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Epidermal Growth Factor Receptor Binding Is Affected by Structural Determinants in the Toxin Domain of Transforming Growth Factor-Alpha–*Pseudomonas* Exotoxin Fusion Proteins

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TGF-alpha–PE40 is a hybrid protein composed of transforming growth factor-alpha (TGF-alpha) fused to a 40,000-dalton segment of *Pseudomonas* exotoxin A (PE40). This hybrid protein possesses the receptor-binding activity of TGF-alpha and the cell-killing properties of PE40. These properties enable TGF-alpha–PE40 to bind to and kill tumor cells that possess epidermal growth factor (EGF) receptors. Unexpectedly, TGF-alpha–PE40 binds approximately 100-fold less effectively to EGF receptors than does native TGF-alpha (receptor-binding inhibition IC_{50} = 540 and 5.5 nM, respectively). To understand the factors governing receptor binding, deletions and site-specific substitutions were introduced into the PE40 domain of TGF-alpha–PE40. Removal of the N-terminal 59 or 130 amino acids from the PE40 domain of TGF-alpha–PE40 improved receptor binding (IC_{50} = 340 and 180 nM, respectively) but decreased cell-killing activity. Substitution of alanines for cysteines at positions 265 and 287 within the PE40 domain dramatically improved receptor binding (IC_{50} = 37 nM) but also decreased cell-killing activity. Similar substitutions of alanines for cysteines at positions 372 and 379 within the PE40 domain did not significantly affect receptor-binding or cell-killing activities. These studies indicate that the PE40 domain of TGF-alpha–PE40 interferes with EGF receptor binding. The cysteine residues at positions 265 and 287 of PE40 are responsible for a major part of this interference.

TGF-alpha–PE40 is a chimeric protein composed of two independent domains that are produced as a single recombinant fusion protein in bacteria (3). The transforming growth factor-alpha (TGF-alpha) domain is derived from a synthetic gene encoding the mature form of human TGF-alpha (7). The *Pseudomonas* exotoxin 40 (PE40) domain is derived from the *Pseudomonas* exotoxin A gene of *Pseudomonas aeruginosa* (10). *Pseudomonas* exotoxin A (PE) consists of three functional domains positioned in tandem along the primary amino acid sequence. At the N terminus, domain I encodes the cellular binding activity of native PE. At the C terminus, domain III encodes the toxin's ADP-ribosylating activity (14, 17). Domain II lies between domains I and III and is responsible for the intracellular translocation of PE proteins from the lysosomal compartment into the cytoplasm (4). Unlike the 66,000-dalton PE protein, PE40 is an approximately 40,000-dalton protein which is missing the N-terminal cellular binding domain of PE. PE40 does retain functional translocation and enzymatic domains.

TGF-alpha–PE40 is a bifunctional molecule that possesses both "cell-targeting" and cell-killing activities. TGF-alpha functions as the cell-targeting domain of TGF-alpha–PE40 by binding specifically to the surface of cells that possess epidermal growth factor (EGF) receptors (3). PE40 functions as the cell-killing domain. PE40 kills mammalian cells by ADP-ribosylating elongation factor 2, which results in the inhibition of new protein synthesis (15). This combination of cell-killing and cell-targeting properties makes TGF-alpha–PE40 potentially useful as an anticancer agent. Many human tumors and tumor cell lines possess increased numbers of EGF receptors on their cell surfaces (12, 21, 32). This difference in numbers of EGF receptors between tumor cells and normal cells may provide a therapeutic window for the selective killing of tumor cells by TGF-alpha–PE40. Pastan and his colleagues (3) have shown that TGF-alpha–PE40 can selectively kill tumor cells that possess EGF receptors in vitro. However, it is less clear what the therapeutic-to-toxic ratio of the properties of TGF-alpha–PE40 will be in vivo.

Several factors will influence the selectivity of TGF-alpha–PE40 cell killing in vivo, including the numbers of EGF receptors on normal cells, the prevalence and physiologic importance of EGF receptor-bearing tissues, and the affinity with which TGF-alpha–PE40 binds to EGF receptors. Our initial studies indicated that TGF-alpha–PE40 had a much lower affinity for the EGF receptor than does native TGF-alpha. To understand why TGF-alpha–PE40 binds so poorly to EGF receptors, we prepared a series of TGF-alpha–PE40 muteins that contained deletion or site-specific substitution mutations. These mutations were introduced into the PE40 domain of TGF-alpha–PE40 in an attempt to improve the receptor-binding activity of the entire hybrid protein.

MATERIALS AND METHODS

Cell lines. A431 epithelial carcinoma cells, HeLa cells, and CHO cells were obtained from the American Type Culture Collection (Rockville, Md.) and used here for cell-killing assays (9). A431 cells were also used to prepare plasma membrane vesicles for EGF receptor-binding assays (6). All cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and antibiotics.

Binding and cell-killing assays. The receptor-binding assay was performed as described (27). The cell-killing assays were performed using A431, HeLa, or CHO cells. Each cell line was seeded into 96-well plates at 10,000 viable cells per well. Twenty-four hours later, the cells were washed once and placed in serum-free medium containing the test com-

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pound under study. Forty-eight hours later the number of surviving cells was quantitated by using an MTT [3-(3,4-dimethylthiazol-2yl)-2,5-diphenyltetrazalium bromide] assay as described previously (25). EGF was purchased from Collaborative Research (Waltham, Mass.), and TGF-alpha came from Bachem (Torrence, Calif.).

ADP-ribosylation assay. The ADP-ribosylation assay was adapted from that described by Chung and Collier (5). Partially purified elongation factor 2 was obtained from wheat germ, and [U-^{14}C]NAD was obtained from Amersham Corp. (Arlington Heights, Ill.). The reaction was performed at 25°C in 120 μl of 25 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–0.1% bovine serum albumin in the presence of 1.5 μM elongation factor 2 and 2.0 μM NAD in 96-well tissue culture plates. The reaction was initiated by the addition of enzyme and proceeded for 15 min. Trichloroacetic acid (TCA) precipitation was used to stop the reaction by mixing reactants with an equal volume of 10% TCA for 5 min. The precipitates were washed with 5% TCA three times by centrifugation of the 96-well plate, aspiration of the supernatant, and resuspension in 5% TCA. [^{14}C]NAD incorporation into TCA-precipitable material was measured by solubilizing in 100 μl of 0.1 M NaOH, neutralizing with 100 μl of 0.1 M HCl, and counting in Aquasol. Purified PE40 was used as the standard for the assay in concentrations from 0 to 0.25 nM, and ADP-ribosylating activity was expressed as milligrams of PE40 equivalents per milligram of TGF-alpha–PE fusion protein.

Gel electrophoresis. Sodium dodecyl sulfate (12.5%)-polyacrylamide gel electrophoresis was performed by the method of Laemmli (18). The procedure for Western blots (immunoblots) was that of Towbin et al. (29). The first antibodies used were polyclonal antiserum against whole PE, obtained from List Biological Laboratories, Inc. (Campbell, Calif.), and polyclonal antiserum against the N-terminal seven amino acids of TGF-alpha coupled to thyroglobulin (TGF-alpha 1-7), made in this laboratory. A goat anti-rabbit antiserum linked to alkaline phosphatase (Cooper Biomedical Inc., West Chester, Pa.) was used as a second antibody.

Bacterial and plasmid vectors. All plasmids were grown and all proteins were produced in *Escherichia coli* JM109. All recombinant DNAs were cloned either into pUC-19 or into a TAC promoter vector system described by Linemeyer et al. (22).

Plasmid preparation, hybridizations, and restriction endonucleases. Small-scale plasmid DNA preparations (1.5 ml) were made by the procedure of Holmes and Quigley (13). Colony hybridizations were performed as described by Grunstein and Hogness (11). DNA probes were prepared either by the random hexamer priming method of Feinberg and Vogelsteen (8) with [α-^{32}P]dCTP (New England Nuclear Corp., Boston, Mass.) or by treating specific oligonucleotides with polynucleotide kinase and [α-^{32}P]ATP (New England Nuclear Corp.). All nucleic acid-modifying enzymes were purchased from New England BioLabs, Inc. (Beverly, Mass.), and used according to the manufacturer's directions.

DNA sequencing. All TGF-alpha constructions were sequenced either by the Maxam and Gilbert protocol (24) or by the dideoxy-chain termination method (28). Two different dideoxy kits were used, GEMSeq from Promega-Biotec (Madison, Wis.) or Sequenase from the United States Biochemical Corp. (Cleveland, Ohio). Both kits were used according to the manufacturer's specifications.

Oligonucleotide synthesis. Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.).

Recombinant TGF-alpha–PE constructions. The TGF-alpha DNA clone was constructed using three sets of synthetic oligonucleotides as described previously (7). The PE40 DNA clone was obtained from the plasmid pVC8 (14). pVC8 was a generous gift of Ira Pastan (National Institutes of Health, Bethesda, Md.). The TGF-alpha DNA was excised from its plasmid vector by using *Hin*dIII and *Fsp*I restriction enzymes. This digestion generated a 168-base-pair DNA encoding the entire TGF-alpha synthetic gene plus four additional amino acid residues (three alanines and one methionine) at the 5′ end of the gene. The PE40 DNA was excised from the pVC8 plasmid by using *Nde*I and *Eco*RI enzymes. The *Nde*I-generated end was made flush using a Klenow enzyme reaction. Both the TGF-alpha and PE40 DNA segments were then cloned in tandem into a pUC-19 vector to yield a complete TGF-alpha–PE40 DNA clone. The correct nucleotide composition and in-frame sequence were verified by nucleic acid sequencing. The TGF-alpha–PE34 and TGF-alpha–PE25 deletion mutants were made by cutting the TGF-alpha–PE40 DNA with *Bgl*I and either *Sal*I or *Apa*I. The *Bgl*I site is located at the extreme 3′ end of the TGF-alpha DNA and does not eliminate any TGF-alpha-encoded amino acids. The *Sal*I and *Apa*I sites are unique within the PE40 DNA clone, occurring once each in the translocation domain. Once digested with *Bgl*I and either *Sal*I or *Apa*I, the free DNA ends were made flush using the Klenow reaction and were ligated together using T4 DNA ligase. The effect of these manipulations was to delete the DNA sequences that normally lie between the *Bgl*I site at the 3′ end of the TGF-alpha DNA and the *Sal*I or *Apa*I sites in the 5′ half of the PE40 DNA. The final deletion mutant clones were subjected to DNA sequencing to insure that they specified the proper nucleotide sequences in the same amino acid reading frame.

Site-directed mutagenesis was carried out as described by Winter et al. (31). A segment of DNA was excised from the TGF-alpha–PE40 DNA clone that encoded the entire PE40 gene and the last five amino acid codons of TGF-alpha, using *Bam*HI and *Sph*I. This DNA segment was subcloned into an M13 mp19 vector plasmid. Site-directed mutagenesis was used to introduce unique restriction enzyme sites where needed on either side of the cysteine codons in the PE40 gene. Three sets of complementary oligonucleotide cassettes were synthesized which spanned the regions of the PE40 gene containing the cysteine codons between unique enzyme sites. The oligonucleotide cassettes were designed to reconstitute the nucleotide sequences on either side of the cysteine codons while changing the cysteines to alanines. A single oligonucleotide cassette was used to change the codons specifying amino acid residues 372 and 379 of PE40 (10) to alanines, thus forming the PE40 domain of TGF-alpha–PE40 Ab. (See Results for explanation of the Ab/aB/ab designations.) Separate oligonucleotide cassettes were used to change the codons specifying amino acid residues 265 and 287 to cysteines. These two changes were performed on the same DNA clone, thus forming the PE40 domain of TGF-alpha–PE40 aB. Once these mutated PE40 genes were created and their sequences were verified, they were ligated into their original vector plasmids with the remainder of the TGF-alpha gene to produce complete TGF-alpha–PE40 fusion genes. The TGF-alpha–PE40 ab gene was created by splicing together the 5′ and 3′ halves of the TGF-alpha–PE40 aB and TGF-alpha–PE40 Ab genes, respectively, using the unique *Sal*I restriction site which lies between the N-terminal and C-terminal pairs of cysteine codons in the PE40 gene.

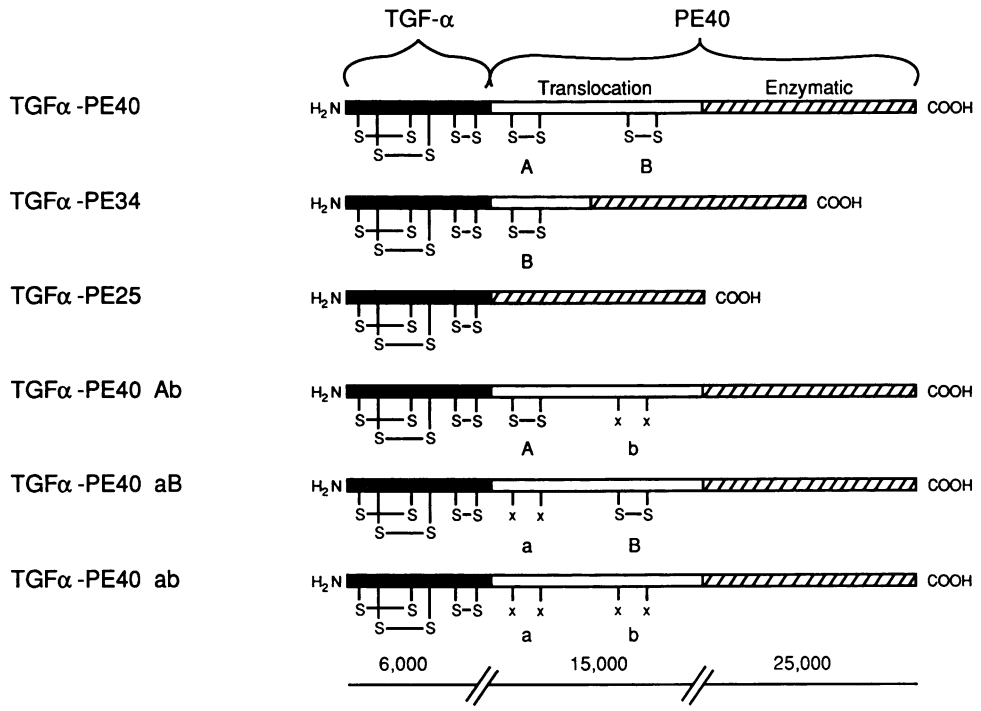

FIG. 1. Schematic representation of TGF-alpha–PE40 and related mutant fusion proteins. Note the position of cysteine residues in the TGF-alpha and translocation domains of TGF-alpha–PE40.

Purification of fusion protein. Transformed *E. coli* cells were cultured in shake flasks in LB-broth in the presence of 100 μg of ampicillin per ml at 37°C. After the A_{600} value reached 0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. The cells were harvested after 2 h of induction and collected by centrifugation. Cell lysis was achieved in 8 M guanidine hydrochloride–50 mM Tris (pH 8.0)–1 mM EDTA with stirring at room temperature for 2 h. All cysteines were converted to the S-sulfonate derivative by the addition of solid sodium sulfite to 0.4 M and sodium tetrathionate to 0.1 M. The pH was adjusted to 9.0 with dilute sodium hydroxide, and the reaction was allowed to proceed at room temperature for 16 h. The protein solution was dialyzed thoroughly against 1 mM EDTA at 4°C before being brought to 6 M urea, 50 mM Tris (pH 8.0), and 50 mM NaCl at room temperature. The protein mixture was stirred for 2 h, cleared by centrifugation (32,000 × *g*, 30 min), and applied to a DEAE-Sepharose Fast Flow column (2.6 by 40 cm; Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) equilibrated with 6 M urea–50 mM Tris (pH 8.0)–50 mM NaCl at a flow rate of 1 ml/min. The column was washed with equilibration buffer until all the unadsorbed materials were removed before the absorbed fusion protein was eluted from the column with a 1,000-ml NaCl gradient (50 to 350 mM). Fractions containing ADP-ribosylating activity associated with protein that was recognized by both anti-TGF alpha and anti-PE antibodies in the Western blots were pooled and then concentrated using a stirred-cell Amicon concentrator fitted with a YM-30 membrane. The concentrated fusion protein (8 ml) was applied to a Sephacryl S-300 column (2.6 by 100 cm; Pharmacia LKB Biotechnology, Inc.) in equilibration buffer at a flow rate of 0.25 ml/min. Fractions (3 ml) were collected and again analyzed for ADP-ribosylation activity and Western-positive protein. Positive fractions were pooled and applied to a Q-Sepharose column (1.6 by 40 cm; Pharmacia LKB Biotechnology, Inc.) in equilibration buffer. After the column was washed to remove unadsorbed protein, bound fusion protein was eluted in a 600-ml NaCl gradient (50 to 450 mM).

Renaturation of fusion proteins. Purified fusion protein from the Q-Sepharose column was dialyzed against 50 mM glycine (pH 9.0) and then diluted into 50 mM glycine (pH 10.5) to give an A_{280} of 0.1. β-Mercaptoethanol was added in a 4:1 molar ratio over theoretical S-sulfonate groups, assuming a 0.1-mg/ml protein solution. Refolding was allowed to proceed for 16 h at 4°C before the protein was transferred to physiologically buffered saline and the concentration was adjusted to 0.5 mg/ml using a Centricon 30 (Amicon Corp., Danvers, Mass.).

Chemical cleavage of fusion proteins. Cyanogen bromide cleavages were performed with a 400-fold molar excess of cyanogen bromide to protein at 22°C for 24 h. The TGF-alpha released by cyanogen bromide treatment was isolated by size-exclusion chromatography on G-75 columns. Dithiothreitol (DTT) treatment (100 mM) was carried out at 37°C for 5 h.

RESULTS

Deletion mutants. Our initial genetic strategy for improving receptor binding was to progressively delete segments of the PE40 domain of TGF-alpha–PE40. Two deletion mutants were constructed by truncating the PE40 domain of TGF-alpha–PE40 at specific unique restriction enzyme sites (see Materials and Methods). These mutants retained the entire coding domain of TGF-alpha fused to either an approximately 34,000-dalton or 25,000-dalton segment from the C terminus of the original PE40 molecule (Fig. 1). Nucleic acid sequencing indicated that both the 34,000- and the 25,000-dalton segments of PE40 retained the entire enzymatic domain required for ADP-ribosylating activity. However, the 34,000-dalton segment is missing the N-terminal 59

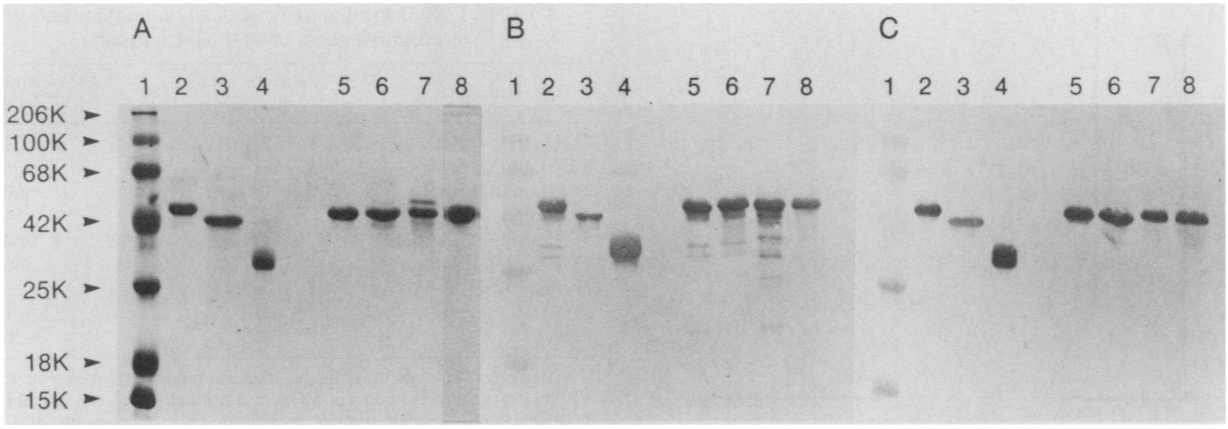

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified TGF-alpha–PE40 fusion proteins. Gels (12.5%) were run under reducing conditions. Each lane was loaded with approximately 0.5 μg of protein. (A) Coomassie-stained gel. (B and C) Western blots reacted with antibodies to PE toxin and to TGF-alpha amino acid residues 1 through 7, respectively. Lanes 1, Molecular weight markers; lanes 2, TGF-alpha–PE40; lanes 3, TGF-alpha–PE34; lanes 4, TGF-alpha–PE25; lanes 5, TGF-alpha–PE40; lanes 6, TGF-alpha–PE40 Ab; lanes 7, TGF-alpha–PE40 aB; lanes 8, TGF-alpha–PE40 ab.

amino acids of PE40, including the first pair of cysteine residues. The 25,000-dalton segment is missing the N-terminal 130 amino acids of PE40, including both pairs of cysteine residues. These deletion mutants were designated TGF-alpha–PE34 and TGF-alpha–PE25, respectively.

Site-specific substitution mutants. Preliminary analysis of TGF-alpha–PE34 and TGF-alpha–PE25 indicated that these proteins had superior receptor-binding activity compared with TGF-alpha–PE40 but were less potent cellular toxins (see below). In an attempt to improve the receptor-binding activity while retaining the cell-killing activity of TGF-alpha–PE40, three additional TGF-alpha–PE40 mutants were prepared. Using site-directed mutagenesis and oligonucleotide cassettes, either the cysteine residues at positions 265 and 287 or the cysteine residues at positions 372 and 379 of PE40 were changed to alanines. The third mutant was constructed with all four cysteine residues changed to alanines (Fig. 1). For convenience, the cysteine residues at positions 265 and 287 were designated the "A" locus and the cysteines at positions 372 and 379 were designated the "B" locus. When cysteines are present in either the A or B locus, uppercase letters are used. When alanines are present in either the A or B locus, lowercase letters are used. Using this nomenclature, the three mutants were designated TGF-alpha–PE40 aB, TGF-alpha–PE40 Ab, and TGF-alpha–PE40 ab.

Isolation of fusion proteins. TGF-alpha–PE40 and each of the mutated fusion proteins were expressed in *E. coli* and isolated through multiple chromatographic procedures (see Materials and Methods). Final purity of the recombinant proteins was assessed by polyacrylamide gel electrophoresis and Coomassie staining of the separated proteins. The major protein species present in each case accounted for 80 to 90% of the total Coomassie-stained protein and exhibited the appropriate gel migration distance anticipated for each fusion protein (Fig. 2A). To confirm that these bands represented TGF-alpha–PE fusion proteins, immunoblotting was performed using both TGF-alpha- and PE-specific antisera. In each case the major protein species was identified by both antisera, indicating that it contained TGF-alpha- and PE-specific amino acid sequences (Fig. 2B and C). The ADP-ribosylating activity of each fusion protein was measured to insure that the enzymatic properties of the mutants were preserved during their isolation. All five of the TGF-alpha–PE40 mutants as well as the parental TGF-alpha–PE40 fusion protein exhibited similar ADP-ribosylating enzyme activities, equivalent to one-quarter of the specific enzymatic activity of native PE or PE40.

Protein structure analysis. To test whether the TGF-alpha domain of TGF-alpha–PE40 had assumed the proper conformation during protein refolding, we examined the high-pressure liquid chromatographic profiles of TGF-alpha isolated from TGF-alpha–PE40 and TGF-alpha–PE40 ab. Each of these fusion proteins was engineered with a single methionine residue located between the TGF-alpha and PE40 domains. Thus cyanogen bromide treatment cleaves these proteins into one TGF-alpha and one PE40 fragment. The TGF-alpha proteins isolated following cyanogen bromide cleavage exhibited tracing patterns and retention times on reversed-phase high-pressure liquid chromatography similar to those of synthetic TGF-alpha (Fig. 3A through C). However, the yield of TGF-alpha obtained following cyanogen bromide cleavage of TGF-alpha–PE40 (84 μg) was approximately 24% less than that from cleavage of an equivalent amount of TGF-alpha–PE40 ab (110 μg). Disulfide bonds formed between the cysteine residues of TGF-alpha and PE40 could hinder the recovery of TGF-alpha from cyanogen bromide-cleaved TGF-alpha–PE40 fusion proteins by cross-linking TGF-alpha to PE40. To disrupt any disulfide bonds that might have interfered with the release of TGF-alpha, the cyanogen bromide-cleaved fusion proteins were separated from free TGF-alpha by size-exclusion chromatography and treated with 100 mM DTT. DTT treatment of the PE40 protein isolated following cyanogen bromide cleavage of TGF-alpha–PE40 resulted in recovery of an additional 24 μg of TGF-alpha (Fig. 3F). As expected, DTT treatment of the PE40 protein isolated following cyanogen bromide cleavage of TGF-alpha–PE40 ab did not release additional TGF-alpha (Fig. 3E).

Receptor-binding activity. The ability of each fusion protein to bind to the EGF receptor was measured as a function of the inhibition of specific binding of radiolabeled EGF to A431 cell membrane vesicles. TGF-alpha–PE40 was 98-fold less potent at blocking EGF receptor binding than TGF-alpha alone (Table 1; for receptor-binding inhibition = 540 and 5.5 nM, respectively). TGF-alpha–PE34 and TGF-alpha–PE25 exhibited higher affinities for the EGF receptor than did TGF-alpha–PE40 as evidenced by IC_{50}s for recep-

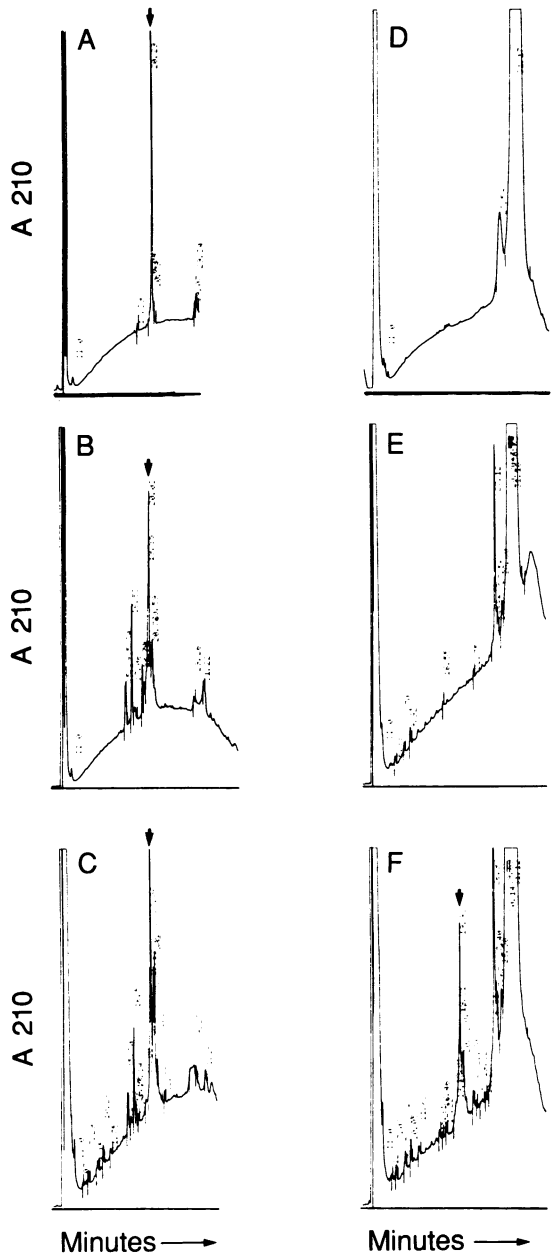

FIG. 3. Reversed-phase high-pressure liquid chromatography tracings of TGF-alpha and PE40 proteins. (A through C) Acetonitrile gradients (10 to 50%) run over 30 min; (D and E) acetonitrile gradients (50 to 60%) run over 15 min. Samples: (A) Synthetic human TGF-alpha; (B) TGF-alpha isolated from TGF-alpha–PE40 ab following cyanogen bromide cleavage; (C) TGF-alpha isolated from TGF-alpha–PE40 following cyanogen bromide cleavage; (D) PE40 containing protein isolated from TGF-alpha–PE40 following cyanogen bromide treatment; (E) PE40 containing protein isolated from TGF-alpha–PE40 ab following cyanogen bromide treatment and exposed to DTT; (F) PE40 containing protein isolated from TGF-alpha–PE40 following cyanogen bromide treatment and exposed to DTT. Arrows denote TGF-alpha species.

tor-binding inhibition of 340 and 180 nM, respectively. TGF-alpha–PE40 aB and TGF-alpha–PE40 ab had approximately equal receptor-binding inhibition activities (IC_{50} = 34 and 37 nM, respectively). TGF-alpha–PE40 Ab exhibited receptor-binding activity (IC_{50} = 575 nM) similar to that of TGF-alpha–PE40.

TABLE 1. Biological activity of deletion mutants and site-specific mutants of TGF-alpha–PE40[a]

Mutant	Receptor binding IC_{50} (nM)	A431 cytotoxicity EC_{50} (pM)
TGF-alpha–PE40	540	21
TGF-alpha–PE34	340	3,400
TGF-alpha–PE25	180	860
TGF-alpha–PE40 Ab	575	54
TGF-alpha–PE40 aB	34	380
TGF-alpha–PE40 ab	37	300
Human TGF-alpha	5.5	
PE40	$>10^3$	$>2 \times 10^5$
PE	$>10^3$	20

[a] All assays were run in triplicate. Data are reported as the mean value of all three tests. Protein concentrations are normalized to the percentage of TGF-alpha–PE40-related protein present in each preparation. IC_{50}, 50% inhibitory concentration; EC_{50}, 50% effective concentration.

Cell-killing activity. The ability of each fusion protein to kill cells bearing EGF receptors was assessed using A431 human tumor cells. TGF-alpha–PE40 and all five of its mutants were effective cytotoxic agents against A431 cells. TGF-alpha–PE40 and TGF-alpha–PE40 Ab were the most potent toxins, with EC_{50}s for cell killing of 21 and 54 pM, respectively. TGF-alpha–PE40 aB and TGF-alpha–PE40 ab were significantly less potent toxins than TGF-alpha–PE40, with EC_{50}s of 380 and 300 pM, respectively. TGF-alpha–PE34 (EC_{50} = 3,400 pM) and TGF-alpha–PE25 (EC_{50} = 860 pM) also killed A431 cells.

A separate series of experiments were performed to confirm that the cell-killing activity of TGF-alpha–PE40 was dependent on binding of the fusion protein to cellular EGF receptors. Both A431 cells and HeLa cells were exposed to TGF-alpha–PE40 in the presence or absence of excess EGF (Fig. 4). In the absence of exogenous EGF, the A431 and HeLa cells were readily killed by TGF-alpha–PE40 (EC_{50} = 31 pM and 1.0 nM, respectively). However, in the presence of 1.0 μM EGF the tumor cells were dramatically less sensitive to the cytotoxic effect of TGF-alpha–PE40 (EC_{50} = 85 and 400 nM, respectively). Similar experiments were carried out using native PE. Both A431 and HeLa cells were equally susceptible to PE-induced cytotoxicity in the presence or absence of added EGF. CHO cells, which lack EGF receptors, were also examined. Native PE killed CHO cells with an EC_{50} of 110 pM, whereas TGF-alpha–PE40 exhibited no cytotoxic effects at concentrations up to 100 nM.

DISCUSSION

TGF-alpha–PE40 fusion proteins bind to and kill mammalian cells (e.g., A431 and HeLa) that possess EGF receptors. The fact that EGF receptor binding is critical to the ability of TGF-alpha–PE40 to intoxicate cells is evidenced by our experiments and those of Chaudhary et al. (3), showing that excess EGF blocks the cytotoxic effect of various TGF-alpha–PE40 fusion proteins. The protective activity of EGF in these studies cannot be attributed to nonspecific or receptor-independent effects since excess EGF did not inhibit the cytotoxicity caused by native PE (Fig. 4). The involvement of receptor binding in the intoxication process suggests that the binding affinity of TGF-alpha–PE40 for the EGF receptor may affect the specificity of TGF-alpha–PE40-induced cytotoxicity.

One of the factors that influence the receptor-binding activity of TGF-alpha and thus TGF-alpha–PE40 is the formation of intrachain disulfide bonds (7, 19, 30). TGF-

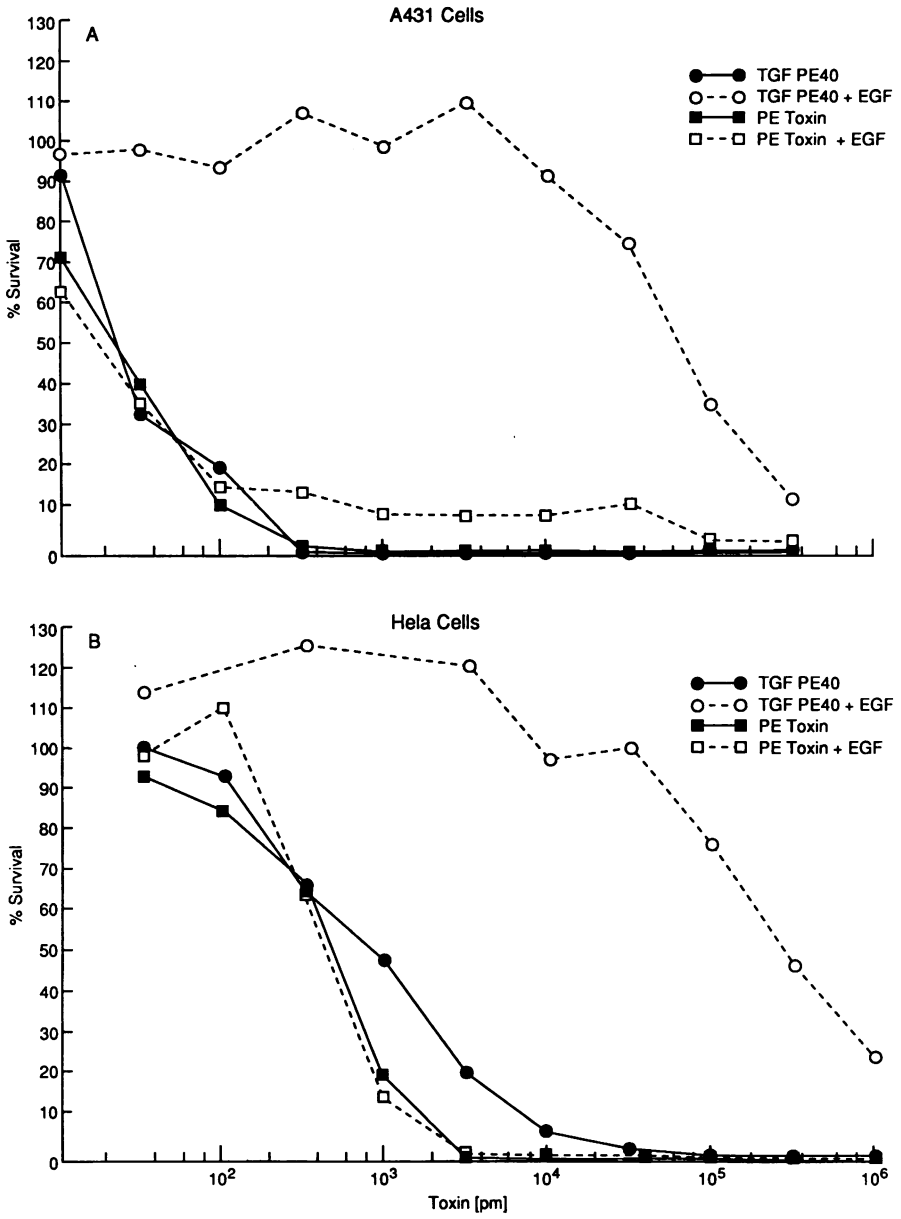

FIG. 4. Cytotoxicity of TGF-alpha–PE40 in mammalian cell lines. A431 cells (A) and HeLa cells (B) were treated with increasing concentrations of either TGF-alpha–PE40 or native PE in the presence or absence of 1.0 μM EGF.

alpha possesses six cysteine residues which form three intrachain disulfide bonds. These cysteine residues must pair properly and form the correct disulfide bonds for TGF-alpha to be biologically active (20, 23). PE40 contains four cysteine residues located in the translocation domain. The x-ray crystallographic analysis of native PE suggests that these cysteines are paired sequentially to form two disulfide bonds (1). Thus, TGF-alpha–PE40 contains 10 cysteine residues that form five disulfide bonds. If any of the cysteines in the PE40 domain interfere with proper disulfide bond formation in the TGF-alpha domain of TGF-alpha–PE40, then the resulting hybrid proteins would be expected to exhibit poor receptor binding. TGF-alpha–PE40 did bind poorly to EGF receptors (IC_{50} = 540 nM). Therefore, we were concerned that some of the disulfide bonds in TGF-alpha–PE40 had formed between incorrect pairs of cysteines, resulting in diminished receptor-binding activity. High-pressure liquid chromatography of TGF-alpha–PE40 following cyanogen bromide cleavage suggested that improper disulfide bonds had formed in at least 22% of the TGF-alpha–PE40 molecules. The TGF-alpha and PE40 domains in this 22% of TGF-alpha–PE40 molecules appeared to be cross-linked to one another through a DTT-labile bond. These cross-links apparently represent incorrectly paired disulfide bonds. The remaining 78% of TGF-alpha–PE40 molecules yielded TGF-alpha species with near normal receptor-binding activity (IC_{50} = 12.0 nM) following treatment with cyanogen bromide. These data suggest that the majority of disulfide bonds in the non-cross-linked TGF-alpha–PE40 molecules were formed between proper pairs of cysteines.

To reduce the possibility of improper disulfide bond formation between cysteine residues in TGF-alpha and PE40, the TGF-alpha–PE40 deletion mutants were engineered to remove DNA segments that contained one or both

pairs of PE40 cysteine residues. TGF-alpha–PE34 contains one pair of cysteine residues in its PE34 domain, and TGF-alpha–PE25 contains no cysteine residues in its PE25 domain (Fig. 1). TGF-alpha–PE34 and TGF-alpha–PE25 exhibited progressively improved receptor-binding properties relative to TGF-alpha–PE40. Unfortunately, the cell-killing activity of these mutants was significantly reduced relative to TGF-alpha–PE40. Presumably, the deletion of large segments of the translocation domain of PE40 interfered with the cytotoxic activity of TGF-alpha–PE34 and TGF-alpha–PE25 by inhibiting their ability to migrate into the target cell's cytoplasm. Interestingly, TGF-alpha–PE34 was less effective at killing A431 cells than was TGF-alpha–PE25. We do not fully understand this observation. However, these results suggest that partial deletion of the translocation domain can be more detrimental to the cytotoxic activity of TGF-alpha–PE40 mutant proteins than complete removal of this domain.

A more direct test of the contribution of PE40's cysteine residues to the receptor-binding properties of TGF-alpha–PE40 was undertaken by changing these amino acids to alanines through site-directed mutagenesis. The cysteine residues were changed two at a time so as to eliminate pairs of residues that normally form disulfide bonds. Both TGF-alpha–PE40 aB and TGF-alpha–PE40 ab had the first two cysteine residues of PE40 replaced with alanines (Fig. 1). Both of these mutants were approximately 15 times more potent at receptor binding than TGF-alpha–PE40. These results suggest that the cysteine residues at positions 265 and 287 of PE40 adversely affect the receptor-binding activity of TGF-alpha–PE40 hybrid proteins. Interestingly, TGF-alpha–PE40 Ab did not bind more efficiently to EGF receptors than did TGF-alpha–PE40. This result suggests that the second set of cysteine residues in PE40 at positions 372 and 379 do not significantly interfere with receptor binding by TGF-alpha–PE40 hybrid proteins. It should be noted that even when all of the cysteine residues in the PE40 domain of TGF-alpha–PE40 were replaced by alanines, as in TGF-alpha–PE40 ab, the receptor-binding activity was still approximately sevenfold less than that of mature TGF-alpha. Therefore, other factors besides the cysteine residues in PE40 must contribute to the reduced binding efficiency of TGF-alpha–PE40. One of these factors may be that the presence of a large protein like PE40 attached to the C terminus of TGF-alpha sterically hinders the binding of TGF-alpha to the EGF receptor. Elimination of the cysteine residues of PE40 also results in TGF-alpha–PE40 mutants with reduced cell-killing activity relative to TGF-alpha–PE40. Again, it is the cysteines at positions 265 and 287 of PE40 that are most important in maintaining the cell-killing activity of TGF-alpha–PE40. Presumably, the disulfide bond that forms between these residues contributes to the conformation of TGF-alpha–PE40 that is optimal for intoxication of mammalian cells.

Several growth factor-toxin hybrid molecules are under investigation as potential therapeutic agents (2, 16, 26). In each case it is unclear whether cytotoxic potency or receptor-binding activity is more important for producing a safe and effective compound. Our studies indicate that TGF-alpha–PE40 is an exceptionally potent cytotoxic agent for EGF-receptor-positive cells, but it exhibits poor receptor-binding activity. TGF-alpha–PE40 aB and TGF-alpha–PE40 ab are significantly less cytotoxic to receptor-positive cells even though their receptor-binding activities are approximately 16-fold better than that of TGF-alpha–PE40. It remains to be seen which version of TGF-alpha–PE40 will be the most effective antitumor agent in vivo and which version will cause the least damage to normal tissues.

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