Immunotoxins That Target an Oncogenic Mutant Epidermal Growth Factor Receptor Expressed in Human Tumors

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ABSTRACT

Human cancers arise from a series of mutations, many of which direct the expression of mutant proteins with altered functions. These aberrant proteins are attractive targets for new therapeutic agents. One such protein is a mutant epidermal growth factor receptor (EGFRvIII) that has an in-frame deletion near the NH_2 terminus of its extracellular domain. This protein was first identified in human gliomas, but has also been shown to be present in lung and breast carcinomas. The deletion results in a receptor with constitutive tyrosine kinase activity that enhances the tumorigenicity of glioblastomas *in vivo*. The deletion also creates a tumor-specific cell-surface sequence at the deletion junction. Three specific anti-EGFRvIII mAbs have been isolated following immunization with a mixture of a deletion junction synthetic peptide and EGFRvIII as present on cell membranes. We have constructed immunotoxins by conjugating a modified version of *Pseudomonas* exotoxin A to these mAbs. Immunotoxins were tested on cells that had been transfected with cDNA for the EGFRvIII receptor and expressed receptor protein at 5×10^5 receptors/cell. All three immunotoxins were cytotoxic to these cells, with 50% inhibition of protein synthesis occurring in a 15–50 pM range. The immunotoxins specifically targeted EGFRvIII, as their cytotoxicity could be blocked by their respective free antibody. They showed little or no cytotoxicity to cells expressing high levels of normal epidermal growth factor receptors, demonstrating that they are able to discriminate between cells expressing the mutant receptor and those expressing the wild-type receptor. Immunotoxins targeted to mutant epidermal growth factor receptors are promising candidates for further development as tumor cell-specific therapeutic agents.

INTRODUCTION

Immunotoxins are experimental therapeutic agents for cancer that have shown promising results in Phase I clinical trials (1). They consist of a targeting moiety, usually an antibody or antibody fragment, joined to a protein toxin. The most widely used toxins are diphtheria toxin, ricin, and PE[2] (2). We have worked with PE, which was the first toxin for which a structural model was available (3). This has facilitated modification of the toxin using the techniques of protein engineering. PE has three domains, each associated with a different function. Domain Ia mediates binding of the toxin to the α_2-macroglobulin receptor (4). Domain II mediates the translocation of the toxin into the cytosol of cells. Domain III catalyses the ADP ribosylation of elongation factor 2, leading to the inhibition of protein synthesis and cell death. The toxin can therefore be targeted to different cell types by substituting domain Ia with protein domains that recognize different cell surface receptors.

Ideally, targets for immunotoxins should be expressed only on tumor cells. In practice, it may be possible to use many targets that are expressed to some extent on normal cells. These cells can be protected by their presence in separate anatomical compartments, by their comparatively low level of expression of the target, or by the fact that they are rapidly regenerated in the body and/or are not life-sustaining. However, reaction of the immunotoxin with these nontumor cells can lead to dose-limiting toxicities that reduce the effectiveness of the immunotoxin as a therapeutic agent.

Recently, mutant versions of the epidermal growth factor receptor have been identified in human glioblastomas (5–7). The most common mutation, designated EGFRvIII, is a deletion of exons 2–7 in the EGFR gene that results in the expression of EGFR mRNA with an 801-base deletion (5). This mutation has been found in about 17% of malignant gliomas (5), in 16% of lung carcinomas (8), and also in breast carcinomas.[3] The fact that the same mutation has been found independently in different patients suggests that the specific mutation contributes to the malignant phenotype. Consistent with this, it has been shown that the mutation results in a receptor with constitutive tyrosine kinase activity (9, 10) and that cDNA for the mutant receptor transforms NIH/3T3 cells as judged by morphological criteria and focus-forming assays (9). Nishikawa *et al.* (11) have shown that EGFRvIII cDNA expressed in the human glioblastoma cell

Received 3/7/95; revised 4/19/95; accepted 4/24/95.

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[2] The abbreviations used are: PE, *Pseudomonas* exotoxin A; EGFR, epidermal growth factor receptor; IC_{50}, 50% inhibitory concentration.

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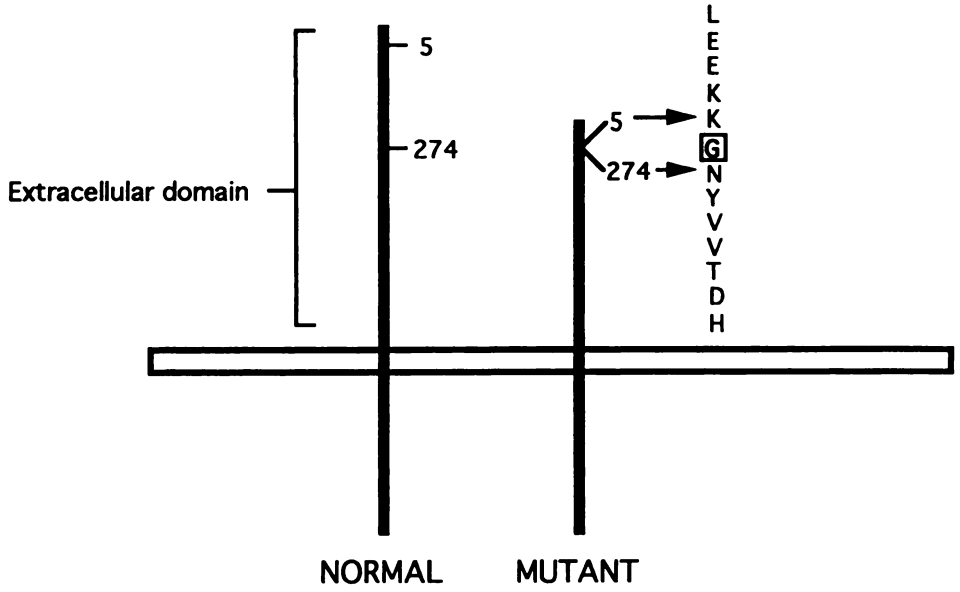

Fig. 1 Mutant EGFR sequence targeted by immunotoxins. The mutant EGFR has an in-frame deletion in its gene that removes the coding sequence for amino acids 6–273 of the extracellular domain, and forms a new glycine codon at the deletion junction. The amino acid sequence of the peptide used to raise the mAbs is shown on the *right*, with the glycine formed at the deletion junction *boxed*.

line U87MG increases the tumorigenicity of these cells when implanted s.c. or intracerebrally in nude mice.

The EGFRvIII mutation results in the expression of an EGFR mRNA that has an in-frame deletion of the sequences coded for by exons 2–7 and a new glycine codon formed at the splice junction between exons 1 and 8. This mRNA directs the expression of a truncated protein in which amino acids 6–273 are replaced by a single glycine residue (Fig. 1), deleting domain I and the cysteine-rich domain II of the extracellular domain of the EGFR (12). The junction sequence shown in Fig. 1 is a new, tumor-specific sequence. We have shown previously that a 14-amino acid peptide spanning the junction can function as an immunogen in rabbits, generating antisera that specifically bind EGFRvIII (5). Thus, the junction sequence is tumor specific, antigenic, and present on the cell surface, making it an attractive target for immunotoxins. Here, we have coupled a modified version of PE to mAbs raised by immunization with a mixture of a junction sequence synthetic peptide and EGFRvIII present on cell membranes. These conjugates were found to be specifically toxic to cells expressing EGFRvIII.

MATERIALS AND METHODS

Cell Lines. NR6 is a Swiss 3T3 mouse fibroblast variant line that expresses no detectable EGFR (13). NR6M cells were made by transfection with cDNA for the mutant EGFRvIII under control of the β-actin promoter. Transfectants were maintained in DMEM containing 750 μg/ml G418 and 10% fetal bovine serum. A431 cells are a human epidermoid carcinoma cell line obtained from the American Type Culture Collection. This line is considered the reference source for normal, intact human EGFR. HC cells (NIH Swiss 3T3 cells transfected with EGFRvIII cDNA) used in the immunization protocol[3] were kindly provided by Dr. Albert Wong.

mAbs. The mAb B3 (IgG1κ), which binds a Lewis Y-related human carbohydrate antigen, has been described previously (14). Monoclonals L8A4 (IgG1), H10 (IgG1), and Y10 (IgG2a) were generated by immunization of BALB/c mice with various combinations of Pep-3 (synthetic 14 mer peptide spanning the deletion fusion junction of EGFRvIII) and EGFRvIII as presented on HC cell membranes.[3] mAbs were purified from murine ascites by protein A (H10, Y10) or protein G (L8A4) affinity chromatography.

Expression and Purification of PE35KDEL. Site-directed mutagenesis was used to change the PE35 carboxyl terminal coding sequence of the plasmid pCT11 (15) from REDLK to KDEL.[4] This plasmid, pRK35K, was used to transform *Escherichia coli* BL21 (λDE3) for expression. Periplasm was prepared as described previously for PE35 (15), and PE35KDEL was purified by sequential Q-Sepharose ion exchange chromatography, Mono Q ion exchange chromatography (both from Pharmacia Biotech AB, Uppsala, Sweden), and TSK G3000SW size exclusion chromatography (TosoHaas, Montgomeryville, PA).

Antibody Conjugation. Antibody (2 mg) in 500 μl PBS/1 mM EDTA was reacted for 1 h at 37°C with a 7–10-fold molar excess of 2-iminothiolane (Pierce Chemical Company, Rockville, IL) and desalted on a PD10 column (Pharmacia Biotech AB). Dithionitrobenzoate (50 μl of a 10 mM solution; Pierce Chemical Company) was then added. Absorbance measurements at 412 nm showed that 1–2 mol of free thiol/mol antibody were produced by the reaction with iminothiolane. The antibody was desalted again on a PD10 column. Two mg

[4] R. Kreitman, unpublished observations.

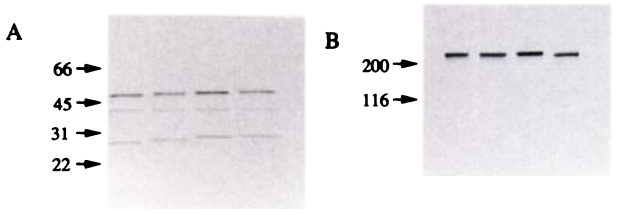

Fig. 2 SDS-PAGE analysis of immunotoxin constructs. One μg of purified immunotoxin per lane was run on a 4–20% SDS-PAGE gel under reducing (*A*) or nonreducing (*B*) conditions. *Lanes from left to right*, B3-PE35KDEL, L8A4-PE35KDEL, H10-PE35KDEL, and Y10-PE35KDEL. *Left of each panel*, molecular weight in kDa.

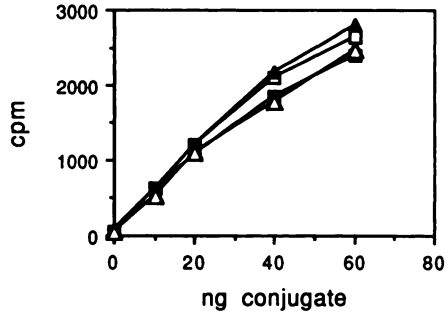

Fig. 3 ADP ribosylation activity of immunotoxins. Different amounts of immunotoxins were assayed for their activity in transferring [^{14}C]ADP-ribose from NAD$^+$ to elongation factor 2 present in wheat germ extract. □, B3-PE35KDEL; ▲, L8A4-PE35KDEL; ■, H10-PE35KDEL; △, Y10-PE35KDEL.

PE35KDEL were treated for 30 min with 20 mM DTT, desalted on a PD10 column, and mixed with the antibody. The mixture was incubated overnight at 10°C and 1:1 antibody: PE35KDEL conjugates were purified by TSK size exclusion chromatography and Mono Q ion exchange chromatography. The concentration of the immunotoxins was determined by absorbance measurements at 280 nm, using ϵ = 1.43 ml/mg · cm, or with Coomassie Plus Protein Assay Reagent (Pierce Chemical Company) using BSA as standard. The two methods were in agreement.

ADP Ribosylation Assays. ADP ribosylation activity was assayed as described by Collier and Kandel (16). Wheat germ extract was used as the source of elongation factor 2.

Cytotoxicity Assays. Cytotoxicity assays measuring the inhibition of protein synthesis were done as described previously (17). Cells in 96-well culture dishes were treated for 24 h with immunotoxins. [^{3}H]leucine (144 Ci/mmol; DuPont/New England Nuclear) was added 2 h before harvesting NR6M and A431 cells.

RESULTS

Construction of Immunotoxins. Immunotoxins were constructed with an engineered version of PE designated PE35KDEL that has the following modifications: (*a*) The protein begins at amino acid 280 of full-length PE. This is the natural endosomal cleavage site of PE (18). PE35KDEL therefore does not require any proteolytic processing to become toxic to target cells. (*b*) Amino acids 365–380 of domain Ib have been deleted. This domain is not required for cytotoxicity. Deletion of this region removes two cysteine residues, so that a single cysteine at position 287 remains for coupling to antibodies. (*c*) The natural carboxyl terminal sequence of PE (REDLK) has been converted to KDEL. This modification has been shown to enhance the cytotoxicity of PE, presumably by enhancing its binding to KDEL receptors (19). PE35KDEL was conjugated via a disulfide linkage to antibodies that had been derivatized with 2-iminothiolane as described in "Materials and Methods." Conjugates (1:1) eluted as the first major peak on Mono Q chromatography, and this fraction was used for cytotoxicity studies. A total of four immunotoxins were constructed: three with the monoclonals L8A4, H10, and Y10 directed against mutant EGFR, and one control immunotoxin made with monoclonal B3 (14). Fig. 2 shows SDS-polyacrylamide gels of the immunotoxins under reducing and nonreducing conditions. Under reducing conditions three bands are seen, corresponding to antibody heavy chain, PE35KDEL, and antibody light chain. Under nonreducing conditions, a single band of about 200 kDa is seen. Fig. 3 shows that the different immunotoxins have very similar ADP-ribosylating activity; any differences in cytotoxicity between the conjugates will therefore be due to differences in the behavior of the antibodies.

Characterization of Cells Transfected with Mutant EGFR cDNA. Although mutant EGFR expression is maintained in xenografts of human gliomas grown in nude mice, it is gradually lost during long-term tissue culture of glioma cells (20). For this reason, we used cell lines transfected with mutant EGFR cDNA to test our immunotoxins. NR6 cells were used because they are a variant of the Swiss 3T3 cells that express no detectable EGFR (13). Transfected cells (designated NR6M) expressed approximately 5 × 10^5 mutant receptors/cell as determined by Scatchard analysis using the mAb L8A4 (data not shown).

L8A4, H10, and Y10 Immunotoxins are Cytotoxic to Cells Expressing Mutant Epidermal Growth Factor. Fig. 4 shows the cytotoxicity of the different immunotoxins when tested on NR6M cells. This was determined by measuring the inhibition of protein synthesis by immunotoxins, which generally correlates well with cytotoxicity. The control immunotoxin made with antibody B3 (14), that recognizes a human Lewis Y-type antigen not present on mouse cells, showed no toxicity to these cells. All three of the immunotoxins made with monoclonals raised against the mutant EGFR junction peptide were toxic to these cells. The concentration at which 50% inhibition of protein synthesis occurred (IC_{50}) was between 3 and 10 ng/ml (15–50 pM; Table 1). The immunotoxins were also assayed on Swiss 3T3 cells (the parent line from which NR6 was originally selected). They showed no cytotoxicity to these cells at 1000 ng/ml (Table 1), demonstrating that the immunotoxins are only toxic to cells transfected with EGFR. The cytotoxicity of the immunotoxins was inhibited by the addition of an excess of their corresponding free antibody, but not by antibody to an unrelated antigen (B3), demonstrating that the cytotoxicity is specific (Fig. 5).

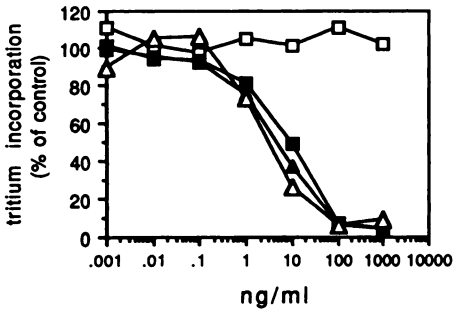

Fig. 4 Cytotoxicity of immunotoxins to NR6M cells. One and five-tenths × 10^4 cells/well were plated in 96-well plates. After overnight growth they were treated for 24 h with immunotoxins. Cells were pulse labeled with [^{3}H]leucine for the final 2 h of the treatment and harvested. Results are expressed as a percentage of ^{3}H incorporated into untreated cells. *Each point*, mean of triplicate determinations. □, B3-PE35KDEL; ▲, L8A4-PE35KDEL; ■, H10-PE35KDEL; △, Y10-PE35KDEL.

Table 1 IC_{50} of immunotoxins on different cell lines[a]

Toxin	Cell lines			
	NR6M	Swiss 3T3	A431	MCF7
B3-PE35KDEL	>1000		0.1	0.3
L8A4-PE35KDEL	5	>1000	200–300	300–400
H10-PE35KDEL	10	>1000	1000	1000
Y10-PE35KDEL	3	>1000	>1000	>1000
PE	0.3–0.5	2–3		

[a] The concentration of immunotoxin at which 50% inhibition of protein synthesis occurs (IC_{50}) is given in ng/ml.

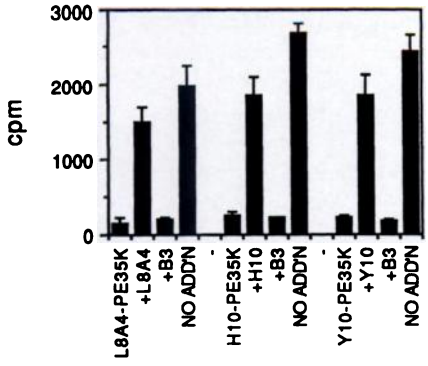

Fig. 5 Inhibition of immunotoxin activities by their respective free antibody. Antibody (20 μg/well) was added immediately before the addition of immunotoxin. Immunotoxin treatment and cell labeling were as described in the legend to Fig. 4.

L8A4, H10, and Y10 Immunotoxins Are Inactive against Cells Overexpressing Wild-Type EGFR. Many tumor cell types overexpress normal EGFRs, and several immunotoxins have been made in an attempt to exploit this property (2). However, normal tissues such as liver also express large amounts of EGFR; therefore, the therapeutic window for these immunotoxins is small (21). To test whether immunotoxins made with mAbs L8A4, Y10, or H10 would be cytotoxic to cells

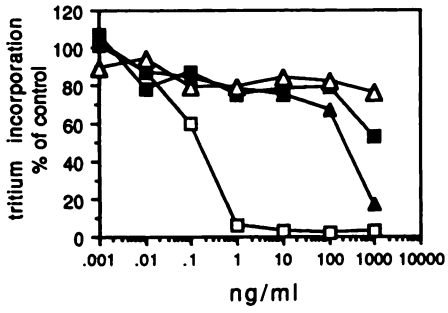

Fig. 6 Cytotoxicity of immunotoxins to A431 cells. One and five-tenths × 10^4 cells/well were plated in 96-well plates and treated as described in the legend to Fig. 4. □, B3-PE35KDEL; ▲, L8A4-PE35KDEL; ■, H10-PE35KDEL; △, Y10-PE35KDEL.

expressing normal EGFRs, we assayed their activity on A431 human epidermoid cancer cells. These cells have been shown to express about 2 × 10^6 wild-type EGFRs/cell (22), about four times higher than the level of mutant receptor expressed in NR6M. Fig. 6 shows the cytotoxicity of the immunotoxins on A431 cells. B3-PE35KDEL was very cytotoxic, with an IC_{50} of 0.1 ng/ml, consistent with previous results (15). However, the L8A4, H10, and Y10 immunotoxins showed little or no cytotoxicity. L8A4 and H10 immunotoxins had IC_{50}s of 200–300 ng/ml and 1000 ng/ml, respectively, whereas Y10 showed no significant cytotoxicity at 1000 ng/ml (Table 1). A similar pattern of weak cytotoxicity was seen with MCF-7 human breast carcinoma cells that express only 1 × 10^4 wild-type receptors per cell (Table 1; Ref. (22)). Since the cytotoxicity does not correlate with receptor number, these immunotoxins are probably being internalized nonspecifically at a very low level.

DISCUSSION

In the past decade there have been enormous advances in our understanding of the pathogenesis of cancer. Human cancers arise from a series of mutations that either activate oncogenes, or inactivate tumor suppressor genes. The structure and function of these oncogene and tumor suppressor proteins is now understood in some detail. Thus far, however, little of this knowledge has been translated into significant improvements in cancer therapy. Here, we have developed a therapeutic agent that is specifically toxic to cells expressing an oncogenic mutation.

The mutant EGFR with a deletion of amino acids 6–273 of the extracellular domain appears to have many of the properties of an ideal immunotoxin target. The deletion junction creates a tumor-specific surface antigen (5). Also, the genetic rearrangement giving rise to the deletion coincides with amplification of the EGFR gene (6, 20), so that the mutant receptor is expected to be overexpressed on the tumor cell surface. The deletion mutant occurs relatively commonly in a range of tumor types including glioblastoma (5), non-small cell lung carcinoma (8), and breast carcinoma.[3] Finally, because the mutant receptor directly contributes to the transformed phenotype, immunotoxins targeting it may destroy the most malignant cells in a tumor population. In this article we have tested the ability of this mutant EGFR to function as an immunotoxin target in cell culture.

Three mAbs were evaluated for their ability to target PE. Each mAb was generated against a mixture of EGFRvIII present on cell membranes and a synthetic peptide shown in Fig. 1 that corresponds to the deletion junction sequence of the mutant receptor. Antibodies were conjugated by a disulfide linkage to a truncated version of PE that does not require proteolytic processing to be cytotoxic (15). All three immunotoxins were cytotoxic to cells expressing mutant EGFR. IC_{50}s were in a range (15–50 pM) similar to other PE-based toxins that have been shown to have therapeutic benefit in mice carrying human tumor xenografts (23).

All three immunotoxins showed little or no toxicity to cells expressing high levels of human wild-type EGFR. The conjugates made with antibodies L8A4 and H10 showed some very weak cytotoxicity to cells expressing wild-type receptor. No cytotoxicity was seen at a concentration of 1000 ng/ml with the Y10 immunotoxin, which also had the highest toxicity for cells expressing mutant receptors. This antibody therefore appears to be the best candidate for further development as a single-chain Fv (24) or disulfide-linked Fv (25) immunotoxin. These types of immunotoxins are preferable for studies on solid tumors in animal models, since their smaller size permits better extravasation and tumor penetration (26). The fact that Y10 immunotoxin shows no reactivity with normal EGFR suggests that there would be little cytotoxicity due to cross-reaction of the antibody part of the immunotoxin with normal tissues. PE itself does show nonspecific liver toxicity at high concentrations (2). However, because the target appears to be truly tumor specific, it should be possible to increase the therapeutic window by enhancing the antibody affinity through protein engineering techniques if required.

To function immunotoxins must be internalized upon binding to their receptors. The first step in internalization is usually the uptake of the immunotoxin-receptor complex into endosomes. With the EGFR, it has been suggested that ligand-induced receptor dimerization is essential for internalization (27). Why are the EGFRvIII-targeted immunotoxins internalized efficiently? The deletion results in a receptor that has constitutive tyrosine kinase activity (9, 10). Perhaps the mutant receptor is also internalized at a high rate due to its activation. The tyrosine kinase activity of the EGFR has been shown to mediate its cellular uptake and intracellular routing (28, 29). Also, there is evidence that constitutively active receptors with large deletions in the extracellular domain already exist as dimers in the membrane in the absence of ligand (30). Thus, the mutation in the EGFR, as well as providing a tumor-specific target, may also promote internalization of the immunotoxin.

REFERENCES

1. Pai, L. H., and Pastan, I. Immunotoxins and recombinant toxins for cancer treatment. *In*: V. T. DeVita, S. Hellman, and S. A. Rosenberg (eds.), Important Advances in Oncology, pp. 3–19. Philadelphia: J. B. Lippincott Company, 1994.

2. Pastan, I., Chaudhary, V., and FitzGerald, D. J. Recombinant toxins as novel therapeutic agents. Annu. Rev. Biochem., *61:* 331–354, 1992.

3. Allured, V. S., Collier, R. J., Carroll, S. F., and McKay, D. B. Structure of exotoxin A of Pseudomonas aeruginosa at 3.0 Angstrom. Proc. Natl. Acad. Sci. USA, *83:* 1320–1324, 1986.

4. Kounnas, M. Z., Morris, R. E., Thompson, M. R., FitzGerald, D. J., Strickland, D. K., and Saelinger, C. B. The α2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes *Pseudomonas* exotoxin A. J. Biol. Chem., *267:* 12420–12423, 1992.

5. Humphrey, P. A., Wong, A. J., Vogelstein, B., Zalutsky, M. R., Fuller, G. N., Archer, G. E., Friedman, H. S., Kwatra, M. M., Bigner, S. H., and Bigner, D. D. Anti-synthetic peptide antibody reacting at the fusion junction of deletion-mutant epidermal growth factor receptors in human glioblastoma. Proc. Natl. Acad. Sci. USA, *87:* 4207–4211, 1990.

6. Sugawa, N., Ekstrand, A. J., James, C. D., and Collins, V. P. Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. Proc. Natl. Acad. Sci. USA, *87:* 8602–8606, 1990.

7. Wong, A. J., Ruppert, J. M., Bigner, S. H., Grzeschik, C. H., Humphrey, P. A., Bigner, D. S., and Vogelstein, B. Structural alterations of the epidermal growth factor receptor gene in human gliomas. Proc. Natl. Acad. Sci. USA, *89:* 2965–2969, 1992.

8. Garcia de Palazzo, I. E., Adams, G. P., Sundareshan, P., Wong, A. J., Testa, J. R., Bigner, D. D., and Weiner, L. M. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. Cancer Res., *53:* 3217–3220, 1993.

9. Yamazaki, H., Ohba, Y., Tamaoki, N., and Shibuya, M. A deletion mutation within the ligand binding domain is responsible for activation of epidermal growth factor receptor gene in human brain tumors. Jpn. J. Cancer Res., *81:* 773–779, 1990.

10. Ekstrand, J. A., Longo, N., Hamid, M. L., Olson, J. J., Liu, L., Collins, V. P., and James, C. D. Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. Oncogene, *9:* 2313–2320, 1994.

11. Nishikawa, R., Ji, X-D., Harmon, R. C., Lazar, C. S., Gill, G. N., Cavanee, W. K., and Huang, H-J. S. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. Proc. Natl. Acad. Sci. USA, *91:* 7727–7731, 1994.

12. Lax, I., Burgess, W. H., Bellot, F., Ullrich, A., Schlessinger, J., and Givol, D. Localization of a major receptor-binding domain for epidermal growth factor by affinity labeling. Mol. Cell. Biol., *8:* 1831–1834, 1988.

13. Pruss, R. M., and Herschman, H. R. Variants of 3T3 cells lacking mitogenic response to epidermal growth factor. Proc. Natl. Acad. Sci. USA, *74:* 3918–3921, 1977.

14. Pastan, I., Lovelace, E. T., Gallo, M. G., Rutherford, A. V., Magnani, J. L., and Willingham, M. C. Characterization of monoclonal antibodies B1 and B3 that react with mucinous adenocarcinomas. Cancer Res., *51:* 3781–3787, 1991.

15. Theuer, C. P., Kreitman, R. J., FitzGerald, D. J., and Pastan, I. Immunotoxins made with a recombinant form of *Pseudomonas* exotoxin A that do not require proteolysis for activity. Cancer Res., *53:* 340–347, 1993.

16. Collier, R. J., and Kandel, J. Structure and activity of diphtheria toxin. I. Thiol dependent dissociation of a fraction of toxin into enzymatically active and inactive fragments. J. Biol. Chem., *246:* 1496–1503, 1971.

17. Prior, T. I., FitzGerald, D. J., and Pastan, I. Barnase toxin: a new chimeric toxin composed of pseudomonas exotoxin A and barnase. Cell, *64:* 1017–1023, 1991.

18. Ogata, M., Fryling, C. M., Pastan, I., and FitzGerald, D. J. Cell-mediated cleavage of *Pseudomonas* exotoxin between Arg279 and Gly280 generates the enzymatically active fragment which translocates to the cytosol. J. Biol. Chem., *267:* 25396–25401, 1992.

19. Seetharam, S., Chaudhary, V. K., FitzGerald, D., and Pastan, I. Increased cytotoxic activity of *Pseudomonas* exotoxin and two chimeric toxins ending in KDEL. J. Biol. Chem., *266:* 17376–17381, 1991.

20. Bigner, S. H., Humphrey, P. A., Wong, A. J., Vogelstein, B., Mark, J., Friedman, H. S., and Bigner, D. D. Characterization of the epidermal growth factor receptor in human glioma cell lines and xenografts. Cancer Res., *50:* 8017–8022, 1990.

21. Pai, L. H., Gallo, M. G., FitzGerald, D. J., and Pastan, I. Antitumor activity of a transforming growth factor α-*Pseudomonas* exotoxin fusion protein (TGF-α-PE40). Cancer Res., *51:* 2808–2812, 1991.

22. Theuer, C. P., FitzGerald, D., and Pastan, I. A recombinant form of *Pseudomonas* exotoxin directed at the epidermal growth factor receptor that is cytotoxic without requiring proteolytic processing. J. Biol. Chem., *267:* 16872–16877, 1992.

23. Brinkmann, U., Pai, L. H., FitzGerald, D. J., Willingham, M., and Pastan, I. B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. Proc. Natl. Acad. Sci. USA, *88:* 8616–8620, 1991.

24. Chaudhary, V. K., Queen, C., Junghans, R. P., Waldmann, T. A., FitzGerald, D. J., and Pastan, I. A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. Nature (Lond.), *339:* 394–397, 1989.

25. Reiter, Y., Brinkmann, U., Webber, K., Jung, S-H., Lee, B., and Pastan, I. Engineering interchain disulfide bonds into conserved framework regions of Fv fragments: improved biochemical characteristics of recombinant immunotoxins containing disulfide-stabilized Fv. Protein Eng., *7:* 697–704, 1994.

26. Yokota, T., Milenic, D. E., Withlow, M., and Schlom, J. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res., *52:* 3402–3408, 1992.

27. Ullrich, A., and Schlessinger, J. Signal transduction by receptors with tyrosine kinase activity. Cell, *61:* 203–212, 1990.

28. Felder, S., Miller, K., Moehren, G., Ullrich, A., Schlessinger, J., and Hopkins, C. R. Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body. Cell, *61:* 623–634, 1990.

29. Glenney, J. R. J., Chen, W. S., Lazar, C. S., Walton, G. M., Zokas, L. M., Rosenfeld, M. G., and Gill, G. N. Ligand-induced endocytosis of the EGF receptor is blocked by mutational inactivation and by microinjection of anti-phosphotyrosine antibodies. Cell, *52:* 675–684, 1988.

30. Kwatra, M. M., Bigner, D. D., and Cohn, J. A. The ligand binding domain of the epidermal growth factor receptor is not required for receptor internalization. Biochim. Biophys. Acta, *1134:* 178–181, 1992.