Functional Analysis of Domains II, Ib, and III of Pseudomonas Exotoxin*

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Pseudomonas exotoxin is composed of three structural domains that are responsible for cell recognition, membrane translocation, and ADP-ribosylation. The substitution of the cell recognition domain (domain Ia) with a growth factor such as transforming growth factor α (TGFα), creates a cell-specific cytotoxic agent, TGFα-PE40, which kills cells bearing epidermal growth factor (EGF) receptors. We have used TGFα-PE40 to define the role of sequences in domains II, Ib, and III. Various mutations were made in these domains and mutant forms of TGFα-PE40 expressed in Escherichia coli. Mutant proteins were then tested for their ADP-ribosylation, EGF receptor-binding, and cell-killing activities. Additionally, the amino boundary of domain III, which contains the ADP-ribosylation activity, was determined by deletion analysis. Data indicate that (i) the functional amino terminus of domain III is near amino acid 400; (ii) deletion of various regions in domain II or conversion of cysteines 265 and 268 to serines results in a loss of cytotoxicity which ranged from 10-fold to more than 150-fold, indicating that domain II is essential for full expression of cytotoxicity; (iii) deletion of the amino terminus of domain Ib results in a molecule with somewhat increased cytotoxic activity, indicating that domain Ib is not essential for the cytotoxic effect of TGFα-PE40; and (iv) TGFα-PE40, produced by denaturing and refolding of insoluble material from inclusion bodies, binds better to EGF receptors and is about 10-fold more cytotoxic to cells bearing EGF receptors than is the secreted form of soluble TGFα-PE40.

Pseudomonas exotoxin A is made up of three domains as shown by x-ray crystallography (1). The physical boundaries indicated that domain Ia is composed of residues 1–252; domain II, residues 253–364; domain Ib, residues 365–404; and domain III, residues 405–613. It has been shown that domain Ia is responsible for cell recognition, domain II is likely to be involved in translocation of the toxin across membranes, and domain III catalyzes the ADP-ribosylation of elongation factor 2, which arrests protein synthesis and results in cell death (2, 3). Domain Ib, whose function has so far not been investigated, is a small domain which, from the crystal structure, is located between domains II and III and is very close to domain Ia (1). Based on DNA and amino acid sequence, however, domain Ib is encoded by sequences that lie between domains II and III (see Fig. 1). Removal of domain Ia results in a truncated Pseudomonas exotoxin molecule (PE40), which retains full ADP-ribosylation activity (4). PE40 is a 364-amino acid polypeptide with a M, of 40,000, which contains domains II, Ib, and III of the Pseudomonas exotoxin molecule. By fusing PE40 with growth factors such as TGFα, interleukin 2, and interleukin 6, we have created chimeric toxins that are cell-specific cytotoxic agents (5–7).

TGFα recognizes and binds to cells that display EGF receptors (8). The fusion protein TGFα-PE40 is cytotoxic to EGF receptor-bearing cells. To study the function of residues within PE40 which might be involved in the translocation process, we have modified the TGFα-PE40 molecule by creating mutations in domains II and Ib. The cytotoxic activity of the TGFα-PE40 fusion proteins was assessed using A431 human epidermoid carcinoma cells that contain large numbers of EGF receptors. The binding activities of TGFα-PE40 and two of the TGFα-PE40 mutant proteins were assessed by determining the amount of these proteins needed to displace 125I-EGF when added to A431 cells.

Mutations in the region adjoining domain III could affect ADP-ribosylation activity in a negative way and thereby decrease cytotoxicity. Therefore, it was necessary to determine the functional boundary between domains Ib and III. To do this, various portions of the Pseudomonas exotoxin gene adjoining and within domain III were deleted and the amino boundary of domain III defined. Our studies indicate that the functional boundary required for full ADP-ribosylation activity starts near amino acid 400 in Pseudomonas exotoxin.

Based on results of cytotoxicity assays, mutant forms of TGFα-PE40 were placed into one of four groups. Two groups have greatly reduced cytotoxicity: one due to reduction in ADP-ribosylation activity, and one due to mutations in domain II. A third group shows about a 10-fold decrease in cytotoxicity, and a fourth group shows a slight increase in cytotoxicity.

**MATERIALS AND METHODS**
Enzymes and chemicals were purchased from standard chemical sources. A cDNA coding for TGFα was a gift from Allan Oliff of Merck, Sharp and Dohme Research Laboratories (West Point, PA). 125I-EGF was purchased from Du Pont-New England Nuclear.

**Bacterial Strains, Plasmids, and Cell Lines**—HB101 from Bethesda Research Laboratories was used for the propagation of the plasmids. BL21 (DE3), which carries an inducible T7 RNA polymerase gene on a λ prophage (5, 9), was used as the host for fusion protein expression. Oligonucleotide-directed mutagenesis (for pCS8) was carried out using the method of Kunkel (10). Mutants were identified by DNA sequencing (11). The plasmids pVC17, pVC8, and pVC7 (Fig. 1) were described previously (12). The plasmids pCS11 and pCS8 were constructed by inserting at the BglII site of pCS8 or pCS11 an oligonucleotide corresponding to a 1.5-kb EcoRI fragment of TGFα DNA (1). Oligonucleotide-directed mutagenesis was used to generate the pCS11 and pCS8 deletion mutants that were used in this study.

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1 The abbreviations used are: TGFα, transforming growth factor α; EGF, epidermal growth factor.
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Fig. 1. Representations of Pseudomonas exotoxin (PE) and various PE40 derivatives. Deletion mutants are shown, and numbers indicate amino acids present in PE40. The first three amino acids (1-3) from Pseudomonas exotoxin were retained in all constructions to give them all the same amino end.

Fig. 2. Representations of various TGFα-PE40 derivatives. Deletion mutants are shown, and numbers indicate amino acids present in PE40. The OmpA signal sequence is located directly after the T7 promoter in all the constructs. The cDNA for TGFα codes for amino acids 1-50 of all the constructions. Dotted lines in Pseudomonas exotoxin (PE) represent disulfide bonds.

pCS12 (Fig. 1) were created from pVC4 (12). The plasmids pCS1–pCS10 (Fig. 2) were created from pYX3205 or pVC3875 and carry an OmpA signal sequence (13). The plasmids pYX3205 and pVC3875 will be described elsewhere. Briefly, pYX3205 contains the human TGFα cDNA sequence (Merck, Sharp and Dohme Research Laboratories) ligated to the 5' end of the gene encoding PE43 carried on pVC20 (12). pVC3875 contains the human TGFα cDNA sequence ligated to the 5' end of the gene encoding PE40 (12). The plasmids pVC3875, pCS2, and pCS10 were also modified to encode fusion proteins without the OmpA signal sequence.

Expression of Recombinant Fusion Proteins—Expression of Pseudomonas exotoxin containing fusion proteins was done using the host E. coli EL21 (ADE3) as described previously (5, 7, 12). Cells were incubated for 90 min following induction, and the periplasm fraction was prepared (12) for the mutant proteins expressed from pCS1-10. For proteins expressed from plasmids pVC17, pVC8, pVC7, pCS11, and pCS12, the total cell pellet was used for analysis. The modified plasmids pCS2, pCS10, and pVC3875, in constructions without OmpA signal sequence, were expressed and fusion proteins purified from inclusion bodies (7) for use in the EGF receptor-binding displacement assay.

 Gel Electrophoresis and Immunoblotting—Buffers contained a sulfate-polyacrylamide gel electrophoresis was performed on 12.5% gels using the method of Laemmli (14). For immunoblotting, samples were transferred from gels to nitrocellulose paper, and antibodies to Pseudomonas exotoxin (2) and Vecta stain kits (Vector Laboratories, Burlington, CA) were used to visualize Pseudomonas exotoxin fusion proteins. Quantitation of fusion proteins was done by comparison with standards (purified PE40) on Western blots.

ADP-ribosylation Assay—ADP-ribosylation activity of protein samples was measured by following the procedure of Collier and Kandel (16) using wheat germ extract enriched in elongation factor 2.

Binding Assay—[125I]-EGF-binding assays were performed using A431 cells plated at 8 x 10^6 cells/ml. Competitions were done using buffer containing 2 ng of [125I]-EGF/ml with or without the potential inhibitors as described (16).

Protein Synthesis Inhibition Assay—The cytotoxic activity of TGFα-toxin fusion proteins was determined by assaying the inhibition of protein synthesis using A431 cells (12). Protein synthesis was assayed by determining the incorporation of [3H]leucine in trichloroacetic acid precipitates of the cells (5, 17).

RESULTS

Preparation of TGFα-PE40 and Other Mutants—To express TGFα-PE40, PE40, and derived mutant proteins, Escherichia coli BL21 (DE3) were transformed with the appropriate plasmid, cultured, and induced with isopropyl-β-D-thiogalactoside. PE40, TGFα-PE40, and its mutant forms, expressed using constructs containing OmpA signal sequences, were directed to the periplasm in a soluble form and were isolated as described under “Materials and Methods.” Mutant proteins composed of domain II, III, and nearby amino acids remained within the cell and were isolated from sucrose-washed spheroplasts. TGFα-PE40 and its mutant forms were also expressed from constructs without OmpA signal sequences and were isolated and purified from inclusion bodies (7). Toxin-containing fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed with a rabbit polyclonal antibody to Pseudomonas exotoxin. Several typical examples are shown in Fig. 3, A and B. For each construction, the major band reacting with antibodies to Pseudomonas exotoxin migrated at the size expected for the gene fusion protein. The amount of recombinant toxin present was estimated visually by comparison of serial dilutions with known standards (purified PE40) on immunoblots using an antibody to Pseudomonas exotoxin.

Amino-terminal Boundary of Domain III—Before making mutations in domains II and I, it was essential to determine how much of the amino terminus of domain III was necessary for full ADP-ribosylation activity. Therefore, the ADP-ribosylation activities of toxin proteins beginning at amino acids 381, 400, and 408 were measured and compared with PE40 (amino acids 253–613) (Table I). Full ADP-ribosylation activity was present in the proteins beginning at amino acids 381 and 400. The deletion of five additional amino acids to 405 resulted in loss of more than 50% of the ADP-ribosylation activity. This result is in apparent conflict with a previous report in which a protein believed to contain amino acids 405–613 retained no ADP-ribosylation activity (2). Further examination of the plasmid pH7, which was used previously to express this truncated Pseudomonas exotoxin protein, revealed that it actually contained amino acids 406, 408–613, and was missing amino acids 405 and 407. We conclude,
that TGFα was interfering with access of some substrate to a protein expressed from pCS11 (Fig. 1), which lacks the same region of domain III important in catalysis. A protein with TGFα-PE40. This was somewhat unexpected because a band (32 kDa in lane 2, TGFα-PE40; lane 4, PE40A253-405; lane 5, PE40A253-408) containing deletions retained full or almost full (over 75%) ADP-ribosylation activity. A protein with a deletion of amino acids 253-407, which deleted a portion of domain III (pCS3), retained less than 2% of ADP-ribosylation activity. Thus, all the mutants except for one (pCS3) had high ADP-ribosylation activity (Table II).

Cytotoxic Activity of Mutant Proteins—To determine the effect of the mutations in domains II and Ib on cytotoxicity, protein synthesis inhibition assays on A431 cells were performed using a secreted form of the protein which was readily prepared from the periplasm. A431 cells contain about 2 × 10⁶ EGF receptors/cell and are very sensitive to the secreted form of TGFα-PE40 with an ID₅₀ of about 1 ng/ml (Fig. 4 and Table II). The mutant proteins fell into four groups. The first group, which had very low cytotoxic activity, contained deletion mutants that either removed a large part of the amino end of domain II (amino acids 253–308), all of domain II (amino acids 253–380), or a large portion of the carboxyl end of domain II (amino acids 337–362 or 337–380). The second group consisted of one mutant encoded by pCS3 (deletion of amino acids 253–407). It had very little cytotoxicity as well as very little ADP-ribosylation activity because the mutation extended into domain III.

A third group of mutants had about 10% of wild-type cytotoxicity. One mutant in this group had a deletion of amino acids 365–399 (pCS9). Another had a deletion of amino acids 254–263 (pCS7), and the third had cysteine residues at positions 265 and 288 converted to serines (pCS8).

The fourth group was different and displayed a small increase in cytotoxic activity compared with wild-type TGFα-PE40. This group contained pCS6 (deletion of amino acids 361–380) and pCS10 (removal of amino acids 364–380). These deletions remove large parts of domain Ib, which begins at amino acid 364 and ends at 400 and contains one disulfide bridge.

The low activity of the domain II mutants could have been due to a decrease in entry of the toxin into the cytosol after endocytosis or a decrease in binding to the EGF receptor. To examine the binding activity of the secreted forms of the chimeric toxin, several different fusion proteins were isolated from the periplasm and tested for their ability to displace I²⁵⁻EGF from A431 cells using standard binding conditions (16). Unexpectedly, neither TGFα-PE40 nor the mutants showed a substantial displacement of I²⁵⁻EGF, although excess EGF was able to compete for the cytotoxic activity of secreted form of TGFα-PE40 (data not shown). This result suggested that the TGFα portion of these fusion proteins was not folded correctly and therefore reacted poorly with the EGF receptor. Because TGFα-PE40 itself bound poorly to the EGF receptor, there was no way to tell if the binding of the mutant forms was diminished relative to TGFα-PE40.

To prepare TGFα-PE40 with more normal binding prop-

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**Table I**

| Mutant | Amino acids | ADP-ribosylation activity % PE40 |
|--------|-------------|---------------------------------|
| pVC17  | 381–613     | 95                              |
| pCS11  | 400–613     | 107                             |
| pCS12  | 405–613     | 38                              |
| pVC7   | 408–613     | <2                              |

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**Fig. 3. Immunoblot analysis of PE40 (A) and TGFα-PE40 (B) fusion proteins using an antibody to Pseudomonas exotoxin. A, lane 1, PE40; lane 2, PE40A253–380; lane 3, PE40A253–400; lane 4, PE40A253–405; lane 5, PE40A253–408. B, lane 1, PE40; lane 2, TGFα-PE40; lane 3, TGFα-PE40A365–399; lane 4, TGFα-PE40A337–380; lane 5, TGFα-PE40A365–380; lane 6, TGFα-PE40 Ser-265 and -288. The doublet bands indicate processed and unprocessed forms of TGFα-PE40 produced during expression of the chimeric protein (11). The smallest immunoreactive molecular mass band (32 kDa in lane 1) represents a bacterial proteolytic cleavage product formed during expression.
ADP-ribosylation and cell-killing activity of various mutant forms of TGFα-PE40

The amount of soluble fusion protein produced from each construct was approximately 1.2 μg/ml of original culture. For ADP-ribosylation activity assays, 10 μl of 100-fold dilution of periplasm (containing soluble protein) was used in a 250-μl reaction mixture containing 2.4 mM [14C]NAD (6 × 10⁶ cpm) and 10 μl of wheat germ extract. ID₅₀ is the concentration of toxin which is required to inhibit protein synthesis by 50% as measured by [3H]leucine incorporation.

| Plasmid | Group | Amino acids deleted or altered in Pseudomonas exotoxin in region from 253-613 | Relative ADP-ribosylation activity | ID₅₀ ng/ml |
|---------|-------|---------------------------------------------------------------------------------|-----------------------------------|-----------|
| pVC3875 |       |                                                                                  |                                   | 100       |
| pCS1    | I     | 253-308                                                                          | 108                               | 114       |
| pCS2    | I     | 253-380                                                                          | 79                                | >180      |
| pCS4    | I     | 337-380                                                                          | 81                                | >50       |
| pCS5    | I     | 337-382                                                                          | 78                                | 50        |
| pCS3    | II    | 253-407                                                                          | <2                                | >150      |
| pCS7    | III   | 254-263                                                                          | 100                               | 10        |
| pCS8    | III   | 265-288                                                                          | 95                                | 10        |
| pCS9    | III   | 365-399                                                                          | 47                                | 12        |
| pCS6    | IV    | 361-380                                                                          | 75                                | 0.3       |
| pCS10   | IV    | 365-380                                                                          | 89                                | 0.6       |

Fig. 4. Toxic activity of TGFα-PE40 and various mutant proteins on A431 cells. TGFα-PE40 was prepared from the periplasm of E. coli. The concentration of the various mutant toxins was estimated as described under "Materials and Methods." [3H]Leucine incorporation into cellular protein was measured as described previously (11). Results are expressed as percent of control cells not exposed to TGFα-PE40, TGFα-PE40 (○), TGFα-PE40Δ253-308 (●), TGFα-PE40Δ254-263 (■), and TGFα-PE40Δ365-380 (□).

Fig. 5. Competition binding assay: displacement of a trace amount of 125I-EGF by TGFα-PE40, TGFα-PE40 mutants, or EGF. TGFα-PE40 and mutant proteins were purified from the inclusion bodies of E. coli. The concentration of the fusion proteins was estimated using the Bradford assay. 125I-EGF bound to A431 cells was measured as dpm. EGF equivalents were calculated by estimating the molar concentrations of TGFα in each gene fusion protein. TGF-PE40 (○), TGFα-PE40Δ253-380 (●), TGFα-PE40Δ365-380 (□), EGF (■).

TABLE II
Cell-killing activity of refolded fusion proteins

Protein was determined by the Bradford assay. The amount of purified refolded fusion protein produced for each construct was approximately 1.5 μg/ml of original culture.

| Plasmid (protein) | ID₅₀       | Ratio of renatured to secreted |
|-------------------|------------|-------------------------------|
| pVC3875 (TGFα-PE40) | 0.18       | 1.0                           |
| pCS2 (TGFα-PE40Δ253-380) | >180       | <0.5                          |
| pCS10 (TGFα-PE40Δ365-380) | 0.10       | 0.6                           |
inclusion bodies are shown in Table III, and these activities are compared with the activities of the chimeric toxins isolated from the periplasm. In each case, the renatured material was more cytotoxic to A431 cells than was the secreted material, and the mutant toxins were consistently less active than TGFα-PE40.

Displacement of [125I]-EGF from EGF Receptors Using TGFα-PE40 and Mutant Forms—Since the renatured toxins were more active, it seemed likely that they would bind to the EGF receptor better than the secreted forms of the toxin. This prediction is confirmed in the binding assays shown in Fig. 5. The refolded chimeric proteins (normal and mutant) competed for [125I]-EGF binding equally well. Comparison of the competition data on a molar basis with native EGF (6 kDa) demonstrates that the refolded TGFα-fusion proteins bind to the EGF receptor approximately 10-fold less well than native EGF.

DISCUSSION

In this study, we have used a chimeric protein composed of TGFα fused to mutant forms of Pseudomonas exotoxin to study the role of domains II and Ib in the cytotoxic activity of this molecule. To determine the regions involved in the translocation process, we created a series of deletion mutants in domains II and Ib. To ascertain that the mutants preserved other biological activities, they were tested for ADP-ribosylation and EGF-binding activities. We also partially determined which region of domain III was necessary for full activity. Previous studies have indicated that His-426, Tyr-481, and Glu-553 in domain III were essential for the full ADP-ribosylation activity of Pseudomonas exotoxin (3, 18-20). Our results indicate that full ADP-ribosylation activity was found in a protein containing residues 400–613 of Pseudomonas exotoxin, partial activity in a protein containing residues 405–613, and no activity in a protein containing residues 408–613 (Table I). Therefore, the functional region of domain III is similar to the structural region deduced from x-ray crystallography, which consists of amino acids 404–613 (1). Using these data, we constructed and tested a series of mutations in domains II and Ib using the chimeric molecule TGFα-PE40 (Table II). The mutants fell into four activity groups based on their cytotoxicity for EGF receptor-containing A431 cells. The first two groups had very low cytotoxic activity (less than 2% of TGFα-PE40), the third had about 10% of the cytotoxic activity of TGFα-PE40, and the fourth had a slight increase in activity.

The proteins in group I had various portions of domain II deleted. Removal of the amino-terminal portion of domain II (residues 253–308) or internal portions of domain II (residues 337–380 and 337–362) dramatically reduced cytotoxicity although retaining full ADP-ribosylation activity (Table II). Removal of all of domain I and a portion of Ib up to amino acid 380 also produced a molecule with extremely low cytotoxicity but full ADP-ribosylation activity (Table II). The fact that these molecules had intact binding domains and full ADP-ribosylation activities indicates that domain II is required for a step after binding and internalization and prior to ADP-ribosylation. Since unlike native Pseudomonas exotoxin, TGFα-PE40 does not require treatment with urea and dithiothreitol for activation, it is very likely that these mutants are defective in the translocation step. This conclusion is in agreement with previous results in which a portion of domain II was deleted from Pseudomonas exotoxin and produced a mutant toxin with very low cytotoxic activity (2).

The second group contained only one member (deletion of 253–407) which contained extremely low ADP-ribosylation activity and, as would be expected, was not cytotoxic to A431 cells.

The third group consisted of three proteins with considerably different mutations. Two of the proteins, one missing residues 254–263 and the other in which Cys-265 and Cys-288 were changed to serine, both contained full ADP-ribosylation activity but showed a 10-fold reduction in cytotoxicity (Table II). From these data, we conclude that small changes in domain II will dramatically reduce the cytotoxic activity of TGFα-PE40 fusion proteins. Once again, this indicates the requirement of domain II for the translocation step. A third protein in this group, missing residues 365–399, contained 47% of full ADP-ribosylation activity, which in part accounted for its reduced cytotoxic activity (Table II). The reduced ADP-ribosylation activity in this mutant may be a result of the proximal location of domains II and III, which can potentially interfere with each others structure. Deletion of sequences including 365–399 (pCS11) in a molecule containing neither TGFα nor domain II (Table I) did not reduce ADP-ribosylation activity.

In the fourth group of mutant proteins, we removed the amino portion of domain Ib (pCS10, missing residues 365–390) in addition to a few amino acids in domain II (pCS6, missing residues 361–380). Unexpectedly, these proteins resulted in a slight increase in cytotoxicity although retaining full ADP-ribosylation activity (Table II). These results clearly indicate that most of domain Ib is not essential for the cell-killing activity of TGFα-PE40. Since domain Ib is closely associated with domain Ia, it may only be required for cytotoxicity when domain Ia is present and may act with domain Ia to help the full length molecule but not the chimeric molecules carry out its cytotoxic action. Alternatively, domain Ib may not have any role in the cytotoxic action of Pseudomonas exotoxin but may be required for secretion of Pseudomonas exotoxin by Pseudomonas aeruginosa or serve yet another function.

Initially, we studied soluble secreted forms of TGFα-PE40 and mutants with alterations in domain II. Since these proteins did not bind well to the EGF receptor, we isolated TGFα-PE40 expressed by plasmids without signal sequences in an insoluble form and purified the protein by denaturation and renaturation. Renatured TGFα-PE40 bound reasonably well to the EGF receptor (Fig. 5) and was generally 10-fold more cytotoxic to A431 cells than the secreted form (Table III). TGFα-PE40 that was prepared by renaturation and two representative mutants showed no differences in their binding to the EGF receptor. Altogether our data indicate that deletions within domain II reduce the cytotoxic activity of TGFα-PE40 by altering entry of the mutant toxins into the cytosol.

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