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A specific targeting domain in mature exotoxin A is required for its extracellular secretion from *Pseudomonas aeruginosa*

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A number of Gram-negative bacteria, including *Pseudomonas aeruginosa*, actively secrete a subset of periplasmic proteins into their surrounding medium. The presence of a putative extracellular targeting signal within one such protein, exotoxin A, was investigated. A series of exotoxin A truncates, fused to β-lactamase, was constructed. Hybrid proteins, which carry at their N-termini 120, 255, 355 or the entire 613 residues of the mature exotoxin A, were stable and were secreted into the extracellular medium. Hybrid proteins which carry residues 1–30 and 1–60 of the mature exotoxin A were unstable; however, they could be detected entirely within the cells after a short labeling period. A hybrid with β-lactamase was constructed which carried only the N-terminal residues 1–3 and region 60–120 of exotoxin A. It was also secreted into the culture medium, suggesting that a specific 60 amino acid domain contains the necessary targeting information for translocation of exotoxin A across the outer membrane. The secretion of the hybrid proteins is independent of the passenger protein, since a similar exotoxin A–murine interleukin 4 hybrid protein was also secreted. The extracellular targeting signal between amino acids 60 and 120 is rich in anti-parallel β-sheets. It has been shown previously to be involved in the interaction of the exotoxin A with the receptors of the eukaryotic cells. In the three-dimensional view, the targeting region is on the toxin surface where it is easily accessible to the components of the extracellular secretion machinery.

Keywords: exotoxin A/extracellular secretion/outer membrane/periplasm/*Pseudomonas aeruginosa*

Introduction

While bacteria lack intracellular compartments, the relatively simple cell envelope still necessitates targeting of proteins into or across hydrophobic membrane barriers. The double membrane which surrounds Gram-negative microorganisms defines three compartments (inner and outer membrane and the periplasm) and the external medium, each of which contains a specific set of proteins. Extracellular secretion is a complex targeting process, which directs a protein across the two hydrophobic membranes, as well as the aqueous periplasm. Several distinct mechanisms have been described recently which promote extracellular protein secretion from Gram-negative bacteria by direct targeting from the bacterial cytoplasm (Wandersman, 1992; Salmond and Reeves, 1993; Cornelis, 1994) or by release from the outer membrane (Pohlner *et al.*, 1987). Another common targeting mechanism functions as an extension of the general secretory pathway, where the secreted proteins, synthesized with typical N-terminal signal peptides, are first secreted into the periplasm, with a concomitant cleavage of their leader peptides (Pugsley, 1993). The subsequent secretion across the outer membrane utilizes a specialized secretion machinery. This machinery, first described in *Klebsiella oxytoca*, is encoded in a multigene operon, which is responsible for secretion of a single enzyme, pullulanase (Pugsley *et al.*, 1990). Similar multicomponent extracellular protein secretion systems have been identifed in a number of different Gram-negative microorganisms, including *Erwinia carotovora*, *E.chrysanthemi*, *Vibrio cholera*, *Aeromonas hydrophila*, *Xanthomonas campestris* and *Pseudomonas aeruginosa* (Lory, 1992; Tommassen *et al.*, 1992; Overbye *et al.*, 1993; Barras *et al.*, 1994). Moreover, genes encoding certain components of this secretion machinery have been identified in *Escherichia coli*; however, the secreted substrate, if any, is still unknown (Hobbs and Mattick, 1993). Besides their conserved function as the secretory components, some homologs of the extracellular secretion machinery are also involved in assembly of type 4 pili in Gram-negative bacteria (Strom and Lory, 1993) and play an unknown role in DNA uptake in Gram-positive bacteria (Albano *et al.*, 1989).

Pseudomonas aeruginosa is an opportunistic human pathogen, which secretes a number of extracellular proteins utilizing a machinery which is homologous with that of *K.oxytoca*. The exotoxin A is synthesized as a precursor with a 25 amino acid signal peptide, which is processed and secreted into the periplasm (Lory *et al.*, 1988). Here, it folds into what appears to be its final conformation (Strom *et al.*, 1991). The machinery of extracellular secretion, encoded by 12 *xcp* genes, then translocates the mature exotoxin A across the outer membrane into the surrounding medium (Bally *et al.*, 1992; Nunn and Lory, 1992; Akrim *et al.*, 1993). This process, which is capable of sorting exotoxin A from other proteins permanently retained in the periplasm, must recognize sequences or specific structures within this mature, folded polypeptide. The secretion from the periplasm does not appear to involve a periplasmic chaperone-like function.

The structure–function characterization of exotoxin A was aided greatly by the availability of its crystal structure (Allured *et al.*, 1986). The 613 amino acid exotoxin A polypeptide can be divided into several distinct functional domains (Wick *et al.*, 1990). Domain I, which encompasses amino acids 1–252 and 365–404, is involved in binding to the eukaryotic cell receptors. Domain II (amino acids

253–364) is the translocation domain, allowing the catalytic domain III (amino acids 400–613) to enter the cytoplasm. The fragment containing the catalytic domain kills cells by inhibiting protein synthesis, by ADP-ribosylation of elongation factor 2. Since exotoxin A appears to be secreted from the *P.aeruginosa* periplasm in a folded form (Strom *et al.*, 1991), the recognition signal for the Xcp machinery must be on the surface of the protein overlapping one of the functional domains of the mature toxin.

In this study, we report mapping of a domain within the mature exotoxin A, which contains all the information necessary for extracellular secretion of the toxin from the periplasm of *P.aeruginosa*. We constructed a series of exotoxin A–β-lactamase hybrid proteins, and determined the minimal sequence of exotoxin A which can promote the secretion of these fusions into the culture medium. Our study indicates that the region corresponding to amino acid residues 60–120 can target the secretion of the mature *E.coli* β-lactamase across the outer membrane of *P.aeruginosa*. Moreover, the secretion signal of exotoxin A could also drive the extracellular secretion of murine interleukin (IL)-4 in *P.aeruginosa*. This work thus defines a new functional domain for exotoxin A, which may be present in other polypeptides that utilize a similar apparatus for extracellular secretion.

Results

Construction of the exotoxin A–β-lactamase hybrid proteins

We have reported previously that a pair of acidic residues near the signal peptide cleavage site plays an important role in the passage of exotoxin A across the outer membrane, by maintaining the mature toxin in a specific export-competent conformation (Lu *et al.*, 1993). In order to identify the extracellular targeting signals within the mature exotoxin A, a series of plasmids (which contained the coding sequence for the signal peptide and different portions of the N-terminal region of exotoxin A fused to the mature portion of *E.coli* β-lactamase) were constructed. These plasmids were introduced into *P.aeruginosa* PAK-NT which contains a mutation in the chromosomal exotoxin A gene. The synthesis of exotoxin A–β-lactamase fusion protein is dependent upon isopropyl-1-β-thiogalactopyranoside (IPTG) induction of the *tac* promoter of the vector. Figure 1 shows the schematic structures of the hybrid proteins that were used in this study.

The levels of synthesis of the hybrid proteins in *P.aeruginosa* were assessed after induction, by pulse labeling with radioactive methionine and cysteine, followed by a chase with non-radioactive amino acids. The total cultures were subjected to immunoprecipitation with rabbit anti-β-lactamase serum. As shown in Figure 2, most of the exotoxin A–β-lactamase hybrid proteins were synthesized, and migrated on SDS–PAGE with the expected mobilities. The sizes calculated from gel mobility were 26.5, 30, 33, 39.5, 53, 64 and 93.5 kDa for the fusions F1, F30, F60, F120, F255, F355 and F613, respectively. These results indicated that the signal peptides of each of the hybrid proteins, except one, have been removed correctly by the *P.aeruginosa* signal peptidase during secretion of the proteins across the bacterial inner membrane. The single

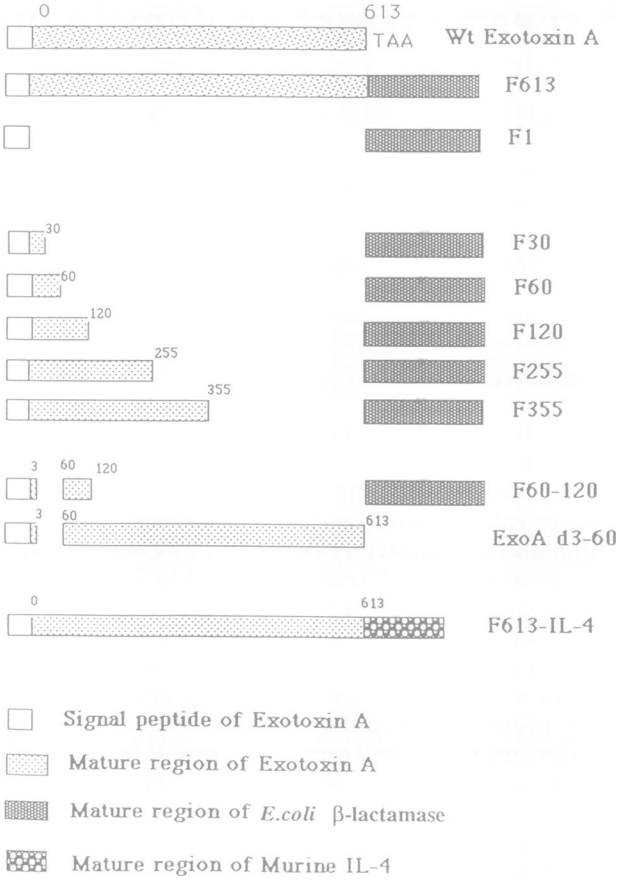

Fig. 1. Representation of the various exotoxin A hybrid proteins used in this study. The numbers above the exotoxin A-specifying region indicate the junction point of the mature exotoxin A sequence and that of β-lactamse or IL-4.

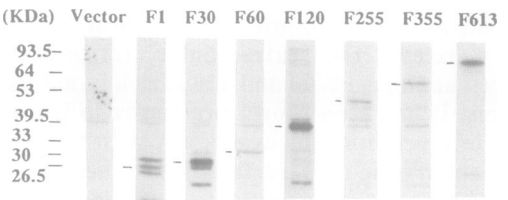

Fig. 2. Synthesis of the hybrid proteins. Pulse labeling and immunoprecipitation of exotoxin A–β-lactamase hybrid proteins from cultures of *P.aeruginosa* PAK-NT. The predicted sizes of the hybrid proteins (from F1 to F613) were 26.5, 30, 33, 39.5, 53, 64 and 93.5 kDa, respectively (marked in the left lane).

exception is fusion F1, which carries only the signal sequence of exotoxin A followed by the β-lactamase mature domain, where three different size bands, within the size range predicted from the sequence, were detected which reacted with anti-β-lactamase antibody. Following prolonged chase, the intensity of the upper band gradually diminished with the corresponding increase in the intensity of the middle band (data not shown). This suggested that the upper band may be the unprocessed precursor form of F1 while the middle band is the mature portion. This is the only hybrid protein which showed an apparent delay in cleavage of the signal peptide. In addition, bands which were smaller than predicted from the size of the polypeptides were seen with the fusion products F1, F613, F30 and F120. These smaller bands are most likely

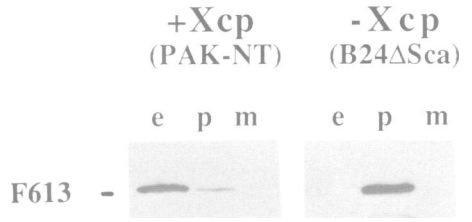

Fig. 3. Xcp-dependent extracellular secretion of F613. Cells of the wild-type *xcp* strain (PAK-NT) and the *xcp R–Z* deletion mutant B24ΔSca harboring plasmids expressing one of the exotoxin A–β-lactamase fusion proteins, F613, were fractionated into extracellular (e), periplasmic (p) and membrane/cytosol mixed (m) components. Equal volumes of each fraction were analyzed by SDS–PAGE, transferred to nitrocellulose and analyzed by immunoblotting with mouse anti-exotoxin A serum followed by goat peroxidase-labeled anti-mouse serum. The bands were detected by ECL protocols.

non-specific proteolytic degradation products of the hybrid proteins.

Secretion of the exotoxin A–β-lactamase fusion protein requires the Xcp machinery

In order to assess the feasibility of using the fusion approach to delineate the extracellular targeting signal within exotoxin A, the plasmid expressing the fusion F613 (a hybrid consisting of the full-length exotoxin A and β-lactamase) was introduced into wild-type *P.aeruginosa* and into *P.aeruginosa* B24ΔSca, a mutant lacking most of the *xcp*-encoded machinery of extracellular protein secretion. The amount of the F613 protein was determined in the extracellular, periplasmic and membrane/cytoplasmic fractions by Western immunoblotting with antibodies to exotoxin A. Figure 3 shows the distribution of F613 in the various fractions, following a 2 h induction of synthesis with IPTG. In wild-type *P.aeruginosa*, the majority of the hybrid protein is extracellular, with a small fraction retained in the periplasm. In contrast, expression of F613 in the *xcp* mutant strain B24ΔSca leads to complete periplasmic localization of the hybrid protein, with none detectable in either the extracellular medium or in the membrane/cytoplasmic fraction. These results indicate that the presence of the passenger protein (β-lactamase) fused to exotoxin A does not interfere with the recognition of the extracellular targeting signal, and that secretion across the outer membrane requires the functional Xcp machinery.

Localization of the extracellular targeting signal to the N-terminal 120 amino acid region of exotoxin A

The subcellular location of a series of hybrid proteins, consisting of carboxy-terminal deletions of exotoxin A and full-length β-lactamase, was determined in *P.aeruginosa*. Bacteria carrying the various fusion plasmids were induced with IPTG for 2 h, the cells were harvested and separated into extracellular (e), periplasmic (p) and mixed membrane–cytoplasmic fractions (m). As shown in Figure 4B, a substantial amount of F120, F255, F355 and F613 could be detected in the extracellular fraction as well as in the periplasmic and cellular fractions. Based on the intensity of the bands, the extent of extracellular secretion varied from 50% (F255) to >75% (F613). This represents true

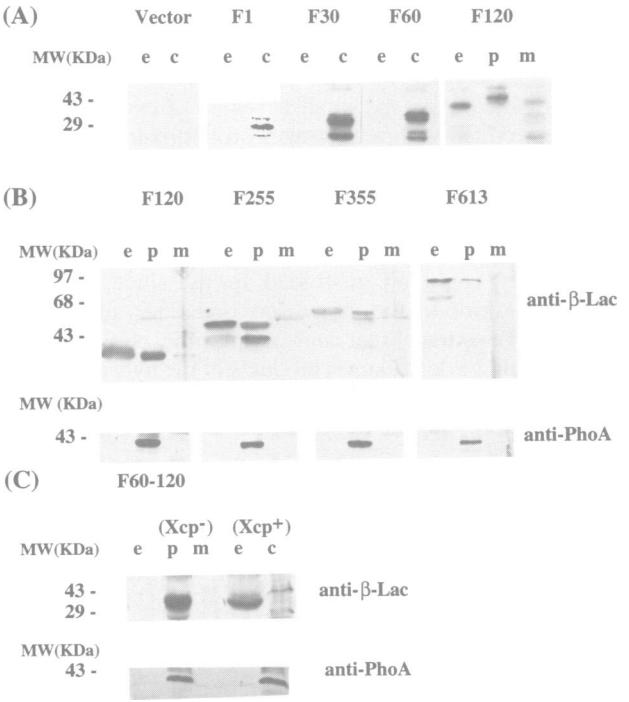

Fig. 4. The extracellular secretion of the exotoxin A–β-lactamase hybrid proteins. (**A**) Pulse labeling and immunoprecipitation of fusions F1, F30, F60 and F120. After pulse–chasing, cells harboring plasmids which express fusion proteins F1, F30, F60 and F120 were separated into extracellular (e) and cellular fraction (c). For F120, a cellular fraction was further fractionated into periplasmic (p) and membrane/cytosol mixed fractions (m). Immunoprecipitation was performed using rabbit anti-β-lactamase serum as described in Materials and methods. The position of each fusion is indicated. (**B**) Immunoblot analysis of F120, F255, F355 and F613. After IPTG induction of the various *P.aeruginosa* strains harboring plasmids, those expressing hybrid proteins F120, F255, F355 and F613 were fractionated into extracellular (e), periplasmic (p) and membrane/cytosol mixed fraction (m) compartments, and then equal volumes of each fraction were analyzed by SDS–PAGE and immunoblotting, using rabbit anti-β-lactamase serum (primary antibody), and goat perioxidase-labeled anti-rabbit serum (secondary antibody) and detected by ECL protocols. (**C**) Immunoblot analysis of the construct F60–120. The bacterial fractions were prepared as described for (B) and immunoblots were reacted with antibody to β-lactamase. The distribution of the periplasmic marker alkaline phosphatase (PhoA) in the extracellular (e) and cellular (c) fractions of the wild-type bacteria (Xcp^+), and in extracellular (e), periplasmic (p) and membrane/cytosol (m) fractions of the *xcp* mutant (Xcp^-), was determined by the treatment of blots with rabbit antibody to alkaline phosphatase (anti-PhoA).

secretion, since the periplasmic marker, the *E.coli* alkaline phosphatase, was retained completely in the periplasm.

In contrast to the larger fusions, under the same conditions, F1, F30 and F60 could not be detected in any fractions. To test whether these short fusions are degraded after synthesis, but perhaps detectable in the short period immediately after protein synthesis, cells were labeled with [^{35}S]Met–Cys and chased with excess amounts of non-radioactive Met–Cys mixture for 15 min. The total culture was then separated into extracellular (e) and cell-associated fractions (c), followed by immunoprecipitation with antibodies to β-lactamase, in the presence of protease inhibitors. The periplasmic fraction was also isolated for fusion F120. As shown in Figure 4A, hybrid proteins F1, F30 and F60 were not secreted into the extracellular medium and could only be detected in the cell-associated

fraction, in their mature form. A small amount of the precursor form of F1 remained unprocessed. In contrast, F120 was secreted into the extracellular fraction. These results indicate that the smallest region of exotoxin A that can be secreted with a passenger protein is located within the N-terminal 120 amino acids. The proteins in the periplasm of fusions F255, F355 and F613 are thought to be the export intermediates, which accumulate in the periplasm due to IPTG-induced overproduction. Alternatively, they could be misfolded forms, since prolonged induction prior to the assay does not alter significantly the relative extracellular and periplasmic pools. Smaller bands could be degradation products of the hybrid proteins, while the smaller fusions, which are probably retarded in the periplasm, appear to be degraded very rapidly.

The region corresponding to residues 60–120 is the secretion signal for translocation across the outer membrane

Since F120 could be secreted into the extracellular media while F60 was completely cell associated, the targeting signal is either located between residues 60 and 120 or the region 1–60 does not contain sufficient sequence information for extracellular secretion. Fusion F60–120, which contains the exotoxin A signal peptide with the first three residues of the mature protein, followed by residues 60–120, and mature β-lactamase, was constructed. When this fusion was introduced into *P.aeruginosa* and analyzed by Western immunoblotting, antibodies to β-lactamase detected this protein entirely in the extracellular medium (Figure 4C). The periplasmic marker alkaline phosphatase remained largely cell associated. The extracellular secretion of F60–120 required a functional Xcp machinery since, in the *xcp* mutant *P.aeruginosa* B24ΔSca, the F60–120 hybrid protein was retained completely in the periplasm. This finding localizes the extracellular targeting signal to residues 60–120 of the mature exotoxin A.

In order to directly implicate the region between amino acids 60 and 120 in extracellular targeting, we engineered an in-frame deletion of exotoxin A, resulting in a protein lacking this 60 amino acid domain. This form of exotoxin A was highly unstable in *P.aeruginosa*. Analysis by short pulse labeling and immunoprecipitation showed that only the periplasm contained a small amount of detectable immunoreactive protein; however, multiple bands were seen, and all were smaller than the predicted size of the deletion (data not shown). This suggested that the region between 60 and 120 not only contains the targeting information but contributes greatly to the intrinsic stability of the protein during periplasmic transit.

Murine IL-4 can be translocated across the outer membrane of P.aeruginosa with the secretion signal of exotoxin A

In order to determine whether a passenger protein other than β-lactamase can be secreted into the extracellular space by the Xcp machinery and by the exotoxin A targeting signal, a hybrid protein F613–IL-4 consisting of exotoxin A and murine polypeptide hormone IL-4 was constructed. In this construct, the coding sequence for the entire 613 amino acid exotoxin A (including the signal peptide) is fused, at its C-terminus, to the coding region

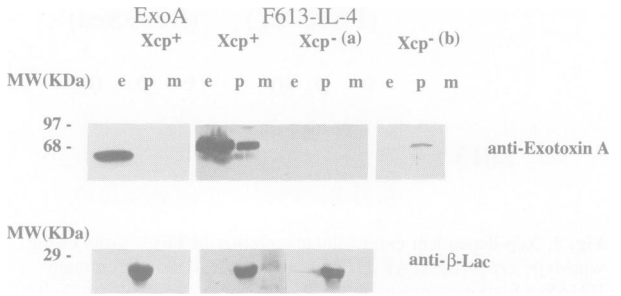

Fig. 5. The extracellular secretion of the exotoxin A–IL-4 fusion protein across the outer membrane of *P.aeruginosa* (immunoblots). After IPTG induction, cells carrying plasmids expressing wild-type exotoxin A and exotoxin A–murine IL-4 fusion protein were fractionated into extracellular (e), periplasmic (p) and membrane/cytosol mixed components (m). Equal volumes of each fraction were analyzed by SDS–PAGE followed by immunoblotting, using mouse anti-exotoxin A serum, or rabbit anti-β-lactamase serum. Also shown is fractionation of wild-type exotoxin A. Autoradiograph for Xcp⁻ of F613–IL-4 (a), is also shown overexposed (b).

of IL-4 at the 9th amino acid. Figure 5 shows the fractionation of F613–IL-4 in *P.aeruginosa* following induction of its synthesis for 2 h with IPTG. As predicted from the sequence, an 81 kDa protein was synthesized, approximately two thirds of which was secreted into the extracellular medium with the remainder retained in the periplasm. Moreover, extracellular secretion of this hybrid protein required a functional Xcp machinery, since no immunoreactive protein was detected in any fractions of the *xcp* mutant *P.aeruginosa* B24 ΔSca, but a small amount of this protein was found in the periplasm when autoradiographs were overexposed. The loss of the F613–IL-4 protein in the *xcp* mutant is very likely due to susceptibility of this hybrid protein to periplasmic proteases. This finding confirms the observations made with β-lactamase as a passenger protein, which suggested that the targeting signal on exotoxin A functioned independently of the rest of the molecule or the fused passenger protein.

Discussion

Extracellular secretion of a number of proteins by Gram-negative bacteria follows a coupled pathway through the bacterial periplasm. These proteins, synthesized as precursors with typical signal peptides, are secreted into the periplasm utilizing the normal secretion machinery that includes removal of the signal peptide by the inner membrane signal peptidase. The second step of this pathway is translocation across the outer membrane, which requires the activities of an apparatus of extracellular protein secretion (Pugsley, 1993). Since many proteins remain in the bacterial periplasm, the machinery of extracellular secretion must recognize some information encoded by the proteins targeted to the extracellular space. This information must be absent in the proteins retained in the periplasm. Little is known about the nature of the targeting signal in the extracellularly secreted proteins, other than that it must be contained in the mature polypeptide, and is very likely presented to the secretion apparatus, in the periplasm, after the protein is folded into what may be its final functional conformation (Strom *et al.*, 1991;

Poquet *et al.*, 1993). For several proteins, a set of disulfide bonds is formed in the periplasm (Pugsley, 1992; Bortoli-Greman *et al.*, 1994) and, in the case of multisubunit toxins, the periplasm is the site of assembly of holotoxins prior to their transfer across the outer membrane (Hirst and Holmgren, 1987).

We have undertaken a study aimed at delineating the extracellular targeting signal in exotoxin A, a protein toxin which is secreted from *P.aeruginosa* utilizing the machinery encoded by the *xcp* genes. This protein was chosen because its crystal structure is known, and it may provide additional clues to the nature of the polypeptide chain which is recognized by the Xcp machinery. We have taken a deletion–fusion approach to define the minimal information in the mature exotoxin A that can direct a passenger protein (β-lactamase) to the extracellular space. β-Lactamase is ideally suited for this type of study since the enzyme with its signal peptide is secreted efficiently into the *P.aeruginosa* periplasm, indicating that it does not contain any sequences which would be incompatible with secretion across the cytoplasmic membrane, nor does it contain extracellular targeting signals. This strategy was chosen also because problems with the stability of the generated constructs were anticipated. Alterations of the polypeptide by mutations or deletions could give rise to an unstable protein susceptible to degradation by the various bacterial proteases, and we reasoned that the presence of a passenger protein may stabilize truncated forms of exotoxin A when part of a hybrid protein. The problems with protein stability were encountered with some fusions, especially those carrying shorter portions of exotoxin A, such as fusions F1, F30 and F60. Furthermore, since the available antiserum against exotoxin A recognizes immunogenic determinants in the C-terminus of the toxin, the fusion to β-lactamase and subsequent probing with anti-β-lactamase provides a reliable measure of the levels of the hybrid protein expression.

In this study, we report identification of a region between amino acids 60 and 120 of the mature exotoxin A that is sufficient to direct secretion of β-lactamase across the outer membrane of *P.aeruginosa*. We therefore postulate that this region contains the extracellular targeting information for the entire exotoxin A. It is very likely that exotoxin A interacts with the Xcp through this discrete domain, resulting in the transfer of the entire polypeptide, in its folded form, across the outer membrane and its subsequent release into the extracellular medium.

Distinct structural domains were assigned to the various portions of exotoxin A, based on structure–function analysis and its crystal structure (Allured *et al.*, 1986; Wick *et al.*, 1990). Domain I, the eukaryotic cell recognition domain, comprises the amino acids 1–252 (a) and 365–404 (b) of the mature protein and is composed primarily of anti-parallel β-sheets (Guidi-Rontani and Collier, 1987). Domain II, the translocation domain, is centrally located, includes residues 253–364, and is composed of six α-helices. Domain III, the ADP-ribosylating domain, includes the C-terminal residues 405–613. The targeting signal 60–120 amino acid region is entirely within domain I and is highlighted in Figure 6. This sequence does not contain any recognizable motifs and lacks any unusual features apparent from the analysis of the primary or secondary structure, other than the three anti-parallel β-strands. Most of the 60–120 amino acid sequence appears in the structure as a protruding knob, which may present a relatively unobstructed surface for interaction with the extracellular secretion machinery. Moreover, this domain is involved in the recognition of cellular receptors during intoxication of the eukaryotic cells. Since the fine structure of the receptor recognition domain of exotoxin A has not been studied as yet, it is difficult to speculate whether the receptors on eukaryotic cells recognize a region which includes the extracellular targeting signal. Nevertheless, the positioning of the domain on the surface of the exotoxin A molecules seems to allow for a free interaction with other molecules during secretion from a bacterial cell or at the initial stages of entry into the target eukaryotic cell.

Two earlier reports attempted to define the regions of exotoxin A which are important for toxin secretion, through the examination of secretion phenotypes using truncated proteins and internal deletions. The work of Chaudhary *et al.* (1988) indicated that domain II of exotoxin A contains important information for the secretion of the toxin to the periplasm of *E.coli*. We find that secretion across neither the cytoplasmic membrane nor the outer membrane of *P.aeruginosa* requires any information from domain II. Hamood *et al.* (1989) suggested that the toxin A signal peptide and the first 30 amino acids (within domain I) of the mature toxin A are required for the extracellular secretion of toxin A by *P.aeruginosa*. This study is in contrast to our observations, where the region 60–120 is located in domain I, but not at the extreme amino-terminus of the protein. The reason for this discrepancy is unclear. We constructed an internal exotoxin A deletion (ExoA d3–60, see Figure 1) which lacks the putative signal identifed by Hamood *et al.* (1989) but contains the targeting signal identified in this work. This protein can be detected only in the periplasm, after pulse labeling and immunoprecipitation, and it appears fragmented into bands, all of which are smaller than the predicted 557 amino acid deletion derivative. We suggest that this deletion causes destabilization of the protein and leads to misfolding of the targeting signal. An independently folding passenger protein may provide the necessary context for presentation of the targeting information to the extracellular secretory apparatus. While a deletion of 56 amino acids completely destabilizes exotoxin A and prevents the recognition of the targeting signal, the fusion F60–120, which not only lacks the same 56 amino acids but has the C-terminal 493 amino acids of exotoxin A sequence replaced by the β-lactamase, is stable and is secreted quite effciently from the bacteria.

Two previous studies addressed the location of the extracellular targeting signals in secreted proteins which utilize a machinery of extracellular secretion homologous to the *P.aeruginosa* Xcp machinery. The targeting information within EGZ, the endoglucanase Z protein of *E.chrysanthemi*, was analyzed using a deletion or peptide insertion approach to investigate the targeting signal within the two known functional domains involved in catalysis and cellulose binding (Py *et al.*, 1993). While many of the derivatives retained at least one of the functional domains, none was secreted from *E.chrysanthemi*, suggesting that the targeting signal is found within both of the functional domains of EGZ, or that it is possible to disrupt the signal

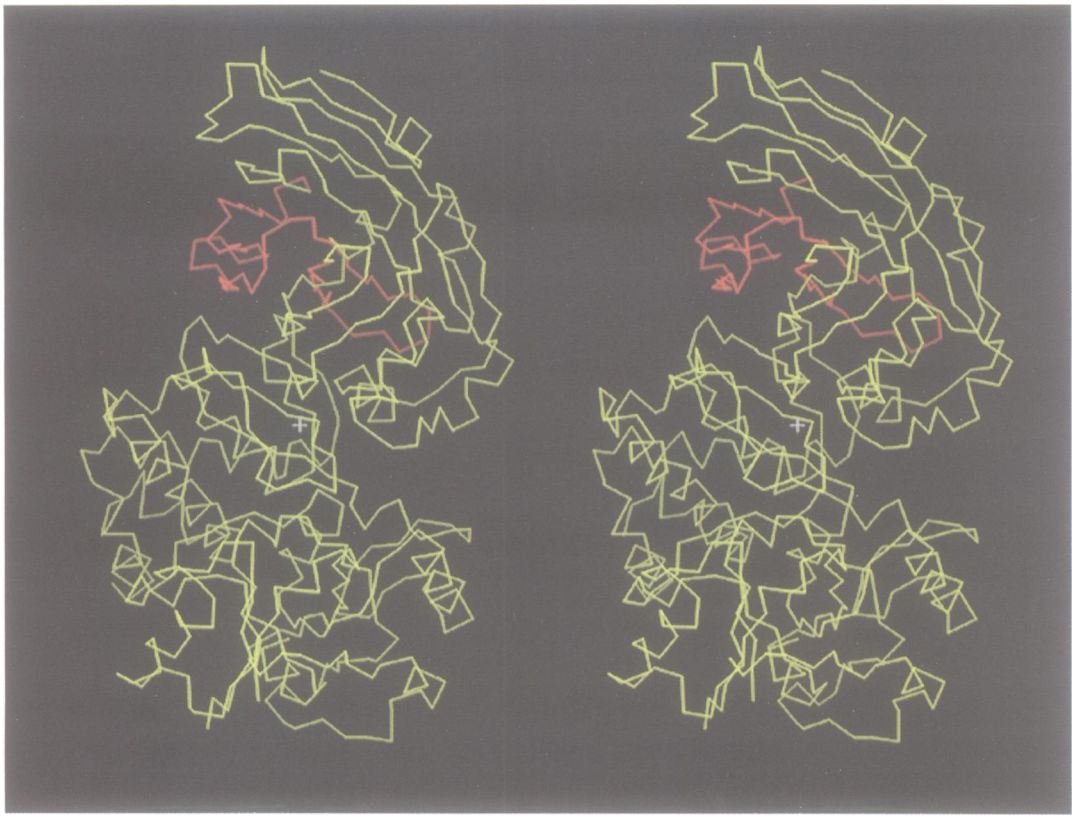

Fig. 6. The three-dimensional structure of the polypeptide backbone of exotoxin A. The region 60–120, containing the extracellular targeting signal, is shown in red.

by mutations without affecting the catalytic or cellulose binding activities. Similarly, point mutations in the aerolysin of *A.salmonicida* lead to disruption of the normal extracellular secretion process at the second stage, and the aerolysin remained periplasmic (Wong and Buckley, 1991). Since concomitant alterations in the activity of the periplasmic enzyme were detected, the mutations resulted in subtle perturbations in the overall structure, leading to a loss of the targeting information, which may be located in an entirely different portion of the molecule.

The precise role of a specific domain in extracellular targeting is not known at this time. The most direct function of such a targeting signal would be to initiate translocation across the outer membrane by a specific interaction with the machinery of extracellular secretion, which in *P.aeruginosa* is encoded by the *xcp* genes. In addition to exotoxin A, several other extracellular enzymes of *P.aeruginosa* are secreted by the Xcp pathway, yet there is no apparent sequence similarity between the 60–120 amino acid sequence of exotoxin A and any regions of these proteins. At this time, it is difficult assess how these different proteins fold in order to present the conserved targeting signal for the Xcp machinery. Alternatively, the interaction of the targeting signal on secreted proteins with the Xcp machinery may be indirect. It is conceivable that, in the periplasm, the targeting information is recognized by an as yet uncharacterized chaperone-like protein, which then directs the secreted protein toward the Xcp pathway. Individual proteins would interact with specific chaperones, which in turn would contain common information recognized by the Xcp machinery. Protein-specific chaperones have been shown to be required for secretion of Yop proteins by *Yersinia* (Wattiau *et al.*, 1994). To date, however, extensive genetic analysis in *P.aeruginosa*, or in other bacteria containing the Xcp-like pathway, has failed to identify such chaperones.

Materials and methods

Bacterial strains, plasmids and media

Escherichia coli DH5α (*HsdR*, *recA*, *lacZYA*F80, *lacZ*M15), from Bethesda Research Laboratories (Gaithersburg, MD), was used as a host for all cloning experiments. *Escherichia coli* XL1 blue [*recA1*, *endA1*, *gyrA 96*, *thi1*, *hsdR17*, *supE44*, lac (F′*proAB*, *lacI*^{q}Z M15, Tn*10*)] and *E.coli* RZ 1032 were hosts for M13 in site-directed oligonucleotide mutagenesis. *Escherichia coli* RK2073 was used as a helper in triparental mating (Kunkel *et al.*, 1987). The exotoxin A mutants of *P.aeruginosa* strain PAK-NT (Starnbach and Lory, 1992) and the *xcp*(*R–Z*) deletion mutant of PAK, B24ΔSca (Turner *et al.*, 1993) were used as hosts for the plasmids that carry the various fusion constructs. Plasmid pMMD4 (Lu *et al.*, 1993) expresses exotoxin A under the control of the *tac* promoter and was used as a source of DNA for the mutagenesis and cloning experiments. pMMB67HE (Furste *et al.*,. 1986) or pMMB67tet (Jiang and Howard, 1992) were used as vectors for expression of various constructs in *P.aeruginosa*, where they were introduced by triparental mating (Leong *et al.*, 1982). Plasmid ptacphoA, expressing the *E.coli* alkaline phosphatase, was a generous gift of Dr Colin Manoil.

Bacterial cells were grown in L broth (Maniatis *et al.*, 1982), or in the minimal medium A (Davis and Mingioli, 1950) supplemented with 1% glycerol, 50 mM monosodium glutamate and 50 mg/ml of an equal mixture of 18 amino acids lacking methionine and cysteine. The antibiotic supplements in the various media were: tetracycline at 100 μg/ml or carbenicillin at 150 μg/ml for *P.aeruginosa*, and ampicillin at 100 μg/ml or tetracycline at 20 μg/ml for *E.coli*.

Construction of plasmids expressing exotoxin A–β-lactamase fusion proteins

Hybrid protein F1 is composed of the signal peptide of exotoxin A and the mature portion of β-lactamase. Plasmid pF1 expressing this protein

was constructed as follows. The unique *Pst*I site in the multiple cloning site of plasmid pMMD4, which carries the *toxA* gene, was eliminated by treatment with T4 DNA polymerase, following digestion with *Pst*I. From the resulting plasmid (pMMD5), the *toxA*-containing *Hind*III–*Eco*RI fragment was subcloned into M13mp18 replicative form DNA. A mutagenic oligonucleotide was used to generate a new *Pst*I site at the cleavage site (+1 position of the mature exotoxin A protein) without any changes in the amino acid sequence (pMMD5-pstI-1). Oligonucleotide-directed mutagenesis was carried out by the method of Kunkel *et al.* (1987). To generate fusion plasmid pF1, PCR was used to generate a *Pst*I–*Eco*RI DNA fragment which contains the coding sequence of the *E.coli* β-lactamase (from the 13th amino acid of the mature protein to its C-terminus), using plasmid pUC18 as the template DNA. The fusion in pF1 was first created by replacing the small *Pst*I–*Eco*RI fragment of pMMD5-pstI-1 with the β-lactamase-encoding *Pst*I–*Eco*RI PCR product. To construct the fusion plasmid pF1 in pMMB67HEtet vector, the shortest *Mlu*I–*Dra*I fragment containing the exotoxin A signal sequence gene was subcloned into the large fragment of *Mlu*I–*Dra*I of pMMB67HEtet vector and then the *Pst*I–*Eco*RI PCR product (with the filled in *Eco*RI site) containing the β-lactamase gene was cloned into *Pst*I–*Dra*I sites of the resulting plasmid.

Plasmid pF613, which expresses the hybrid protein F613 composed of the entire signal sequence plus the mature sequence of exotoxin A followed by the *E.coli* β-lactamase fused to its C-terminus, was constructed by a procedure similar to that described above, with some exceptions. A *Pst*I site was generated after the translation stop codon by site-directed mutagenesis and was ligated to a *Pst*I–*Eco*RI DNA fragment of *E.coli* β-lactamase gene which was created by PCR, generating an in-frame fusion between full-length exotoxin A and the mature β-lactamase.

Plasmids pF30, pF60, pF120, pF255 and pF355, which express the hybrid proteins F30, F60, F120, F255 and F355 that contain the signal sequence and N-terminal residues 30, 60, 120, 255 and 355 of mature of exotoxin A protein respectively, with the mature β-lactamase fused to their C-terminus, were constructed as follows. A *Pst*I site was created at the site corresponding to the positions of +30, +60, +120, +255 and +355 of the mature exotoxin A in pMMD5 by site-directed mutagenesis. The amino acid changes resulting from the engineered *Pst*I site were: in F30 (Ala–Ile→Ala–Ala), in F60 (Ala–Ile→Ala–Ala), in F120 (Asn–Ala→Thr–Ala), and in F255 (Ala–Leu→Ala–Val). There was no change in F355.

To construct the fusion plasmids pF30, pF60, pF120, pF255 and pF355, the smaller *Mlu*I–*Pst*I fragments from each pMMD5-related plasmid were used to substitute for the *Mlu*I–*Pst*I fragment of pF1.

Plasmid pexoAd3–60, in which the region corresponding to residues 3–60 of the mature exotoxin A protein was removed from pMMD5, was constructed as follows. A *Pst*I site was generated at the site corresponding to the +3 position of the mature exotoxin A protein in pMMD5 by site-directed mutagenesis. To construct the deletion mutant of exotoxin A, the small *Mlu*I–*Pst*I fragment from the modified pMMD5 plasmid (*Pst*I at +3) was used to replace the corresponding fragment in pMMD5 (*Pst*I at +60).

Plasmid pF60–120 (which encodes the region corresponding to the exotoxin A signal peptide and the first three amino acids of the mature protein, fused to the exotoxin A region 60–120, with the C-terminal β-lactamase) was constructed by substituting the *Mlu*I–*Kpn*I small fragment of pAd3–60 with that of pF120.

Plasmid pexoA-IL-4 was constructed as follows. A DNA fragment, containing the coding sequence of mature IL-4 was amplified by PCR from the plasmid pIck-IL-4 (a gift of Dr David Lewis). The primers contained engineered *Pst*I–*Eco*RI sites, such that the *Pst*I site, corresponding to the 6th codon of IL-4, was in-frame with the exotoxin A sequence in pF613 (*Pst*I at amino acid 613). The PCR product was designed with *Eco*RI and *Pst*I and cloned into the same sites of pF613, resulting in pexoA-IL-4.

The schematic representations of all of the above hybrid proteins are shown in Figure 1.

Radioactive labeling of proteins and immunoprecipitation

The cells carrying the various fusion plasmids were grown at 37°C in L broth with tetracycline until they reached an optical density of 0.8–1.0, and they were collected by brief centrifugation. The cells were washed twice with minimal A salts supplemented with 1% glycerol and 50 mM monosodium glutamate and were resuspended in the same volume of the above minimal medium plus a mixture of 18 amino acids (except methionine and cysteine). After the cell suspension was incubated for 3 min at 37°C, IPTG was added to a final concentration of 2 mM to induce the fusion protein synthesis, immediately followed by 20–40 μCi/ml of [^{35}S]Met–Cys mixture. After a 5 min labeling period, the chase was initiated with excess amounts of a non-radioactive Met–Cys mixture for 15 min. Samples were then boiled immediately in 0.2% SDS for 3 min, followed by dilution in immunoprecipitation buffer (2% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 8.0) to lower the SDS concentration to 0.04%. Insoluble material was removed by brief centrifugation, and 2.5 μl of rabbit anti-β-lactamase serum or 5 μl of mouse anti-exotoxin A serum was added . After overnight incubation at 4°C, 50 μl of a 10% cell suspension of protein A was added and incubated on ice for 50 min. The precipitate was collected by centrifugation and washed twice in washing buffers (buffer I: 50 mM Tris pH 8.0, 1 M NaCl, 1% Triton X-100, and buffer II: 50 mM Tris pH 8.0). Samples were boiled in 0.4% SDS protein loading buffer, electrophoresed on 10% SDS–polyacrylamide gels (Laemmli, 1970) and bands were visualized by fluorography.

Cell fractionation

A culture of cells, in L broth with appropriate antibiotics, was grown at 37°C until it reached an optical density of 0.4, when IPTG was added to a final concentration of 2 mM, followed by an additional 2 h incubation. The (1.5 ml) culture was centrifuged, and the supernatant and cell pellets were taken as the extracellular fraction (e), and the cell-associated fraction (c), respectively. The periplasmic fraction was prepared by the method of Hoshino and Kageyama (1980). The cell-associated fraction was washed once with 50 mM Tris (pH 7.5) and was resuspended in 0.5 ml of the same buffer supplemented with 0.2 M $MgCl_2$. Suspensions were incubated in a 30°C water bath for 30 min with gentle shaking, immediately chilled on ice for 5 min, and incubated at room temperature for 15 min. This treatment was then repeated once. The mixture was centrifuged and the supernatant was taken as the periplasmic fraction (p). The resultant pellets were saved as the mixed membrane–cytoplasmic fraction (m). Extracellular and periplasmic fractions were precipitated by addition of 13% trichloroacetic acid with 0.07% non-fat dry milk as a carrier and incubated on ice for 30 min. The pellets were washed once with 100% acetone, and dry pellets were solubilized in 20 μl of SDS–PAGE sample buffer (0.4% SDS). The mixed cytoplasmic/membrane fraction was washed once with 50 mM Tris–HCl (pH 7.5) buffer and suspended in 20 μl of SDS–PAGE sample buffer.

SDS–PAGE and Western immunoblot analysis

Protein samples were analyzed by 10% SDS–PAGE and transferred to nitrocellulose membranes by the method of Towbin *et al.* (1979). The membranes were blocked in phosphate-buffered saline (PBS) buffer containing 5% dry non-fat milk and 0.05% sodium azide for 1 h at room temperature. Depending on the analysis, the membranes were then incubated overnight either with mouse antiserum against purified exotoxin A (Strom *et al.*, 1991), rabbit anti-β-lactamase serum (5′ to 3′, Inc., Boulder, CO), rat anti-mouse IL-4, monoclonal antibody (Upstate Biotechnology Inc.) or rabbit anti-alkaline phosphatase serum (gift of Colin Manoil). The membranes were washed four times with PBS within 1 h and reacted with peroxidase-labeled affinity-purified goat anti-rabbit IgG (H+L), goat anti-mouse IgG (H+L) (Kirkegaard and Perng Laboratories, Inc., Gaithersburg, MD) for 1 h, followed by four more washes with PBS. The bands in the membranes were detected by ECL Western blotting protocols (Amersham International, Buckinghamshire, UK).

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