Monoclonal Antibody Cetuximab Binds to and Down-regulates Constitutively Activated Epidermal Growth Factor Receptor vIII on the Cell Surface

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Abstract. *Background: The epidermal growth factor receptor (EGFR) plays an important role in the growth and survival of many human tumors of epithelial origin. EGFR variant III (EGFRvIII) is a truncated form of EGFR that does not bind ligand, is constitutively active, and is reported to be co-expressed with EGFR in some human tumors including breast, glioblastoma, lung, and prostate. Materials and Methods: Here we have tested the anti-EGFR monoclonal antibody cetuximab for its interaction with EGFRvIII. Chinese hamster ovary (CHO), 32D (non-tumorigenic murine hematopoetic cells), and U87-MG stable transfectants were generated to express EGFRvIII. Results: Analysis of receptor phosphorylation showed that the EGFRvIII was constitutively phosphorylated in transfected cells. Flow cytometry, direct binding, and immunoprecipitation analysis of EGFRvIII transfectants showed specific binding of cetuximab to EGFRvIII. Cetuximab bound to EGFRvIII with a K_D of 0.38 nM determined by Scatchard analysis and 1.1 nM determined by Biacore analysis respectively. In internalization studies, binding of cetuximab to the EGFRvIII on the cell surface led to at least 50% of the cetuximab-EGFRvIII complex internalized from cell surface of CHO-EGFRvIII after 3 hours. This internalization led to a reduction in phosphorylated EGFRvIII in transfected cells. Furthermore, incubation of cells expressing EGFRvIII with cetuximab resulted in 40-50% inhibition of cell proliferation. Conclusion: These data suggest that cetuximab may be a potential candidate for the treatment of tumors that also express EGFRvIII.*

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It is well known that EGFR (epidermal growth factor receptor) is often overexpressed in a variety of tumors such as colon, head and neck, lung, renal, and pancreatic carcinomas (1). EGFR activation by EGF triggers the activation of ras and mitogen-activated protein kinase and further activates cyclin D1, a protein required for cell cycle progression from the G1- to the S-phase (2). EGFR plays an important role in tumor proliferation, division and angiogenesis. Transfection of cells with the EGFR gene leads to transformation and enhanced cell proliferation *in vitro* (3). *EGFR* transfected cells are tumorigenic and grow more aggressively in animal models than non-transfected cells (4). EGFR overexpression in tumors may be associated with gene amplification. For example, the EGFR gene is amplified in 50% of human grade IV gliomas (5). High levels of EGFR expression in tumors correlate with poor prognosis (6).

EGFR gene arrangement has been studied extensively and may also play an important role in tumor proliferation. The type III EGF receptor deletion mutant (*EGFRvIII*), resulting from a gene rearrangement, is a major variant of EGFR and was first identified in primary glioblastoma tumors (7-9). *EGFRvIII* is characterized by in-frame deletion of exons 2-7 of coding sequence, which leads to removal of 267 amino acids from the extracellular domain. This deletion renders EGFRvIII unable to bind to EGF or TGF-α (10, 11). However, EGFRvIII is constitutively activated and autophosphorylated. Unlike wild-type EGFR, the constitutively active EGFRvIII is not down-regulated from the cell surface, suggesting that the altered conformation of the mutant does not result in exposure of receptor sequence motifs required for endocytosis and lysosomal sorting (12). Expression of EGFRvIII in glioma, breast, leukemia, and NIH3T3 cells has resulted in enhanced growth and tumorigenicity in athymic mice (13, 14). EGFRvIII expression is also detected in tumors other than glioma (15), including breast (16), non-small cell lung (17), ovarian (10), and prostate (18). Several studies have

shown that EGFRvIII was related to a shorter interval to relapse and decreased survival rate in glial tumor patients (19). Given the oncogenic potential and surface expression of EGFR and EGFRvIII, they serve as ideal targets for antibody-based therapy. Several EGFRvIII-specific antibodies have been developed (20-22). These antibodies were shown to be able to decrease autophosphorylation of EGFRvIII from tumors and suppress the growth of tumor xenografts expressing EGFRvIII.

Erbitux® (cetuximab), a human-mouse chimeric monoclonal antibody to EGFR, was recently approved for head and neck and refractory colorectal patients with EGFR positive tumors (23-25). Cetuximab has been shown to effectively inhibit the growth of tumor expressing EGFR in animals and cancer patients (24). However, we intended to test whether cetuximab can interact with EGFRvIII, even though it lacks amino acids 6-274. In this study, we demonstrated that cetuximab binds to EGFRvIII and reduces the amount of the receptor on the cell surface as well as the autophosphorylation levels of EGFRvIII. This suggests that cetuximab may be able to block EGFRvIII-mediated signaling in cancer cells.

Materials and Methods

Antibodies and cell lines. Anti-EGFR monoclonal antibody cetuximab was produced by the ImClone Systems Incorporated manufacturing facility (Somerville, NJ, USA). Mouse anti-human EGFR monoclonal antibody 111.6 was purchased from GeneTex (San Antonio, TX, USA). Mouse anti-phosphotyrosine monoclonal antibody PY20 was purchased from Invitrogen (Carlsbad, CA, USA). MDA-MB-231, CHO, 32D, and U87-MG cells were obtained from American Type culture Collection (Manassas, VA, USA). Tumor cell lines, MDA-MB-231, CHO and U87-MG, were maintained in RPMI 1640 (Invitrogen) with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, USA). 32D cells were cultured in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate supplemented with: 10% heat-inactivated fetal bovine serum and 10% mouse Interleukin-3 culture supplement (Becton Dickinson, CA, USA).

Construction of vectors encoding EGFRvIII gene. EGFRvIII gene was constructed from a cDNA encoding full-length EGFR which was isolated from A431 cells. To PCR-clone full-length EGFR, mRNA was prepared from A431 cells and cDNA was synthesized using reverse transcriptase (Invitrogen). To generate a DNA fragment encoding EGFRvIII, the sequence of exons 2-7 was eliminated from full length EGFR cDNA. Two DNA fragments corresponding to nucleotide 175 and 274 were generated by PCR using two pairs of primers, GCG CGC AAG CTT CGG GGA GCA GCG ATG CGA CCC TCC GGG/CTG TCA CCA CAT AAT TAC CTT TCT TTT CCT CCA GAG CCC GAC and CTC TGG AGG AAA AGA AAG GTA ATT ATG TGG TGA CAG ATC ACG/GCG TCT GAG TTG GTC CTG GGT ATC GAA AGA GTC. The two fragments were mixed and underwent a second PCR reaction resulting in an EGFRvIII DNA fragment. The EGFRvIII DNA fragment was digested with *Hind* III and *Xho* I before cloning into pcDNA3 (Invitrogen). To clone EGFRvIII DNA into pIND (Invitrogen), DNA was digested with *EcoR* V and *Xho* I.

Transfection and maintenance of cell lines. Ten micrograms of the pcDNA3-EGFRvIII construct was transfected into $5x10^6$ U87-MG and 32D cells by electroporation. 32D cells were subcloned into 96-well plates and selected in 500 μg/ml G418 for 2-3 weeks. U87-MG cells were subcloned in 10 cm plates and selected in 500 μg/ml G418. Cell clones were picked with cloning rings. Clones were screened for expression of EGFRvIII *via* FACS analysis. CHO cells were transfected with pIND/EGFRvIII vector and clones were selected in the presence of Zeocine. The expression of EGFRvIII was induced with 10 μM muristrone A. For transient expression of EGFRvIII, COS cells were plated in 10 cm plates and transiently transfected with 4 μg pcDNA3-EGFRvIII using Lipofectamine 2000 (GIBCO BRL). Cells were examined for EGFRvIII expression 48 hours after transfection.

Generation of soluble EGFRvIII from baculoviral expression system. The EGFR vIII gene was cloned into the pFastBac Dual expression vector (Invitrogen), following the polyhedrin promoter. The EGFR vIII gene contains its natural, mammalian, secretion signal sequence rather than an insect signal sequence. The construct ends at alanine 647 (mature, wt EGFR numbering) followed by six histidines. The EGFR vIII/pFastBac Dual plasmid was cloned into DH10Bac bacteria (Invitrogen) to produce bacmid DNA following the manufacturer's instructions. Sf9 insect cells (Invitrogen) were transfected with the bacmid DNA, and the resulting baculovirus was amplified three times following the manufacturer's recommendations. The high-titer virus stock was used to infect 4 L of Hi5 insect cells (Invitrogen). After three days, the conditioned media was harvested from the infected cells, $NiCl_2$ and $CaCl_2$ were added to 1 mM and 10 mM respectively, and the pH was adjusted to 7.3 by addition of Tris base. Precipitate was removed by centrifugation and filtration, and the cleared supernatant was loaded onto a 5 ml nickel sepharose column (GE Healthcare). The column was washed with 50 mM Tris, pH 8.0; 1 M NaCl, followed by 50 mM Tris, pH 8.0; 250 mM NaCl, then the protein was eluted with a 10 column volume gradient from 0 to 0.4 M imidazole. Fractions containing purified EGFRvIII were pooled and concentrated, then passed over a Superdex-200 gel filtration column (GE Healthcare) in 50 mM Tris, pH 8.0; 250 mM NaCl. Fractions corresponding to monomeric protein were pooled and left in the gel filtration buffer.

Phosphorylation assay. Cells were serum starved overnight, washed with cold HBSS containing 0.1 mM Na_2VO_4, and treated with 5 μg/ml cetuximab or hIgG for 30 mins at 37˚C, followed by stimulation with 20 ng/ml EGF for an additional 30 mins at 37˚C. Cells were then washed 2x with cold PBS/50 nm Na_2VO_4, and lysed with Buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.25% Deoxycholic acid, 1 mM PMSF, 0.1 mM Na_2VO_4, and 1 mM NaF. Lysates were cleared by centrifugation and equal amounts of each lysate were immunoprecipitated with 5 μg/ml cetuximab for 1 h at 4˚C followed by Protein G beads for 1 h at 4˚C. Immunoprecipitates were washed and separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots

were probed with HRP conjugated anti-phosphotyrosine monoclonal antibody PY20. Immunoreactive bands were detected *via* ECL (Chemicon, CA, USA).

Flow cytometric analysis. EGFRvIII-transfected 32D cells and CHO-EGFRvIII cells were analyzed for cell surface expression of EGFRvIII. A total of $1x10^6$ cells were incubated with anti-EGFR mAb cetuximab or an isotype matched human IgG1 control at 5 μg/ml for 30 mins on ice and washed 3x with staining buffer (1% BSA/PBS), followed by a 30 minute incubation with 5 μg/ml of a FITC-labeled goat anti human mAb. Cells were washed, fixed with 0.1% paraformaldehyde for 10 mins and analyzed with an EPICS XL flow cytometer (Coulter).

Immunoprecipitation and Western Blots. Cells plated in 10 cm dishes were washed with cold PBS and incubated in methionine/cysteine-free DMEM with 100 μCi/ml ^{35}S for 3-4 h at 37˚C. After incubation, cells were washed 3x with cold PBS, lysed with 1 ml RIPA buffer (Pierce) and precleared with Protein A/G sepharose beads for 1 h on ice. Lysates were then incubated with 5 μg/ml cetuximab, followed by Prot A/G sepharose beads for 1 h at 4˚C. Immunoprecipitates were washed and separated by SDS-PAGE. The gel was fixed in methanol/acetic acid for 15 mins and then dried at 80˚C for 1 h in a gel dryer. Bands were detected using a Phospho Imager (Molecular Dynamics).

ELISA. Falcon flexible 96-well flat bottomed plates were coated with recombinant human EGFR or EGFRvIII (2 μg/ml x 100 μl) at 4˚C overnight. The next day the plate was blocked with 1% BSA in PBS containing 0.1% Tween-20 for 2 h at RT. Various amounts of cetuximab in 100 μl were added. The plate was then washed 3x with PBS/Tween and 100 μl of HRP conjugated goat anti-human antibody (Biosource, Camarillo, CA, USA) diluted at 1:5000 in 100 μl was added to the plate and incubated for 1 h at RT. The plate was then washed 3x and 50 μl/well of TMB (KPL, Gaithersburg, MD, USA) substrate was added to the plate. The plates were read at 450 nm using a microplate reader (Molecular Devices).

Biacore analysis. The binding kinetics of the antibodies to recombinant human EGFRvIII were measured using the Biacore biosensor (Pharmacia). EGFRvIII protein was immobilized onto a CM5 research grade sensor chip and cetuximab was injected at concentrations ranging from 0.5 nM to 100 nM. Sensorgrams were acquired for each concentration and were evaluated using the BIA Evaluations 3.2 program to determine the rate constants k_{on} and k_{off}. K_D was calculated from the ratio of the rate constants k_{off}/k_{on}.

Binding and internalization assay. Cetuximab was iodinated by the chloramine T method. Specific radioactivity for ^{125}I-cetuximab was 2000 cpm/ng. For binding studies, $1x10^5$ 32D/EGFRvIII cells were collected in 1 ml Eppendorf tubes and washed with 1xPBS. Cells were then incubated for 2 h at 4˚C in 100 μl of binding buffer (DMEM, 25 mM Hepes, 0.1%BSA) with ^{125}I cetuximab or 200-fold cold cetuximab to determine non-specific binding. Cells were then rinsed three times with PBS and lysed with 1 N NaOH. Radioactivity of cell homogenates was determined using a gamma counter (PerkinElmer, CT, USA). For internalization studies, U87-MG, CHO-EGFRvIII and untransfected CHO cells plated in 6-well plates were washed 3x with cold PBS. ^{125}I-cetuximab radiolabeled antibody 20 nM was added to each well. Non-specific binding was determined by adding a 200 M excess of cold cetuximab. At each time-point relative amounts of binding were determined by acid stripping antibody bound to receptor with 0.2 M acetic acid and 0.5 M NaCl. Internalization of the antibody was determined by solubilizing cells with 1 N NaOH and 0.1% Triton-X 100.

Proliferation assay. Cells were plated at $5x10^3$ cells/well in RPMI1640 with 1% serum in 96-well flat bottom plates. Cetuximab or human IgG was added at varying concentrations in duplicates and incubated for 4 days. CellTiter 96 Aqueous One Solution (Promega, NJ, USA) was added at 20 μl/well and plates were read at 490 nm on an ELISA plate reader (Molecular Devices, CA, USA).

Results

Cetuximab specifically binds to EGFRvIII. To confirm that the constructed EGFRvIII vector expressed EGFRvIII, COS cells were transiently transfected with pcDNA3/EGFRvIII. The transfected cells were then harvested, lysed, and subjected to a Western analysis using monoclonal antibody specific for EGFR and EGFRvIII. As shown in Figure 1A, mAb 111.6 bound to full length EGFR from U87-MG cell and EGFRvIII from transfected COS cells. The molecular weight of EGFR and EGFRvIII are expected to be 180 and 145 kDa, respectively. We further tested if EGFRvIII expressed in COS cells could bind to our EGFR-specific mAb cetuximab in an immuno-precipitation assay. The transfected COS cells were labeled with ^{35}S methionine before precipitation with cetuximab. The precipitates were then loaded onto SDS-PAGE and detected by X-ray film (Figure 1B). As expected, cetuximab precipitated out full length EGFR. But it also precipitated out EGFRvIII in our experiments. The same experiments were repeated three times with identical results.

Tumor cells tend to lose EGFRvIII expression *in vitro* (10, 11). To analyze the effect of cetuximab on EGFRvIII further, we established three stable cell lines expressing EGFRvIII (Figure 1C). CHO cells were transfected with pIND/EGFRvIII vector and clones were selected in the presence of Zeocine. The expression of EGFRvIII was induced with 10 μM muristrone A. The second cell line chosen for expression of EGFRvIII was 32D, which is a murine IL-3-dependent hematopoietic cell line. It was considered an ideal cell line for the expression of Erb-B family receptors since it does not express ErbB family members. The IL-3-dependent pathway could be abrogated by introduction of ErbB-family genes, followed by stimulation with the appropriate growth factors. Tang C *et al.* have shown that expression of EGFRvIII in 32D cells abrogated the IL-3 requirement and caused them to become tumorigenic in nude mice (13). 32D cells were transfected with pcDNA3/EGFRvIII. The positive cells were selected in the presence of 500 μg of G418 and 5% IL-3 condition medium. The third cell line transfected with EGFRvIII is

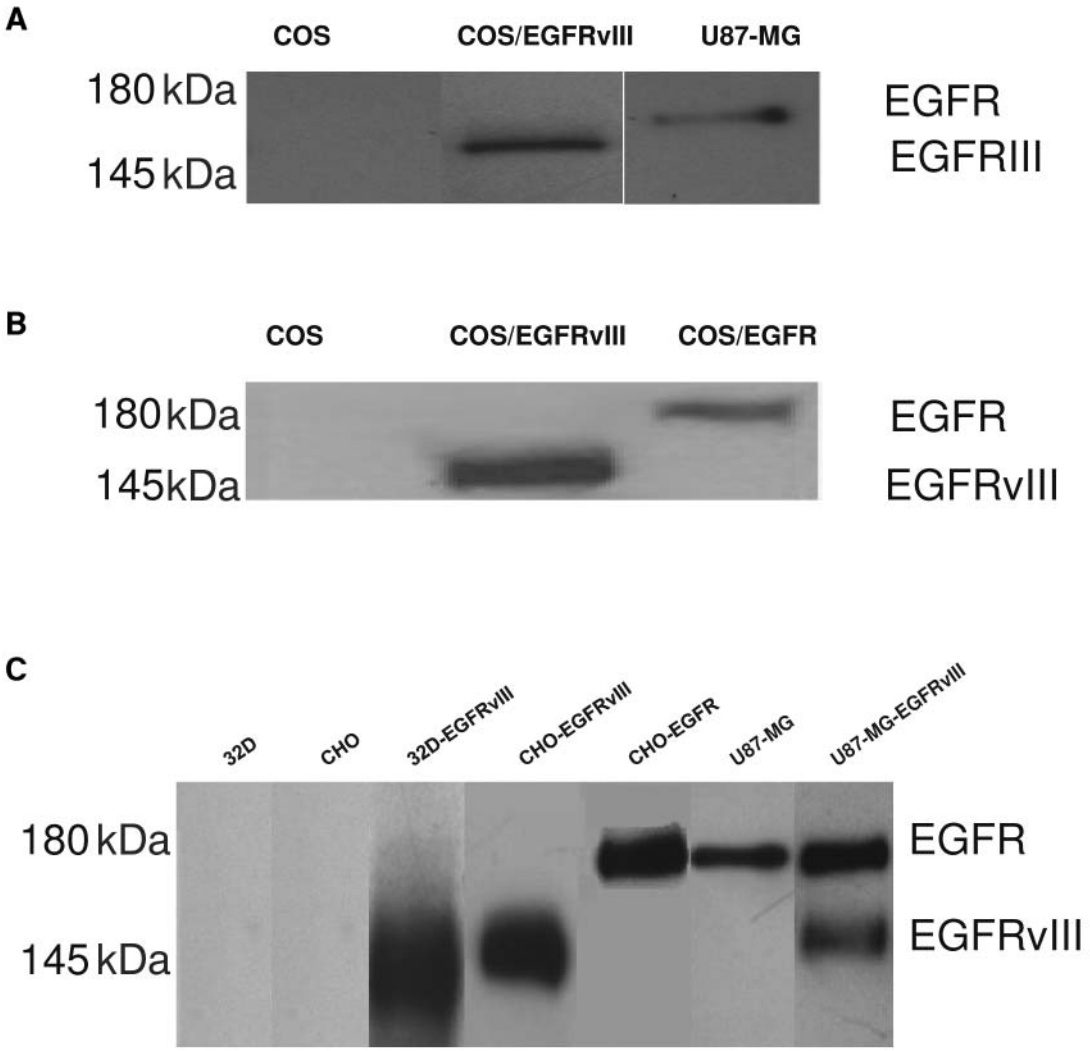

Figure 1. *Immunoprecipitation and Western analysis of the expression of EGFRvIII. U87-MG cells expressing full length EGFR was used as positive control. (A) U87-MG cells, COS cells transfected with pcDNA3/EGFRvIII, and COS cells were lysed with lysis buffer, run on 10% SDS-PAGE, and transfected onto nitrocellulose membranes. The membrane then probed with mAb 111.6 specific for EGFR and EGFRvIII. (B) EGFR and EGFRvIII were immunoprecipitated from COS cells transfected with pcDNA3/EGFR or pcDNA3/EGFRvIII using cetuximab. (C) Cetuximab specifically binds and immunoprecipitates EGFRvIII and EGFR from cell lysates of stable cell lines. Transfected cells were harvested, lysed, and subjected to an IP-Western analysis. Lysates were incubated with cetuximab and followed by protein A/G sepharose beads. The precipitates were then loaded onto SDS-PAGE and transferred to PVDF membranes. Blots were probed with anti-EGFR mAb 111.6 and detected via ECL.*

human glioblastoma cell line U87-MG, which expresses wild type EGFR. Since EGFRvIII is expressed on cell surfaces, we tested if cetuximab can bind to EGFRvIII expressed by stable cell lines using Flow Cytometry. As shown in Figure 2, cetuximab specifically stained 32D-EGFRvIII and CHO-EGFRvIII cells. Furthermore, the specificity of cetuximab binding to EGFRvIII was confirmed by IP-Western analysis (Figure 1C). Cetuximab was able to immunoprecipitate EGFR and EGFRvIII from 32D-EGFRvIII, CHO-EGFRvIII, and U87-MG-EGFRvIII. The molecular weight of EGFR and EGFRvIII detected were 180 kDa and 145 kDa respectively, which are in agreement with the predicted molecular weight for both.

Direct binding studies using ^{125}I cetuximab also confirmed that cetuximab binds to EGFR and EGFRvIII specifically (Figure 3). MDA-MB-231 and 32D-EGFRvIII cells were used in binding studies. MDA-MB-231 is an EGFR expressing human breast tumor cell line (27). The binding affinities to EGFR (0.38 nM) and EGFRvIII (0.4 nM) were very similar, suggesting that cetuximab does not

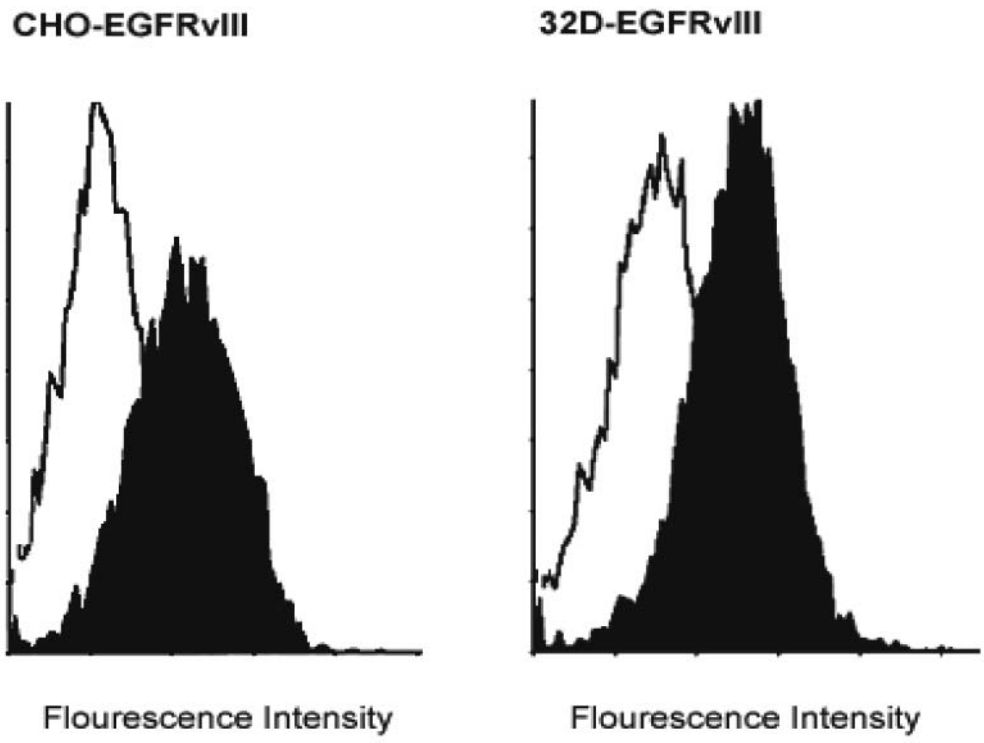

Figure 2. *Flow Cytometric analysis of CHO and 32D cells expressing EGFRvIII. CHO and 32D cell were transfected with vector encoding EGFRvIII as described in Materials and Methods section. Stable cell lines were selected by cloning cells. Cells were stained with control human IgG or cetuximab. The experiments were performed three times with reproducible results.*

bind to domain I of EGFR. MDA-MB-231 and 32D-EGFRvIII cells express $9.7x10^5$ of EGFR and $3.4x10^5$ of EGFRvIII on the cell surface, respectively. ELISA and Biacore analysis further demonstrated the interaction of cetuximab and EGFRvIII (Figure 4). As shown in Figure 4A, cetuximab bound to recombinant soluble EGFR and EGFRvIII on ELISA with an identical EC_{50} (0.1 nM). Biacore analysis also showed that cetuximab bound to EGFR and EGFRvIII with a K_D of 5 nM and 1.1 nM, respectively (Figure 4B).

Cetuximab internalizes cetuximab-EGFRvIII complex from cell surface. Binding of EGF to EGFR triggers rapid dimerization, conformational change, activation of intrinsic tyrosine kinase activity, and autophosphorylation of receptor. It also induces receptor internalization and down-regulation from the cell surface. Cetuximab blocks ligand induced activation and induces internalization of EGFR (25, 26). Deletion of the portion encoded by exons 2-7 in EGFRvIII causes the structural change and activation of EGFRvIII, which mimics the structural changes caused by ligand binding. But the EGFRvIII activation signaling is not attenuated or down-regulated. We were curious to know if cetuximab can trigger the internalization of the cetuximab-EGFRvIII complex. Cetuximab was labeled with ^{125}I and incubated with CHO/EGFRvIII or control cells. The bound and internalized cetuximab were determined at various times (Figure 5). Cetuximab bound to EGFR and EGFRvIII with similar kinetic fashion, reaching 80% of maximum binding within 1 h. The internalization of EGFRvIII started as soon as cetuximab bound to EGFRvIII (Figure 5B). These data suggest that cetuximab can effectively bind to EGFRvIII and internalize the cetuximab-EGFRvIII complex from the cell surface.

Cetuximab reduces autophosphorylation of EGFRvIII. Cetuximab blocks EGF binding to and inhibits EGF-induced phosphorylation of wild-type receptor. We investigated if the autophosphorylation activity of EGFRvIII could be influenced by cetuximab in 32D-EGFRvIII and CHO-EGFRvIII cells (Figure 6). EGFRvIII in both cell types undergoes autophosphorylation without EGF stimulation; addition of EGF had no effects on autophosphorylation of EGFRvIII. Cetuximab treatment dramatically reduced EGFRvIII autophosphorylation in both cell types but to different degrees (Figure 6). EGFRvIII autophosphorylation was stronger in CHO-EGFRvIII cells than that in 32D-EGFRvIII cells. This may be due to different EGFRvIII

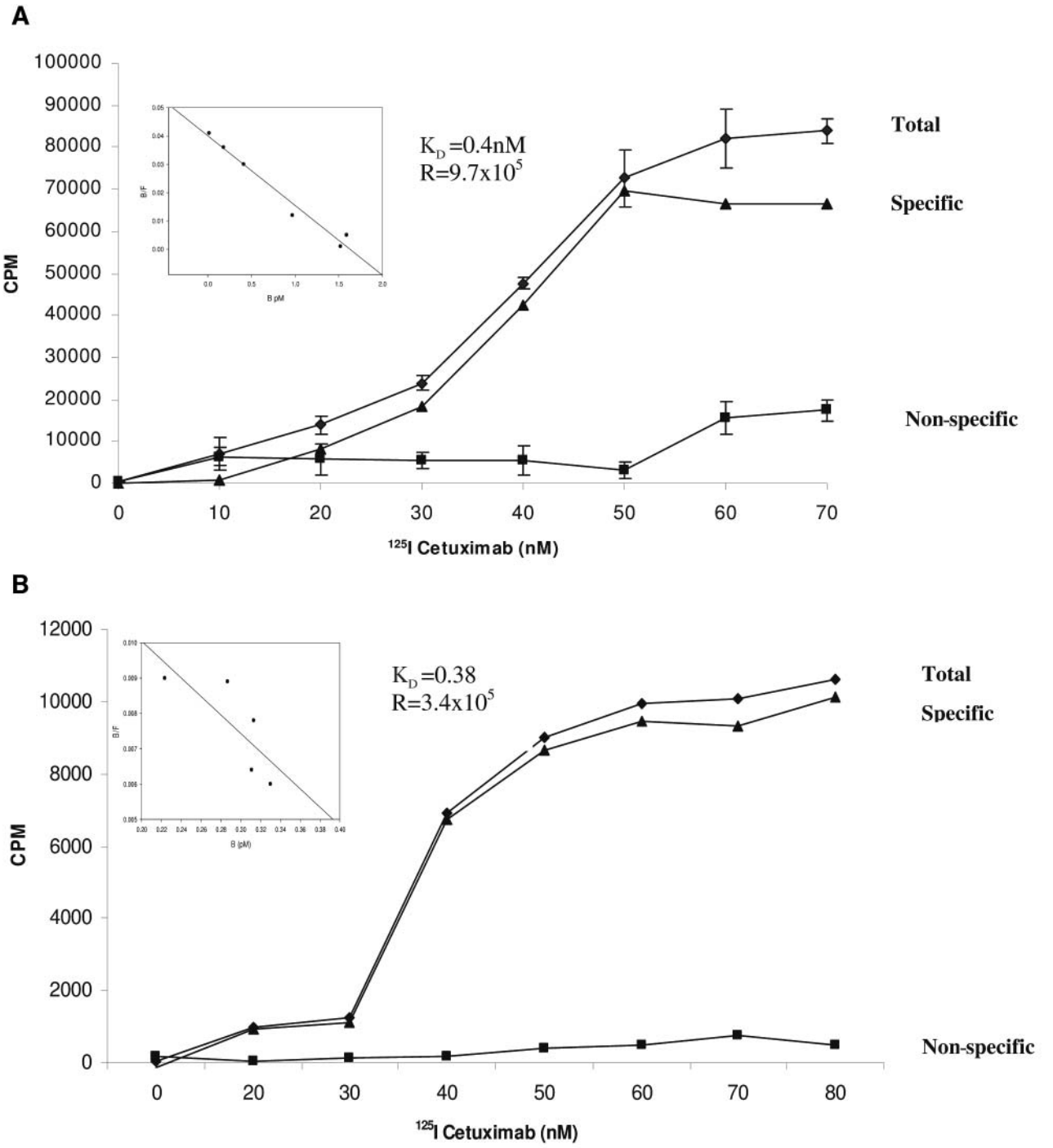

Figure 3. *Direct binding of ^{125}I cetuximab to 32D/EGFRvIII cells (A) and MDA-MB-231 cells (B). Cells were incubated for 2 h at 4°C with ^{125}I cetuximab at various concentrations as shown. Binding was determined in the absence of any competitors (total) or in the presence of unlabeled cetuximab in 200-fold excess (nonspecific). The differences between total and nonspecific binding represent specific binding.*

expression level or other factors in different cell types. The lower basal level of autophosphorylation in 32D-EGFRvIII cells was almost abolished by cetuximab and 80% reduced in CHO-EGFRvIII cells.

Cetuximab inhibits the proliferation of EGFRvIII transfected cells. To evaluate effect of cetuximab on the growth of EGFRvIII expressing cells, a proliferation assay was performed on cetuximab-treated CHO-EGFRvIII and U87-MG-EGFRvIII

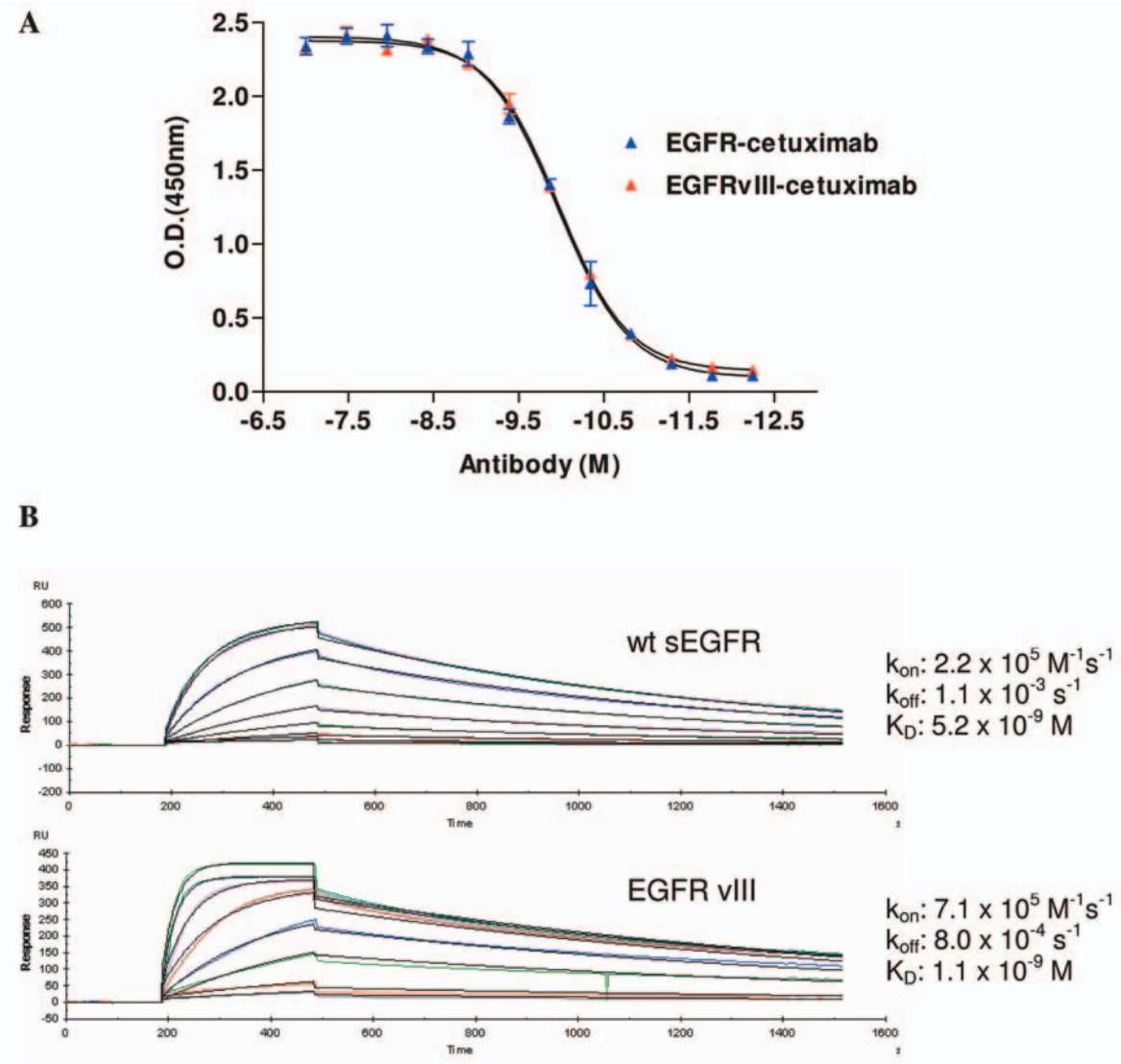

Figure 4. *Cetuximab binds to EGFR and EGFRvIII on ELISA and Biacore analysis. (A) Soluble EGFR and EGFRvIII were coated on ELISA plates and cetuximab binding was detected as described in methods section. EC_{50} were 0.1 nM for EGFR and EGFRvIII. (B) Biacore analysis. Recombinant human EGFR or EGFRvIII were immobilized onto sensor chips and cetuximab was injected at concentrations ranging from 0.5 nM to 100 nM. K_{on} and K_{off} were determined.*

cells (Figure 7). Cetuximab had no direct effect on the growth of untransfected CHO cells, but inhibited the proliferation of CHO-EGFRvIII cells by 51% and U87-MG-EGFRvIII cells by 39%. Since U87-MG cells express full-length EGFR, a 34% inhibition of growth by cetuximab also was observed.

Discussion

EGF binds to the epitopes on domain I and domain III of EGFR (28, 29). The crystal structure of the complex of cetuximab and extracellular domain of EGFR has been resolved recently (30). Cetuximab binds exclusively to domain III of EGFR, covering an epitope that overlaps the EGF binding site on this domain (Figure 8) (30). Both the heavy chain and light chain of cetuximab participate in the interaction with domain III (Figure 8) (30). Cetuximab is able to block EGF binding to its receptor and inhibits the activation of EGFR completely (23-25), suggesting that EGF must be in contact with the sites on domain I and III for high affinity binding and that blockage of one site is sufficient to abolish the ligand binding. Since EGFRvIII lacks domain I and cetuximab does not interact with domain I, II and IV, (Figure 8), it explains why cetuximab binds to EGFRvIII with a similar affinity to that with EGFR.

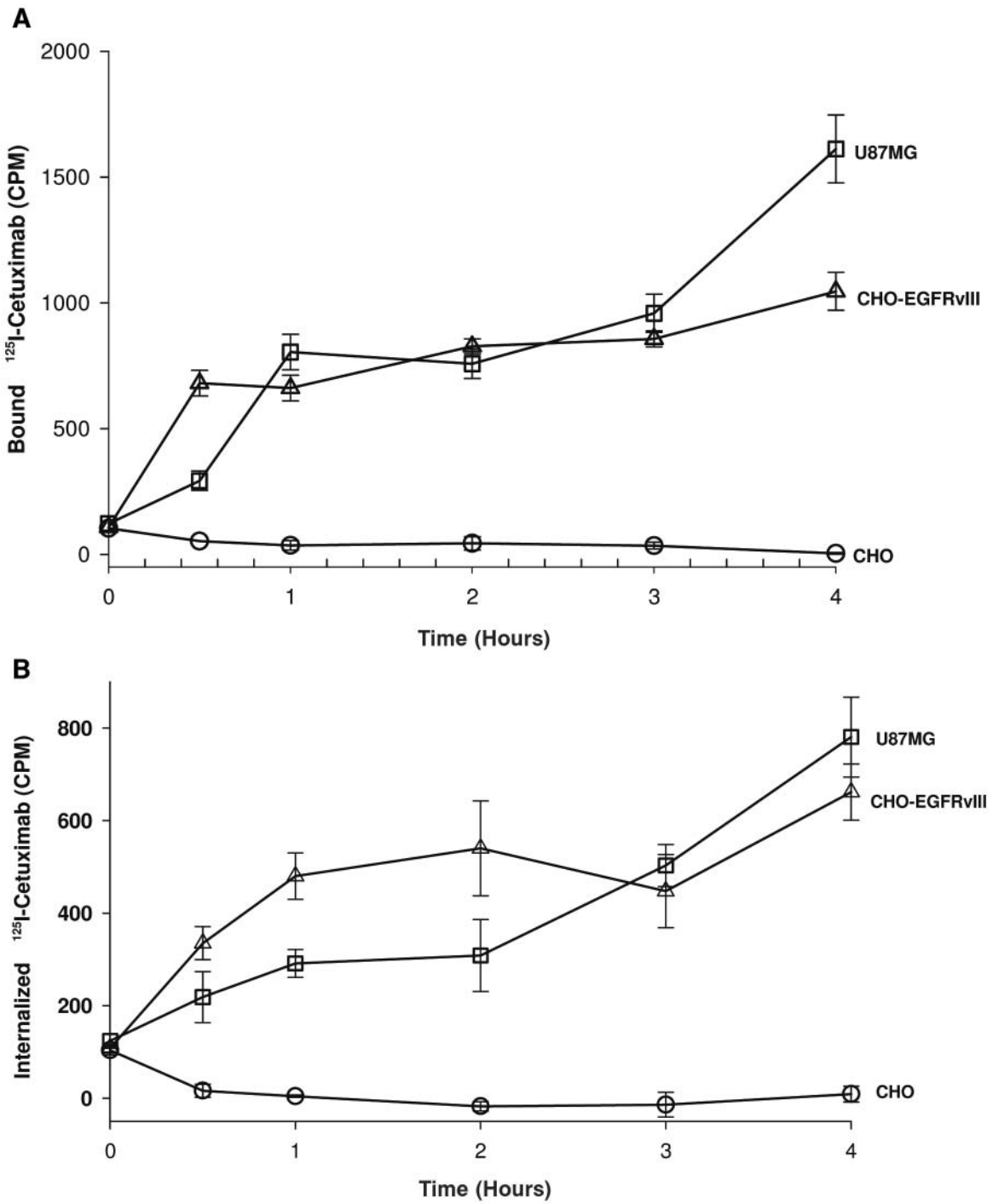

Figure 5. *(A) The time course of cetuximab binding to EGFRvIII. cetuximab was labeled with ^{125}I and incubated with CHO, CHO/EGFRvIII or U87 cells at various times as indicated. The surface bound cetuximab were then stripped with acid and counted. (B) The time course of cetuximab induced internalization. cetuximab was labeled with ^{125}I and incubated with CHO, CHO/EGFRvIII or U87 cells at different times as indicated. The surface bound cetuximab were then stripped away with acid and the internalized cetuximab was released in 1 M NaCl.*

Internalization of the activated receptors is the major mechanism by which cells down-regulate or balance growth signals. Once ligands bind to receptors it triggers a series of events including dimerization, phosphorylation, exposure of cryptic motif for endocytosis, and internalization of receptors. In the case of EGFRvIII, the structural changes

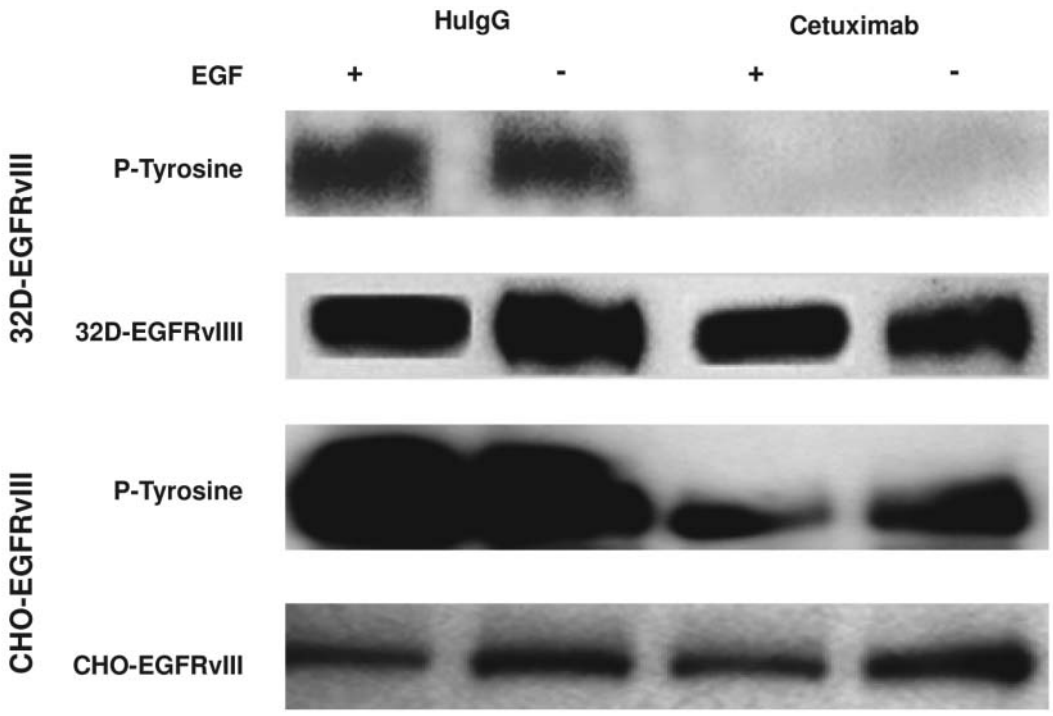

Figure 6. *cetuximab reduced the autophosphorylation of EGFRvIII. 32D-EGFRvIII or CHO-EGFRvIII cells were treated with cetuximab or control hIgG for 2 h and then incubated in the presence (+) or absence (-) of EGF (20 ng/ml) for 30 min before being lysed with lysis buffer. Blots were then probed with antiphosphotyrosine mAb PY20.*

resulting from the deletion of amino acids 6-273 allow dimer formation and autophosphorylation without ligand stimulation, but the cryptic sequence for endocytosis is apparently not exposed (9, 10). Cetuximab not only blocks EGF binding to EGFR, but also induces internalization of EGFR (25, 26). It has been reported that cetuximab Fab fragment incorporated in liposomes induces liposome internalization into cells expressing EGFR or EGFRvIII (31). Our results show that cetuximab induces internalization of the complex of cetuximab-EGFRvIII from cell surface. Although EGF and cetuximab both induce the receptor internalization, the fate of the internalized ligand:receptor or antibody:receptor complex may be different. The EGF:EGFR complex is completely dissociated at endosomal pH levels of ~5 (32). The dissociated EGFR is then recycled to the cell surface (32). On the other hand, cetuximab: EGFR is stable when pH is reduced from 7 to 5 (30). It suggests that cetuximab is unlikely to dissociate from EGFR or EGFRvIII within the endosome, and antibody:receptor complex is likely to be targeted to the lysosome for degradation.

For unknown reasons, tumor cells tend to lose the expression of EGFRvIII *in vitro*. Even in transfected cell lines, EGFRvIII expression is unstable. The expression of

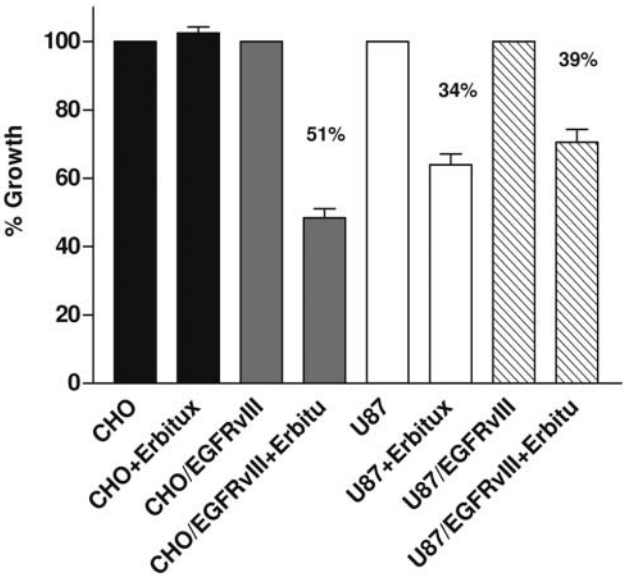

Figure 7. *Cetuximab inhibits proliferation of EGFRvIII-transfected cells. CHO-EGFRvIII, U87-MG-EGFRvIII or control cells were plated 5x10^3 cells/well in RPMI 1640 with 1% serum in 96-well flat bottom plates. Cetuximab or control human IgG was then added at various concentrations in duplicates and incubated four days. CellTiter 96 Aqueous One Solution was then added and plates were read at 490 nm on an ELISA plate Reader.*

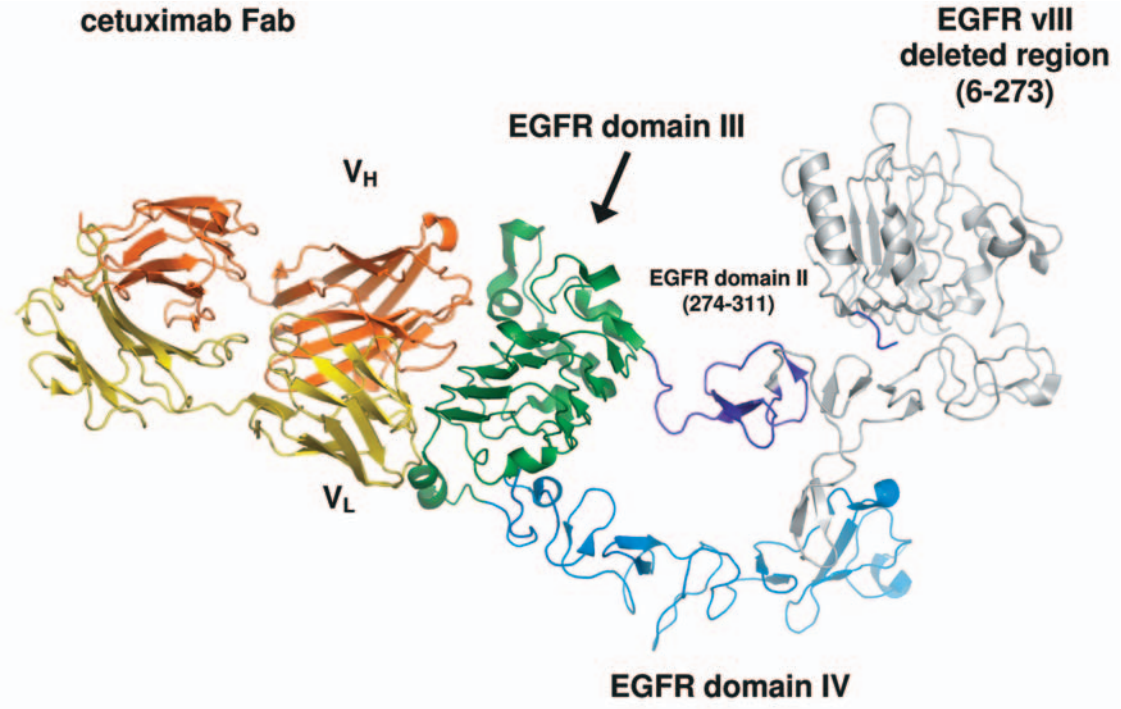

Figure 8. *The cetuximab binding epitope is preserved in EGFR vIII. The ribbon representation of cetuximab Fab-EGFR interaction was drawn based on the crystal structure of cetuximab Fab:sEGFR (27). The V_L chain of cetuximab is shown in yellow, the V_H chain in orange, domain II of sEGFR in purple, domain III of sEGFR in green, domain IV in blue, and EGFRvIII deleted region (domain I and part of domain II) in gray.*

EGFRvIII *in vivo* was determined by flow cytometry of the freshly isolated tumor cells or immunochemical assay of frozen and formalin-fixed tissue (33). EGFRvIII is expressed at 2.7-6.8x10^5/cell in most tumors tested (33), which is lower than the EGFR expression level when both receptors are co-expressed in the same cells. Our stable cell lines expressed approximately 1.09x10^6 and 0.97x10^6 EGFRvIII/cell in CHO and 32D transfectants respectively, which is comparable to that of primary tumors. EGFRvIII may also form heterodimers with other members of Erb family (13). Such heterodimers may influence EGFRvIII activity and expression pattern. Whether these dimers can be phosphorylated without ligand stimulation and what effects of EGF or cetuximab have on these dimers remain to be determined. The blockage of the EGFR pathway by cetuximab in tumor cells *in vitro* cannot predict efficacy of cetuximab *in vivo*. Cetuximab can inhibit the growth of EGFR-expressing tumor dramatically (24) or has no effect on the growth of EGFR-expressing tumor *in vivo* (34). Although we have shown that cetuximab down-regulates EGFRvIII activation, the effect of cetuximab on EGFRvIII-expressing tumors *in vivo* needs to be further investigated.

EGFRvIII is expressed exclusively in tumor cells. The in-frame deletion of amino acids 6-273 has generated new tumor-specific epitopes near the amino acid terminus of the receptor extracellular domain, which provides the opportunity to develop tumor-specific antibodies for therapy. Several EGFRvIII specific antibodies have been generated (20-22). These EGFRvIII-specific antibodies have been used to assess both the quantitative and qualitative expression of EGFRvIII in cells and tissue samples derived from human tumor biopsies. Some EGFRvIII-specific antibodies, such mAb ICR62 and mAb 806, also bound to EGFR and have shown antitumor effects *in vivo* (20, 21). The binding affinity and epitopes of these antibodies on EGFR may be different from that of cetuximab. For example, mAb 806 bound to disulfide-bonded loop within domain II of EGFR (amino acids 287-302) (35). It bound to EGFRvIII with high affinity (1 nM), but did not bind to parental cells that express wild-type EGFR. Only when EGFR is expressed in cells containing amplification of the EGFR gene can it be recognized by mAb 806 and the binding affinity was lower than that determined for EGFRvIII (35). On the other hand, cetuximab bound to EGFR and EGFRvIII with same affinities (Figures 3 and 4).

In summary, to our surprise we found that the EGF blocker, cetuximab, not only binds to EGFRvIII but also down-regulates its phosphorylation. The mechanism of this action needs to be further investigated. The finding that cetuximab can inhibit both EGFR and EGFRvIII activation

is clinically important. EGFRvIII always co-exists with EGFR on tumor cells and both receptors are oncogenic on tumor cells. Antibodies that target both EGFR and EGFRvIII will have tremendous advantage over single receptor blockers.

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