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Substitution of Foreign Protein Sequences into a Chimeric Toxin Composed of Transforming Growth Factor α and *Pseudomonas* Exotoxin

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TGFα-PE40 is a chimeric toxin made by replacing domain Ia of *Pseudomonas* exotoxin (PE) with transforming growth factor α (TGFα). We have now replaced a portion of domain Ib of PE with different polypeptides or an extra domain III of PE in transforming growth factor α-PE40 and maintained cell killing. Thus, TGFα-PE40 can be used to transport foreign protein sequences into the cytosol of cells.

Pseudomonas aeruginosa exotoxin A (PE) is composed of three structural domains: Ia and Ib, II, and III (1). Each domain has a particular function: domain Ia contains sequences that bind to the cellular receptor for PE, domain II is involved in processing and translocation into the cytosol, and domain III is the catalytic subunit responsible for ADP-ribosylation of elongation factor 2, which halts protein synthesis and results in cell death (8). No function has been ascribed to domain Ib, and much of it can be deleted without affecting toxin function (14). PE40, a mutant form of PE containing domains II, Ib, and III, can be genetically fused or chemically conjugated to protein ligands or antibodies to create cytotoxic chimeric toxins (2, 4, 5, 9, 10, 13, 15). In the present work, our goal was to explore whether the cytotoxicity of the transforming growth factor α (TGFα)-PE40 chimeric toxin (2, 15) would be changed if foreign protein sequences were introduced into the portion of PE that is normally transported to the cytosol. The region of choice was the N-terminal part of domain Ib (Ala-365 to Ala-380 of PE), which, when deleted, did not significantly alter the cytotoxic activity of TGFα-PE40 (14). We also determined whether increasing the number of ADP-ribosylating domains placed in this portion of the molecule would enhance TGFα-PE40 cytotoxicity.

Expression, purification, and identification of recombinant chimeric toxins. The constructions were propagated in *Escherichia coli* HB101 and expressed in strain BL21(λDE3) under the control of the T7 late promoter (16). Proteins were isolated and purified as described previously (13). The sequences and strategies for the preparation of all the plasmids are available from the authors upon request. Toxicity was measured as inhibition of [^{3}H]leucine incorporation into cellular proteins in the presence of various derivatives of chimeric toxin (8). ADP-ribosylation activity was assayed by the method of Collier and Kandel (6). Protein concentration was estimated by the Bradford assay with the Pierce (Rockford, Ill.) Coomassie blue G-250-based reagent. To determine purity, chimeric toxins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as shown in Figure 1.

All the purified proteins exhibited the expected molecular masses (Fig. 1a; see also Fig. 2), and their purity was greater than 90%. Immunoreactivity of Western immunoblots with a polyclonal antibody to PE revealed bands corresponding to the Coomassie blue-stained protein bands (Fig. 1b). TGFα-PE40 derivatives which had different versions of somatostatin-14 inserted into domain Ib (Fig. 1c, lanes 2 and 3) were identified by using an antisomatostatin antibody; this antibody did not react with any of the other TGFα-PE40 toxins. Proteins reacting with anti-PE or antisomatostatin antibodies were identified with a Vecta-Stain kit (Vector Laboratories, Burlingame, Calif.).

Cytotoxic and ADP-ribosylating activities of chimeric toxins with substitutions of somatostatin and a methionine-rich peptide. The plasmids, their encoded chimeric proteins, their molecular masses, and their cytotoxicities are shown in Fig.

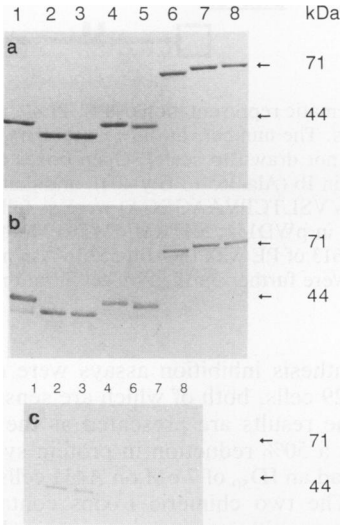

FIG. 1. SDS-PAGE and immunoblots of chimeric proteins. (a) SDS-PAGE followed by staining with Coomassie blue. (b and c) Corresponding immunoblots with (b) anti-PE antibody and (c) antisomatostatin antibody. Lanes: 1, TGFα-PE40 (pWD156); 2, TGFα-PE40[Thr-1]somatostatin (pWD150); 3, TGFα-PE40[Thr-1, Ser-3, Ser-14]somatostatin (pWD154); 4, TGFα-PE40Asp-553 (pWD152); 5, TGFα-PE40 methionine-rich peptide (pWD163); 6, TGFα-PE40 domain III (pWD148); 7, TGFα-PE40Asp-553 domain III (pWD149); 8, TGFα-PE40 domain IIIAsp-553 (pWD151).

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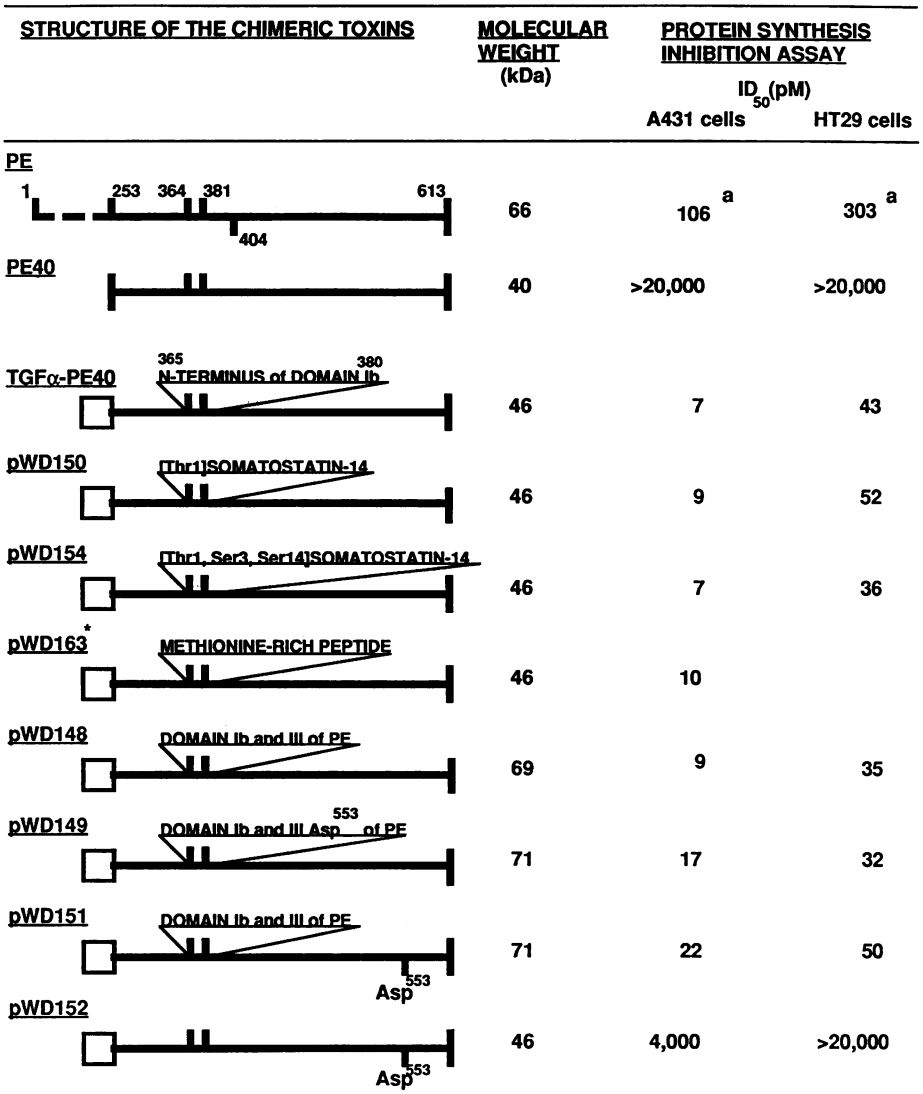

FIG. 2. Schematic representation of PE, PE40, and chimeric toxins and their cytotoxicity on A431 and HT29 cells. Solid lines correspond to PE sequences. The numbers at the vertical bars indicate amino acids as present in PE. The dotted line represents domain I of PE (amino acids 1 to 253; not drawn to scale). Open boxes at the N terminus represent the TGFα molecule. PE40 contains domain II (Gly-253 to Asn-364), domain Ib (Ala-365 to Gly-404), and domain III (Gly-405 to Lys-613) of PE. The sequences of substitutions in the N terminus of domain Ib (ADVVSLTCPVAAGECA) were as follows: TGCKNFFWKTFTSC for somatostatin-14 in pWD150; TGSKNFFWKTFTSS for somatostatin-14 in pWD154; MDMMMMTCPMMMGTCM for methionine-rich peptide in pWD163; Gly-381 to Lys-613 of PE in pWD148; Ala-365 to Lys-613 of PE with the Glu-553 to Asp mutation in pWD149; Ala-365 to Lys-613 of PE in pWD151. *Purified on Mono-Q column; other proteins were further purified by gel filtration. [a] From reference 15.

2. Protein synthesis inhibition assays were carried out on A431 and HT29 cells, both of which are sensitive to TGFα-PE40 (15). The results are presented as the concentration that produced a 50% reduction in protein synthesis (ID_{50}). TGFα-PE40 had an ID_{50} of 7 pM on A431 cells and 43 pM on HT29 cells. The two chimeric toxins containing somatostatin-14 with or without cysteines were also active and demonstrated activities similar to that of TGFα-PE40 on both cell lines. In addition, they had similar ADP-ribosylation activities (77 and 89% of TGFα-PE40 activity, respectively; 100% = 102 cpm/ng/15 min). The cytotoxic activity of a fusion protein containing a peptide enriched in methionine residues (56% of the inserted amino acids) was also very close to that exhibited by TGFα-PE40. These findings show that foreign amino acid sequences can be introduced into the toxin structure without altering membrane translocation and killing. As expected, TGFα-PE40Asp-553 did not have any activity on HT29 cells, although it did exhibit weak cytotoxic activity (ID_{50} = 4 nM) on A431 cells. The ADP-ribosylating activity of this fusion protein was extremely low (0 to 4% of the activity seen with TGFα-PE40), which is consistent with the residual ADP-ribosylating activity present in the PEAsp-553 mutant molecule (7).

Activity of TGFα-PE40 derivatives with a duplication of domain III. In an attempt to increase the cytotoxicity of the toxin, an additional catalytic domain III was substituted for part of domain Ib in TGFα-PE40 (TGFα-PE40 domain III). This made a chimeric toxin of considerably larger size (69 kDa) than TGFα-PE40 (46 kDa). When TGFα-PE40 with two wild-type domain IIIs in tandem was compared with

TGFα-PE40, it had only slightly lower cytotoxicity than TGFα-PE40 itself on A431 cells (9 versus 7 pM) and its activity on HT29 cells was about the same as that of TGFα-PE40 (Fig. 2).

We tried to determine which of the two domain IIIs placed in tandem is responsible for the cytotoxic activity by placing an Asp at Glu-553 in one or the other domain. A chimeric toxin with an Asp-553 mutation in domain III placed proximal to domain II (pWD149) was 50% less active on A431 cells, but it had equal activity on HT29 cells. The protein with an Asp-553 mutation in domain III distal to domain II (pWD151) was 20 to 40% less active than TGFα-PE40Asp-553 domain III. Interestingly, TGFα-PE40 with two domain IIIs in tandem did not have twice the ADP-ribosylating activity as TGFα-PE40, as one might expect, but the same activity (101%). This may be due to an unfavorable conformation of this protein resulting in the inability to use efficiently the two active sites of domain III at the same time. Proteins with mutation Asp-553 in either domain III had similar ADP-ribosylating capabilities and capabilities similar to that of the unmutated fusion protein (93 and 66%, respectively). However, even if the mutation of Glu-553 to Asp in the distal domain III decreased its activity versus the nonmutated version of the protein, the cytotoxic effect was still relatively high. Therefore, we suspect that the translocation process and movement of the molecule into the cytosol occurred equally well for both molecules. We speculate that once the process of translocation is initiated, it proceeds uninterrupted despite the increased length of the molecule to be transported. In addition, the protein synthesis inhibition results and the ADP-ribosylating activity measurements indicate that either of the domains, but not both, can be utilized for catalysis at the same time.

During the course of this work, two recent findings shed new light on the mechanism of biological action of PE and hybrid toxins derived from it. First, it was established that in order to be active, the toxin has to be proteolytically cleaved within domain II (11). The other finding showed the importance of a proper C-terminal sequence in PE for it to possess full cytotoxicity (3). Since the constructions studied in this work always had one intact domain II and a normal C-terminal end of PE40, any changes made in the Ib region should not influence the process of proteolysis or C-terminal recognition. Therefore, it should be possible to introduce specific peptides, enzymes, or single-chain antibodies into the cell cytoplasm by using PE or its hybrids. By making such molecules without ADP-ribosylating activity and leaving the other features of domain II and the C terminus of PE unchanged, insertion into domain Ib may be an efficient way to accomplish this goal. In fact, the fusion protein which had a mutated domain III distal to a normal domain III serves as a first example of such an approach.

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REFERENCES

1. **Allured, V. S., R. J. Collier, S. F. Carroll, and D. B. McKay.** 1986. Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0-Ångstrom resolution. Proc. Natl. Acad. Sci. USA **83**:1320–1324.
2. **Chaudhary, V. K., D. J. P. FitzGerald, S. Adhya, and I. Pastan.** 1987. Activity of a recombinant fusion protein between transforming growth factor alpha and Pseudomonas toxin. Proc. Natl. Acad. Sci. USA **84**:4538–4542.
3. **Chaudhary, V. K., Y. Jinno, D. J. FitzGerald, and I. Pastan.** 1990. Pseudomonas exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. Proc. Natl. Acad. Sci. USA **87**:308–312.
4. **Chaudhary, V. K., T. Mizukami, T. R. Fuerst, D. J. FitzGerald, B. Moss, I. Pastan, and E. A. Berger.** 1988. Selective killing of HIV-infected cells by a recombinant human CD4-pseudomonas exotoxin hybrid protein. Nature (London) **335**:369–372.
5. **Chaudhary, V. K., C. Queen, R. P. Junghans, T. A. Waldmann, D. J. FitzGerald, and I. Pastan.** 1989. A recombinant immunotoxin consisting of two antibody variable domains fused to Pseudomonas exotoxin. Nature (London) **339**:394–397.
6. **Collier, R. J., and J. Kandel.** 1971. Structure and activity of diphtheria toxin. I. Thiol-dependent dissociation of a fraction of toxin into enzymically active and inactive fragments. J. Biol. Chem. **246**:1496–1503.
7. **Douglas, C. M., and R. J. Collier.** 1987. Exotoxin A of *Pseudomonas aeruginosa*: substitution of glutamic acid 553 with aspartic acid drastically reduces toxicity and enzymatic activity. J. Bacteriol. **169**:4967–4971.
8. **Hwang, J., D. J. P. FitzGerald, S. Adhya, and I. Pastan.** 1987. Functional domains of pseudomonas exotoxin identified by deletion analysis of the gene expressed in *E. coli*. Cell **48**:129–136.
9. **Lorberboum-Galski, H., D. J. P. FitzGerald, V. Chaudhary, S. Adhya, and I. Pastan.** 1988. Cytotoxic activity of an interleukin 2-*Pseudomonas* exotoxin chimeric protein produced in *E. coli*. Proc. Natl. Acad. Sci. USA **85**:1922–1926.
10. **Ogata, M., V. Chaudhary, D. J. P. FitzGerald, and I. Pastan.** 1989. Cytotoxic activity of a recombinant fusion protein between interleukin 4 and Pseudomonas exotoxin. Proc. Natl. Acad. Sci. USA **86**:4215–4219.
11. **Ogata, M., V. K. Chaudhary, I. Pastan, and D. J. FitzGerald.** 1990. Processing of *Pseudomonas* exotoxin by a cellular protease results in the generation of a 37,000-Da toxin fragment that is translocated to the cytosol. J. Biol. Chem. **265**:20678–20685.
12. **Pastan, I., M. C. Willingham, and D. J. FitzGerald.** 1986. Immunotoxins. Cell **47**:641–648.
13. **Siegall, C. B., V. K. Chaudhary, D. J. P. FitzGerald, and I. Pastan.** 1988. Cytotoxic activity of an interleukin 6-*Pseudomonas* exotoxin fusion protein on myeloma cells. Proc. Natl. Acad. Sci. USA **85**:9738–9742.
14. **Siegall, C. B., V. K. Chaudhary, D. J. FitzGerald, and I. Pastan.** 1989. Functional analysis of domains II, Ib, and III of pseudomonas exotoxin. J. Biol. Chem. **264**:14256–14261.
15. **Siegall, C. B., Y.-H. Xu, V. K. Chaudhary, S. Adhya, D. J. P. FitzGerald, and I. Pastan.** 1989. Cytotoxic activities of a fusion protein comprised of TGFα and *Pseudomonas* exotoxin. FASEB J. **3**:2647–2652.
16. **Studier, F. W., and B. A. Moffat.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.