Recombinant Immunotoxin Containing a Disulfide-Stabilized Fv Directed at erbB2 That Does Not Require Proteolytic Activation

Chien-Tsun Kuan and Ira Pastan*

Laboratory of Molecular Biology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Building 37, Room 4E16, 37 Convent Drive, MSC 4255, Bethesda, Maryland 20892-4255

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ABSTRACT: PE35/e23(dsFv)KDEL is a recombinant immunotoxin composed of a recombinant form of *Pseudomonas* exotoxin that does not need proteolytic activation and a disulfide-stabilized Fv fragment of the anti-erbB2 monoclonal antibody e23. In this molecule, the variable heavy (V_H) domain is inserted near the carboxyl terminus of PE at position 607 and the variable light (V_L) domain is connected to the V_H domain by a disulfide bond engineered into the framework region. The disulfide bond forms between cysteines introduced at position 44 of V_H and position 99 of V_L [Reiter *et al*. (1994) *J. Biol. Chem. 269*, 18327−18331]. In contrast to other PE-derived Fv fusion proteins, this type of recombinant toxin does not need proteolytic activation of the toxin domain. PE35/e23(dsFv)KDEL is very cytotoxic toward erbB2 antigen-expressing N87 cells (IC_{50} = 0.8 ng/mL) despite the fact that it binds to the erbB2 protein only 25% as well as e23(dsFv)PE38KDEL, in which the dsFv moiety is located at the amino terminus of the toxin. The lower binding affinity is probably due to interference by domain III of PE with the amino terminus of e23(V_H), possibly where the antigen binding sites are located. Nevertheless, the specificity of immunotoxin is still retained, and it is very stable at 37 °C. Because of its small size, stability, and activity without proteolytic processing, this immunotoxin may be advantageous for tumor treatment. PE35/e23(dsFv)KDEL was also used to gain information about whether reduction of the disulfide bonds connecting V_H and V_L occur in the endoplasmic reticulum (ER) or in a proximal compartment. To do this, we switched the ER retention sequence KDEL from the toxin−V_H subunit to the V_L subunit. Our results suggest that reduction of the disulfide bond connecting the dsFv heterodimer occurs before the immunotoxin reaches the ER, where translocation to the cytosol appears to occur.

Immunotoxins were initially produced by chemically coupling antibodies to toxins (Vitetta & Uhr, 1985; Pastan et al., 1992). The antibody portion mediates selective binding and the toxin mediates killing of target cells. Several toxins have been used to make immunotoxins, including ricin A chain, blocked ricin, saporin, pokeweed antiviral protein, diphtheria toxin, and *Pseudomonas* exotoxin A (PE)[1] (Pastan & FitzGerald, 1991; Vitetta & Thorpe, 1991; Tazzari et al., 1992; Uckun et al., 1992). Several clinical trials with immunotoxins have been carried out and they have shown activity against lymphomas and other cancers derived from the hematopoietic system (Vitetta et al., 1991; Grossbard et al., 1993). However, these immunotoxins are heterogeneous and their large size limits penetration into solid tumors. Second-generation immunotoxins are totally recombinant molecules made by fusing the smallest functional module of an antibody, the Fv fragment, to a truncated toxin which lacks the cell-binding domain (Brinkmann et al., 1991; Kreitman et al., 1992). The small size of single-chain Fv immunotoxins should make them much more useful than chemical conjugates of whole antibodies for certain therapeutic applications because their small size increases tumor penetration and efficacy (Fukimori et al., 1989; Jain, 1990; Sung et al., 1990).

Several types of recombinant Fv immunotoxins containing PE have been made and tested *in vitro* as well as in animal models (Brinkmann et al., 1991; Kreitman et al., 1992; Batra et al., 1992; Reiter et al., 1994d; Brinkmann et al., 1993a). Initially, the Fv regions of the immunotoxins were arranged in a single-chain form (scFv immunotoxin) with the V_H and V_L domains connected by a linking peptide. More recently, disulfide-stabilized forms of Fv immunotoxins (dsFv immunotoxins) have been generated in which the V_H and V_L domains are connected by a disulfide bond engineered into the framework region (Brinkmann et al., 1993c; Reiter et al., 1994c). We have compared the stability of three different single-chain and dsFv immunotoxins, and in all three cases the dsFv immunotoxins were more stable (Reiter et al., 1994b,c).

Recombinant immunotoxins containing PE must be proteolytically activated within the cell by cleavage in domain II between amino acids 279 and 280 (Ogata et al., 1992). To eliminate the need for intracellular proteolytic activation and thereby increase cytotoxic activity, the toxin moiety of recombinant toxins has been modified. This was initially done with recombinant toxins containing TGFα by producing a truncated toxin (PE280−613) with TGFα inserted near the end of domain III at position 607 (Figure 1) (Theuer et al., 1993a,b). Because the toxin begins at position 280, it does not need proteolytic activation within the cell (Ogata et al., 1992; Theuer et al., 1992). In addition, these molecules had

* To whom correspondence should be addressed.

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[1] Abbreviations: V, variable; H, heavy; L, light; scFv and dsFv, single-chain and disulfide-stabilized Fv, respectively; CDR, complementary-determining region; PE, *Pseudomonas* exotoxin; MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline; ER, endoplasmic reticulum.

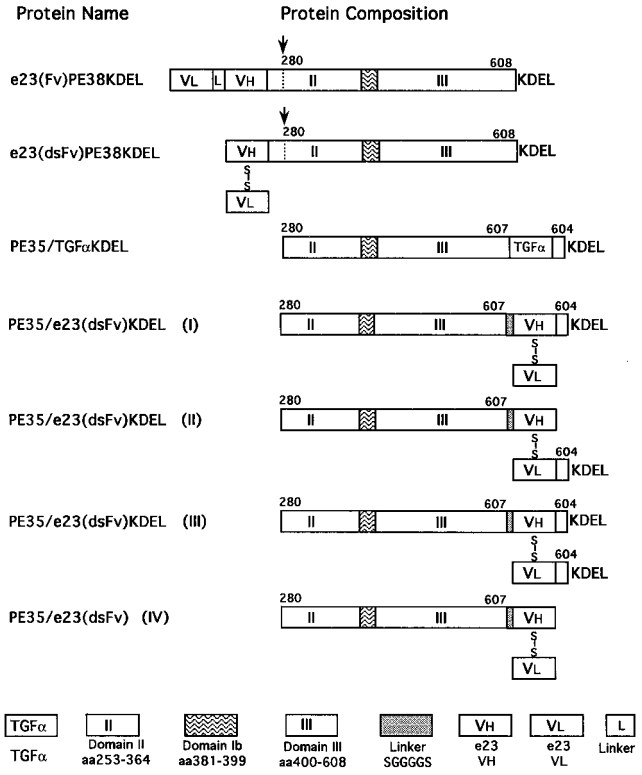

FIGURE 1: Schematic of expressed proteins. Positions of amino acids that span PE sequences are numbered. The amino acids listed in the one-letter code are the C-terminal residues. The arrow sign marks the proteolytic site of PE for activation. S−S shows the disulfide bond linkage between the Fv fragments.

two other mutations (Figure 1). One was deletion of unnecessary residues in domain Ib (365−380). The other was to change the carboxyl terminus from REDLK to KDEL to increase cytotoxic activity (Seetharam et al., 1991). This molecule, termed PE35/TGFαKDEL, is 10−700-fold more active than TGα-PE40 on several human bladder cancer cell lines (Theuer et al., 1993a).

Our goal in this study was to construct a recombinant immunotoxin that was smaller in size than the current recombinant PE40/38-derived immunotoxins and that did not need intracellular proteolytic processing to convert it to an active form. Therefore, we inserted the V_H fragment of monoclonal antibody e23 into domain III near its carboxyl terminus at the position used for TGFα (Theuer et al., 1993a) and linked the V_H domain to the V_L domain of e23 Fv, directed to erbB2, with a disulfide bond (Figure 1), located in conserved framework regions of the Fv as previously described (Reiter et al., 1994a). We report here the successful construction of a dsFv immunotoxin that does not need proteolytic processing.

EXPERIMENTAL PROCEDURES

Construction of Plasmids. All plasmids listed in Figure 2 use an isopropyl 1-thio-β-D-galactopyranoside-inducible T7 promoter expression system (Studier & Moffatt, 1986). pCT12 encodes a protein, termed PE35/TGFαKDEL, starting with a Met at position 280 of PE and amino acids 281−364 and 381−607, with a gene encoding TGFα inserted after residue 607 and followed by residues 604−608 and KDEL, which are substituted for the carboxyl-terminal REDLK sequence of PE (Theuer et al., 1993a). pYR39 encoding e23(V_HCys$_{44}$)PE38KDEL is the expression plasmid for the

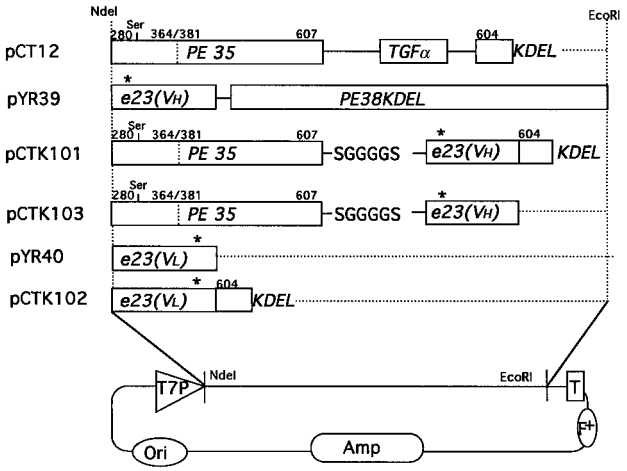

FIGURE 2: Plasmids for expression of dsFv immunotoxins. Positions of cysteine replacement (shown as asterisks) in the framework region of e23(Fv) are Asn$_{44}$ → Cys in V_H and Gly$_{99}$ → Cys in V_L as described previously (Reiter et al., 1994). pCT12 encodes a protein, termed PE35/TGFαKDEL, containing a Met at position 280 and amino acids 281−364 and 381−607 with a gene encoding TGFα inserted between amino acids 607 and 604 of PE, and the carboxyl-terminal amino acids KDEL are substituted for the native REDLK sequence. pCTK101 and pCTK103, encoding PE35/e23(V_HCys$_{44}$)KDEL and PE35/e23(V_HCys$_{44}$), respectively, are the expression plasmids for the toxin−V_H components of the dsFv immunotoxin PE/e23(dsFv)KDEL. pYR39 and pYR40 encode e23(V_HCys$_{44}$)PE38KDEL and e23(V_LCys$_{99}$), respectively. They are the expression plasmids for the component of the dsFv immunotoxin (Reiter et al., 1994a). pCTK102 encodes a protein containing e23(V_LCys$_{99}$), PE amino acids 604−608, and the carboxyl-terminal sequence KDEL.

V_H−toxin components of the dsFv immunotoxin e23(dsFv)-PE38KDEL (Reiter et al., 1994). pCTK101 and pCTK103, encoding PE35/e23(V_HCys$_{44}$)KDEL and PE35/e23(V_HCys$_{44}$), respectively, are the expression plasmids for the toxin−V_H components of the dsFv immunotoxin PE/e23(dsFv)KDEL. They were constructed by cloning the *Stu*I−*Eco*RI-digested PCR fragments into *Stu*I−*Eco*RI restriction sites in pCT12. The PCR was carried out using 10 ng of pYR39 as template and 100 pmol of primers 5′-AAACCGAGGCCTTCCGGAGGTGGTGGATCCGAAGTGCAGCTGCAGGAGTCAGGA-3′ and 5′-TTAGCAGCCGAATTCTTAGAGCTCGTCTTTCGGCGGTTTGCCGGAGGAGACGGTGACCGTGGTCCCTG-3′ for PE35/e23(V_HCys$_{44}$)KDEL or 5′-AAACCGAGGCCTTCCGGAGGTGGTGGATCCGAAGTGCAGCTGCAGGAGTCAGGA-3′ and 5′-GATCGCTCGGAATTCTTAGGAGACGGTGACCGTGGTCCCTGC-3′ for PE35/e23(V_HCys$_{44}$). The protein encoded by pCTK101 is a single-domain immunotoxin in which e23(V_HCys44) was introduced between residue 607 of PE, followed by a peptide linker SGGGGS, and residues 604−608 and KDEL. The protein encoded by pCTK103 was the same as the pCTK101-encoded protein except without amino acids 604−608 and KDEL. pYR40 encodes e23(V_LCys$_{99}$), the V_L component of the dsFV immunotoxin (Reiter et al., 1994a). pCTK102 encodes e23(V_LCys$_{99}$) fused to PE amino acids 604−608 and carboxyl-terminal sequences KDEL. This plasmid was constructed by subcloning a *Nde*I−*Eco*RI-digested PCR product, which used pYR40 as template and a T7 promoter primer as well as 5′-TTAGCAGCCGAATTCTTAGAGCTCGTCTTTCGGCGGTTTGCCGGAGGAGACGGTGACCGTGGTCCCTG-3′ as PCR primers, into *Nde*I−*Eco*RI restriction sites found in pYR40. Positions of cysteine replacement in the framework region of e23(Fv) are Asn$_{44}$

→ Cys in V_H and Gly_{99} → Cys in V_L were described previously (Reiter et al., 1994a). All plasmids were confirmed by DNA sequencing.

Production of Recombinant Proteins. The components of the disulfide-stabilized immunotoxins PE35/e23(V_HCys_{44})-KDEL, PE35/e23(V_HCys_{44}), e23(V_HCys_{44})PE38KDEL, e23-(V_LCys_{99}), and e23(V_LCys_{99})KDEL or single-chain immunotoxins were produced in separate *Escherichia coli* BL21(λDE3) (Studier & Moffatt, 1986) cultures harboring the corresponding expression plasmid (see Figure 2). All recombinant proteins accumulated in inclusion bodies. Properly folded disulfide-stabilized immunotoxins were obtained by mixing equimolar amounts of solubilized and reduced inclusion bodies into redox-shuffling refolding buffer containing an aggregation-preventing additive, as described previously (Reiter et al., 1994d), except that the final oxidation step was omitted and refolding was carried out at pH 9.5. As illustrated in Figure 1, PE35/e23(dsFv)KDEL (**I**) was produced by mixing PE35-e23(V_HCys_{44})KDEL and e23(V_LCys_{99}); PE35/e23(dsFv)KDEL (**II**) was producing by mixing PE35-e23(V_HCys_{44}) and e23(V_LCys_{99})KDEL; PE35/e23(dsFv)KDEL (**III**) was produced by mixing PE35-e23(V_HCys_{44})KDEL and e23(V_LCys_{99})KDEL; and PE35/e23(dsFv) (**IV**) was produced by mixing PE35-e23(V_HCys44) and e23(V_LCys99). Properly folded disulfide-stabilized and single-chain immunotoxins were purified by sequential ion-exchange chromatography (Q-Sepharose and Mono Q) followed by size-exclusion chromatography on a TSK G3000SW (Toso Haas) column (Reiter et al., 1994b).

Analysis of Immunotoxins. The cytotoxic activity of immunotoxins was determined by inhibition of protein synthesis. Target cells were incubated for 20 h in 96-well plates containing 1.5×10^4 cells in 200 μL of medium each, with various dilutions of immunotoxin in PBS containing 0.2% human serum albumin (HSA). Inhibition of protein biosynthesis by the toxins was determined by measuring the incorporation of tritium-labeled leucine as described before (Kuan et al., 1994). Thermal stability of the immunotoxins was determined by incubating them at 100 μg/mL in PBS at 37 °C for 2 or 8 h, followed by analytical chromatography on a TSK G3000SW (Toso Haas) column to separate the monomers from dimers and larger aggregates. Relative binding affinities of the immunotoxins were determined by adding ^{125}I-labeled e23IgG to 10^5 N87 cells as a tracer with various concentrations of the competitor. The binding assays were performed at 4 °C for 2 h in RPMI medium containing 1% bovine serum albumin and 50 mM MES (Sigma) as described (Batra et al., 1992).

RESULTS

Our goal was to construct an active recombinant immunotoxin that was smaller than the current generation of recombinant immunotoxins and that did not need intracellular proteolytic cleavage for activation. To do that we inserted e23(dsFv) near the carboxyl terminus of PE35KDEL, a truncated form of PE that contains only the portion of the toxin that undergoes translocation to the cytosol (Figure 1).

Plasmid Constructions and Production of PE35/e23(dsFv)-KDEL. Because the V_H−toxin was found to precipitate during dialysis (Reiter et al., 1994b), this dialysis step led to reduced contamination with the single-domain toxin during purification of the dsFv toxin. Therefore we inserted V_H rather than V_L near the carboxyl terminus of PE35KDEL to make PE35/e23(V_H)KDEL, which was connected to e23-(V_L) by a disulfide bond. The disulfide bond forms between cysteines introduced at position 44 of the V_H and position 99 of V_L (Reiter et al., 1994a). In the toxin portion, cysteine 287 was changed to a serine to reduce the chance of incorrect disulfide-bond formation (Theuer et al., 1993a; Figure 2). The location we chose for e23(V_HCys_{44}) insertion was after amino acid 607 of PE and it was preceded by a small peptide linker SGGGGS. Following the V_H domain are amino acids 604−608 and KDEL. Diagrams of this molecule, PE35/e23(dsFv)KDEL (I), constructed as described in Materials and Methods are shown in Figures 1 and 2. Immunotoxins were expressed in *E. coli* BL21(λDE3); cultures for expressing the components of the dsFv immunotoxin were prepared separately. The immunotoxins were purified by refolding of inclusion bodies in a redox-shuffling buffer and sequential ion-exchange and gel-filtration chromatography as described in Materials and Methods. As shown in lane 1 of Figure 3, the proteins obtained were over 95% homogeneous and had the expected molecular mass on SDS−PAGE (60 kDa). This new immunotoxin, e23(dsFv)PE35KDEL, is 3 kDa (5%) smaller than e23(dsFv)PE38KDEL. In the presence of the reducing agent β-mercaptoethanol, the dsFv immunotoxin PE35/e23(dsFv)KDEL (**I**), as shown in lane 5, was reduced into two species; one is e23(V_LCys_{99}) and the other is a single-domain toxin PE35/e23(V_HCys_{44})KDEL. The apparent molecular masses of these components, as expected, 13 and 47 kDa, respectively.

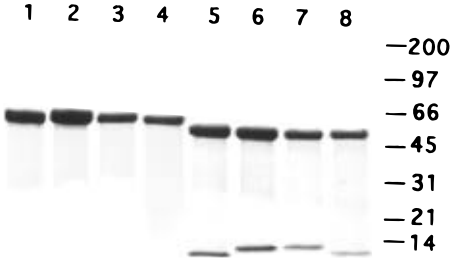

FIGURE 3: Purification of PE35/e23(dsFv)KDEL by SDS−4−20% PAGE. Lane 1, PE35/e23(dsFv)KDEL (**I**), nonreduced; lane 2, PE35/e23(dsFv)KDEL (**II**), nonreduced; lane 3, PE35/e23(dsFv)-KDEL (**III**), nonreduced; lane 4, PE35/e23(dsFv) (**IV**), nonreduced; lane 5, PE35/e23(dsFv)KDEL (**I**), reduced; lane 6, PE35/e23(dsFv)-KDEL (**II**), reduced; lane 7, PE35/e23(dsFv)KDEL (**III**), reduced; lane 8, PE35/e23(dsFv) (**IV**), reduced.

Specific Cytotoxic Activity of PE35/e23(dsFv)KDEL toward e23-Antigen-Expressing Cell Lines. The cytotoxicity of PE35/e23(dsFv)KDEL was determined by measuring the reduction in the incorporation of [^{3}H]leucine by various human cancer cell lines after treatment with serial dilutions of the immunotoxin as described in Materials and Methods. e23(scFv)PE38KDEL and e23(dsFv)PE38KDEL were included for comparison. Figure 4 and Table 1 show a comparison of the activity of the immunotoxin PE35/e23-(dsFv)KDEL (**I**) and the other two reference molecules, e23-(scFv)PE38KDEL and e23(dsFv)PE38KDEL, and indicates that all three proteins are cytotoxic to cells expressing erbB2 (e.g., N87 and A431) but not to cells (e.g., HUT-102) that do not bind monoclonal antibody e23 (Table 1). In this assay, PE35/e23(dsFv)KDEL(**I**) had an IC_{50} of 0.8 ng/mL of N87 cells. Although its activity is slightly less than those of the two other molecules [IC_{50} of 0.5 ng/mL for e23(scFv)-PE38KDEL and 0.1 ng/mL for e23(dsFv)PE38KDEL], it is still extremely active.

Table 1: Cytotoxicity of Recombinant e23 Immunotoxins toward Various Cell Lines[a]

cell line	cancer type	e23 antigen	cytotoxicity $IC_{50} \pm SD$ (ng/mL) e23(Fv)PE38KDEL	e23(dsFv)PE38KDEL	PE35/e23(dsFv)KDEL (**I**)
N87	gastric	+++	0.5 ± 0.04	0.1 ± 0.02	0.8 ± 0.01
A431	epidermoid	+	2.9 ± 0.16	1.0 ± 0.05	3.0 ± 0.14
Hut102	leukemia	−	>1000	>1000	>1000

[a] Cytotoxicity data are given as IC_{50} values, the concentration of immunotoxin that causes a 50% inhibition of protein synthesis after a 20-h incubation with immunotoxin. The level of antigen expression is marked +++, +, and − for high, lower, and no detectable expression, respectively. Each protein was assayed on each cell line in triplicate and repeated three times.

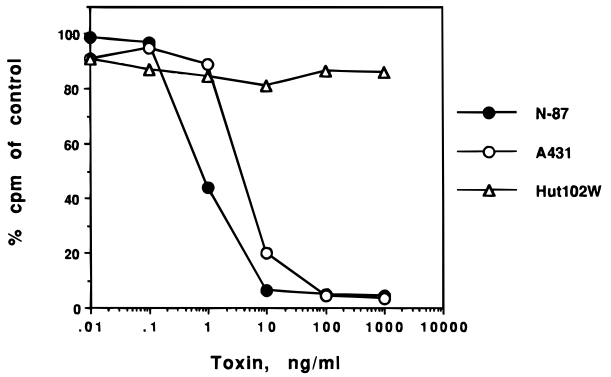

FIGURE 4: Specific cytotoxicity of e23-related Fv immunotoxins toward different cell lines. Cytotoxicity of PE35/e23(dsFv)KDEL (**I**) toward cell lines N-87 (●), A431 (○), and Hut-102 (△) is shown.

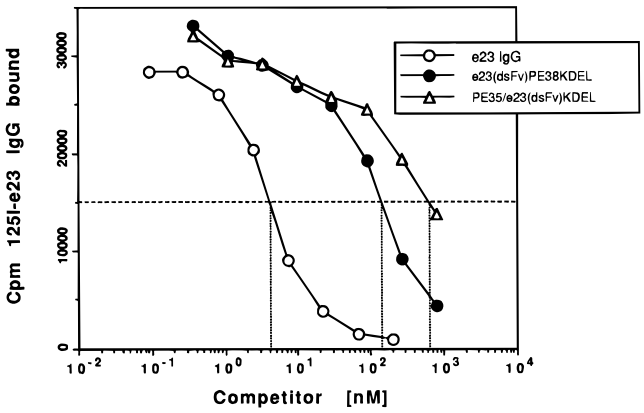

FIGURE 5: Binding of PE35/e23(dsFv)KDEL and e23(dsFv)-PE38KDEL to N87 cells. Competitive binding analysis of the ability of purified PE35/e23(dsFv)KDEL (**I**) and e23(dsFv)-PE38KDEL to inhibit the binding of ^{125}I-labeled e23 IgG to cells overexpressing erbB2 is shown: e23 IgG (○); e23(dsFv)-PE38KDEL (●); PE35/e23(dsFv)KDEL (△).

*Antigen-Binding Analysis of PE35/e23(dsFv)KDEL (**I**).* To investigate the reason for the decreased cytotoxicity of PE35/e23(dsFv)KDEL (**I**), we analyzed its antigen binding affinity on antigen-positive cells (e.g., N87 cells) by competition assays, in which increasing concentrations of each immunotoxin were present to compete for the binding of ^{125}I-e23-IgG to N87 cells at 4 °C. The results shown in Figure 5 indicate that e23 IgG, e23(dsFv)PE38KDEL, and PE35/e23(dsFv)KDEL competed for the binding of ^{125}I-e23 IgG to N87 cells by 50% at 4 nM, 140 nM, and 500 nM, respectively. Thus, the binding affinity of PE35/e23(dsFv)-KDEL (**I**) is 4-fold less than that of e23(dsFv)-PE38KDEL on N87 cells. Hence, the lower cytotoxicity of PE35/e23-(dsFV)KDEL (**I**) is associated with a lower binding affinity. As previously reported, the bivalent e23 IgG had a higher apparent affinity than e23(dsFv)PE38KDEL, which is monovalent (Reiter et al., 1994a).

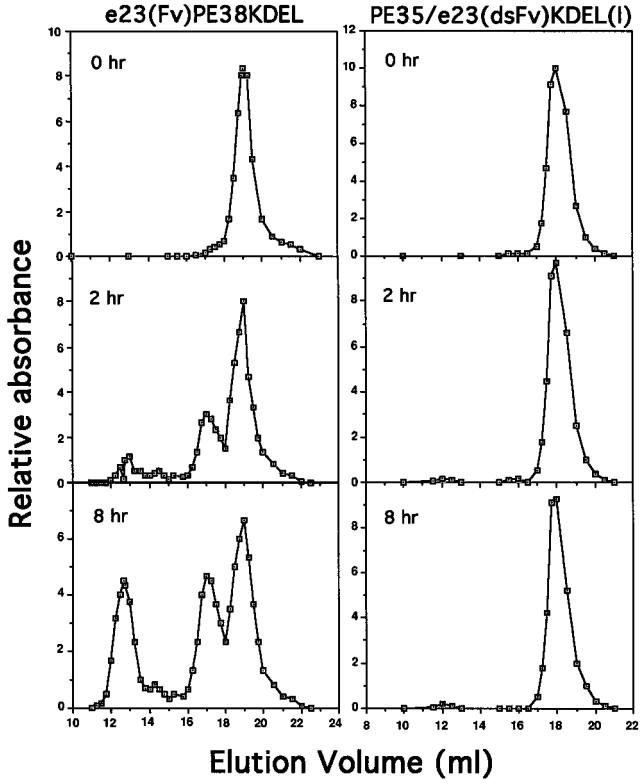

FIGURE 6: Stability of PE35/e23(dsFv)KDEL immunotoxin in buffered saline. PE35/e23(dsFv)KDEL (**I**) and e23(Fv)PE38KDEL were diluted in PBS to 0.1 mg/mL, incubated at 37 °C for 2 and 8 h, and then analyzed by size-exclusion chromatography at 4 °C, as described in Materials and Methods. Monomers elute at 18−20 mL, dimers elute at 16−18 mL, and aggregates elute at 11−13 mL. Proteins prior to incubation at 37 °C were shown as 0 h.

*Improved Stability of Immunotoxin PE35/e23(dsFV)KDEL (**I**).* The thermal stability of PE35/e23(dsFv)KDEL (**I**) was examined and compared with that of e23(Fv)PE38KDEL by measuring the amount of aggregation and inactivation at 37 °C as described in Materials and Methods. Figure 6 shows the chromatographic profiles of scFv and dsFv immunotoxins after incubation in PBS for 2 or 8 h at 37 °C. PE35/e23-(dsFv)KDEL (**I**) was a monomer before incubation in PBS at 37 °C and remained monomeric for 2 or 8 h. In contrast, the single-chain immunotoxin e23(Fv)PE38KDEL formed 30% aggregates and 25% dimers after an 8-h incubation at 37 °C. As shown in Figure 7, following the 8-h 37 °C treatment, PE35/e23(dsFv)KDEL (**I**) retained almost the same cytotoxic activity as before treatment, while e23(Fv)-PE38KDEL had an IC_{50} of 3.1 ng/mL on N87 cells, which is only 16% of its cytotoxic activity before treatment. This result indicates that the purified PE35/e23(dsFv)KDEL, like e23(dsFv)PE38KDEL (Reiter et al., 1994c), is very stable and has a low propensity to aggregate.

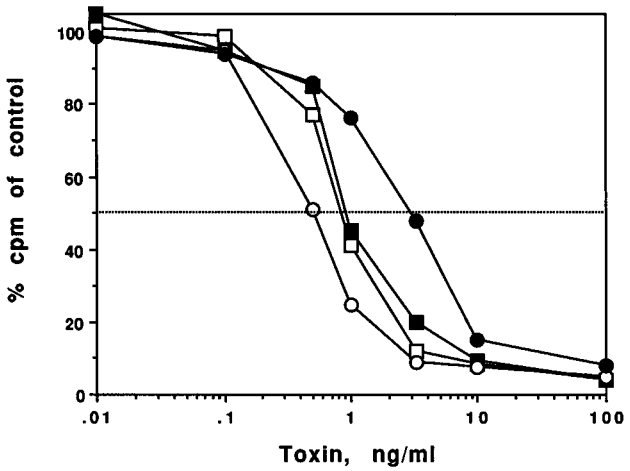

FIGURE 7: Cytotoxicity of immunotoxins assayed on N87 cells before or after an 8-h incubation in PBS at 37 °C: e23(scFv)-PE38KDEL, 0 h (○); e23(scFv)PE38KDEL, 8 h (●); PE35/e23-(dsFv)KDEL, 0 h (□); and PE35/e23(dsFv)KDEL, 8 h (■). Each protein was assayed on the N87 cell line in triplicate and repeated two times.

Table 2: Comparison of Four Different Types of PE35/e23(dsFv) Immunotoxins

construct	activity,[a] IC_{50} (ng/mL)	relative binding[a,b] (nM)
PE35/e23(dsFv)KDEL (**I**)	0.8 ± 0.01	500 ± 8.3
PE35/e23(dsFv)KDEL (**II**)	1000 ± 8.2	400 ± 11.4
PE35/e23(dsFv)KDEL (**III**)	1.2 ± 0.05	530 ± 37
PE35/e23(dsFv) (**IV**)	>1000	610 ± 28

[a] Cytotoxicity and binding assays were measured on the N87 cell line. Each protein was assayed on the N87 cell line in triplicate and repeated two times, and standard errors are shown. [b] The concentration of competitor which caused 50% inhibition of the binding of ^{125}I-e23 IgG. The compositions of **I**−**IV** are shown in Figure 1.

Importance of the Position of KDEL for Cytotoxicity. In PE35/e23(dsFv)KDEL (**I**), the KDEL is on the same polypeptide chain as the toxin moiety. The KDEL sequence is considered to mediate transport of the toxin moiety of the immunotoxin to the ER where it can translocate. We asked whether it was important to have the KDEL sequence on the C-terminus of the toxin or whether it could be attached to the C-terminus of the V_L, which is attached to V_HPE35 by a disulfide bond. To address this question, we constructed molecules with KDEL on V_L instead of the V_H−toxin, with KDEL on both the V_H−toxin and the V_L, and with KDEL on neither (Figure 1). These were termed PE35/e23(dsFv)-KDEL (**II**−**IV**) (Table 2 and Figure 1). Table 2 shows that, for the recombinant toxin to inhibit protein synthesis on target cells, it is important to have the KDEL on the same polypeptide as the toxin moiety. If no KDEL is present, toxicity is lost. If KDEL is on the V_L domain, cytotoxicity is also lost. The presence of KDEL on V_L in addition to V_H−toxin does not change cytotoxic activity. Thus the KDEL sequence must be on the same polypeptide chain as the toxin.

To investigate the possibility that the affinity of the four different types of PE35/e23(dsFv)KDEL to target cells may be different, we conducted binding assays to antigen-positive cells (N87) for each molecule. Table 2 shows that there is very little difference in binding affinities among the four molecules. Thus the differences in cytotoxicities can be attributed to whether the KDEL sequence is on the toxin molecules or not.

DISCUSSION

In order to kill cells, PE and recombinant toxins containing PE need to undergo a series of steps. Intoxication starts with cell binding followed by internalization via coated pits into the endocytic compartment where the toxin is cleaved between residues 279 and 280. After reduction of a disulfide bond, the C-terminal fragment is transported to the endoplasmic reticulum, where translocation to the cytosol occur s. In the cytosol, elongation factor 2 is inactivated by the translocated fragment, which results in protein synthesis inhibition and cell death (Pastan et al., 1992).

One important step is proteolytic processing, which is catalyzed by the enzyme furin (Chiron et al., 1994). Because some cancer cells might have low furin levels and because this step could be rate-limiting, we sought to produce a recombinant immunotoxin that did not need to be activated by furin. This was achieved by inserting the disulfide-bonded form of the Fv fragment of an antibody to erbB2 (e23) near the carboxyl end of a PE-derived toxin (Figure 1), where it would be translocated into the cytosol with domains II and III. Since proteolysis is not needed, one might expect this toxin to be more active than a toxin which needs to be proteolytically processed like e23(dsFv)-PE38KDEL, but in fact when tested on two cell lines, it was found to be 5−10-fold less active. Several factors may contribute to the observed decrease in activity. One is that the binding of the dsFv to erbB2 is reduced 3−4-fold, probably due to the close proximity of the dsFv to domain III of PE. A second possibility is that the size of the fragment of PE that must be translocated to the cytosol is increased by 12 kDa by including the Fv, and this 12-kDa Fv domain has two internal disulfide bonds that may need to be reduced. The third possibility is that the dsFv fragment is located close to KDEL and could interfere with binding of the toxin to the KDEL receptor that is required for transport to the endoplasmic reticulum. Insertion of the dsFv at other locations such as in the location of domain Ib region (amino acid 365−394) in PE35 may improve binding and overcome this loss of activity.

To determine whether the KDEL sequence that is hypothesized to promote transfer of the toxin to the endoplasmic reticulum must be on the toxin-containing moiety or could be on the Fv that is disulfide-linked to the toxin, we made several other constructions (Figure 1). We found that the KDEL must be peptide-bonded to the toxin as in PE35/e23-(dsFv)KDEL (**I**) (Figure 1). If it is on the V_L as in PE35/e23(dsFv)KDEL (**II**), cytotoxic activity is abolished. This finding strongly suggests that the disulfide bond connecting V_H and V_L is reduced before PE35/e23(dsFv)KDEL reaches the ER. If the KDEL sequence becomes separated from the toxin moiety before it reaches the ER, then the toxin moiety cannot reach the ER from which compartment translocation takes place. However, other interpretations are possible but we think less likely. One is that disulfide bond reduction occurs in the ER (e.g., by protein disulfide isomerase) but that the toxin fragment without KDEL rapidly leaves the ER before translocation occurs. Another is that KDEL is required as part of the translocation process.

In this study, we also have shown that PE can deliver an antibody V_H domain to the cytosol of cells. Unlike previous immunotoxins with dsFv at the amino terminus, in PE35/e23(V_H)KDEL V_H is not removed by proteolysis but appears

to be translocated along with domain II and III of PE. The ability to deliver antibody V_H domains, which can show specific antigen-binding properties, to the cytosol might greatly expand the therapeutic application of engineered antibody fragments.

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