

An EGF-pseudomonas exotoxin A recombinant protein with a deletion in toxin binding domain specifically kills EGF receptor bearing cells

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We constructed two chimeric toxins; one composed of epidermal growth factor (EGF) and pseudomonas exotoxin A (PE), designated EGF-PE and the other composed of EGF and PE with a deletion of the Ia domain (cell-binding domain), designated EGF-PE (Δ Ia). Both chimeric toxins reacted with anti-EGF and anti-PE antibodies. The cell-killing experiments showed that EGF-PE, but not EGF-PE(Δ Ia), was cytotoxic to the murine fibroblast cell line NR6, which carried the PE receptor, but not the EGF receptor. However, after NR6 was transfected with DNA for the expression of human EGF receptor, the transfected cell line, designated NRHER5, over-expressed human EGF receptors and became sensitive to EGF-PE(Δ Ia). The cytotoxicity of EGF-PE(Δ Ia), but not EGF-PE, to NRHER5 can be completely blocked by an excess amount of EGF. To completely reverse the cytotoxicity of EGF-PE on NRHER5, both the EGF receptor pathway and the PE receptor pathway need to be blocked. These results suggest that EGF-PE exhibits both EGF and PE binding activities, while EGF-PE(Δ Ia) possesses only EGF binding activity. Thus, EGF-PE(Δ Ia) may be a better chimeric toxin than EGF-PE in terms of target specificity to EGF receptor bearing cells. We, therefore, examined the cytotoxicity of EGF-PE(Δ Ia) to various human cancer cell lines. We find that human cancer cells containing more EGF receptors are more sensitive to EGF-PE(Δ Ia).

Key words: chimera/EGF/pseudomonas exotoxin A

Introduction

Treatment with chemotherapeutic drugs have resulted in the amelioration of some types of human cancer. However, solid tumors such as colon, kidney and prostate tumors are only weakly responsive to conventional drugs. In addition, cancer patients develop multiple drug resistance (Fojo *et al.*, 1987; Gottesman *et al.*, 1988a,b) after the conventional drug treatment. Thus, the simultaneous development of resistance to multiple drugs and the lack of target-cell specificity by antitumor drugs appear to be the major impediments to successful chemotherapy of human tumors. Therefore, an alternative treatment which employs a targeted toxin as a new approach to specific cytotoxic therapy will be desirable. Since several types of cancer cells have been found to contain unusually large numbers of growth factor receptors (Xu *et al.*, 1986), toxins coupled to growth factors have been proposed as agents for the treatment of human cancer (FitzGerald *et al.*, 1986; Chaudhary *et al.*, 1987, 1989, 1990;

Lyall *et al.*, 1987; Lorberboum-Galski *et al.*, 1988; Siegall *et al.*, 1989, 1990). It is believed that this treatment method will supplement conventional chemotherapeutic agents to which cancer cells often become resistant (Pastan *et al.*, 1986).

We, therefore, attempted to design a tumor-specific toxin composed of epidermal growth factor (EGF) and pseudomonas exotoxin A (PE). Since the intoxication process of PE is thought to contain at least three steps, namely binding to the cell, translocation across the cell membrane and ADP-ribosylation of elongation factor 2 (Eidels *et al.*, 1983; Middlebrook and Dorland, 1984), the strategy in altering PE to become a tumor-specific toxin involved retention of the PE translocation and ADP-ribosylation activities, while at the same time altering PE binding specificity to become only EGF receptor specific. The altered toxin would then bind preferentially to cancer cells overexpressing the EGF receptor instead of normal cells and, consequently, kill only cancer cells. In this study, we have applied recombinant DNA techniques to construct the plasmids pEJ-4 and pEJ-8, which could be used for the expression of the chimeric toxins, EGF-PE and EGF-PE(Δ Ia) respectively. This study has demonstrated that both EGF-PE and EGF-PE(Δ Ia) could effectively kill human cancer cells. However, EGF-PE kills cancer cells through both the EGF receptor and PE receptor pathways, while EGF-PE(Δ Ia) kills cancer cells only through the EGF receptor pathway. Thus, EGF-PE(Δ Ia) may be a better tumor-specific toxin than EGF-PE in terms of target specificity.

Materials and methods

Materials

Pseudomonas exotoxin A was obtained from ICN Biomedicals. Restriction endonucleases, T4 DNA ligase and other enzymes used in cloning DNA were obtained from New England Biolabs or Bethesda Research Laboratories and used under the conditions recommended by the supplier. Synthetic oligomers were prepared using Pharmacia Gene Assembler. Reagents for gel electrophoresis were from Bio-Rad. Taq DNA polymerase, 5-bromo-4-chloro-3-indolyl-phosphate, nitro blue tetrazolium and goat antimouse-IgG alkaline phosphatase conjugate were purchased from Promega. All other chemicals were analytical grade reagents.

Bacterial strains and plasmids

Escherichia coli strain HB 101: [F^- , *hdsS20* (r_B^- , m_B^-), *supE44*, *ara14*, *galK2*, *lacY1*, *proA2*, *rpsL20* (str^R), *xyl5*, *leu*, *mill*, l^- , *recA13*] was used as the host for the propagation of the plasmid. *Escherichia coli* strain BL21(DE3) containing the T₇ RNA polymerase gene under the control of the *lac* promoter was used for chimeric toxin expression (Studier and Moffat, 1986). Plasmid pJH4, which encoded the full length of PE and plasmid pJH8, which encoded PE with deletion of the Ia domain, were obtained as described previously (Hwang *et al.*, 1987). Synthetic EGF DNA, which carries the sequence coding for human mature EGF peptide, was kindly provided by Dr R.A. Weinberg of Whitehead Institute, MIT (Stern *et al.*, 1987).

Expression of the recombinant toxin in BL21(DE3)

Escherichia coli strain BL21(DE3) containing the appropriate plasmid for the expression of the chimeric toxin was cultured in LB broth with 50 µg/ml ampicillin at 37°C. When absorbance at 600 nm reached 0.3, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mM. Cells were harvested 90 min later. These chimeric toxins were examined by SDS-PAGE, immunoblotting and cell-killing activity.

SDS-PAGE and immunoblotting

Samples were dissolved in Laemmli sample buffer and boiled for 5 min prior to application to a 0.1% SDS, 10% acrylamide slab gel and stained with Coomassie blue after electrophoresis as described by Laemmli (1970). For immunoblotting, electrophoresed samples were transferred from gel to Millipore polyvinylidene difluoride paper, followed by blotting with antisera directed against intact PE or EGF. The chimeric toxin was visualized by treatment with alkaline phosphatase-conjugated goat antirabbit-IgG, followed by staining with substrate, 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium. The antiserum to PE was generated in female New Zealand White rabbits as described previously (Hwang and Chen, 1989). The antiserum to EGF was purchased from the Collaborative Research Corporation.

Isolation and purification of EGF-PE and EGF-PE(ΔIa)

Host cells, BL21 (DE3), after transforming with pEJ8, which encoded EGF-PE(ΔIa) or pEJ4, which encoded EGF-PE, were cultured in LB broth with 50 µg/ml of ampicillin. When absorbance at 600 nm reached 0.3, IPTG was added to induce chimeric toxin expression. After 90 min, the cells were harvested and resuspended in 20 vol of lysis buffer (1.5 mg/ml lysozyme, 1 mM EDTA, 50 mM Tris, pH 8.1). DNase I, MgCl₂ and MnCl₂ were then added to final concentrations of 50 µg/ml, 10 mM and 1 mM respectively. The incubation was performed at room temperature for 15 min. In order to completely lyse the cells, EDTA and Triton X-100 were added to final concentrations of 50 mM EDTA and 1% Triton X-100 and the incubation was continued for a further 2 min. The lysates were then centrifuged at 13 000 *g* for 10 min to pellet down the inclusion bodies. The pellets were washed twice with 20 vol of wash buffer (1% Triton X-100, 5 mM EDTA, 5 mM EDTA, 10 mM Tris, pH 8.1) in order to remove the residual DNA contaminant. Finally, the pellets were extracted with 10 vol of 8 M urea. The purity of the chimeric toxins in the 8 M urea extracts reached approximately 70–80%. Further purification of the chimeric toxin was performed as follows. First, the 8 M urea extracts were dialyzed at 4°C against 500 vol of 1 mM EDTA, 10 mM Tris, pH 8.1 and the dialysis buffer was changed every 3 h. After dialysis against five changes of the dialysis buffer, samples were loaded onto a DEAE-sephacel column, which was pre-equilibrated with 1 mM EDTA, 10 mM Tris, pH 8.1 and eluted with an NaCl gradient up to 1 M NaCl. The chimeric toxins were eluted out at 0.4 M NaCl. The fractions containing EGF-PE or EGF-PE(ΔIa) were then collected and examined by SDS-PAGE. Usually for each purification, 4 l of culture was used and the recovery of purified EGF-PE and EGF-PE(ΔIa) was ~50 and 20 mg respectively, as determined by the method of Bradford (1976).

Cell lines

NR6, originating from mouse fibroblast, does not express any EGF receptor and NRHER5, originating from NR6 transfected with plasmid pC012-EGFR for the expression of human EGF

receptor (Velu *et al.*, 1988), expresses unusually large numbers of EGF receptors on the cell surface. Unless specified, cells were purchased from the American Type Culture Collection. A431 epidermoid carcinoma cells and KB-3-1 cells were generous gifts from Dr Ira Pastan (Laboratory of Molecular Biology, National Cancer Institute). Human esophageal carcinoma cell lines CE48T/VGH and CE81T/VGH (Hu *et al.*, 1984; Chen *et al.*, 1991), human lung carcinoma cell line CaLu-1 (Fogh and Trempe, 1975) and human hepatoma cell line HA22T/VGH (Chang *et al.*, 1983) were generous gifts from Dr Cheng-po Hu (Department of Medical Research, Veterans General Hospital, Taipei, Taiwan). All the cell lines were cultured as monolayers or suspension using Dulbecco's Modified Eagles Medium or RPMI 1640 medium containing 10% fetal bovine serum, penicillin-streptomycin (50 IU/ml and 50 µg/ml respectively) and glutamine at 1 mM in a controlled atmosphere of 5% CO₂ (v/v) and 95% air (v/v) at 37°C.

Assay of cytotoxic activity

The cytotoxic activities of EGF-PE and EGF-PE(ΔIa) were tested on NR6, NRHER5 and human cancer cells. The cells were plated in a 24-well tissue culture dish at a density of 4 × 10⁴ cells per well for 24 h prior to the cytotoxicity assay. Tested cells were incubated with various concentrations of EGF-PE and EGF-PE(ΔIa) in the presence or absence of excess amounts of EGF and/or PE with deletion of the C-terminal 38 amino acid residues, designated PE(Δ576-613), which was previously shown to be able to block PE cytotoxicity (Chow *et al.*, 1989). After 72 h, the monolayers of the cells were stained with methylene blue to detect surviving cells and to measure the cytotoxicity of the chimeric toxins. Additionally, relative cloning efficiency was determined by plating 300 cells in 60 mm dishes overnight in complete medium and then adding the indicated amounts of the chimeric toxins. After incubation at 37°C for 7 days, the dishes were stained with methylene blue and the number of colonies was counted. The relative cloning efficiency is the number of colonies formed in the presence of the chimeric toxin being tested divided by the number of colonies formed in the absence of the toxin. To determine the amount of chimeric toxin required to inhibit protein synthesis by 50% (*ID*₅₀), cells were treated with various concentrations of the chimeric toxin for 24 h and the incorporation of [³H]leucine into the cellular protein was then measured as described (Chaudhary *et al.*, 1987).

Displacement of [¹²⁵I]EGF binding by EGF and EGF-containing chimeric toxin

A431 cells were plated in triplicate in 24-well tissue culture dishes at a cell density of 4 × 10⁴ cells per well in 1.0 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The following day, the medium was replaced by fresh Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin and incubation was continued at 37°C for 1 h. Cells were chilled on ice for 15 min prior to the addition of various amounts of EGF-containing chimeric toxins (final concentration up to 200 nM) and a fixed amount of [¹²⁵I]EGF (1 nM; sp. act. 10⁸ c.p.m./nmol). Binding was carried out on ice for 2 h. Following this period, cells were washed three times with ice cold medium and then solubilized in 0.2 ml of 1 N NaOH prior to scintillation counting.

Results*Construction of plasmids expression EGF-PE and EGF-PE(ΔIa)*

Plasmid pJH4, which encoded full-length PE and plasmid pJH8, which encoded PE with deletion of the Ia domain, were used

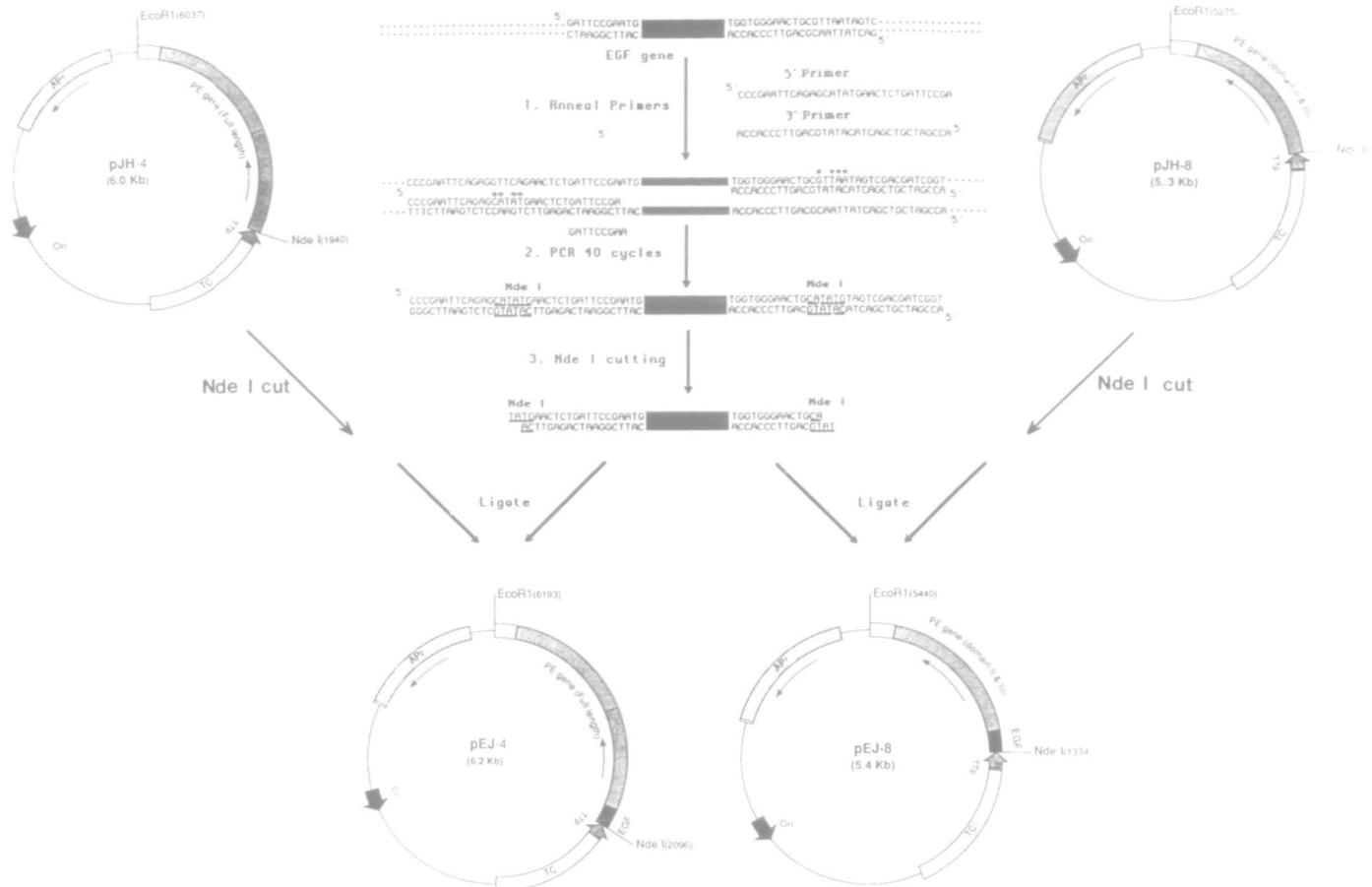


Fig. 1. Construction of plasmids expression of EGF-PE and EGF-PE(Δ Ia). Mutagenesis through polymerase chain reaction was performed to introduce two *NdeI* sites on both ends of EGF DNA. After 30 cycles of polymerase chain reaction, a EGF DNA fragment containing two *NdeI* sites was generated. The polymerase chain reaction product was cleaved with restriction enzyme *NdeI*. The *NdeI* cleaved EGF DNA fragment was ligated with *NdeI* linearized pJH4 or pJH8. The resulting plasmids, pEJ4 and pEJ8, which are used for the expression of EGF-PE and EGF-PE(Δ Ia) respectively, are constructed as shown.

as starting materials to construct plasmids expressing EGF-PE and EGF-PE(Δ Ia). Since both pJH4 and pJH8 have an *NdeI* site at the initiation codon of the toxin structural gene, the strategy used to construct plasmids expressing EGF-PE and EGF-PE(Δ Ia) was to insert synthetic EGF DNA, which carries the sequence coding for human mature EGF peptide, at the *NdeI* site. However, there is no *NdeI* sequence at the ends of the human EGF DNA clone. We, therefore, conducted mutagenesis of the human EGF DNA clone through a polymerase chain reaction to introduce two *NdeI* sites on both ends of the EGF DNA (Figure 1). In addition, the EGF DNA, after insertion into pJH4 or pJH8 in front of the PE structural gene, also left the toxin gene in the right reading frame after the EGF DNA. Two primers containing the *NdeI* sequence, which were complementary to both ends of EGF DNA with four mismatches, were used in a polymerase chain reaction to generate modified EGF DNA. After 30 cycles of polymerase chain reaction, modified EGF DNA fragments were produced. The polymerase chain reaction product was cleaved with the restriction enzyme *NdeI* and the *NdeI* cleaved EGF DNA fragment was ligated to *NdeI* linearized pJH4 or pJH8. The resulting plasmids, pEJ4 encoding EGF-PE and pEJ8 encoding EGF-PE(Δ Ia), carry the EGF coding sequence at the 5' end of the toxin structural gene. DNA sequencing was performed to confirm the correct sequence and orientation (data not shown). The plasmids pEJ4 and pEJ8 were then used to express the chimeric toxins, EGF-PE and EGF-PE(Δ Ia), in BL21(DE3).

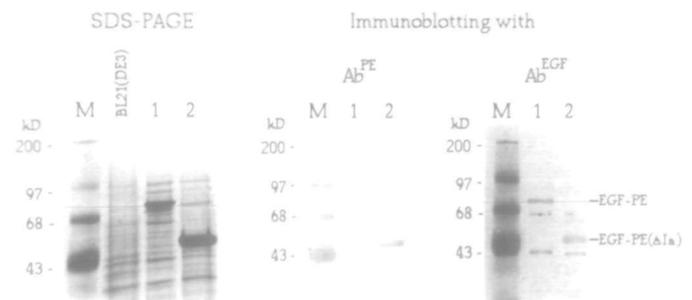


Fig. 2. Expression characterization of EGF-PE and EGF-PE(Δ Ia). (A) Coomassie blue stained SDS-PAGE protein gel. M stands for protein mol. wt marker and BL21(DE3) stands for host cell lysate. Lane 1, crude lysate of BL21(DE3)/pEJ4; lane 2, crude lysate of BL21(DE3)/pEJ8. (B) Immunoblotting with anti-PE antibody. Lane 1, crude lysate of BL21(DE3)/pEJ4; lane 2, crude lysate of BL21(DE3)/pEJ8. (C) Immunoblotting with anti-EGF antibody. Lane 1, crude lysate of BL21(DE3)/pEJ4; lane 2, crude lysate of BL21(DE3)/pEJ8.

Expression and identification of EGF-PE and EGF-PE(Δ Ia)

For the expression of the chimeric toxins EGF-PE and EGF-PE(Δ Ia), BL21(DE3) containing the appropriate plasmid was cultured in LB medium with ampicillin at a concentration of 50 μ g/ml. When cell density reached approximately 5×10^7 cells/ml, IPTG was added to a final concentration of 0.4 mM. After 90 min of IPTG induction, the cells were harvested and examined by SDS-PAGE. As shown in Figure 2(A), both EGF-

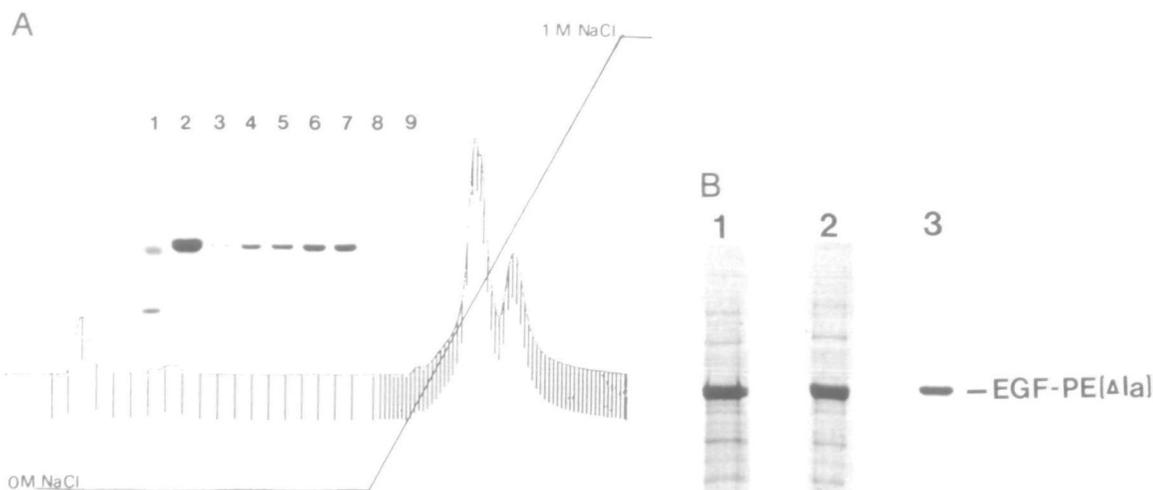


Fig. 3. Purification of EGF-PE(Δ Ia) through (A) a DEAE-sephacel column chromatography and (B) SDS-PAGE of samples at various stages of purification of EGF-PE(Δ Ia). (A) Renatured EGF-PE(Δ Ia) extracts were loaded onto a DEAE-sephacel column, which was pre-equilibrated with 1 mM EDTA, 10 mM Tris, pH 8.1. The column was eluted with an NaCl gradient up to 1 M NaCl and fractions (1 ml per fraction) were collected. Active monomeric EGF-PE(Δ Ia) was eluted at 0.4 M NaCl and an aggregated form of EGF-PE(Δ Ia) was eluted at 0.5 M NaCl. The vertical axis represents the absorbance at 280 nm. The inset represents the SDS-PAGE result of samples from different fractions of DEAE-sephacel column chromatography. Lane 1, protein marker, lane 2, sample before DEAE-column chromatography; lanes 3–7, fractions of the first peak containing active monomeric EGF-PE(Δ Ia), lanes 8 and 9, fractions of the second peak containing the aggregated form of EGF-PE(Δ Ia). The maximum absorbances at OD₂₈₀ for peak I and peak II are 1.6 and 0.8 respectively. The amount of protein obtained from the second peak is not proportional to the magnitude of the OD₂₈₀ absorbance. OD₂₈₀ in peak II may represent impurities from the trailing peak I or contamination with nucleic acids in EGF-PE(Δ Ia) aggregates. (B) Lane 1, crude cell lysate of BL21(DE3)/pEJ8; lane 2, 8 M urea extracts of BL21(DE3)/pEJ8; lane 3, EGF-PE(Δ Ia) after DEAE-sephacel column chromatography.

PE and EGF-PE(Δ Ia) were overexpressed in BL21(DE3)/pEJ4 and BL21(DE3)/pEJ8 and they could be visualized directly as a major band at the expected mol. wts after Coomassie blue staining. Further identification of these chimeric toxins by immunoblotting with anti-PE and anti-EGF antibodies was also performed [Figure 2(B) and (C)]. EGF-PE and EGF-PE(Δ Ia) reacted positively to both anti-EGF and anti-PE antibodies. These results demonstrate that the chimeric toxins contain both EGF and PE moieties.

Purification of the EGF-PE and EGF-PE(Δ Ia)

Chimeric toxins, EGF-PE and EGF-PE(Δ Ia), were isolated from BL21(DE3) cells carrying pEJ4 and pEJ8 respectively. Cells after IPTG induction were harvested by centrifugation and lysed with lysozyme, followed by DNase I treatment and 1% Triton X-100 extraction to remove DNA contaminant and unwanted proteins. Since both EGF-PE and EGF-PE(Δ Ia) existed as forms of inclusion bodies, 8 M urea was thus used to extract the chimeric toxins from the inclusion bodies. The 8 M urea extracts were then dialyzed to renature the chimeric toxins. Using this approach, EGF-PE(Δ Ia) was purified to approximately 70% purity [Figure 3(A), lane 2]. The renatured EGF-PE(Δ Ia) was further purified through a DEAE-sephacel column to obtain over 95% purity [Figure 3(A), lanes 4–7]. The improvement of the purity of EGF-PE(Δ Ia) after 8 M urea extraction and DEAE-sephacel column chromatography is shown in Figure 3(B). The purity of EGF-PE(Δ Ia) reached 70% after urea extraction and over 95% purity after DEAE-sephacel column chromatography [Figure 3(B), lanes 2 and 3]. The procedures for the purification of EGF-PE(Δ Ia) were applied to purify EGF-PE. We also obtained EGF-PE at over 95% purity (data not shown). For each purification, 4 l of culture was used and the recoveries of purified EGF-PE and EGF-PE(Δ Ia) were approximately 50 and 20 mg respectively.

Target specificity of EGF-PE and EGF-PE(Δ Ia)

To examine the target specificity of EGF-PE and EGF-PE(Δ Ia), two cell lines, NR6 and NRHER5, were used for this study. NR6

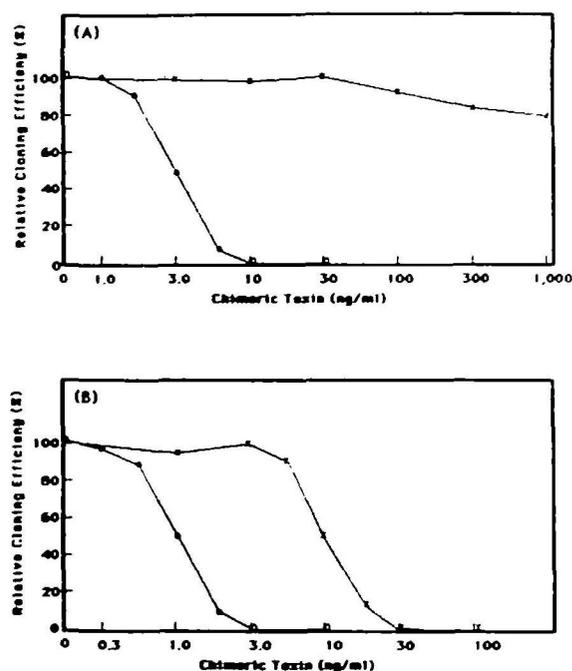


Fig. 4. Cytotoxicity of EGF-PE and EGF-PE(Δ Ia) to (A) NR6 and (B) NRHER5 cell lines. The y-axis represents the relative cloning efficiency. The relative cloning efficiency is the number of colonies formed in the presence of the chimeric toxin being tested divided by the number of colonies formed in the absence of the toxin. The test concentrations were in the range 0.1–1000 ng/ml. EGF-PE (○) and EGF-PE(Δ Ia) (×).

cells that originated from mouse fibroblast had no EGF receptors (Schneider *et al.*, 1986a), while NRHER5, derived from NR6 cells transfected with the human EGF receptor gene, expressed 1×10^6 EGF receptors on the cell surface (Bjorge *et al.*, 1990). Thus, NR6 cells can be killed by chimeric toxins through the PE receptor pathway only, while NRHER5 cells can be killed through both the PE receptor and the EGF receptor pathways.

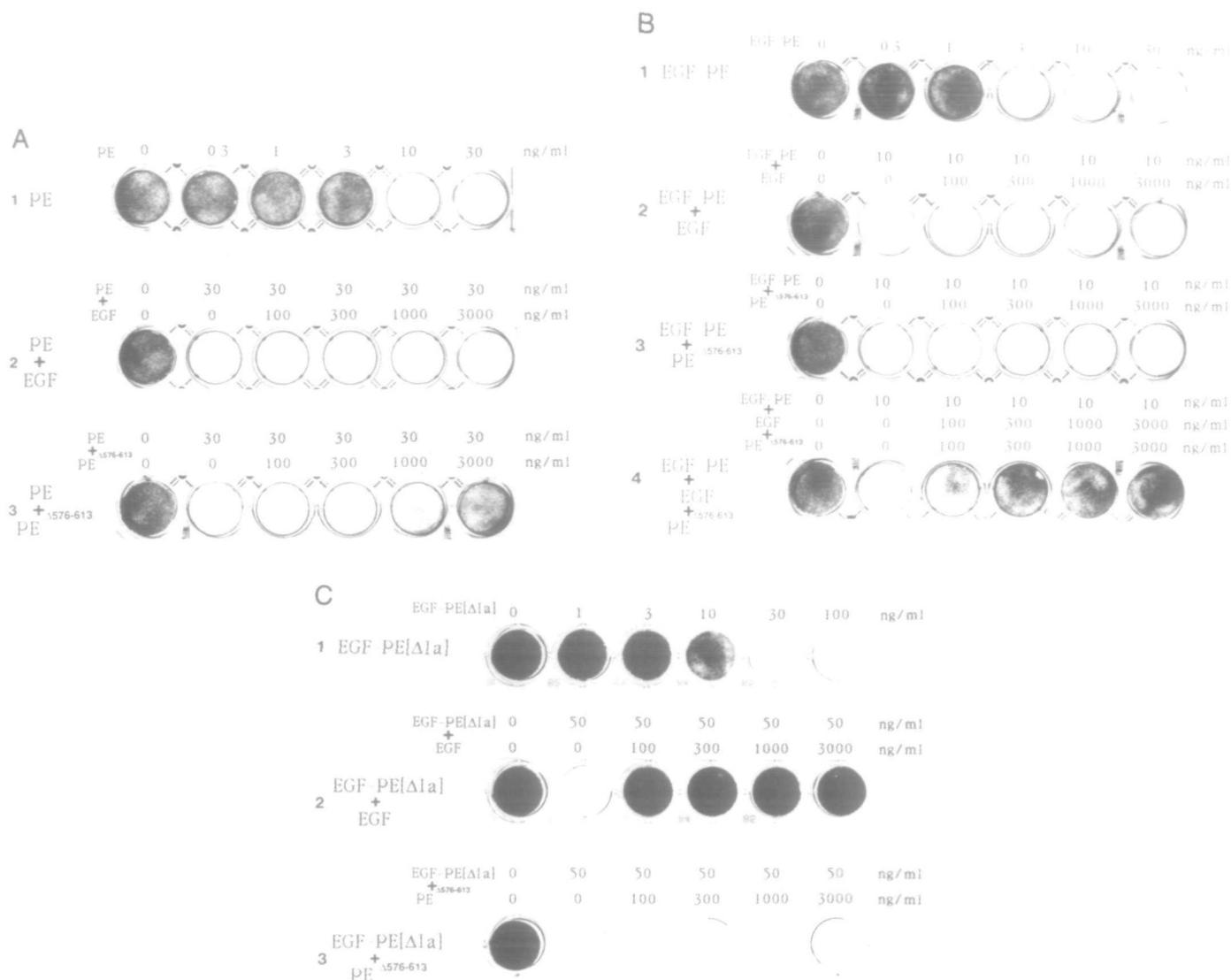


Fig. 5. (A) Cytotoxicity of PE to NRHER5 cells and its reversal. NRHER5 cells were seeded in a 24-well microtiter plate 24 h prior to cytotoxic assay. Lane 1, NRHER5 cells challenged with various concentrations of PE in the range 0–30 ng/ml; lane 2, NRHER5 cells simultaneously incubated with 30 ng/ml of PE and various concentrations of EGF; lane 3, NRHER5 cells simultaneously incubated with 30 ng/ml of PE(Δ 576–613) and various concentrations of EGF; lane 4, NRHER5 cells simultaneously incubated with 30 ng/ml of PE(Δ 576–613) and various concentrations of EGF. (B) Cytotoxicity of EGF-PE to NRHER5 and its reversal. NRHER5 cells seeded in a 24-well microtiter plate 24 h prior to cytotoxic assay. Lane 1, NRHER5 cells challenged with various concentrations of EGF-PE in the range 0–30 ng/ml; lane 2, NRHER5 cells simultaneously incubated with 10 ng/ml of EGF-PE and various concentrations of EGF; lane 3, NRHER5 cells simultaneously incubated with 10 ng/ml of EGF-PE and various concentrations of PE(Δ 576–613); lane 4, NRHER5 cells simultaneously incubated with 10 ng/ml of EGF-PE and various concentrations of EGF and PE(Δ 576–613). (C) Cytotoxicity of EGF-PE(Δ 1a) to NRHER5 and its reversal. NRHER5 cells were seeded in a 24-well microtiter plate 24 h prior to cytotoxic assay. Lane 1, NRHER5 cells challenged with various concentrations of EGF-PE(Δ 1a) in the range 0–100 ng/ml; lane 2 NRHER5 cells simultaneously incubated with 50 ng/ml of EGF-PE(Δ 1a) and various concentrations of EGF; lane 3, NRHER5 cells simultaneously incubated with 50 ng/ml of EGF-PE(Δ 1a) and various concentrations of PE(Δ 576–613).

As expected, we found that EGF-PE, which exhibited both EGF and PE binding activities, could effectively kill both NR6 and NRHER5 cells with IC_{50} s of 3 ng and 1 ng/ml respectively (Figure 4). Since the IC_{50} of PE to NRHER5 cells is 3 ng/ml, this result indicates that NRHER5 cells are more sensitive to EGF-PE than to PE. This may be due to the fact that EGF-PE uses both the EGF receptor and PE receptor pathways, while PE uses only the PE receptor pathway to kill NRHER5 cells. On the other hand, although EGF-PE(Δ 1a) can effectively kill NRHER5 cells with an IC_{50} of 10 ng/ml, it fails to kill NR6 cells. The IC_{50} of EGF-PE(Δ 1a) to NR6 cells is >1000 ng/ml (Figure 4). In order to further examine the target specificity of EGF-PE and EGF-PE(Δ 1a), a cytotoxicity blocking assay was performed. A modified non-toxic PE, PE(Δ 576–613), which has

Table I. The IC_{50} of EGF-PE, EGF-PE(Δ 1a) and PE to NRHER5 cells under conditions where either the EGF receptor or PE receptor pathway was blocked

	IC_{50} (ng/ml)		
	EGF-PE	EGF-PE(Δ 1a)	PE
–	1.0	10	3.0
EGF (3 μ g/ml)	5.0	>100	3.0
PE(Δ 576–613) (30 μ g/ml)	1.8	10	>30

NRHER5 cells were plated on 60 mm dishes at a density of 300 cells per dish for 24 h prior to cytotoxic assay. NRHER5 cells were challenged with various concentrations of EGF-PE, EGF-PE(Δ 1a) or PE in the presence of excess amounts of EGF or PE(Δ 576–613). IC_{50} is the toxin concentration required to inhibit relative cloning efficiency by 50%.

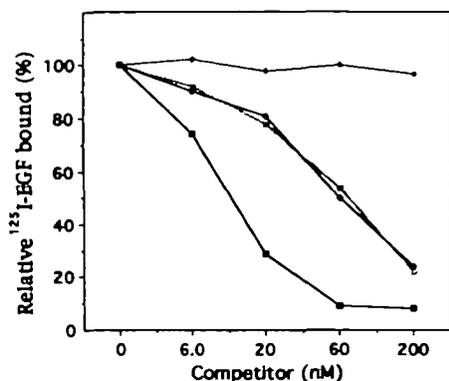


Fig. 6. Competition binding assay: displacement of a trace amount of [125 I]EGF by EGF, PE, EGF-PE, and EGF-PE(Δ Ia). [125 I]EGF, bound to A431 cells in the absence of a competitor, was used as a control and represented 100% [125 I]EGF binding activity. The relative [125 I]EGF bound to A431 cells in the presence of various amounts of EGF, PE, EGF-PE and EGF-PE(Δ Ia) was measured by comparison with that in the absence of a competitor. EGF-PE (\diamond), EGF-PE(Δ Ia) (\square), EGF(\blacklozenge) and PE(\blacklozenge).

Table II. Inhibition of protein synthesis (24 h) by PE, EGF-PE and EGF-PE(Δ Ia) in cell lines with various numbers of EGF receptors

	Number of EGF binding sites per cell	ID_{50} (ng/ml)		
		PE	EGF-PE	EGF-PE(Δ Ia)
A431	2.5×10^6	15	0.5	3.0
CE48T/VGH	5.1×10^5	20	3.0	5.5
CE81T/VGH	3.6×10^5	20	3.5	6.5
CaLu-1	1.6×10^5	30	4.0	8.5
KB-3-1	1.5×10^5	15	3.0	9.0
HA22T/VGH	1.3×10^5	10	3.0	9.0
MCF-7	7.0×10^3	25	25	>200
HUT102	$< 10^3$	60	65	>200
U266	$< 10^3$	70	80	>200

Cells were seeded in a 24-well microtiter plate at a density of 4×10^5 cells per well for 24 h prior to cytotoxic assay. Cells were challenged with various concentrations of PE, EGF-PE or EGF-PE(Δ Ia) in the range 0–200 ng/ml at 37°C for 24 h, followed by examining the [3 H]leucine incorporation. ID_{50} is the protein concentration required to inhibit protein synthesis by 50%. Protein concentrations of PE, EGF-PE and EGF-PE(Δ Ia) were determined by the method of Bradford (1976).

been shown to be able to block PE cytotoxicity (Chow *et al.*, 1989), was used to block the PE receptor pathway during treatment with the chimeric toxins. As shown in Figure 5(A), PE(Δ 576–613), as expected, can effectively block the cytotoxic effect of native PE [Figure 5(A), lane 3]. However, EGF fails to block the cytotoxic effect of native PE, suggesting that the cytotoxicity of PE is through a specific PE receptor pathway [Figure 5(A), lane 2]. We have also observed that neither EGF nor PE(Δ 576–613) alone can block EGF-PE cytotoxicity to NRHER5 [Figure 5(B), lanes 2 and 3]. In order to completely block EGF-PE cytotoxicity in NRHER5, both EGF and PE(Δ 576–613) are required [Figure 5(B), lane 4], suggesting that EGF-PE kills NRHER5 cells through both the EGF receptor and PE receptor pathways. In addition, the cytotoxic effect of EGF-PE(Δ Ia) on NRHER5 can be completely blocked by excess amounts of EGF, but not by excess amounts of PE(Δ 576–613), suggesting that EGF-PE(Δ Ia) exhibits EGF binding activity only [Figure 5(C)]. These results indicate further that EGF-PE(Δ Ia) exhibits target specificity to EGF receptor bearing cells.

We also compared the cytotoxicity of EGF-PE with that of

EGF-PE(Δ Ia) to NRHER5 cells and found that a deletion of the Ia domain of PE in EGF-PE resulted in a 10-fold decrease in cytotoxicity. One speculation concerning the 10-fold decrease in the cytotoxicity of EGF-PE(Δ Ia) was that the Ia domain of PE might play some role in enhancing the cytotoxicity of EGF-PE in addition to the toxin binding activity. To test this possibility, we have measured the IC_{50} of EGF-PE NRHER5 cells under conditions where either the EGF receptor pathway or the PE receptor pathway was blocked. As shown in Table I, when the PE receptor pathway was blocked, the IC_{50} s to NRHER5 cells by EGF-PE and EGF-PE(Δ Ia) were 1.8 and 10 ng/ml respectively. This observation suggests that the Ia domain of PE may make a contribution to the stability of the tertiary structure through direct or indirect interdomain contacts and thereby enhances the activity of EGF-PE during the cytotoxic process. Alternatively, EGF-PE may have a stronger binding affinity than EGF-PE(Δ Ia) for the EGF receptor of NRHER5 cells.

The EGF receptor binding activity of EGF-PE and EGF-PE(Δ Ia)

We have used a binding displacement assay to measure the relative binding activities of EGF, PE, EGF-PE and EGF-PE(Δ Ia) to the EGF receptor. As shown in Figure 6, both EGF-PE and EGF-PE(Δ Ia) were less efficient than EGF, while PE was completely ineffective, in competing with [125 I]EGF for the binding to the EGF receptor, indicating that both EGF-PE and EGF-PE(Δ Ia) use the EGF moiety to bind the EGF receptor and that EGF-PE and EGF-PE(Δ Ia) were weaker ligands for the EGF receptor than EGF (Figure 6). The observation that EGF-PE and EGF-PE(Δ Ia) become weaker ligands may be due to the toxin moiety causing a steric hindrance to EGF, which leads to a decreased affinity for the EGF receptor. Since EGF-PE and EGF-PE(Δ Ia) have equal ability in replacing [125 I]EGF in binding to the EGF receptor, this result suggests that EGF-PE and EGF-PE(Δ Ia) have similar binding affinities for the EGF receptor. Thus, it ruled out the possibility that the enhanced cytotoxicity of EGF-PE is due to a stronger affinity of EGF-PE than EGF-PE(Δ Ia) for the EGF receptor. In this study, we also observed that the IC_{50} s of EGF-PE and PE to NRHER5 cells were 5 and 3 ng/ml respectively, when the EGF receptor pathway was blocked. This difference is due to the fact that EGF fused in front of PE results in steric hindrance to the Ia domain of PE, therefore, decreasing the affinity of EGF-PE to the PE receptor.

Cytotoxicity of EGF-PE(Δ Ia) to human cancer cells

Since EGF-PE(Δ Ia) can specifically kill EGF receptor bearing cells, we thus examined the cytotoxicity of EGF-PE(Δ Ia) to various human cancer cell lines. These cancer cells carried EGF receptors ranging from several hundreds to millions. For example, the A431 cell contains $\sim 2.5 \times 10^6$ EGF receptors per cell, the KB-3-1 cell has $\sim 1.5 \times 10^5$ EGF receptors per cell and the HUT-102 cell carries less than 1000 EGF receptor molecules per cell. Since these human cancer cell lines carried various numbers of EGF receptors, the cytotoxicity of EGF-PE(Δ Ia) to these cancer cells could be used to evaluate the relative sensitivity of cancer cells as a function of EGF-receptor number. The cytotoxicity of PE, EGF-PE and EGF-PE(Δ Ia) to these cancer cells was assayed by incubating 4×10^5 cells in a 1 ml medium with various concentrations of PE, EGF-PE and EGF-PE(Δ Ia) ranging from 0.1 to 200 ng/ml at 37°C for 24 h, followed by examining [3 H]leucine incorporation. We find that A431 cells are very sensitive to EGF-PE(Δ Ia). CE48T/VGH, CE81T/VGH, CaLu-1, KB-3-1 and HA22T/VGH cells are only slightly less sensitive, while MCF-7, HUT102 and U266 cells are resistant to EGF-PE(Δ Ia) (Table II). This result further

supports the previous notion that EGF-PE(Δ Ia) specifically kills EGF receptor bearing cells and that cells with more EGF receptors are more sensitive to EGF-PE(Δ Ia).

Discussion

Pseudomonas exotoxin A, a single-chain polypeptide toxin with a mol. wt of 66 kDa, is one of the most potent cytotoxic agents produced by *Pseudomonas aeruginosa* (Liu, 1966). Because PE is highly toxic to mammalian cells, PE has been chemically conjugated to monoclonal antibodies or growth factors to generate specific cytotoxic agents that are potentially useful in killing a specific population of cells, including cancer cells (FitzGerald, *et al.*, 1983a,b, 1984, 1986; Bjorn *et al.*, 1986; Lyall *et al.*, 1987). However, the chimeric toxins obtained by chemical conjugation were found to retain residual PE activity (Pirker *et al.*, 1985). In addition, the chemical conjugation gave a very low yield due to the inefficient coupling procedures. To overcome these problems, it was proposed to use recombinant DNA techniques to generate chimeric toxins (Pastan and FitzGerald, 1989, 1991). Generation of target-specific toxins have been reported in several successful cases (Chaudhary *et al.*, 1987, 1989, 1990; Lorberboum-Galski *et al.*, 1988; Siegall *et al.*, 1989, 1990). These studies thus prompted us to apply recombinant DNA techniques to generate an EGF-containing chimeric toxin. It may be argued that construction of an EGF-containing chimeric toxin is redundant in terms of engineering a targeting toxin, since TGF α -PE40, an EGF receptor targeting toxin, has already been constructed (Chaudhary *et al.*, 1987). However, as we recently reported, the cytotoxicity of an EGF-containing chimeric toxin may be used as an indicator for the screening of mutations in EGF which are important for EGF-receptor interactions (Shiah *et al.*, 1992). Construction of an EGF-containing chimeric toxin is thus an initial step for the study of structure-function relationships of EGF.

For example, EGF and TGF α display a 35% homology with conservation of all six cysteine residues. Both growth factors interact similarly with the EGF receptor (Marquardt *et al.*, 1984), which results in many common biological activities, such as the stimulation of cell growth, eyelid opening and inhibition of gastric acid secretion (Smith *et al.*, 1985; Rhodes *et al.*, 1986; Carpenter and Cohen, 1990). However, the actions of EGF and TGF α differ subtly in a number of bioassay systems. For instance, TGF α is more effective than EGF in the stimulation of bone resorption *in vitro* (Stern *et al.*, 1985; Ibbotson *et al.*, 1986) and in the promotion of angiogenesis *in vivo* (Schreiber *et al.*, 1986). On the other hand, EGF is more potent than TGF α in the longitudinal muscle bioassay (Yang *et al.*, 1990). In addition, EGF, but not TGF α causes a rapid desensitization of the contractile response in the circular muscle bioassay (Gan *et al.*, 1987). These differences may be due to a difference in the magnitude of receptor binding affinities or in receptor-mediated signal transduction. Since EGF and TGF α have been reported to have the same affinity for the EGF receptor (Marquardt *et al.*, 1984; Schreiber *et al.*, 1986), this subtle variation in biological activities may reflect differences in receptor-mediated signal transduction. As mentioned earlier, EGF and TGF α share only 35% homology with conservation of all six cysteine residues, which presumably contributes to receptor binding. Therefore, it would be reasonable to assume that the subtle differences in biological activities may result from the non-homologous region. Construction of an EGF-containing chimeric toxin may be an initial step in testing this idea.

Using recombinant DNA techniques, we have constructed two fusion proteins, EGF-PE and EGF-PE(Δ Ia). The major difference

between these two chimeric toxins is that EGF-PE contains full-length PE, while EGF-PE(Δ Ia) contains PE with a deletion of the cell recognition domain. Thus, EGF-PE possessed PE binding activity in addition to EGF binding activity. However, EGF-PE(Δ Ia) exhibits EGF binding activity only. As expected, EGF-PE(Δ Ia) was able to kill cells only through the EGF receptor pathway. The target specificity of EGF-PE(Δ Ia) to EGF receptor bearing cells is supported further by the cytotoxicity blocking experiment where the cytotoxicity of EGF-PE(Δ Ia), but not EGF-PE, to NRHER5 can be completely blocked by excess amounts of EGF. Previous studies have shown that PE with a deletion of the Ia domain lost cell binding activity (Hwang *et al.*, 1987). Thus, replacing the cell recognition domain of PE with the EGF moiety will result in the modified chimeric toxin exhibiting EGF binding activity instead of PE binding activity. However, comparing the cytotoxicity of EGF-PE with that of EGF-PE(Δ Ia), we found that a deletion of the Ia domain of PE in EGF-PE not only abolished PE binding activity but also produced a 10-fold decrease in cytotoxicity. Since the removal of PE binding activity from EGF-PE cannot totally account for the 10-fold decrease in EGF-PE(Δ Ia) cytotoxicity, this may indicate that the domain Ia of PE possibly makes a contribution to the stability of the tertiary structure through direct or indirect interdomain contacts to enhance the cytotoxicity of EGF-PE in addition to the toxin binding activity. This notion was further supported by studies of the cytotoxic effect of mutated EGF-PE, designated EGF-PE(Lys57-Glu57), in which the lysine at residue 57 of PE, critical for PE binding activity, was mutated to glutamine, resulting in a loss of PE binding activity. The cytotoxicity of EGF-PE(Lys57-Glu57) on NRHER5 decreased by only 3-fold, when compared to that of EGF-PE (unpublished result), while a deletion of the Ia domain of PE in EGF-PE decreased cytotoxicity to NRHER5 by 10-fold. This result thus suggests that the Ia domain in EGF-PE may play another role in addition to the toxin binding activity in the cytotoxic process. To examine the functional role of the Ia domain of PE, we have constructed PE-EGF molecules with sequential deletions at the N-terminal of the Ia domain, followed by examination of their EGF receptor-binding and cytotoxicity to A431 cells. Our results show that the functional role of the Ia domain in the cytotoxicity of EGF-containing chimeric toxin in A431 cells is to insert a toxin molecule into the plasma lipid bilayers (Liao and Hwang, unpublished result).

Previous studies from Pastan's laboratory have shown that a single chain immunotoxin, OVB3 158-2, which is composed of variable regions of anti-OVB antibody and PE with a deletion of the Ia domain, could effectively bind to OVCAR3 cells. However, this immunotoxin was less toxic to the OVCAR3 cells (Chaudhary *et al.*, 1990), but when the Ia domain of PE was added to OVB3 158-2, the new immunotoxin became highly cytotoxic to OVCAR3 cells. This further strengthens our suggestion that the Ia domain of PE may have another role in addition to the toxin binding activity during the cytotoxic action. Since domain II of PE has been demonstrated to play an important role in mediating translocation of PE into the cytosol (Pastan and FitzGerald, 1989; Siegall *et al.*, 1989; Theuer *et al.*, 1992), one possible role of the Ia domain may be to make a contribution to the stability of the tertiary structure through direct (to translocation domain II) or indirect interdomain contacts and thereby enhance the cytotoxicity of EGF-PE chimeric toxin.

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