 1995). Thus, it would be necessary for toxin to be highly concentrated from the extracellular space to the Golgi compartment to allow significant binding. This would be achieved, for example, if 1000 molecules of toxin could be concentrated within a cell to a volume of $\sim 3.3 \times 10^{-13}$ mL (5 μM), which is ~0.1% of the volume of a cell of radius 5 μm. However, since only one or a few molecules delivered to the cytoplasm are necessary to kill a cell, PE may not require intracellular concentrations of this magnitude for cell killing.

In conclusion, we have shown that the removal of the terminal lysine of PE is an early step in its cellular intoxication which is required for its binding to the KDEL receptor. The fact that this step occurs in human plasma makes it an early step in cellular intoxication by PE. Our results also are consistent with our model that the KDEL receptor is instrumental for transporting truncated PE intracellularly toward the ER, from which it can translocate to the cytosol and catalytically inactivate EF-2, causing cell death.

ACKNOWLEDGMENT

We wish to thank Dr. Duncan Wilson for helpful conversations regarding Golgi binding assays. We also appreciate the anti-carboxypeptidase N antibody kindly supplied by Drs. B. Grimwood and T. Plummer, Jr. We thank Drs. Ira Pastan and David FitzGerald for helpful discussions and critical reading of the manuscript.

REFERENCES

Allured, V. S., Collier, R. J., Carroll, S. F., & McKay, D. B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1320−1324.
Bailey, J. M., & Shively, J. E. (1990) *Biochemistry 29*, 3145−3156.
Brinkmann, U., Brinkmann, E., Gallo, M., & Pastan, I. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10427−10431.
Campbell, W., & Okada, H. (1989) *Biochem. Biophys. Res. Commun. 162*, 933−939.
Chaudhary, V. K., Jinno, Y., FitzGerald, D., & Pastan, I. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 308−312.
Chiron, M. F., Fryling, C. M., & FitzGerald, D. J. (1994) *J. Biol. Chem. 269*, 18167−18176.
Debinski, W., & Pastan, I. (1992) *Cancer Res. 52*, 5379−5385.
Grimwood, B. G., & Plummer, T. H., Jr. (1988) *J. Biol. Chem. 263*, 14397−14401.
Grimwood, B. G., Plummer, T. H., Jr., & Tarentino, A. L. (1989) *J. Biol. Chem. 264*, 15662−15667.
Hwang, J., FitzGerald, D. J., Adhya, S., & Pastan, I. (1987) *Cell 48*, 129−136.
Kounnas, M. Z., Morris, R. E., Thompson, M. R., FitzGerald, D. J., Strickland, D. K., & Saelinger, C. B. (1992) *J. Biol. Chem. 267*, 12420−12423.
Kreitman, R. J., & Pastan, I. (1995) *Biochem. J. 307*, 29−37.
Kreitman, R. J., Chaudhary, V. K., Kozak, R. W., FitzGerald, D. J. P., Waldmann, T. A., & Pastan, I. (1992) *Blood 80*, 2344−2352.
Kreitman, R. J., Batra, J. K., Seetharam, S., Chaudhary, V. K., FitzGerald, D. J., & Pastan, I. (1993) *Bioconjugate Chem. 4*, 112−120.
Kreitman, R. J., Bailon, P., Chaudhary, V. K., FitzGerald, D. J. P., & Pastan, I. (1994) *Blood 83*, 426−434.
Miesnbock, G., & Rothman, J. E. (1995) *J. Cell Biol. 129*, 309−319.
Ogata, M., Chaudhary, V. K., Pastan, I., & FitzGerald, D. J. (1990) *J. Biol. Chem. 265*, 20678−20685.
Ogata, M., Fryling, C. M., Pastan, I., & FitzGerald, D. J. (1992) *J. Biol. Chem. 267*, 25396−25401.
Seetharam, S., Chaudhary, V. K., FitzGerald, D., & Pastan, I. (1991) *J. Biol. Chem. 266*, 17376−17381.
Skidgel, R. A. (1988) *Trends Pharmacol. Sci. 9*, 299−304.
Tabas, I., & Kornfeld, S. (1979) *J. Biol. Chem. 254*, 11655−11663.
Theuer, C. P., Buchner, J., FitzGerald, D., & Pastan, I. (1993a) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7774−7778.
Theuer, C. P., Kreitman, R. J., FitzGerald, D. J., & Pastan, I. (1993b) *Cancer Res. 53*, 340−347.
Theuer, C., Kasturi, S., & Pastan, I. (1994) *Biochemistry 33*, 5894−5900.
Wilson, D. W., Lewis, M. J., & Pelham, H. R. B. (1993) *J. Biol. Chem. 268*, 7465−7468.

BI971447W