Deletions within the structural exotoxin A gene of 27 or 119 amino acids in domain I of the mature polypeptide, or of 88 or 105 amino acids in domains I and II, resulted in the synthesis of exotoxin A (ETA) polypeptides that were not secreted from Pseudomonas aeruginosa hosts but were localized in the cell membrane. Insertions of a hexanucleotide sequence, either pCGAGCT or pCGAATT, at TaqI sites within the gene resulted in variant exotoxin A polypeptides which were secreted normally. pCGAGCT causes insertion of either Glu-Leu or Ser-Ser in the amino acid sequence of the toxin, while pCGAATT causes insertion of either Glu-Phe or Asn-Ser dipeptides. Although the cytotoxicity of eight variants was unimpaired, that of four others was reduced, and one variant which had a Glu-Phe insert between residues 60 and 61 (ETA-60EF61) was 500-fold less cytotoxic than wild-type exotoxin A. Purified ETA-60EF61 dissociated much faster from mouse LMTK cells than wild-type ETA, suggesting that the insertion impaired the ability of ETA-60EF61 to interact with exotoxin A receptors. The location of the insert is within a major concavity on the surface of domain I of the exotoxin A molecule, suggesting that this concavity is important for toxin-receptor interaction.

The exotoxin A of Pseudomonas aeruginosa (\(M_r = 66,583\)) kills mammalian cells by a mechanism involving three major steps (Pastan et al., 1986). The first step is the binding of the toxin to cell surface receptors. The second is entry of the toxin into target cells, which involves endocytosis and passage of the toxin out of the endocytic compartment into the cytosol. The third step is the enzymatic transfer of the adenosine diphosphate ribosyl (ADPR) moiety of NAD+ to elongation factor 2 in the cytosol, which inactivates elongation factor 2, arrests protein synthesis, and kills the cell. The ADPR transferase activity of exotoxin A (ETA) appears identical to that of diphteria toxin (Iglewski and Kabat, 1975; Vasil et al., 1977).

The three-dimensional structure of the proenzyme form of exotoxin A, solved by Allured et al. (1986), comprises three domains. Domain III, the carboxyl-terminal domain (residues 405–613 of the mature protein), carries the catalytic site for ADPR transferase activity (Chen et al., 1987; Douglas et al., 1987; Gray et al., 1984; Lukac et al., 1988), although the catalytic activity is latent in the secreted proenzyme form of the toxin. The function of domain II (residues 253–364) has been inferred to involve membrane penetration (Allured et al., 1986; Chaudhary et al., 1988). Domain I, the amino-terminal domain, which is subdivided into domains Ia (residues 1–252) and Ib (residues 365–404), has been concluded to contain the receptor-binding site of the toxin (Guidi-Rontani and Collier, 1987; Hwang et al., 1987). We are interested in more precisely defining the region(s) of domain I required for the receptor binding activity of exotoxin A. Evidence that the receptor-binding site is located in domain I is based on the failure of exotoxin polypeptides with extensive deletions of domain I to inhibit the cytotoxic activity of native exotoxin A, suggesting that these deleted polypeptides did not bind to cell surface receptors (Guidi-Rontani and Collier, 1987; Hwang et al., 1987). The deleted polypeptides were derived by eliminating appropriate regions of the exotoxin A structural gene, which has been previously cloned on a 2760-bp PstI-EcoRI fragment (Fig. 1) from the chromosome of P. aeruginosa strains PA103 (Chen et al., 1987; Gray et al., 1984) and PA01 (Chen et al., 1987). The deleted exotoxin polypeptides were expressed in Escherichia coli hosts and were extracted from cell lysates for analysis (Guidi-Rontani and Collier, 1987; Hwang et al., 1987). In this report we have used Pseudomonas hosts and have made smaller deletions in domain I, and in domains I and II, but the exotoxin polypeptides were not secreted, even in Pseudomonas. To derive modified exotoxins with more conservative changes that did not impair secretion, we have inserted dipeptides into various locations within domain I and have obtained several variants that are secreted and that have reduced cytotoxicity. One variant containing a Glu-Phe dipeptide insert between residues 60 and 61 had a reduction in cytotoxicity of approximately 500-fold. We present evidence that the proenzyme form of this variant is defective in binding to cell surface receptors. The site of the dipeptide insertion in this variant lies within a major concavity on the surface of domain I of the exotoxin A molecule, suggesting that this concavity may be important for receptor binding. A preliminary report of this work has appeared (Clowes et al., 1988).

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains and Plasmids—*P. aeruginosa* wild-type strain PA103 and PA0286, a meta-28 trp6 mutant of *P. aeruginosa* FA01, were used as sources of wild-type exotoxin A. Escherichia coli strains HB101 (hsdS20 recA13 araI4 proA2 lacY1 galK2 rpsL20 xyl-5 mitr supE44 λ F (Bayer and Roulland-Dussoix, 1969) and A102 (recA...“}
pro thi supE endA hsdR ΔIgal-chld-pgi-atl0 (Ahmed, 1985) and MAM4, a recA102 metS toxA mutant of P. aeruginosa PA01 (Chen et al., 1987), were used as hosts for the construction and expression of the cloned exotoxin A gene. The vector pRC357 was constructed from pUC9 (Vieira and Messing, 1982) by insertion of a 1.85-kilobase PstI fragment from pRR1614 which permits replication in Pseudomonas hosts (Olsen et al., 1982), and subsequent deletion of the PstI site distal to the multiple cloning site of pUC9 (Chen et al., 1987). pRC360 was constructed by inserting pUC9 the 2760-bp PstI-EcoRI chromosomal segment of P. aeruginosa PA103 that carries the exotoxin A gene (Gray et al., 1984), and pRC362 carries the same PstI-EcoRI segment inserted into pRC357 (Chen et al., 1987). PUC4-KISS (Pharmacia LKB Biotechnology Inc.) was used as a source of a 1.3-kilobase kanamycin resistance (Km-r) cassette, excised by SstI or EcoRI digestion (Barany, 1985).

Media and Biologicals—The media used comprised Oxoid no. 2 nutrient broth and agar and tryptic soy broth dialysate (TSBD) as previously described (Tsaur and Clowes, 1989). Solid TSBD was prepared by the addition of 0.8% agarose (SeaKem). In later experiments leading to the partial purification of exotoxin products from culture supernatants, the glycerol concentration in TSBD was increased from 1 to 1.7% (w/v), and that of monosodium glutamate was decreased from 0.1 to 0.06 M. Where appropriate, disodium carbobenicol (Sigma) was added to a concentration of 300 μg ml⁻¹, and kanamycin was added to 50 μg ml⁻¹ for the selection of P. aeruginosa strains. Restriction enzymes were obtained from Bethesda Research Laboratories and Fisher/Promega, and hexanucleotides from Pharmacia.

Methods—Large scale preparation of plasmid DNA was as described by Birnboim and Doly (1979). Isolation of plasmid DNA from transformant clones for screening procedures used essentially the alkaline method (Maniatis and Fisher/Promega, and hexanucleotides from Pharmacia).

Restriction enzyme cleavage, ligation, and transformation of HBlOl and λ were carried out as previously described (Olsen et al., 1982), and subsequent deletion of the PstI site distal to the multiple cloning site of pUC9 (Chen et al., 1987). pRC360 was constructed by inserting pUC9 the 2760-bp PstI-EcoRI chromosomal segment of P. aeruginosa PA103 that carries the exotoxin A gene (Gray et al., 1984), and pRC362 carries the same PstI-EcoRI segment inserted into pRC357 (Chen et al., 1987). PUC4-KISS (Pharmacia LKB Biotechnology Inc.) was used as a source of a 1.3-kilobase kanamycin resistance (Km-r) cassette, excised by SstI or EcoRI digestion (Barany, 1985).

Deletions extending into domain II were similarly derived following separation of the BglII (1488) and Apal (1965) cleaved fragments, secondary Sau3AI cleavage at 1753 and 1803 bp, and ligation of appropriate fragments to derive pRC362-ΔD (deleted from nucleotides 1488 to 1753, i.e. 88 amino acids from 224 to 311) and pRC360-ΔDE, deleted from nucleotides 1488 to 1803, or 105 amino acids from 224 to 328 (Fig. 1).

In all four mutants, the cleavage sites occur after the same nucleotide position of the reading frame, so that the amino acid sequence of the carboxyl-terminal segment in all deleted polypeptides is identical to that of the native polypeptide.

**Barany Hexanucleotide Mutagenesis**—The method of Barany (1985) was used to insert hexanucleotides within the coding region of the gene to produce dipeptide insertions in the exotoxin polypeptide. In essence, the method involved cleavage with a restriction enzyme which produces a 2-bp 5' overlap, followed by ligation of a hexamer which in the first two bases are complementary to the overlap, and the remaining four nucleotides are palindromic, so that the hexamers bind to both cleaved strands to form cohesive ends. By appropriate choice of hexanucleotide, a new restriction enzyme cleavage site can thus be constructed. This new cleavage site may be used to identify insertions, and if used in conjunction with a cassette carrying antibiotic resistance which is flanked by cohesive ends produced by the same enzyme, permits selection of the insertion mutant by that resistance. Thus, TaqI cleavage producing 5'-GC overlaps can be ligated to a pCGAATT hexanucleotide to create an EcoRI recognition site.

**FIG. 1.** Plasmid pRC360: the 2760-bp PstI-EcoRI chromatosomal segment from P. aeruginosa PA103, cloned in pUC9, indicating deletion derivatives. The box represents the structural gene, with the numbers below showing distances in nucleotides from the PstI site. The 746-821-bp segment codes for a signal sequence which is cleaved to form the mature protein. The sites for a number of restriction enzymes NcoI, BglII, and Apal, unique to the cloned fragment, and for Sau3AI (S) are shown above the box. The roman numerals at the top indicate the physical domains of the mature protein established by X-ray crystallography (Allured et al., 1986). The thick horizontal lines below show the segments of the gene which are retained in the deletion mutants -ΔB to -ΔDE as indicated at the right. The numbers in parentheses above and below these thick horizontal lines indicate the location of amino acids, numbered from 1 at the NH₂-terminal end and (Allured et al., 1986). (pRC362 is an identical plasmid, except for the insert of a 1.85-kilobase PstI fragment at the PstI site, followed by removal of the distal PstI site.)
site. This EcoRI site can then be used to insert a sequence flanked by EcoRI-generated termini and specifying kanamycin resistance which is used to select mutants carrying the hexanucleotide insert. We chose TaqI since this enzyme has multiple cleavage sites in the structural gene, 10 of which are located in domain I (Gray et al., 1984). Mutants were constructed in pRC362 derivatives in an E. coli K12 host cell strain (either HB101 or AA102) (Fig. 1). When the mutational site had been localized, the plasmid was transformed into P. aeruginosa MAM4 for biological tests on the exotoxin product. Plasmids were designated by the dipeptide inserted and the sequence number of the amino acids flanking the insert, e.g. a mutant of pRC362-60EF61 in an insert of a glu-Phe dipeptide between amino acids 60 and 61 in the mature secreted protein was designated as pRC362-60EF61.

**Growth of Exotoxin-producing Cultures for Purification**—150 ml of prewarmed, deferrated, filter-sterilized (0.22 μm) TSBD medium in each of four 2-liter Erlenmeyer flasks was inoculated with a 1:200 dilution of an overnight culture of the same strain in the same medium and incubated with shaking at 32 °C for 22 h to an OD₆₅₀ of about 5. One drop of antifoam B (J. T. Baker) was added during growth to prevent foaming, and phenylmethanesulfonyl fluoride was added to 1 μg/ml after 15 h of incubation to inhibit protease activity. Cells were removed by centrifugation at 10,000 x g for 20 min at 4 °C, the pH was adjusted to 8.0 with Tris-HCl, and the supernatant was filter-sterilized at 4 °C.

**Partial Purification of Exotoxin Products**—Partial purification of the exotoxin products secreted into the supernatants of MAM4 host strains carrying plasmids pRC362 or pRC362-60EF61 was achieved by the following procedure based on those previously described (Leppla, 1976) carried out at 4 °C. During purification, exotoxin levels in the supernatants from hosts carrying pRC362 or pRC362-60EF61 were monitored by ADPR transferase activity (Chen et al., 1987). Sepharose CL-4B was added immediately to the filtered supernatant followed by (NH₄)₂S0₄ to 70% saturation over a 2-3-h period. The beads were allowed to settle overnight, the supernatant was decanted, and the beads were poured into a column. Fractions were eluted with buffer A (20 mM Tris, 1 mM EDTA, 2 mM 2-mercaptoethanol, pH 8.0), and those containing toxin were pooled and extensively dialyzed. The pooled dialyzed fractions were added to a DE52 column, and the column was extensively washed in buffer A until the A₆₅₀ of the eluate was the same as buffer A. The washed column was then eluted with a linear NaCl gradient (0.0-0.35 M). Toxin-containing fractions were again pooled, the pH adjusted to 8.0, and extensively dialyzed against buffer A. The proteins were precipitated with (NH₄)₂S0₄ to 70% saturation, separated by centrifugation, resuspended in about 2 ml of buffer A, again dialyzed against buffer A. The toxin from purified preparations of MAM4(pRC362) is termed ETA (as is purified exotoxin A) and that from MAM4(pRC362-60EF61) is termed ETA-60EF61.

**Cell Pellet Fractions**—These were prepared by centrifuging cooled 1-ml samples of 20-h cultures of cells grown in TSBD in an Eppendorf centrifuge for 5 min. The cell pellets were resuspended in an equal volume of 50 mM Tris at pH 7.4. 50-μl samples were removed from supernatants and cell suspensions, mixed with an equal volume of 2 × Laemml SDS sample buffer (Laemmli, 1970), and heated for 2 min at 100 °C, then repeatedly drawn through a tuberculin syringe to cleave the DNA. Immunoblot analysis of exotoxin-related polypeptides was carried out following SDS-polyacrylamide gel electrophoresis separation as described previously (Chen et al., 1987), except that subsequent transfer to nitrocellulose used a Khyse-Anderson (1984) graphite electrolotter.

**Cell Membrane Fractions**—These were prepared by centrifuging 20 ml of a similar 20-h culture, resuspending the cell pellet in 4 ml of 50 mM Tris-HCl (pH 8.5), and sonicating at 4 °C (HSE sonicator for six 10-s pulses). Following removal of nonbroken cells by sedimentation, the supernatant was centrifuged (Beckman SW 50.1 rotor) at 27,000 rpm (80,000 × g) for 2 h to produce a "membrane" supernatant and pellet fraction.

**Cytotoxicity Tests**—These used thymidine kinase-deficient mouse L (LMTK) cells grown at 37 °C in humidified 10% CO₂/90% air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 4.5 g/liter glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml Fungizone (amphotericin B), and in early experiments, 10 mM HEPES at pH 7.4 (Marnell et al., 1982). Protein synthesis was measured by the incorporation of L-[4,5-³H]leucine into tri chloroacetic acid-precipitable material. To test cytotoxicity, 1 to 2 × 10⁵ LMTK cells/well were transferred after trypsin treatment to 24-well assay plates, and after 20-24 h of growth, the DMEM growth medium was replaced with DMEM lacking bicarbonate and 99% of L-leucine and supplemented with 5 mM HEPES (assay medium). After 1 h of incubation in assay medium, toxin was added, and 3 h later, labeled leucine was added, and incubation continued for 1 h. Cells were then washed twice in phosphate-buffered saline and lysed in 0.1% SDS, 0.2 mg/ml DNase I, 1 mM MgCl₂, and 1 mM CaCl₂. Samples were transferred to Whatman 3MM squares, soaked in 5% trichloroacetic acid, 0.5 mg/ml leucine for 30 min, dried, and counted in a liquid scintillation counter (Marnell et al., 1982) (see Fig. 3).

**Binding Dissociation Tests**—Tests measuring the rate of dissociation of bound exotoxin were carried out on ETA and ETA-60EF61. LMTK- cells were incubated with toxin (46 ng/ml ETA or 20 μg/ml ETA-60EF61) for 2 h at 4 °C to permit initial binding. The unbound toxin was then removed by replacing the assay medium with fresh medium, and the cells were held for varying periods of time (5-60 min) at 4 °C to permit dissociation of bound toxin. The cells were...
Effects of dipeptide insertions in domain I on cytotoxicity of exotoxin polypeptides

| Hexamer inserted | Nucleotide site | Amino acid site | Peptide inserted | Designation of variant | Relative cytotoxicity |
|------------------|----------------|----------------|------------------|------------------------|----------------------|
| pCGGAGTT         | 894–895        | 5–6            | Glu-Phe          | 5EF6                   | 1.0                  |
| pCGGAGCT         | 909–910        | 30–31          | Glu-Leu          | 30EL31                 | 1.0                  |
| pCGGAGTT         | 909–910        | 30–31          | Glu-Phe          | 30EF31                 | 1.0                  |
| pCGGAGCT         | 999–1000       | 60–61          | Glu-Leu          | 60EL61                 | 0.01                 |
| pCGGAGCT         | 1044–1045      | 75–76          | Glu-Phe          | 60EF61                 | 0.003                |
| pCGGAGCT         | 1151–1152      | 111–112        | Ser-Ser          | 111SS112               | 1.0                  |
| pCGGAGTT         | 1224–1225      | 135–136        | Asn-Ser          | 111NS112               | 0.12                 |
| pCGGAGCT         | 1467–1468      | 216–217         | Glu-Phe          | 135EF136               | 1.0                  |
| pCGGAGCT         | 999–1000       | 60–61          | Glu-Leu-Glu-Leu  | 60EL6EL61              | 0.01                 |

*Compared to wild-type exotoxin (see Fig. 4).

This mutant was further cleaved with SstI, followed by insertion of a second pCGGAGCT to result in a mutant shown as.

again transferred to fresh assay medium to remove dissociated toxin and then incubated at 37°C for 3 h at which time [3H]leucine was added and incorporation measured in the usual way (see Fig. 5).

Three-dimensional Analysis—Analysis of the exotoxin A protein used the x-ray crystallographic coordinates (Allured et al., 1986) with an Evans and Sutherland model PS300 graphics system and “Insight” (Biosym Tech) software (see Fig. 6).

RESULTS

Deletion Mutants—Fig. 1 indicates the segments eliminated in the four deletion mutants, A, B, C, and DE, that were constructed as described under "Experimental Procedures." Supernatants taken from cultures of MAM4 harboring plasmids encoding each of the four mutated proteins had no cytotoxic activity (data not shown). To test whether deleted proteins were present in the culture supernatants, samples were electrophoresed in an SDS-polyacrylamide gel, and the proteins were transferred to nitrocellulose and probed by immunoblotting with antibodies to native exotoxin A. The results, shown in Fig. 2A, indicated that the deletion variants were not present in the supernatants while native exotoxin A, included as a control, was found in the supernatant of MAM4 carrying the unmutated exotoxin gene. In contrast, when the cell pellets were lysed and examined by immunoblotting, exotoxin-related proteins of sizes consistent with the deletions were detected in the lysates of each mutant (Fig. 2B). A membrane fraction prepared from MAM4(pRC362-AB), the mutant with the smallest deletion, showed that most of the protein was membrane-associated, although some material of lower molecular weight, presumably degraded protein, was soluble (Fig. 2C). The failure of the deletion variants to be secreted prompted us to use other less damaging methods of creating lesions in domain I, and we turned to the TAB insertion technique.

Barony Hexanucleotide Mutants—TaqI cleaves the exotoxin A structural gene at 16 sites, 10 in domain I, 1 in domain II, and 5 in domain III. We were successful in inserting hexanucleotides at 7 of the 10 TaqI sites in domain I. The hexanucleotide insertions are listed in Table I. Radioimmunoassays of supernatants from MAM4 hosts harboring plasmids encoding each of the insertion variants indicated that the variant exotoxin proteins were secreted at levels equivalent to normal exotoxin A (data not shown). When the crude supernatants were tested for cytotoxic activity with mouse cells, four of the variants had reduced activity, as indicated in Table I. Variants 60EL61 and 60EF61, which had Glu-Leu or Glu-Phe inserted between amino acids 60 and 61, showed a reduction in cytotoxicity of 100- and 300-fold, respectively. Inserting Glu-Leu between residues 75 and 76 (variant 75EL76) reduced activity 10-fold while placing Asn-Ser between residues 111 and 112 (variant 111NS112) resulted in almost a 10-fold loss in cytotoxicity. None of the other insertions had any detectable effect on the activity of the toxin.

Insertion of Glu-Phe between Residues 60 and 61 Improves Toxin Binding—We chose to further characterize the protein produced by mutant 60EF61 (ETA-60EF61) since it was the most impaired and because inserting a Glu-Leu dipeptide at the same location also significantly reduced cytotoxicity, further suggesting that this site might be important for the activity of the toxin. Wild-type toxin (ETA) and ETA-60EF61 were purified from culture supernatants as described under "Experimental Procedures." The preparations were highly purified as indicated by polyacrylamide gel electrophoresis in the presence of SDS (Fig. 3). The cytotoxic activities of the purified preparations were tested, and ETA-60EF61 was 500-fold less cytotoxic than normal toxin (Fig. 4), in reasonable agreement with the data of Table I.

Treatment of native exotoxin A with urea and a reducing agent causes a conformational change that activates the ADPR transferase activity of the toxin but also strongly reduces its cytotoxic activity with live cells (Leplla, 1976; Vasil et al., 1977). Thus, the impaired cytotoxicity that was expressed by the variant exotoxin A molecules could be the consequence of a mutation that destabilized the conformation of the toxin, activating the ADPR transferase activity, concomitant with reducing its cytotoxicity. In Table II we compare the ADPR transferase activity of wild-type toxin and ETA-60EF61 preparations before and after enzymatic acti-
The effects on protein synthesis of toxin remaining on the cell surface were measured. Sufficient ETA and ETA-60EF61 were used in the initial incubation to reduce protein synthesis by 60–83% when no dissociation at 4 °C was allowed. As seen in Fig. 5, protein synthesis in cells treated with ETA did not recover during incubation at 4 °C, indicating that little or no toxin dissociated from receptors. In contrast, cells treated with ETA-60EF61 returned to normal levels of protein synthesis within 20–30 min. This provides direct evidence that the rate constant for dissociation of ETA-60EF61 from the cell surface is increased. Unless the second-order rate constant for ETA-60EF61 binding to receptors has been increased to compensate for the increase in the rate of dissociation, which seems unlikely, these data lead to the conclusion that the affinity of ETA-60EF61 for receptors is much less than that of wild-type ETA.

**DISCUSSION**

Our original attempts to isolate exotoxin A variants by deleting segments of the cloned DNA between adjacent in-frame Sau3A1 cleavage sites were frustrated due to the fact that in all cases, in contrast to the wild-type toxin, which is secreted into the culture medium of a *P. aeruginosa* nontoxicogenic host (Chen et al., 1987), the variant exotoxins were not secreted. Analysis of cell pellets and membrane-associated fractions showed that a toxin peptide of an appropriate size was synthesized but appeared to be bound to the cell membrane. One of our long-term goals is to produce a variant that is nontoxic because it has lost its ability to bind to cell surface receptors so that it may then be coupled to another receptor binding moiety and thereby form a hybrid toxin that may be useful in cancer therapy (Olnes and Pihl, 1982). We therefore did not wish to use treatments to release the bound toxin which might have produced structural changes that may adversely affect efficient membrane penetration and enzymatic activity. Consequently we turned to variants produced by more conservative changes which were expected to be more efficiently secreted.

The introduction of dipeptides by the Barany hexanucleotide insertion produced variants that were efficiently secreted. In most cases, however, the introduction of a Glu-Leu dipeptide had little effect on the cytotoxicity of the toxin, although in domain I insertion between amino acids 75 and 76 resulted in a reduction in the cytotoxicity of crude supernatants to 10%, and a similar insert between amino acids 60 and 61 led to a reduction to 1%. The choice of the hexanucleotide pCGAATT was made to introduce a Glu-Phe dipeptide, which was anticipated would lead to a greater perturbation in the physical structure due to the larger dimensions and hydrophobicity of the phenylalanine side chain. This rationale appeared to be valid, since a Glu-Phe insertion between amino acids 60 and 61 now led to a variant (designated ETA-60EF61) that was 500-fold less cytotoxic when partially purified than is native ETA. ETA-60EF61 appeared to dissociate from the surface of mouse LMTK− cells much faster than normal ETA, strongly suggesting that the dipeptide insert affects the affinity of the toxin for cell surface receptors. It is important to note that ETA-60EF61 is predominantly in the proenzyme form, as indicated by its latent ADPR transerase activity. Activation of normal ETA causes conformational changes that reduce its cytotoxic activity (Leppla, 1976), possibly by interfering with receptor binding, so it would be difficult to conclude with certainty that a variant toxin was impaired in receptor binding if the variant was activated. Previous studies concluding that domain I of ETA contained information for receptor binding used deletion variants that were (primarily)
Fig. 6. α-Carbon backbone model of domain I of the proenzyme form of the wild-type exotoxin A generated by the Evans and Sutherland model PS300 graphics system and “Insight” software (Biosym Tech) using the coordinates of Allured et al. (1986). The amino-terminal end is at top left. The three highlighted β-sheets span residues 55 (at left) to 81 (at right).

activated (Hwang et al., 1987) or used variants containing deletions (Guidi-Rontani and Collier, 1987) or point mutations (Jinno et al., 1988) whose state of activation was not reported. Our work indicates that a lesion in domain I of ETA that does not activate the toxin also impairs receptor binding, confirming the conclusion that domain I of the proenzyme form of ETA is important for receptor binding.

A computer-assisted projection of the three-dimensional structure of domain I of the wild-type exotoxin A molecule from its x-ray coordinates (Allured et al., 1986) indicates the presence of a concavity, between loop-out projections of the α-carbon backbone between amino acids 20/21 and 81/82. The surface of this concavity is lined by three antiparallel β-strands formed from amino acid residues 55–61, 63–69, and 72–80 (Fig. 6). Insertion of the dipeptide in ETA-60EF61 might alter one or more of these β-sheets, suggesting that the concavity may be an important structural determinant for ETA binding to receptors. It is also notable that ETA-75EL76 expressed a 10% reduction in cytotoxicity whereas insertions at other sites which are outside of this area were without effect (except for 111NS112). Recent data of Jinno et al. (1988) is also consistent with the identification of this region as a receptor binding site. When they independently converted each of 12 lysine residues in domain I to glutamic acid residues, only one substitution had a major effect on cytotoxicity. This substitution was for lysine 57, which also reduced the ability of the variant to bind to cell receptors and is within the same β-sheet as the insert in ETA-60EF61. None of the other lysine residues altered by Jinno et al. (1988) are located between residues 55 and 80. We suggest that these three antiparallel β-sheets between residues 55 and 80 make an important contribution to the site on domain I at which receptor binding occurs.

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