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NOTES

A Periplasmic Intermediate in the Extracellular Secretion Pathway of *Pseudomonas aeruginosa* Exotoxin A

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Pseudomonas aeruginosa **exotoxin A is synthesized with a secretion signal peptide typical of proteins whose final destination is the periplasm. However, exotoxin A is released from the cell without a detectable periplasmic pool, suggesting that additional determinants in this protein are important for recognition by a specialized machinery of extracellular secretion. The role of the N terminus of the mature exotoxin A in this recognition was investigated. A series of exotoxin A proteins with amino acid substitutions for the glutamic acid pair at the +2 and +3 positions were constructed by mutagenesis of the exotoxin A gene. These N-terminal acidic residues of the mature exotoxin A protein were found to be important not only for efficient processing of the precursor protein but also for extracellular localization of the toxin. The mutated exotoxin A proteins, in which a glutamic acid at the +2 position was replaced by a lysine or a double substitution of lysine and glutamine for the pair of adjacent glutamic acids, accumulated in precursor forms in the mixed cytoplasmic and membrane fractions, which was not seen with the wild-type exotoxin A. The processing of the precursor form of one exotoxin A mutant, in which the glutamic acid at the +2 position was replaced with a glutamine, was not affected. Moreover, a substantial fraction of the mature forms of all three mutants of exotoxin A accumulated in the periplasm, while wild-type exotoxin A could be detected only extracellularly. The periplasmic pools of these variants of exotoxin A could therefore represent the intermediate state during extracellular secretion. Kinetic analysis of export of one of the exotoxin A mutants, following inhibition of protein synthesis, showed that the periplasmic intermediate was gradually transported into the medium. These results suggested that the pathway of extracellular secretion of exotoxin A is very likely a two-step process, involving initial translocation of the protein across the cytoplasmic membrane and then transfer of the protein from the periplasm across the outer membrane. This second stage of export requires a specialized machinery of extracellular secretion. The signal for extracellular localization may be located in a small region near the amino terminus of the mature protein or could consist of several regions that are brought together after the polypeptide has folded. Alternatively, the acidic residues may be important for ensuring a conformation essential for exotoxin A to traverse the outer membrane.**

The opportunistic pathogen *Pseudomonas aeruginosa* produces a number of enzymes and toxins which cause extensive damage through degradation of protein and lipid components of host tissue. One of these is exotoxin A, a potent inhibitor of protein synthesis in eukaryotic cells (30). Initial studies on extracellular secretion of exotoxin A suggested a novel mechanism of extracellular secretion (17). According to this model, exotoxin A is inserted into the inner membrane shortly after its synthesis and is transported into the outer membrane through zones of adhesion between the two membranes (3, 7), thus bypassing the periplasm. The hypothesis that these zones of adhesion are responsible for extracellular secretion is supported by demonstration of an outer membrane pool of unprocessed exotoxin A in bacteria that have had their membranes perturbed by ethanol. Moreover, under standard experimental conditions, a periplasmic pool of exotoxin A could not be detected, further arguing for a pathway which bypassed the periplasm. These data collectively suggested that secretion of exotoxin A, and of several selected extracellular proteins made by gram-negative bacteria in which no periplasmic intermediate can be detected, bypasses the periplasm or traverses this compartment extremely rapidly. Mechanistically, this model differs from the two-stage process of the so-called general secretory pathway, where extracellularly secreted proteins are initially translocated from the inner membrane into the periplasm and then translocated across the outer membrane (23). Consistent with the latter model was the observation that mutants defective in one of the components of the extracellular secretion apparatus accumulated exotoxin A in the periplasm (19, 27).

Recently, in several gram-negative bacteria, including *P. aeruginosa*, genetic determinants of extracellular protein secretion have been identified (15, 28). All of these share significant homology with the previously described machinery for exporting *Klebsiella oxytoca* pullulanase, which involves the products of 14 *pul* genes as well as the components of the general secretion pathway (24). No periplasmic intermediate could be demonstrated for the pullulanase lipoprotein, although replacement of its N terminus, including the lipid modification site and the signal peptide, with that of periplasmic maltose-binding protein yielded a hybrid protein that was secreted into the periplasm and subsequently across the outer membrane (20). Moreover, the efficient translocation of wild-type pullu-

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	-2	-1	+1	+2	+3	+4	+5	
pLL1	Ser	Ala	Ala	Glu	Glu	Ala	Phe	(Wild Type)
pLL2	Ser	Ala	Ala	**Gln**	Glu	Ala	Phe	(Mutant)
pLL3	Ser	Ala	Ala	**Lys**	Glu	Ala	Phe	(Mutant)
pLL4	Ser	Ala	Ala	**Lys**	**Gln**	Ala	Phe	(Mutant)

FIG. 1. Amino acid substitutions in exotoxin A. The portions of the N-terminal sequences of wild-type and mutant exotoxin A proteins used in this study are shown. The amino acid substitutions in the various mutants are shown in bold; the arrow indicates the cleavage site for signal peptidase. Plasmid pMS150 (16) and pMMD4 (27) both express exotoxin A under the control of the *lac* or *tac* promoter and were used as a source of DNA for the mutagenesis experiments. Plasmids pLL2 and pLL3 were constructed by mutagenesis of a uracil-containing template containing the 0.8-kb *Hin*dIII-*Kpn*I fragment from pMS150 in M13mp18, propagated in *E. coli* RZ1032 (*dut ung*) (11). The mutagenic primer 5′ GTCCGCCGCC(C/G/A/T)AG (C/G/A/T)AAGCCTTCTGACC 3′ was hybridized to the template and extended with Klenow polymerase. After transfection of *E. coli* XL-1 with the in vitro-synthesized M13 DNA, individual plaques were isolated and DNA inserts were sequenced. Once the desired mutants were identified, a 0.8-kb *Hin*dIII-*Kpn*I fragment carrying the mutated exotoxin A gene was isolated and ligated into the *Hin*dIII-*Kpn*I sites of plasmid pMMD4, which carries the wild-type exotoxin A gene in pMMB66HE (6), replacing the corresponding wild-type sequence. Plasmid pLL4, which carries a mutated gene encoding a double amino acid substitution in exotoxin A, was constructed by synthesizing uracil-containing single-stranded DNA from an M13mp18 derivative which carries the *Hin*dIII-*Kpn*I fragment from pLL2. Plasmid pLL1, which carries genes encoding for the wild-type exotoxin A protein, was constructed by directly cloning the 0.8-kb *Hin*dIII-*Kpn*I fragment of pMS150 to the same sites of plasmid pMMD4. All of the plasmids were introduced into exotoxin A mutant *P. aeruginosa* PAK-NT (26) by triparental mating with pRK2073 as a helper plasmid (13).

lanase across the outer membrane requires the formation of intramolecular disulfide bonds, catalyzed in the periplasm by the *dsbA* gene product of *Escherichia coli* (22), further demonstrating the involvement of periplasmic components in the pathway of extracellular protein secretion.

In *P. aeruginosa*, 12 genes that have homologous counterparts in the *pul* operon of *K. oxytoca* have been identified (28). *P. aeruginosa* secretes exotoxin A, phospholipase C, alkaline phosphatase, elastase, and lipase via the general secretion pathway, with the terminal determinants encoded by the *xcp* genes (1, 2, 19, 27). All of these proteins are synthesized with typical N-terminal secretion signal sequences (4, 5, 8, 21, 31). The localization signals encoded in the mature proteins and recognized during subsequent stages of export have not been defined as yet, although there is evidence that the N-terminal region of mature exotoxin A plays a role in its secretion (9).

In this article, we report that alterations of the acidic N terminus of exotoxin A led to delays in removal of the signal peptide and to periplasmic accumulation of exotoxin A. The finding that the periplasmically located exotoxin A mutant protein can still be released from the cell suggests that a free periplasmic intermediate is part of the normal pathway for extracellular secretion of exotoxin A.

Effect of mutations in the mature region of exotoxin A on processing and extracellular secretion. We introduced several substitutions in the codons for the two adjacent glutamic acids located at positions +2 and +3 relative to the signal sequence cleavage site of the exotoxin A precursor (Fig. 1). These replacements of glutamic acid at +2 in the wild-type sequence with glutamine (on plasmid pLL2) or with lysine (on pLL3)

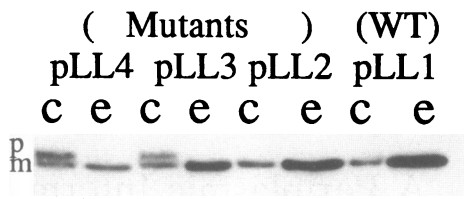

FIG. 2. Extracellular secretion of wild-type (WT) and mutant exotoxin A. *P. aeruginosa* PAK-NT with the plasmids expressing wild-type and mutant exotoxin A were grown in L broth (18) at 37°C until they reached an optical density of 0.4. Synthesis of exotoxin A was induced with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 15 min. The culture was separated into extracellular (e) and cell-associated (c) fractions, and equal volumes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12) and immunoblotting (29) with antibodies to exotoxin A.

and the double mutation at +2 and +3 substituting Lys-Gln for Glu-Glu (on pLL4) resulted in preproteins where the acidic charge (−2 on the wild type) in this region was progressively changed to −1, neutral, and +1, respectively.

The effect of these mutations on the processing and localization of exotoxin A was then examined by immunoblotting of cell fractions (Fig. 2). Densitometric analysis of autoradiographs showed that, under these conditions, 75, 63, and 31% of exotoxin A mutant proteins expressed from plasmids pLL2, pLL3, and pLL4, respectively, were secreted into extracellular media. This contrasts with >90% extracellular secretion of wild-type exotoxin A. While wild-type exotoxin A and one mutant (expressed from pLL2) showed only the processed, mature form in the cell-associated fraction, precursor forms of exotoxin A were detected in *P. aeruginosa* expressing either of the mutant exotoxin A proteins from pLL3 or pLL4. Cell-associated fractions of mutants where a basic residue (Lys) replaced Glu at the +2 position (on plasmid pLL3) or where Lys-Gln replaced Glu-Glu at the +2 and +3 positions (pLL4) showed impaired (ca. 50%) processing of the precursor of exotoxin A.

To examine the subcellular location of exotoxin A, the cells were harvested and separated into extracellular, periplasmic,

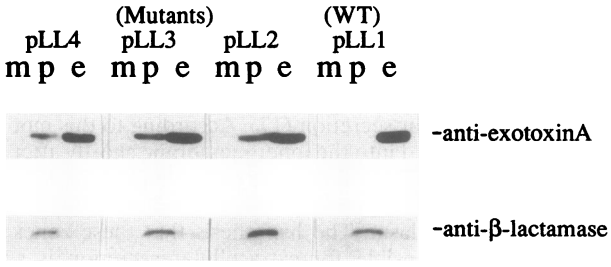

FIG. 3. Subcellular localization of wild-type (WT) and mutant exotoxin A. Cultures were grown as described in the legend to Fig. 1, except that IPTG (isopropyl-β-D-thiogalactopyranoside) induction was increased to 40 min. Each culture was harvested, the extracellular (e) fraction was saved, and cells were further separated into periplasmic (p) and mixed membrane-cytoplasmic (m) fractions by the method of Hoshino and Kageyama (10). Equivalent volumes from each fraction were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. The immunoblots were cut into two sections, and the upper portion of the blot was reacted with antibodies to exotoxin A, while the lower portion was reacted with antibodies to β-lactamase.

and mixed membrane and cytoplasmic fractions (Fig. 3). In contrast to wild-type exotoxin A, which is rapidly secreted into the medium, a portion of each of the mutant variants of exotoxin A was found in the periplasmic fraction, cofractionated with β-lactamase. Some processing of the precursors of exotoxin A might have occurred in mutants during preparation of periplasmic fractions. Under the conditions used in this experiment to induce exotoxin A synthesis, the majority of the mutant protein with a basic residue at +2 (expressed from plasmid pLL3) and the double mutant at the +2 and +3 positions (pLL4) was processed with only a small amount of precursor detectable in the cytoplasmic-membrane fraction upon overexposure of autoradiographs.

Kinetics of release of periplasmic exotoxin A. To examine whether the periplasmic pool of one of the mutants of exotoxin A represents a true intermediate in extracellular secretion, a kinetic study of the release of the periplasmically accumulated exotoxin A was carried out in a culture where further protein synthesis was inhibited by tetracycline. Figure 4A shows that tetracycline at 200 μg/ml completely inhibited synthesis of exotoxin A, while in a control culture containing 0.1% ethanol (solvent carried over from the tetracycline stock solution), levels of exotoxin A increased linearly for 60 min.

At various times, the tetracycline-inhibited culture was separated into extracellular, periplasmic, and mixed membrane-cytoplasmic fractions, and the levels of exotoxin A were determined in each of these compartments by densitometric quantitation of immunoblots. Figure 4B shows a typical autoradiograph of an immunoblot of a fractionation experiment that clearly demonstrates progressive release of the periplasmically accumulated exotoxin A into the medium. Quantitation of the results of several experiments is also shown in Fig. 4B. The accumulated pool of exotoxin A was completely released in 45 min. In contrast, β-lactamase remained in the periplasm throughout the experiment (data not shown). This analysis demonstrates that the mutant exotoxin A, while in the periplasm, remains competent for extracellular secretion, although the rate of translocation across the outer membrane is relatively slow.

The significance of N-terminal acidic residues in protein export is not clear. In *E. coli*, mutations that alter the charge in this region significantly delay cleavage of the signal peptide and the appearance of alkaline phosphatase in the periplasm (14). Yamane and Mizushima (33) have shown that mutants of β-lactamase with amino acid substitutions at the N terminus that replaced the −1 charge of the wild-type protein with a +2 net charge accumulated in the cytoplasmic membrane, while the wild-type protein was efficiently released into the periplasm. An $OmpF^-$ lipoprotein chimera with an uncleavable signal peptide could be secreted into inverted inner membrane vesicles in vitro. However, mutants of the same proteins that increase the net positive charge near the region of signal peptidase recognition site resulted in inhibition of translocation of these mutants into inverted vesicles. By using the in vitro translocation assay, the inhibitory effect of positive charges on translocation was traced to steps following the initial interaction of the mutant protein with cytoplasmic and membrane components. These studies have shown that the inhibition of secretion occurs at the stage of translocation across the lipid bilayer or release to the periplasmic side of the membrane and not at recognition by cytoplasmic or membrane-associated components of the secretion machinery.

In the work described in this article, we have shown that the alteration of negatively charged groups at the extreme N terminus of exotoxin A, an extracellularly secreted protein, results in a delay in the processing of its precursor form. We

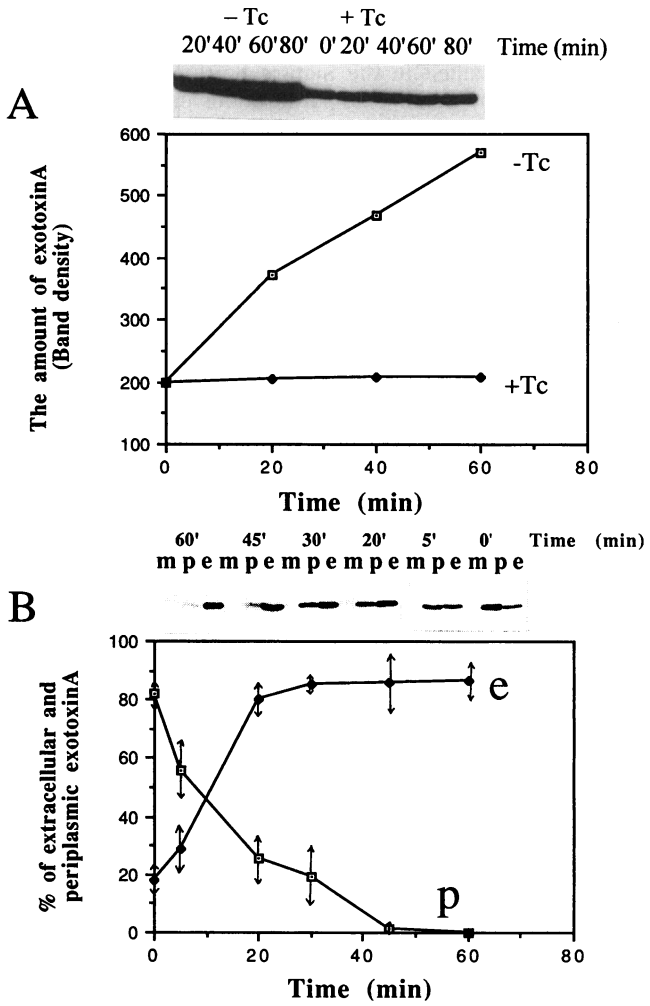

FIG. 4. Effect of inhibition of protein synthesis and extracellular secretion of periplasmic exotoxin A mutant with a Lys-Gln substitution for Glu-Glu at the +2 and +3 positions. (A) A culture of *P. aeruginosa* PAK-NT (pLL4) was grown at 37°C until it reached an optical density at 600 nm of 0.4. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 2 mM for 30 min, and one half of the culture was treated with 200 mg of tetracycline (Tc) per ml. At various times, aliquots were removed and precipitated with 12% trichloroacetic acid. The precipitates were washed with acetone and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer. Equivalent volumes were then analyzed by SDS-PAGE and immunoblotting with antibodies to exotoxin A. The autoradiograph (top) and its densitometer tracing (bottom) are shown. (B) Release of exotoxin A from tetracycline-treated bacteria. After IPTG induction and tetracycline treatment of *P. aeruginosa* PAK-NT (pLL4), aliquots were removed and separated into extracellular (e), periplasmic (p), and mixed membrane-cytoplasmic (m) fractions. Equivalent volumes were analyzed by SDS-PAGE and immunoblotting with anti-exotoxin A antibodies. A radiograph of a typical experiment (top) and a densitometric analysis of exotoxin A in the extracellular (e) and periplasmic (p) compartments (bottom) are shown. The percentage of exotoxin A was calculated from densitometer scans, with the total amount of exotoxin A at the time of addition of tetracycline set at 100%. Each point represents the mean ± standard error of the mean for three independent experiments.

have shown further that mutations in the same negatively charged region of mature exotoxin A resulted in transient accumulation of the protein in the bacterial periplasm. This secretion intermediate could be translocated across the outer membrane, suggesting that information essential for this translocation is contained in the mature portion of exotoxin A. Alternatively, the amino acid substitutions may have affected folding of exotoxin A in the periplasm, thereby interfering with a sorting signal in a different part of the protein. Mutations that altered the negative charge in the acidic terminus could also affect the rate of exotoxin A folding, leading to a delay in achieving a conformation competent for extracellular secretion.

Two previous studies involving proteins secreted via the general secretory pathway have attempted to define export signals by using mutational analysis, targeting the mature portions of these proteins. Substitutions for a tryptophan residue in the middle of the mature aerolysin led to normal processing of its signal peptide and secretion into the periplasm, followed by oligomerization. However, translocation of aerolysin derivatives across the outer membrane was blocked (32), suggesting that the conformation of the central portion of this protein is involved in or influences transport across the outer membrane. Similarly, Py et al. (25) analyzed a series of mutants obtained by altering the coding region of the *Erwinia chrysanthemi* endoglucanase Z, all of which lost their secretability, implying either a distribution of export signals throughout the entire protein or exquisite sensitivity of the signal-containing domain to perturbation of the overall tertiary structure. In this article, we have presented for the first time evidence of mutations that delay secretion of an extracellular protein without completely abolishing its secretability.

We have previously suggested that exotoxin A may utilize a novel pathway for extracellular secretion, in which the precursor form is inserted into the cytoplasmic membrane and carried out of the outer leaflet of the outer membrane through junctions between the inner and outer membranes (17). Cleavage of the signal peptide prior to or during the translocation would result in a spontaneous release of the mature protein into the medium. On the basis of work reported in this article, we now believe that exotoxin A is extracellularly secreted by a different pathway, involving a free periplasmic intermediate having as its only unique feature rapid translocation across the periplasm.

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