DOI:10.1158/1078-0432.CCR-06-1453

Clinical Cancer Research

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Clin Cancer Res 2007;13:1911-1925. Published online March 15, 2007.

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The Efficacy of Epidermal Growth Factor Receptor – Specific Antibodies against Glioma Xenografts Is Influenced by Receptor Levels, Activation Status, and Heterodimerization

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Abstract

Purpose: Factors affecting the efficacy of therapeutic monoclonal antibodies (mAb) directed to the epidermal growth factor receptor (EGFR) remain relatively unknown, especially in glioma.

Experimental Design: We examined the efficacy of two EGFR-specific mAbs (mAbs 806 and 528) against U87MG-derived glioma xenografts expressing EGFR variants. Using this approach allowed us to change the form of the EGFR while keeping the genetic background constant. These variants included the de2-7 EGFR (or EGFRvIII), a constitutively active mutation of the EGFR expressed in glioma.

Results: The efficacy of the mAbs correlated with EGFR number; however, the most important factor was receptor activation. Whereas U87MG xenografts expressing the de2-7 EGFR responded to therapy, those exhibiting a dead kinase de2-7 EGFR were refractory. A modified de2-7 EGFR that was kinase active but autophosphorylation deficient also responded, suggesting that these mAbs function in de2-7 EGFR – expressing xenografts by blocking transphosphorylation. Because de2-7 EGFR – expressing U87MG xenografts coexpress the wild-type EGFR, efficacy of the mAbs was also tested against NR6 xenografts that expressed the de2-7 EGFR in isolation. Whereas mAb 806 displayed antitumor activity against NR6 xenografts, mAb 528 therapy was ineffective, suggesting that mAb 528 mediates its antitumor activity by disrupting interactions between the de2-7 and wild-type EGFR. Finally, genetic disruption of Src in U87MG xenografts expressing the de2-7 EGFR dramatically enhanced mAb 806 efficacy.

Conclusions: The effective use of EGFR-specific antibodies in glioma will depend on identifying tumors with activated EGFR. The combination of EGFR and Src inhibitors may be an effective strategy for the treatment of glioma.

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with intrinsic tyrosine kinase activity. Overexpression of the EGFR is observed in numerous epithelial tumors and is often associated with a poorer clinical prognosis (1 – 3). Overexpression of the EGFR can result from *EGFR* gene amplification, particularly in glioma (4). In glioma, gene amplification is associated with *EGFR* rearrangements with the most common mutation, the de2-7 EGFR (or EGFRvIII), characterized by an in-frame deletion of 801 bp spanning exons 2 to 7 of the coding sequence (4 – 6). This rearrangement results in the deletion of 267 amino acids from the extracellular domain and the insertion of a novel glycine at the fusion site, all of which produces a unique junctional peptide. Although the de2-7 EGFR is unable to bind any known ligand, the receptor displays a low level of constitutive activation and is able to enhance the growth of glioma and breast cancer xenografts (7, 8).

Inhibition of the EGFR is a rational strategy for the development of new cancer therapeutics. Potential therapeutics include monoclonal antibodies (mAb) directed to the EGFR (e.g., C225, ABX-EGF, and EMD55900; refs. 9 – 11) and small molecular weight tyrosine kinase inhibitors (TKI) of the EGFR (e.g., ZD1839 and OSI-774; ref. 12). Indeed, some of these therapeutics have been approved for limited clinical use in lung cancer (ZD1839, Iressa) and colon cancer (C225, Erbitux). From these clinical trials, it is abundantly clear that not all patients positive for the EGFR respond to these targeted therapeutics (Table 1). Determining factors that cause patients to be susceptible to EGFR therapeutics is an important goal from a patient welfare and economic point of view. Likewise, understanding the nature of resistance to EGFR therapeutics may help identify approaches for overcoming it.

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Received 6/14/06; revised 12/15/06; accepted 12/27/06.

Grant support: National Health and Medical Research Council of Australia program grant 280912 and project grant 433615 and National Cancer Institute grant PO1 CA95616 (W.K. Cavenee and F.B. Furnari), Goldhirsh Foundation Scholar Award for Cancer Research (F.B. Furnari), and National Foundation for Cancer Research Fellow Award (W.K. Cavenee).

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Mechanisms causing resistance/susceptibility to EGFR-targeted TKIs have been studied extensively, whereas factors affecting the efficacy of anti-EGFR antibodies remain relatively unknown (see Table 1). A few generalizations can be drawn from these studies with respect to TKIs. First, the sensitivity of cell lines to inhibition by TKIs correlates with increasing cell surface EGFR (Table 1), suggesting that there is some intrinsic level of EGFR expression required for these inhibitors to function. Second, the ability to sustain signaling through the phosphatidylinositol 3-kinase/Akt pathway following EGFR inactivation reduces the efficacy of TKIs (Table 1). The overwhelming number of these studies has been done *in vitro*; thus, it is not known if these observations hold true in the *in vivo* setting. Recently, several studies have analyzed the status of the *EGFR* gene in lung cancer patients treated with Iressa (ZD1839) and found that patients who responded to therapy often had gain-of-function mutations in the kinase domain (Table 1). Furthermore, a secondary kinase mutation that leads to Iressa resistance has also been described (Table 1). Initial studies suggest, however, that these observations are not general and that the mutations described in lung patients are not observed in other tumor types.

The limited number of studies using anti-EGFR antibodies makes it difficult to derive any generalizations about susceptibility to these agents (Table 1). Apart from the lack of *in vivo* studies, many of these susceptibility studies have been done

Table 1. Cellular aspects associated with susceptibility to EGFR therapeutics

EGFR inhibitor	Experimental system	Observation	Comment	References
PD153035	Multiple cell lines *in vitro*	Sensitivity correlated with wt EGFR number	No *in vivo* data	(32)
C225	Renal cell carcinomas *in vitro*	Only cells containing the *VHL* gene were sensitive	No *in vivo* data	(33)
EMD55900 and EMD72000	Multiple cell lines *in vitro* and xenografts	Sensitivity correlated with wt EGFR number		(34)
SU1195 and ZD1839	Multiple cell lines *in vitro* and xenografts	More difficult to inhibit the phosphorylation of EGFR in the presence of ErbB2		(35)
mAbR3 and C225	A431 xenografts	Recurrent xenografts following complete regression were often resistant to further therapy	Overexpression of VEGF was a common observation in resistant cell lines	(36)
ZD1839	A431 and NR6M (express the de2-7 EGFR) xenografts	Xenografts expressing the de2-7 EGFR were resistant	NR6M expresses the de2-7 EGFR in the absence of the wt EGFR, clinically both are coexpressed	(37)
AG1478	Glioma cell lines *in vitro*	Resistant glioma expresses IGFR-I, which is further up-regulated by AG1478. IGFR-I effect seems mediated through PI3K/Akt	Observation restricted to a single cell line *in vitro*	(38)
CGP59326	BT474 breast and MKN7 gastric cancer cells *in vitro*	Activation of erbB2/3 heterodimers by heregulin-generated resistance	No *in vivo* data	(39)
ZD1839	Multiple cell lines *in vitro*	Sensitivity correlated with wt EGFR number Constitutive active MAPK increased resistance.	No *in vivo* data	(40)
AG1478	Large cell panel *in vitro*	Two requirements for sensitivity: high wt EGFR and ability to respond to EGF by entering cell cycle	No *in vivo* data	(41)
ZD1839 and PD153035	Multiple cell lines *in vitro*	Sustained signaling through Akt or Erk may cause resistance	No *in vivo* data	(42)
ZD1839	A431 and MDA-468 breast cancer cells *in vitro*	Sustained signaling through Akt causes resistance. Presence of PTEN increases effectiveness of EGFR therapeutics	No *in vivo* data	(43)
ZD1839 and C225	A431 and multiple NSCLC *in vitro*	No correlation with EGFR number	No *in vivo* data	(44)
ZD1839	Patients with NSCLC	Patients with activating mutations in the EGFR kinase domain more likely to respond	Subsequent data suggest that not all patients with mutations respond	(28, 45)
ZD1839	NR6 fibroblasts and U87MG glioma cells	Cells expressing the de2-7 EGFR were resistant, possibly related to an inability to fully inhibit de2-7 EGFR phosphorylation	No *in vivo* data	(46)
OSI-774	Panel of glioma cell lines	Cells capable of increasing the mRNA for EGFR in response to therapy are more resistant		(47)
ZD1839 and OSI-774	Patients with NSCLC	A secondary mutation in EGFR kinase causes resistance		(48)
C225 and ABX	Patients with colorectal cancer	Response correlated with increase in *EGFR* copy number	Small sample numbers	(26)
OSI-774 and ZD1839	Patients with glioma	Coexpression of EGFRvIII and PTEN is associated with responsiveness		(49)

Abbreviations: IGFR-I, insulin-like growth factor receptor-I; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; NSCLC, non–small cell lung cancer.

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using cell panels, which, given the variation in signaling pathways between cell lines and the presence or absence of other ErbB family members, makes it difficult to identify single factors associated with EGFR sensitivity or resistance. To address some of these issues, we tested the *in vivo* susceptibility of the U87MG glioma cell line, which expresses modest levels of the wild-type (wt) EGFR, to two EGFR-specific antibodies. We then transfected U87MG cells with a variety of wt and de2-7 EGFR constructs to determine what effect receptor number and activation has on susceptibility to antibody therapy.

The two antibodies used in this study are mAbs 806 and 528. MAb 806 is a novel anti-EGFR–specific antibody that was raised against cells expressing the de2-7 EGFR (13). Interestingly, although mAb 806 clearly binds the de2-7 EGFR, it also binds to a subset of the wt EGFR (~10%) expressed on the surface of cells overexpressing the receptor (13). Recent analysis showed that the mAb 806 epitope is only exposed in a conformational form of the EGFR that exists transiently as the receptor moves from its inactive to active state (14). Unlike the wt EGFR, the de2-7 EGFR is constitutively in this transitional conformation and thus available for mAb 806 binding. Our previous studies have shown that treatment of xenografts, which express the de2-7 or overexpress the wt EGFR with mAb 806, causes significant inhibition of tumor growth (15–17). The 528 antibody was produced and isolated at the same time as the murine version of the C225 antibody (Erbitux) and displays very similar properties (18). MAb 528 acts as a ligand antagonist and inhibits the growth of EGFR-expressing cells both *in vitro* and *in vivo* when grown as xenografts (18).

Materials and Methods

Cell lines and mAbs. The U87MG-transfected cell lines U87MG.Δ2-7, U87MG.DK, U87MG.wt, U87MG.DY5, and U87MG.DY2 have been described in detail elsewhere (16, 19). The A431 cell line has also been described previously (20). All cell lines were maintained in either DMEM (DMEM/F12; Life Technologies, Inc., Grand Island, NY) or RPMI 1640 containing 10% FCS (CSL, Melbourne, Victoria, Australia), 2 mmol/L glutamine (Sigma Chemical Co., St. Louis, MO), and penicillin/streptomycin (Life Technologies, Grand Island, NY). In addition, transfected cell lines were maintained in 400 mg/mL geneticin (Life Technologies, Inc., Melbourne, Victoria, Australia).

The mAbs 806 and 528 were produced and purified in the Biological Production Facility (Ludwig Institute for Cancer Research, Heidelberg, Melbourne, Australia). Antibodies to the specific tyrosine phosphorylation sites of the EGFR and a rabbit polyclonal anti-EGFR antibody were obtained from Cell Signaling Technology (Danvers, MA). Src was detected using the mouse mAbs v-Src 327 (Oncogene Research Products, San Diego, CA) or c-Src H-12 (Santa Cruz Biotechnology, Santa Cruz, CA). The rabbit polyclonal antibody PY418 (BioSource International, Inc., Camerillo, CA) was used for the detection of phospho-Src. The anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). The C13 used for detection of both wt and truncated EGFR was obtained from BD Transduction Laboratories (San Diego, CA).

Generation of U87MG.Δ2-7$_{DNSrc}$ cell line. A dominant-negative, kinase dead Src construct (K296R/Y528F) was obtained from Upstate Biotechnology. A *Hin*dIII fragment containing the DNSrc was subcloned into the pcDNA3.1/Hygro(+) vector obtained from Invitrogen Life Technologies (Carlsbad, CA) and the resulting construct was transfected into U87MG.Δ2-7 cells by electroporation. A second cell line transfected with the pcDNA3.1/Hygro vector alone was also generated. Cells were plated out in 1 mL aliquots into 96-well plates, at a density of approximately 2×10^4 cells per well, and incubated at 37°C for 48 h after which 100 μg/mL hygromycin (Roche Diagnostics, Mannheim, Germany) was added. Once clones were obtained (~2 weeks), cells were placed back in 400 μg/mL geneticin as well as the hygromycin.

Transfected cells were initially screened by fluorescence-activated cell sorting analysis to confirm that expression of the de2-7 EGFR had been retained. Clones were then subjected to either whole-cell lysis or immunoprecipitation before Western blotting using Src-specific antibodies (v-Src 327 and c-Src H-12). Several clones showing dramatically increased levels of total Src (Src levels are very low in the original cell line) were identified and expanded. The increased Src levels were further confirmed by immunoprecipitating ^{35}S-labeled cell lysates with the v-Src 327 antibody and subjecting the resulting precipitates to SDS-PAGE and quantitative autoradiography. The clone expressing the highest levels of DNSrc was selected and the DNSrc was shown to be phosphorylated at position Y418, suggesting that it is correctly folded.

In vitro *growth assays.* The antiproliferative effect of mAbs 806 and 528 *in vitro* was examined as described in detail previously (18). Briefly, cells were seeded at 1×10^4 cells per well in 24-well plates in medium containing 0.5% FCS. After 4 days, cells were removed with trypsin and counted using a hemocytometer. Antibodies were used at a final concentration of 100 μg/mL, a concentration consistent with that obtained within xenografts.

Xenograft models. Tumor cells (3×10^6) in 100 μL PBS were inoculated s.c. into both flanks of 4- to 6-week-old, female nude mice (Animal Research Centre, Perth, Western Australia, Australia). All studies were conducted using established tumor models as reported previously (15, 16). Treatment commenced once tumors had reached a mean volume of ~100 mm^3. Tumor volume in cubic millimeter was determined using the formula (length × width^2) / 2, where length was the longest axis and width being the measurement at right angles to the length. Data are expressed as mean tumor volume ± SE for each treatment group. All data were analyzed for significance by Student's *t* test. A minimum of 10 xenografts per group were used in each study.

Immunoblotting. Cells were lysed in cold lysis buffer [30 mmol/L HEPES, 150 mmol/L NaCl, 10 mmol/L NaF, 1% Triton X-100, 200 μmol/L NaO_3V, 0.4% H_2O_2, the protease inhibitor cocktail set 1 (Calbiochem, San Diego, CA) containing 500 μmol/L AEBSF, 150 nmol/L aprotinin, 1 μmol/L E-64 protease inhibitor, 0.5 mmol/L EDTA, and 1 μmol/L leupeptin (pH 7.4)]. Lysates were immunoprecipitated with the mAb 806 or 528, and the resultant precipitates were analyzed by immunoblotting as described by us in detail (21).

Immunofluorescence microscopy. MAbs 806 and 528 were directly labeled with Cyanine 3 (Cy3) dye using the Cy3 mAb Labeling kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, United Kingdom) according to the manufacturer's instructions. Successful labeling of antibody was determined via flow cytometry analysis of binding to U87MG.Δ2-7 cells. The early endosome-specific, antimouse early endosome autoantigen 1 (EEA1) mAb was purchased from Transduction Laboratories. Cyanine 2–conjugated AffiniPure F(ab')^2 fragment donkey anti-mouse IgG secondary antibody and unlabeled AffiniPure Fab fragment goat anti-mouse IgG blocking antibody were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). U87MG.Δ2-7 or NR6.Δ2-7 cells were grown on 12 mm glass coverslips or 12 mm Biocoat Cell Environments Poly-L-Lysine coverslips (Becton Dickinson Labware, Bedford, MA) in MEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, penicillin/streptomycin, and glutamate at 37°C. Antibody binding to cells was carried out in the presence of 0.25% bovine serum albumin (Sigma Chemical). Cy3-conjugated mAbs 806 and 528 were used at concentrations of 5 and 2 μg/mL, respectively, and surface labeling was carried out at 4°C for 20 min under humidified conditions. Cells were washed thrice in ice-cold 0.25% bovine serum albumin/PBS. Internalization of surface-bound antibody was initiated by incubation of individual coverslips at 37°C. Following internalization for varying periods, individual coverslips were removed from 37°C, washed thrice

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in ice-cold bovine serum albumin/PBS to stop internalization, and fixed in 4% paraformaldehyde for 20 min at room temperature. Coverslips were then washed in bovine serum albumin/PBS before washing in double-distilled water and mounted onto glass slides with fluoromount G mounting medium (Southern Biotechnology, Birmingham, AL). Samples were analyzed with confocal microscope (Nikon Instech Co. Ltd., Kanagawa, Japan) using appropriate wavelength settings. For colocalization studies, cells were permeabilized with 0.1% Triton X-100 for 1 min. Samples were then washed and incubated with unlabeled goat anti-mouse Fab fragment to block all existing mouse binding sites (i.e., internalized mAb 806 or 528) for 20 min at room temperature. Samples were then washed in bovine serum albumin/PBS before incubation with anti-EEA1 for 20 min at room temperature. Cells were finally washed and incubated with cyanine 2–conjugated secondary donkey anti-mouse $F(ab')^2$ antibody fragment. DNA vectors for green fluorescence protein (GFP)–tagged lysosomal glycoprotein 120 (lgp-120-GFP) was kindly provided by Prof. Ira Mellman and professor from the Department of Cell Biology, Yale University School of Medicine (New Haven, CT). Cells grown in MatTek glass bottom microwell dishes containing an embedded 14 mm glass coverslip (MatTek Corp. Ashland, MA) were transfected overnight using LipofectAMINE reagent (Invitrogen Life Technologies, Mulgrave, Victoria, Australia) following the manufacturer's instructions. Confocal imaging of positively transfected cells, which fluoresced green when excited with 488 nm wavelength light, was undertaken 24 h after transfection.

Results

Correlation between* in vitro *and* in vivo *sensitivity. Many of the studies described in Table 1 have been conducted *in vitro*. Our experience with both mAb- and TKI-targeted EGFR therapy clearly shows that *in vitro* sensitivity and *in vivo* response do not reliably correlate. Indeed, we published recently an example where two cell lines showing similar sensitivity to the EGFR-specific TKI AG1478 *in vitro* differed notably in their *in vivo* response to the same agent (22). Using a standard *in vitro* growth inhibition assay described previously for C225, and an antibody concentration consistent with that achieved at the xenograft site, we saw little correlation between antibody inhibition *in vitro* and *in vivo* antitumor activity (Table 2). Neither mAb 528 or 806 inhibited the growth of U87MG.Δ2-7 cells *in vitro*, but both antibodies display robust antitumor activity *in vivo* that was independent of immune effector function (see Fig. 2). In addition, even if one EGFR-targeted antibody showed correlation *in vitro* and *in vivo* in a particular cell line (e.g., mAb 528 in A431 cells and xenografts; Table 2), this did not necessarily imply that another EGFR-specific antibody will correlate in the same cell line (e.g., mAb 806 in A431 cells and xenografts; Table 2). This simple analysis, along with our previous observations clearly, shows the limited value of *in vitro* assays in determining sensitivity to EGFR therapeutics.

Table 2. *In vitro* and *in vivo* comparison of sensitivity to EGFR therapeutics

	Cell line			
	U87MG.Δ2-7		A431	
	mAb 528	mAb 806	mAb 528	mAb 806
In vitro	–	–	+	–
*In vivo**	+	+	++	++

**In vivo* data for A431 xenografts from Perera et al. (16).

Antibody therapy of U87MG glioma xenografts expressing different forms of the EGFR. The parental U87MG cells, which express moderate levels of the wt EGFR, or the same cell line transfected with additional wt EGFR, the de2-7 EGFR or various modified forms of the de2-7 EGFR (Fig. 1) were injected s.c. into nude mice and allowed to establish as tumor xenografts. Treatment with antibody commenced once xenografts had reached ~100 mm^3. All tumors were treated with 1 mg mAb 528 or 806 thrice weekly for 2 weeks. This dose and schedule of antibody treatment was chosen as it elicits a strong antitumor response in our standard U87MG.Δ2-7 xenograft model but is not so efficacious that it would obscure any increased antitumor activity that might be seen in other U87MG-derived cell lines containing different variants of the EGFR. As discussed in detail below, the antitumor efficacy of mAbs 806 and 528 was similar in all the U87MG-derived glioma xenografts (Fig. 2).

(*a*) Parental cells (U87MG): neither antibody inhibited the growth of the U87MG xenografts despite the fact that it expresses the EGFR at moderate levels (approximately 5×10^4 receptors per cell; ref. 13).
(*b*) Cells overexpressing the wt EGFR (U87MG.wt): transfection of U87MG cells with the wt EGFR to increase expression (approximately 1×10^6 receptors per cell) did not change the *in vivo* growth rate of the xenografts (Fig. 3A) but caused the tumors to become sensitive to both antibodies. Although this is not surprising for mAb 806, as it preferentially binds to cells overexpressing the wt EGFR, it was somewhat unexpected for mAb 528, as it suggests that even an increase in receptor number in the absence of a phenotypic change can induce a response to antibody therapy. On day 31, when the control group was sacrificed, the inhibition induced by mAb 528 was significant ($P < 0.01$), with xenografts in the vehicle group having a mean tumor volume of 950 mm^3 compared with 450 mm^3 in the mAb 528 treatment group. Analysis of the mAb 806 experiment on day 39 showed that antibody treatment significantly inhibited xenograft growth ($P < 0.001$) with tumor volumes being 960 and 470 mm^3 for the PBS and mAb 806, groups, respectively.
(*c*) Cells expressing the de2-7 EGFR (U87MG.Δ2-7): the growth of U87MG xenografts transfected with the constitutively active, but ligand-independent, de2-7 EGFR was also inhibited by both antibodies (Fig. 2). Unlike overexpression of the wt EGFR, coexpression of the de2-7 EGFR in the presence of endogenous wt EGFR generates a significant growth advantage to U87MG xenografts (Fig. 3B). The constitutive phosphorylation of this receptor was confirmed by immunoblotting (Fig. 4). Treatment with mAb 528 significantly inhibited tumor growth ($P < 0.005$) with the vehicle group having an average tumor volume of 1,170 mm^3 compared with 510 mm^3 for the mAb 528 group at day 20 postinoculation. Given that the primary function of mAb 528 has been presumed to be ligand antagonism, its antitumor activity against a xenograft expressing the ligand-independent de2-7 EGFR was unexpected. Thus, mAb 528 probably disrupts EGFR signaling by other mechanisms apart from its ability to block ligand. Likewise, mAb 806, which only

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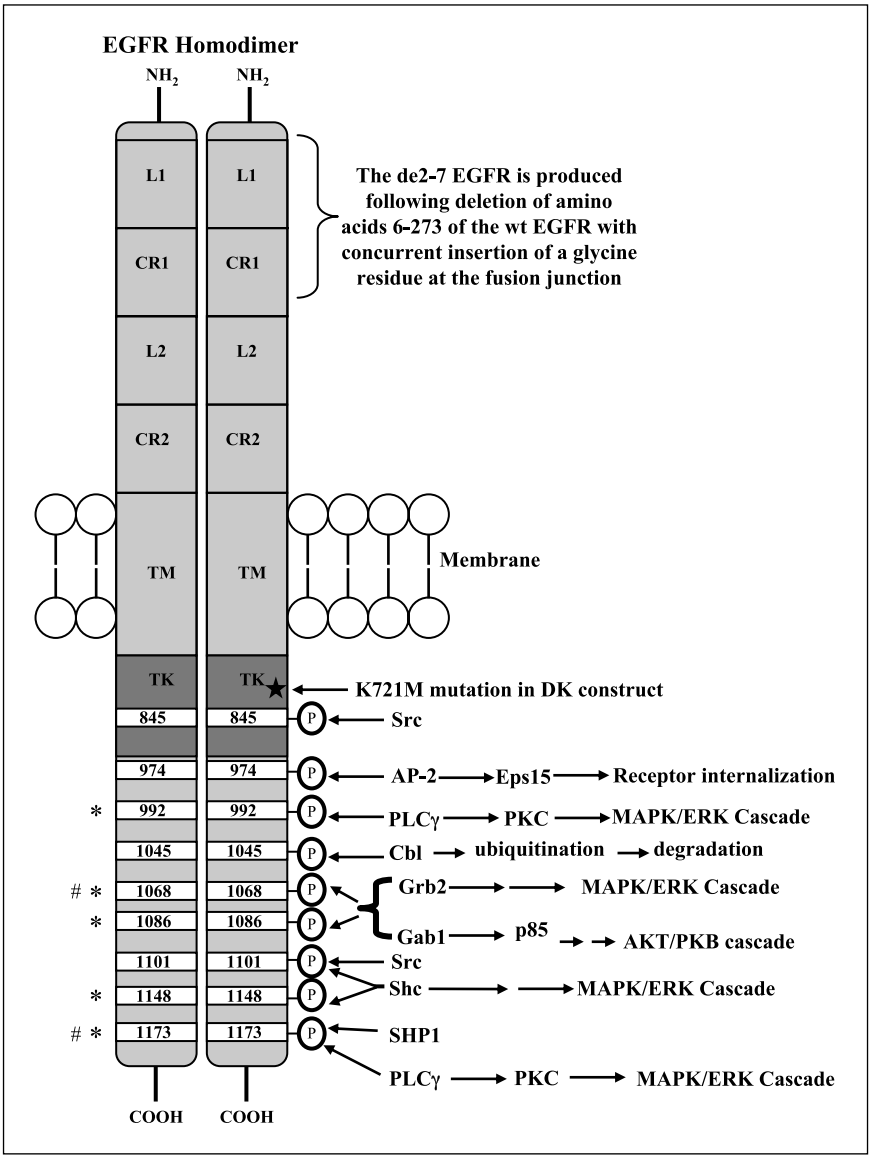

Fig. 1. Schematic representation of the EGFR. The extracellular region deleted in the de2-7 EGFR is identified by parenthesis. The DK version of the de2-7 EGFR contains a single point mutation (K→M) at position 721. The DY2 version of the de2-7 EGFR has Y→F mutations at residues 1068 and 1173, whereas the DY5 variant also has these substitutions plus 992, 1086, and 1148. *, tyrosine residues mutated in DY5; #, tyrosine residues mutated in DY2.

binds the de2-7 EGFR and not the wt EGFR in these cells, must mediate its antitumor activity independent of any effect on ligand interaction as it inhibited the growth of de2-7 EGFR–expressing xenografts to a similar level as mAb 528. At day 21, when the vehicle group was culled, the control xenografts had a mean tumor volume of 1,500 mm^3 compared with a significantly lower 390 mm^2 in the mAb 806–treated group ($P < 0.0001$). Thus, both antibodies can inhibit glioma xenografts expressing a ligand-independent but constitutively active form of the EGFR.

(*d*) Cells expressing a dead kinase (DK) version of the de2-7 EGFR (U87MG.DK): U87MG cells transfected with a DK version of the de2-7 EGFR grew as xenografts at a rate similar to parental cells (Fig. 3B) and were not significantly inhibited by either antibody (Fig. 2). This receptor lacks phosphorylation at the major sites associated with signaling but remains phosphorylated at sites associated with receptor internalization and degradation (Fig. 4). Binding of both antibodies to these cells is similar to that seen in de2-7 EGFR–expressing cells both *in vitro* and *in vivo* (16). Furthermore, because the DK variant of the de2-7 EGFR only contains a single intracellular point mutation, the affinity of mAbs 806 and 528, which bind the extracellular domain, should not be altered. This result shows that any immune effector function mediated by these antibodies *in vivo* is insufficient to initiate an antitumor response. Furthermore, it shows that the antitumor activity of anti-EGFR antibodies require a receptor with a functional kinase domain.

(*e*) Cells expressing a version of the de2-7 EGFR with deletion of two major sites for autophosphorylation (U87MG.DY2): U87MG xenografts expressing a de2-7 EGFR construct unable to autophosphorylate at two major autophosphorylation sites (Tyr^{1068} and Tyr^{1173} changed to phenylalanine) were significantly inhibited by both antibodies when grown as tumor xenografts ($P < 0.01$ and 0.006 for mAbs 528 and 806, respectively; Fig. 2). This observation, combined with the lack of activity seen against the U87MG.DK xenografts, suggests that the kinase activity, as opposed to autophosphorylation, correlates with responsiveness to antibody therapy.

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(*f*) Cells expressing a version of the de2-7 EGFR incapable of autophosphorylation (U87MG.DY5): U87MG cells expressing a de2-7 EGFR construct unable to autophosphorylate at all five major autophosphorylation sites associated with signaling (Tyr^{1173}, Tyr^{1148}, Tyr^{1086}, Tyr^{1068}, and Tyr^{992} changed to phenylalanine) were grown as tumor xenografts. This receptor lacks phosphorylation at the major sites associated with signaling but remains phosphorylated at

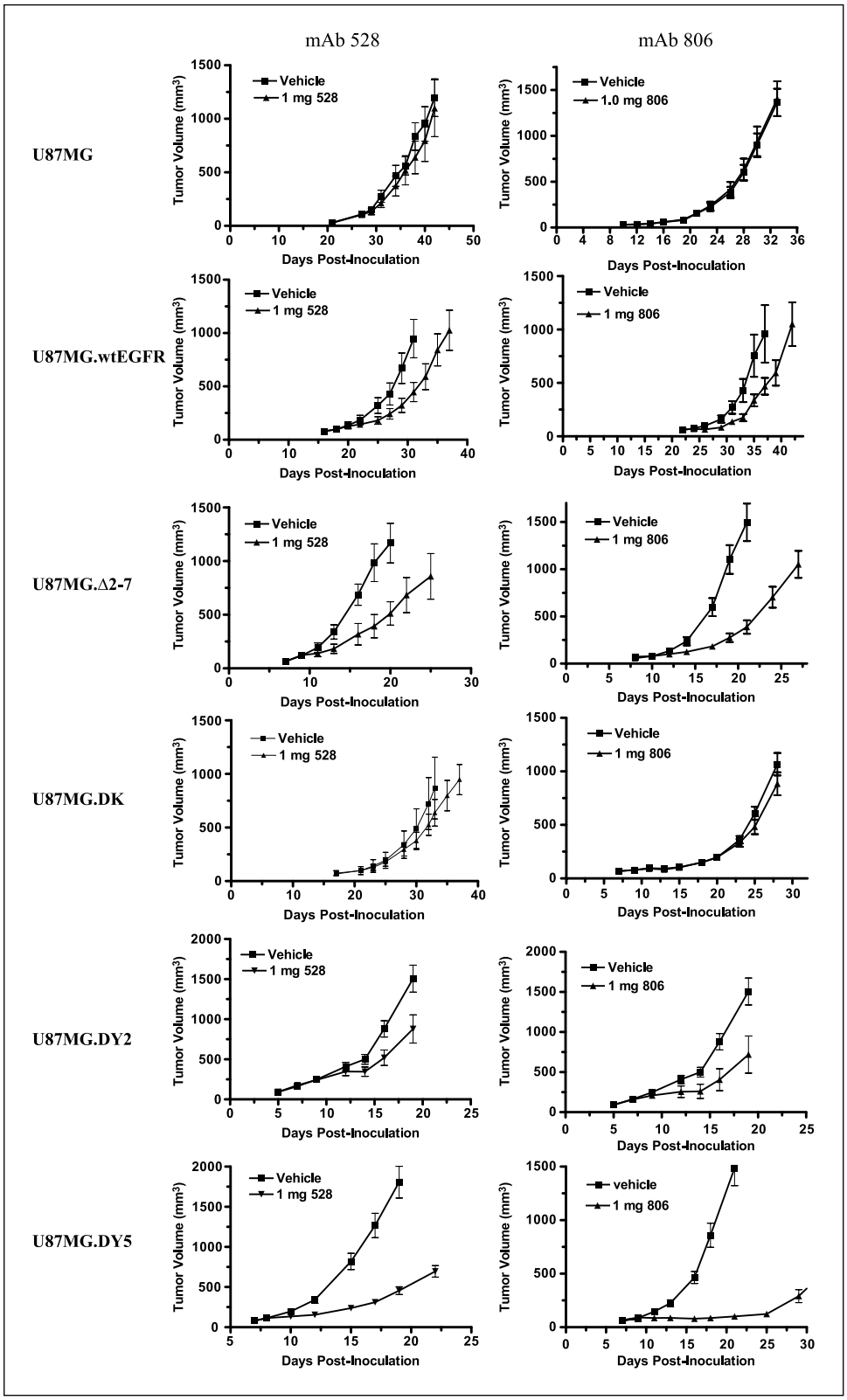

Fig. 2. Sensitivity of different xenografts to EGFR-specific antibodies. Xenografts were established by injection of 3×10^6 cells in both flanks of nude BALB/c mice. Antibody therapy commenced when xenografts reached an approximate mean volume of 100 mm^3. Mice were treated with 1 mg mAb 528 (*left*) or mAb 806 (*right*) thrice weekly for 2 wks (i.e., a total of six injections). Points, mean tumor volume; bars, SE.

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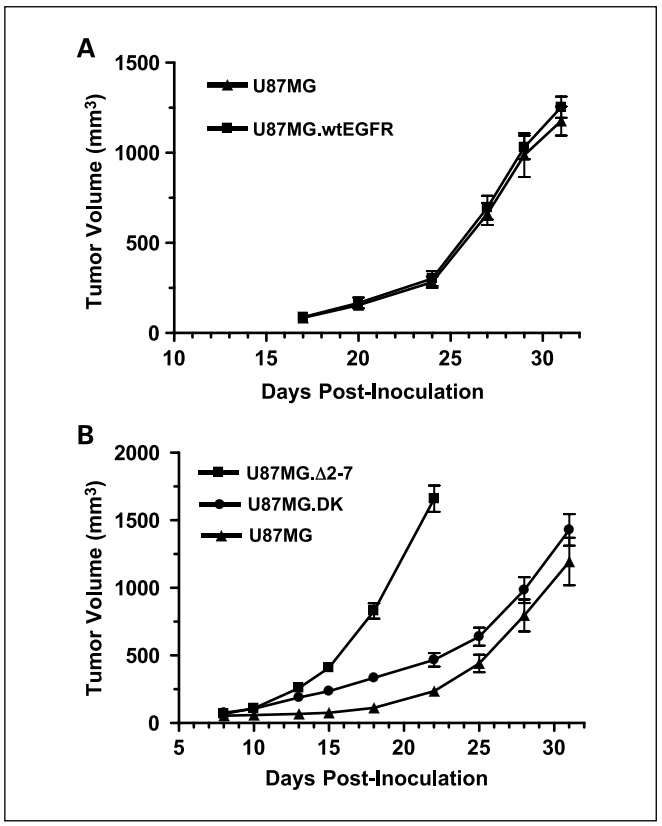

Fig. 3. Xenograft growth curves for U87MG-based cell lines. Xenografts were established by injection of 1×10^6 cells in both flanks of nude BALB/c mice in order to determine growth curves. Points, mean tumor volume; bars, SE.

sites associated with receptor internalization and degradation (Fig. 4). Consistent with the result obtained with DY2 xenografts, both antibodies significantly inhibited the growth of xenografts expressing the DY5 de2-7 EGFR construct ($P < 0.0001$ for both antibodies; Fig. 2). Given this somewhat unexpected result, we repeated this experiment with both antibodies, at a lower dose (0.5 versus 1 mg per injection), and once again obtained significant inhibition of tumor growth in both cases (data not shown). Because the DY5 form of the de2-7 EGFR is incapable of directly binding adapter molecules critical for downstream signaling, it suggests that an active kinase domain rather than the interaction with these molecules is a critical feature that leads to responsiveness to EGFR-specific antibodies.

Treatment of U87MG xenografts expressing high levels of the de2-7 EGFR. The data in Fig. 2 suggest that the more dependent a xenograft becomes to EGFR signaling the more likely it is to respond to EGFR-specific antibody therapy. Therefore, using fluorescence-activated cell sorting, we isolated the cells expressing very high levels of the de2-7 EGFR (U87MG.Δ2-7_{high}; Fig. 5A). U87MG.Δ2-7_{high} xenografts grew faster than the original U87MG.Δ2-7 xenografts (Fig. 5B), suggesting that the rapid growth of these xenografts is reliant on the high levels of the de2-7 EGFR. The levels of de2-7 EGFR expression were retained *in vivo* as determined by immunoblotting of xenograft lysates (Fig. 5C). Treatment with mAb 806 or 528 caused significant inhibition of U87MG.Δ2-7_{high} xenografts that was greater than that observed for any other of the U87MG-derived cell lines (Fig. 5D). On day 18, when the control group was sacrificed for ethical reasons, the mean tumor volume was 1,760, 90, and 90 mm^3 for the vehicle, mAb 806, and mAb 528 groups, respectively ($P < 0.001$). Significantly, although there were no complete regressions in any of the previous U87MG-derived therapy studies (Fig. 2), 40% of the mAb 806–treated and 20% mAb 528–treated U87MG.Δ2-7_{high} xenografts completely regressed. One of the mAb 806 tumors recurred at day 46 postinoculation, whereas other tumors had not recurred by day 126 when the mice were sacrificed. Thus, xenografts driven by the overexpression of a constitutively active form of the EGFR are more sensitive to EGFR-specific antibodies.

mAbs 806 and 528 therapy of established NR6-derived xenografts. The NR6 murine fibroblastic cell line does not endogenously express any members of the ErbB family (23), an observation we confirmed by fluorescence-activated cell sorting for EGFR, ErbB2, and ErbB3 (data not shown). These cells were then stably transfected with human de2-7 EGFR (NR6.Δ2-7). Because all the U87MG-derived cell lines used to test the efficacy of mAbs 806 and 528 against the de2-7 EGFR also coexpress the wt EGFR, we assessed their therapeutic efficacy in mice with established NR6.Δ2-7 xenografts. MAb 806 treatment resulted in a reduction in overall tumor growth rate compared with treatment with vehicle that was highly

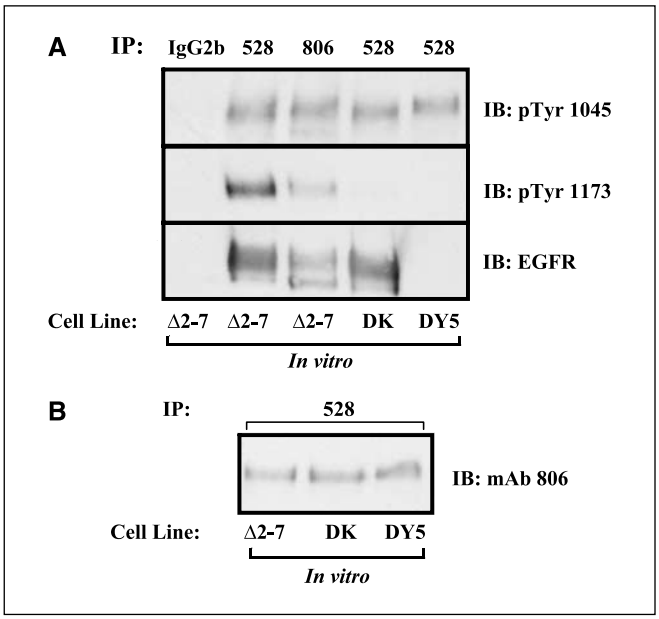

Fig. 4. *In vitro* phosphorylation of de2-7 EGFR Variants in U87MG.Δ2-7, U87MG.DK, and U87MG.DY5 cells. *A,* the de2-7 EGFR protein was immunoprecipitated (*IP*) with mAb 806, mAb 528, or an irrelevant isotype-matched control antibody, and resulting samples were immunoblotted (*IB*). All de2-7 EGFR variants were positive for phosphorylation at Y1045, the major site associated with ubiquitination and degradation (*top*). Whereas the de2-7 EGFR was constitutively phosphorylated at position Y1173, both the DK and DY5 variants were negative for phosphorylation at this site as expected (*middle*). The presence of EGFR was confirmed using the rabbit COOH-terminal polyclonal antibody to the EGFR (*bottom*). This COOH-terminal antibody did not recognize the DY5 variant because it contains a Y1068F mutation, which turns out to be a critical residue for antibody binding. Thus, the presence of total DY5 protein was confirmed in (*B*) by immunoblotting with mAb 806.

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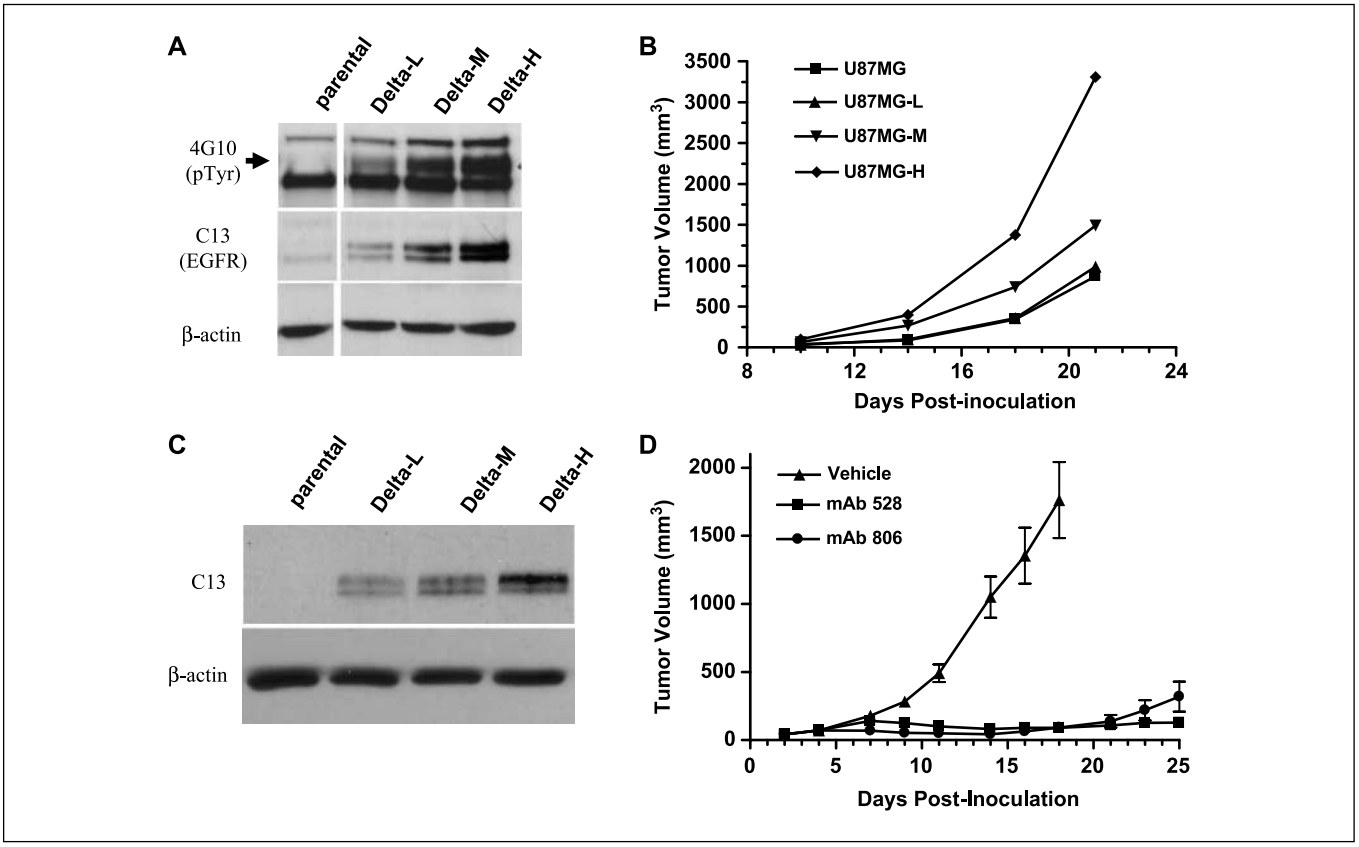

Fig. 5. U87MG cells expressing high levels of de2-7 EGFR. U87MG.Δ2-7 cells were FACS sorted into low (*L*), medium (*M*), and high (*H*) expressing populations. *A,* cells were lysed following 36 h of serum starvation and analyzed by immunoblotting for de2-7 expression (*C13*) and tyrosine phosphorylation (*4G10*) of the de2-7 EGFR. Levels of phosphorylation correlated with de2-7 EGFR. *B,* parental U87MG, U87MG-L, U87MG-M, and U87MG-H xenografts were established by injection of 1×10^6 cells in both flanks of nude BALB/c mice in order to determine growth curves. Points, mean tumor volume; bars, SE. *C,* tumors from (*B*) were analyzed by immunoblotting for expression of de2-7 EGFR (C13). *D,* mice with U87MG-H xenografts were treated with 1 mg mAb 528 or 806 thrice weekly for 2 wks (d 4, 6, 8, 11, 13, and 15). Points, mean tumor volume; bars, SE.

significant at day 42 postinoculation ($P < 0.003$; Fig. 6). The average tumor volume on the final day of therapy (day 39) was 1,520 and 670 mm^3 for the vehicle and mAb 806 treatment groups, respectively (Fig. 6A).

Mice bearing established NR6.Δ2-7 xenografts were also treated with mAb 528. On day 56 postinoculation, when animals were killed for ethical reasons, the size of tumors treated with mAb 528 did not differ from that of vehicle-treated xenografts (Fig. 6B). We conducted a second therapy experiment with mAb 528 using a slightly varied protocol whereby mice received antibody twice weekly for 3 weeks. Once again, mAb 528 failed to inhibit the growth of established NR6.Δ2-7 xenografts under these conditions (Fig. 6C). Thus, unlike mAb 806, mAb 528 is unable to inhibit xenografts expressing the de2-7 EGFR in the absence of the wt EGFR.

Src activity modulates the responsiveness of de2-7 EGFR–expressing xenografts to antibody therapy. Because mAbs 806 and 528 inhibit xenografts expressing the DY5 version of the de2-7 EGFR and because neither antibody decreases de2-7 EGFR phosphorylation as a single agent *in vivo* (16), it is likely that these antibodies mediate their antitumor activity by disrupting the transphosphorylation of a target downstream of the de2-7 EGFR. Our observations with the NR6.Δ2-7 xenografts suggest that the antitumor activity of mAb 528 is dependent on the coexpression of the de2-7 EGFR with another member of the ErbB family, whereas mAb 806 activity is independent of this interaction. Therefore, we examined if the de2-7 EGFR could interact with Src, as is the case for the wt EGFR, and if this potential interaction is related to mAb 806 efficacy.

Activation of the wt EGFR leads to the transient activation of Src kinase. In a synergistic manner, activation of Src leads to phosphorylation Tyr^{845} (Y845) on the EGFR, which is not an autophosphorylation site rather a target for Src phosphorylation (24). Using an antibody specific to Y845, we examined the phosphorylation of Y845 in the de2-7 EGFR. When expressed in U87MG glioma cells, the de2-7 EGFR showed robust phosphorylation of Y845 (Fig. 7A). Phosphorylation at Y845 was rapidly blocked by incubating cells with PP1 and PP2, inhibitors of the Src-family kinases, whereas the autophosphorylation site at Y1173 was relatively unaffected (Fig. 7A).

Given that the de2-7 EGFR seems to be a target for Src kinase phosphorylation in a manner analogous to that of the wt EGFR, we sort to determine if this interaction was critical to mAb 806 activity. Initially, we constructed a de2-7 EGFR containing a Y845F substitution; however, this protein showed reduced phosphorylation at multiple sites[4] and was therefore

[4] T.G. Johns, unpublished observation.

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considered unsuitable for these studies. Thus, as described in Materials and Methods, we developed a U87MG cell line coexpressing the de2-7 EGFR and a DNSrc (U87MG.Δ2-7$_{DNSrc}$). U87MG.Δ2-7$_{DNSrc}$ xenografts grew as tumor xenografts in nude mice but at a rate slower than U87MG.Δ2-7 transfected with a vector control (Fig. 7B). Treatment of U87MG.Δ2-7$_{DNSrc}$ with mAb 806 resulted in robust inhibition of tumor growth (Fig. 7C). At day 34 postinoculation, the average xenograft volume was 1,220 mm^3 in the vehicle group compared with 100 mm^3 in the mAb 806–treated group ($P < 0.001$; Fig. 7C). Furthermore, 60% of all U87MG.Δ2-7$_{DNSrc}$ xenografts in the mAb 806–treated group completely regressed and had not recurred by day 50 postinoculation. Thus, inhibition of Src signaling seems to increase the efficacy of mAb 806 therapy (Fig. 2; Fig. 7C).

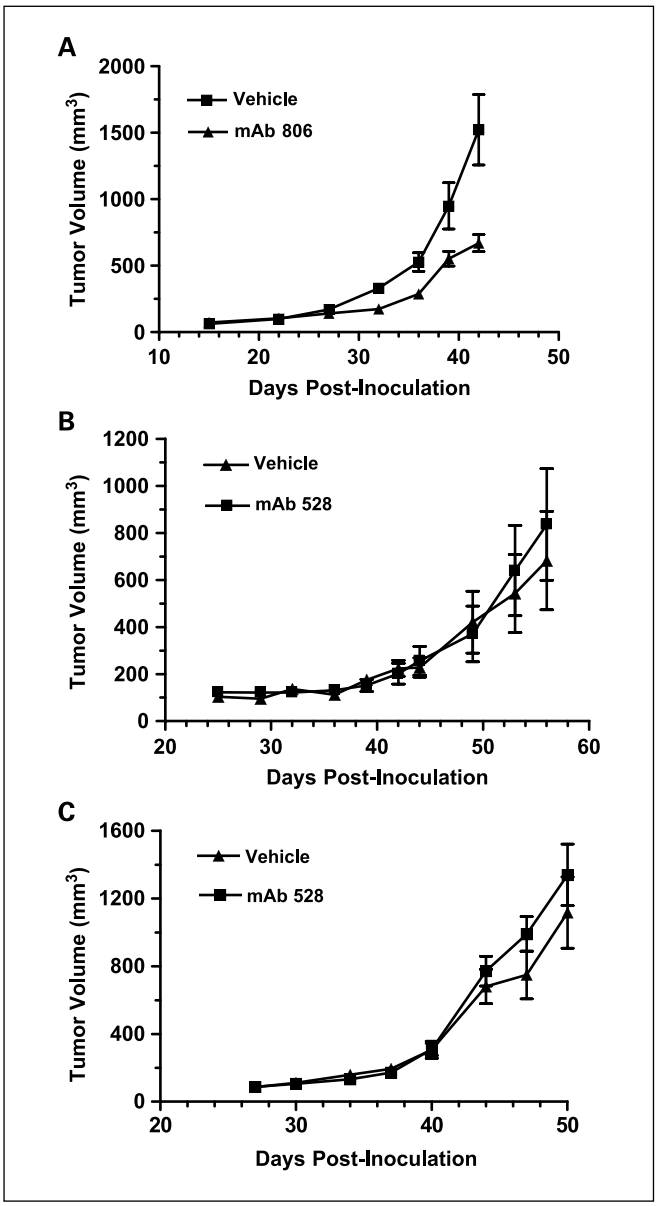

Fig. 6. Treatment of NR6.Δ2-7 xenografts with EGFR-specific antibodies. Xenografts were established by injection of 3×10^6 cells in both flanks of nude BALB/c mice. Antibody therapy commenced when xenografts reached an approximate mean volume of 100 mm^3. Mice were treated with 1 mg mAb 806 (*A*) or mAb 528 (*B*) thrice weekly for 2 wks (days 22, 25, 29, 32, 36, and 39) or with mAb 528 (*C*) twice weekly for 3 wks (days 27, 30, 34, 37, 41, and 44). Points, mean tumor volume; bars, SE.

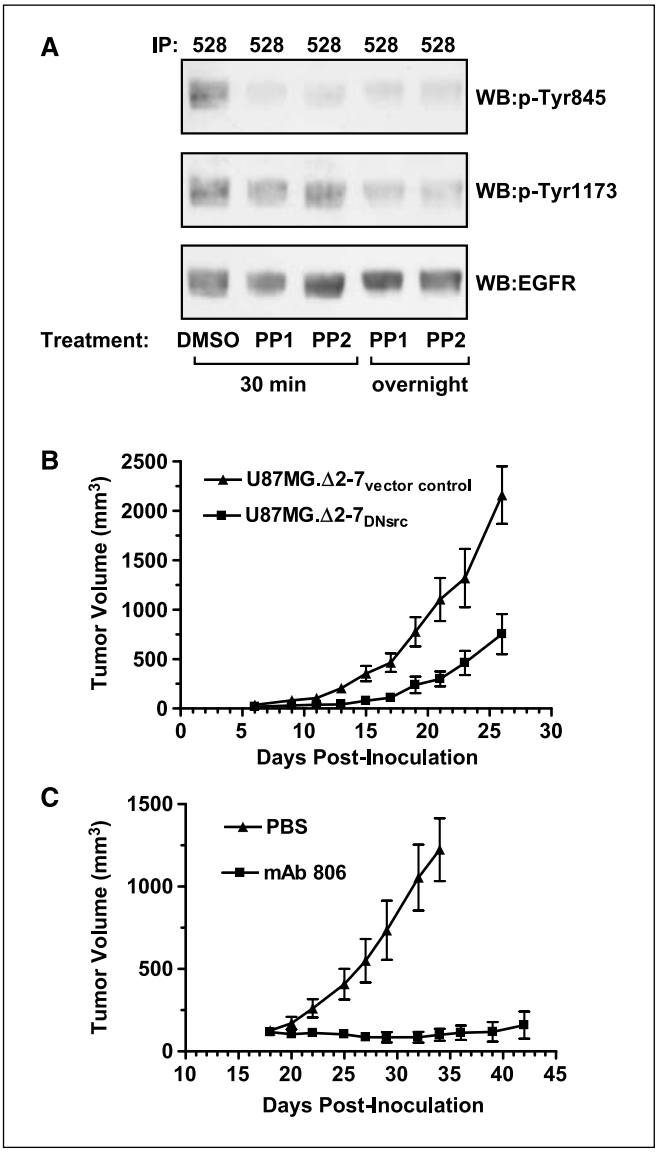

Fig. 7. Interaction between de2-7 EGFR and Src. *A,* cells were serum starved overnight before treatment with 10 μmol/L PP1 or PP2 or vehicle (DMSO) for 30 min or 24 h before immunoprecipitation with mAb 528, mAb 806, or an irrelevant isotype control. Immunoblotting (*WB*) was done with an antibody specific for Y845 of the EGFR, whereas total de2-7 EGFR was visualized with the rabbit COOH-terminal polyclonal antibody. Results are representative of four independent experiments. *B,* U87MG.Δ2-7$_{vector\ control}$ and U87MG.Δ2-7$_{DNSrc}$ xenografts were established by injection of 1×10^6 cells in both flanks of nude BALB/c mice in order to determine growth curves. Points, mean tumor volume; bars, SE. *C,* U87MG.Δ2-7$_{DNSrc}$ xenografts were established by injection of 3×10^6 cells in both flanks of nude BALB/c mice. Antibody therapy commenced when xenografts reached an approximate mean volume of 100 mm^3. Mice were treated with 1 mg mAb 806 thrice weekly for 2 wks (days 18, 20, 22, 25, 27 and 29). Points, mean tumor volume; bars, SE.

Internalization of mAb 806 in U87MG.Δ2-7 cells. The intracellular trafficking of mAb 806 following binding to de2-7 EGFR expressed in U87MG.Δ2-7 cells was investigated by confocal microscopy. Following incubation of mAb 806-Cy3 at 4°C and before chase at 37°C, mAb 806 bound to de2-7 EGFR

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was located along the plasma membrane (Fig. 8A, *0 min, mAb 806-Cy3*). Following incubation at 37°C, mAb 806 (Fig. 8A, *mAb 806-Cy3*) was observed to translocate to small, punctate, cytoplasmic vesicles. Subsequent immunostaining with anti-EEA1, which identifies early endosomes (Fig. 8A, *EEA1*), showed partial colocalization with mAb 806 as visualized by the presence of yellow fluorescence (Fig. 8A, *Merge*). Following 60 min of chase at 37°C, the colocalization was minimal (Fig. 8A, *Merge, 60 min*), suggesting that the majority of antibody has moved out of early endocytic compartments. These observations indicate that mAb 806 localizes to early endocytic compartments immediately following internalization before moving to an alternative location later in its intracellular trafficking cycle.

Lysosomal localization of mAb 806 following binding and internalization of de2-7 EGFR in U87MG.Δ2-7 cells was accomplished via colocalization analysis in cells transiently transfected with lgp-120-GFP (Fig. 8B). Cells positively transfected for lgp-120-GFP displayed cytoplasmic perinuclear green fluorescence consistent with localization to lysosomal compartments as expected (Fig. 8B, *lgp-120-GFP*). Before induction of internalization, mAb 806-Cy3 was only detected on the cell surface (Fig. 8B, *0 min, mAb 806-Cy3*) and did not colocalize with lgp-120-GFP (Fig. 8B, *0 min, Merge*). Following warming to 37°C for 30 min, small intracellular vesicular structures corresponding to internalized mAb 806 were observed (Fig. 8B, *30 min, mAb 806-Cy3*). Some of these structures colocalized with lgp-120-GFP; however, the majority of red and green signal remained segregated (Fig. 8B, *30 min, Merge*). Longer incubation at 37°C for 60 and 120 min resulted in increased colocalization of internalized mAb 806-Cy3 and lgp-120-GFP (Fig. 8B, *60-120 min, Merge*). These observations are consistent with the hypothesis that mAb 806 initially traverses through early endocytic compartment but after longer periods moves into lysosomal compartments where it accumulates.

The internalization of mAb 806 following binding to the de2-7 EGFR expressed on U87MG.Δ2-7 cells was also analyzed by electron microscopy. Following 5 min of incubation at 37°C, gold particles, corresponding to mAb 806, were observed in structures resembling clathrin-coated pits (Fig. 9A and B). Gold particles were also detected in free clathrin-coated vesicles located within the cytoplasm (Fig. 9C). No gold particles were

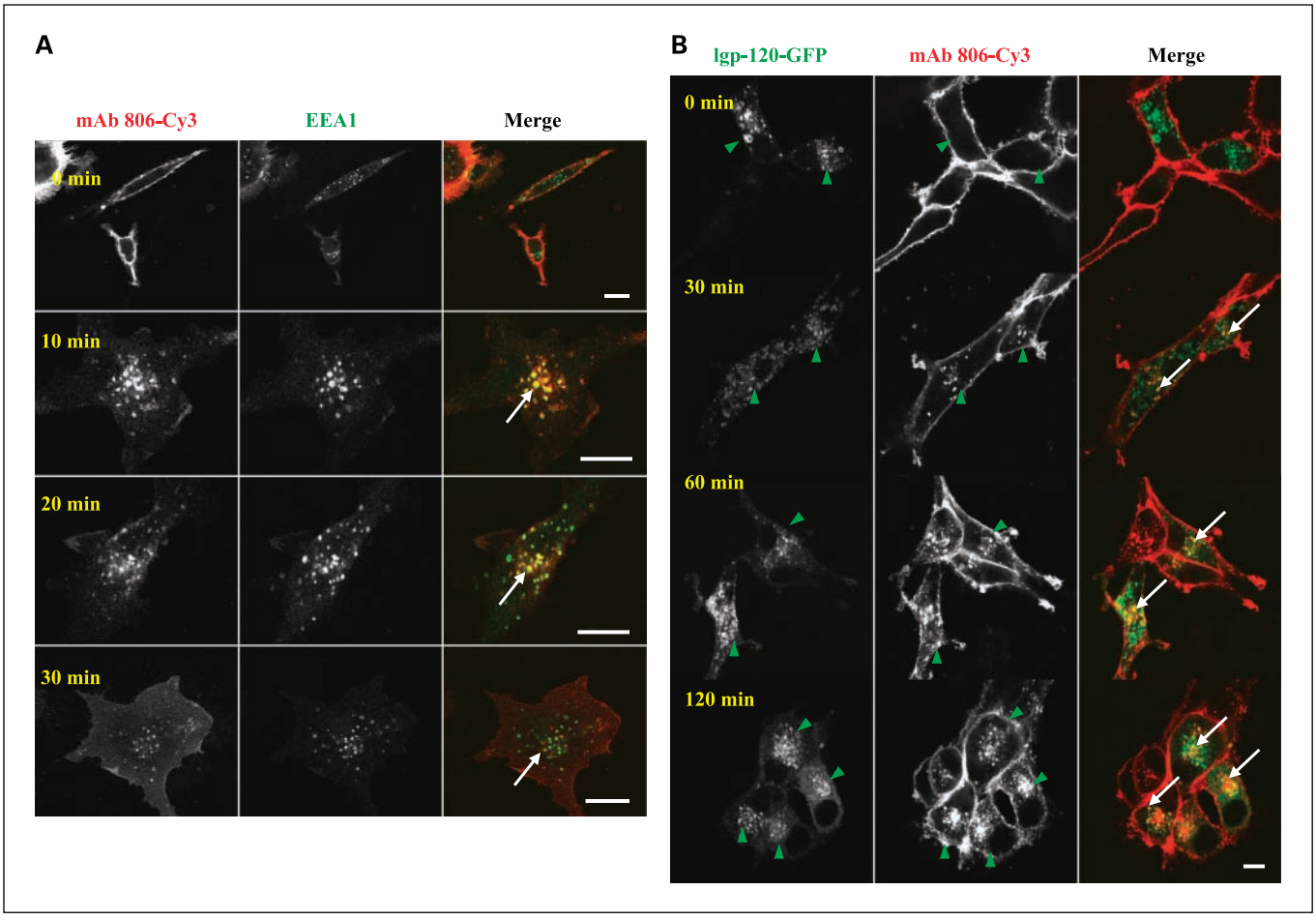

Fig. 8. Colocalization of internalized mAb 806-Cy3 and EEA1 or lgp-120 in U87MG.Δ2-7 cells. *A,* cells seeded on glass coverslips were preincubated with mAb 806-Cy3 (*red*) at 4°C (0 min). Internalization was stimulated by incubation at 37°C for 10, 20, and 30 min. Cells were fixed and permeabilized and then stained with anti-EEA1 followed by Cy2-conjugated donkey anti-mouse antibody (*green*). Colocalization (*yellow in the merged images, arrows*). Bar, 20 μm. *B,* cells were transiently transfected with lgp-120 tagged with GFP (lgp-120-GFP; *green*). Positively transfected cells (*lgp-120-GFP, green arrowheads*). Following transfection, cells were incubated with mAb 806-cy3 at 4°C (*red*; 0 min), before induction of internalization by incubating at 37°C for 30, 60, and 120 min. Samples were subsequently fixed. Colocalization of mAb 806-Cy3 and lgp-120-GFP (*yellow in the merged images, white arrows*). Bar, 10 μm.

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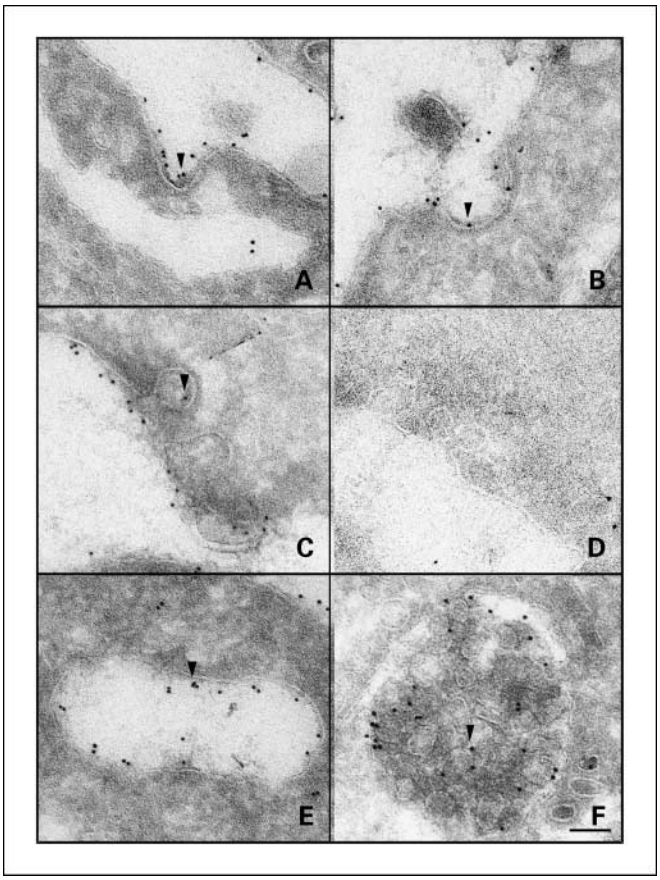

Fig. 9. Electron microscopic analysis of clathrin-mediated endocytosis and intracellular trafficking of mAb 806 following binding to de2-7 EGFR in U87MG.Δ2-7 cells. Gold particles (mAb 806-Au; *arrowheads*) were readily detected in clathrin-coated pits (*A* and *B*) and vesicles (*C*) following induction of internalization for 5 min. No gold particles were present in structures resembling caveolae (*open arrowheads*) (*D*). After 10 to 15 min of internalization, gold particles were detected in tubular vesicular structures resembling early endosomes (*E*). After longer periods of internalization, gold particles were seen in multivesicular bodies (*F*). Bar, 100 nm.

observed in structures resembling caveolae (Fig. 9D). Following 10 min of chase at 37°C, mAb 806 localized to large tubular-vesicular structures resembling early endocytic compartments (Fig. 9E). Longer chase periods of 30 min resulted in antibody localization in structures resembling multivesicular bodies (Fig. 9F). These observations are consistent with the immunofluorescence microscopy data that indicated colocalization of mAb 806 with lgp-120 between 30 and 60 min.

Internalization of mAbs 806 and 528 in NR6.Δ2-7 cells. Given the differences in therapeutic efficacy of mAbs 806 and 528 against NR6.Δ2-7 xenografts, the internalization characteristics of each antibody was investigated in this cell line. Furthermore, because NR6.Δ2-7 cells do not express any endogenous members of the ErbB family, this cell line can determine if the presence of wt EGFR is required for internalization of these antibodies. Cells incubated with mAb 806-Cy3 at 4°C showed membrane staining with no intracellular fluorescence as expected (Fig. 10, *left, mAb 806, 0 min*). In contrast to U87MG.Δ2-7 cells (Fig. 8), membrane staining was not uniform. More intense staining was associated with membrane junctions between cells (Fig. 10, *left, mAb 806, 0 min*) and focal adhesions (Fig. 10, *left, mAb 806, 0 min*). Some cells showed very little membrane staining (Fig. 10, *left, mAb 806, 0 min*). Following induction of internalization by raising the temperature to 37°C, characteristic intracellular punctate vesicular structures were observed. These accumulated in a perinuclear pattern (Fig. 10, *left, mAb 806, 15-60 min*) consistent with rapid lysosomal localization. Initial localization (Fig. 10, *right, mAb 528, 0 min*) and subsequent internalization (Fig. 10, *right, mAb 528, 15-60 min*) of mAb 528 was identical to that of mAb 806. Thus, both antibodies were rapidly internalized to the lysosomal compartment following binding to the de2-7 EGFR even in the absence of the wt EGFR.

Discussion

mAb 528. Many, but not all, previous studies have suggested that EGFR number on the cell surface is one factor that influences the efficacy of EGFR-targeted therapeutics, especially TKIs (Table 1). However, these experiments have always compared antitumor activity using different cell lines and thus are not controlled with respect to genetic background, the presence of other ErbB family members, and the occurrence of other functional receptors/kinases capable of modulating the EGFR signaling pathway. Furthermore, many of these studies have been conducted *in vitro*, which we have shown does not correlate with *in vivo* activity. Increasing the wt EGFR number 10-fold converts U87MG glioma xenografts from mAb 528 resistant to antibody responsive. Because the increase in wt EGFR number did not alter the growth rate of the U87MG xenografts, the advent of antitumor activity was not simply the result of mAb 528 inhibiting an induced growth advantage. The presence of more wt EGFR within U87MG.wtEGFR xenografts would almost certainly lead to increased antibody localization at the tumor site. Given that mAb 528 possesses low, but measurable, immune effector function (25), the increased level of antibody at the tumor site may result in increased complement deposition and recruitment of immune cells that contribute to inhibition of tumor growth. However, a role for immune effector function in initiating the antitumor activity of mAb 528 seems unlikely given our data with the U87MG.DK xenografts. These xenografts have as many mAb 528 binding sites as the U87MG.wtEGFR xenografts but are not inhibited by the antibody. One intriguing possibility is that overexpression of the wt EGFR leads to ligand-independent EGFR signaling (the parental U87MG seems not to have a strong autocrine ligand loop), which in turn causes the cells to become more dependent on the EGFR signaling system. Thus, U87MG.wtEGFR xenografts respond to mAb 528 therapy because, unlike the parental cell line, the EGFR signaling pathway is active and functional. Therefore, overexpression of the wt EGFR is a surrogate marker of cells dependence on EGFR signaling and therefore such cells are more likely, but not guaranteed, to respond to EGFR therapeutics (26).

It has been presumed that the antitumor activity of antibodies, such as mAb 528, is predominantly mediated by their ability to antagonize ligand activation of the EGFR. Given that mAb 528 inhibited the growth U87MG.wtEGFR xenografts in the absence of significant ligand expression suggests that other mechanisms may contribute to the antitumor effect. Furthermore, mAb 528 displayed significant efficacy against xenografts expressing the ligand-independent de2-7 EGFR. This antitumor activity could not directly result from mAb 528 binding the

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endogenous wt EGFR coexpressed in these xenografts, as it did not inhibit the growth of parental U87MG or U87MG.DK xenografts, both of which express identical levels of the wt EGFR. Excluding immune effector function, alternate antitumor mechanisms could include receptor down-regulation, induction of inappropriate signaling, translocation of the receptor to unsuitable membrane domains, and interference with receptor dimerization and/or oligomerization. Indeed, some TKIs directed to the EGFR not only function by inhibiting kinase activity but also induce inactive dimers capable of "mopping" up excess ligand, an unanticipated antitumor mechanism (27).

Interestingly, a recent immunohistochemistry study analyzing EGFR expression in colon patients showing differential response to C225 reported that several patients "negative" for EGFR had clinical responses to this EGFR-specific antibody (26). Presumably, these patients have levels of EGFR below the detection sensitivity of immunohistochemistry, yet the EGFR present is activated and contributes to tumor growth/survival. This observation suggests that EGFR activation is at least as important, if not more so, than simply the level of EGFR expression. Our data showing that mAb 528 did not inhibit the growth of U87MG xenografts expressing a DK version of this truncated receptor (U87MG.DK) support the view that the efficacy of EGFR-specific antibodies is intimately associated with kinase active receptors. As suggested above, EGFR overexpression represents one mechanism by which this activation can occur; the expression of a constitutively active mutant, such as the de2-7 EGFR, denotes another. This continuous activation of the EGFR causes cells to become "addicted" to EGFR signaling, which in turn makes them susceptible to anti-EGFR therapy. This concept is analogous to the situation in lung cancer patients, where most patients who respond to EGFR-specific TKIs carry activating mutations in the EGFR kinase domain (28).

The ability of mAb 528 to inhibit the growth of U87MG.DY2 or DY5 xenografts highlights the significance of an active kinase domain as opposed to autophosphorylation as a determinant of efficacy. Thus, it is an active kinase that determines the response to antibody therapy, not the direct interaction of phosphorylated tyrosines with adapter or signaling molecules. One corollary to this result is that mAb 528 seemingly inhibits the growth of U87MG.Δ2-7/DY2/DY5 xenografts by preventing the transphosphorylation of a downstream target (Fig. 11). Because all these U87MG-derived cell lines coexpress the wt EGFR, and given that we showed recently that the de2-7 EGFR can form dimers and phosphorylate the wt EGFR (29), the wt EGFR is a likely candidate for this secondary target. This proposition is supported by the fact that NR6 cells expressing the de2-7 EGFR in the absence of the wt EGFR were completely refractory to the antitumor effects of mAb 528. Taken together, these studies suggest that, along with its ligand blocking properties, mAb 528 functions in part by preventing the homodimerization of the overexpressed wt EGFR and heterodimerization between the wt and de2-7 EGFR. Interestingly, the structure of C225 (an antibody very similar to mAb 528) in complex with the EGFR suggests that, apart from ligand blockade, this antibody may prevent EGFR dimerization by partially inhibiting EGFR untethering (30).

mAb 806. Responsiveness of U87MG-derived cell lines *in vivo* to mAb 806 completely mirrored that observed with mAb 528, indicating that many of the above principles apply, although there are some important differences. This study confirms and extends our previous studies showing that mAb 806 reactivity is associated with EGFR activation (16). Unlike mAb 528, and all current antibodies in clinical evaluation, mAb

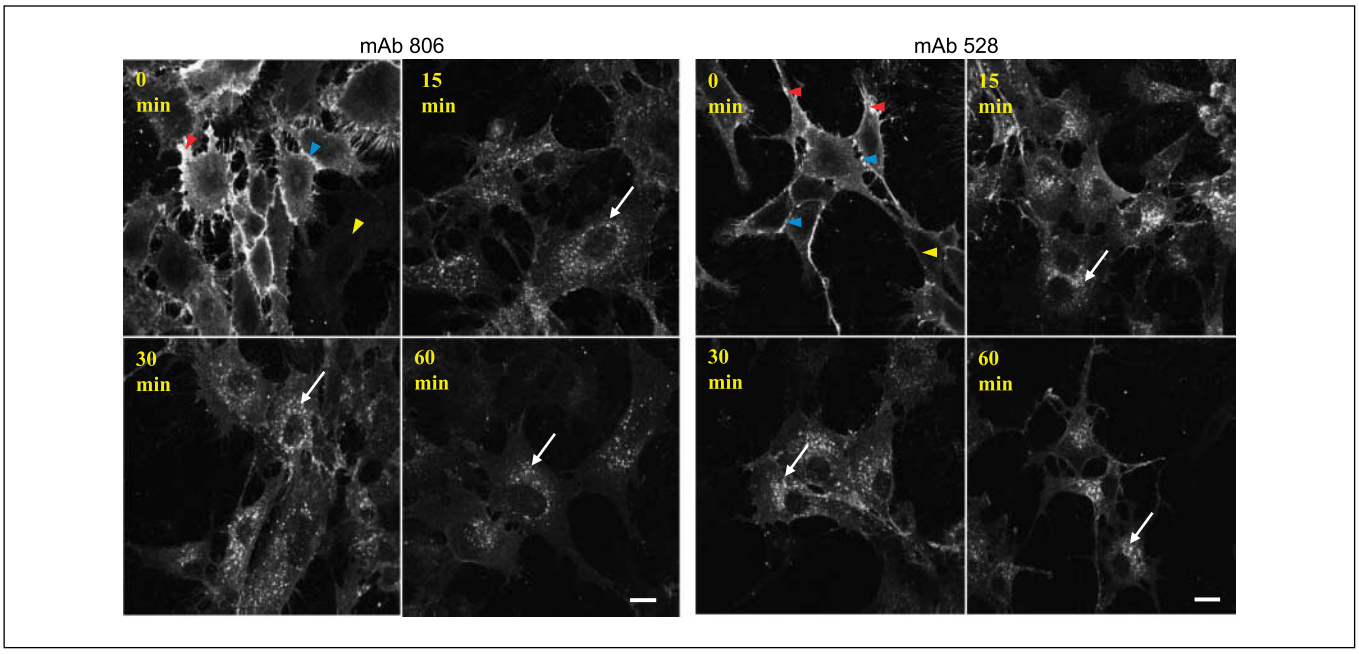

Fig. 10. Internalization of mAbs 806 and 528 in NR6.Δ2-7 cells. Cells were preincubated with mAb 806-Cy3 (*left*) or mAb 528-Cy3 (*right*) at 4°C (0 min), before incubation at 37°C for varying periods of time to induce internalization. Images representing 15, 30, and 60 min of incubation at 37°C. Staining with both antibodies before internalization was associated with membrane junctions between cells (*blue arrowhead*) and focal adhesions (*red arrowhead*), whereas some cells showed very little membrane staining (*yellow arrowhead*). Internalized antibody at later time points (*white arrows*). Bar, 20 μm.

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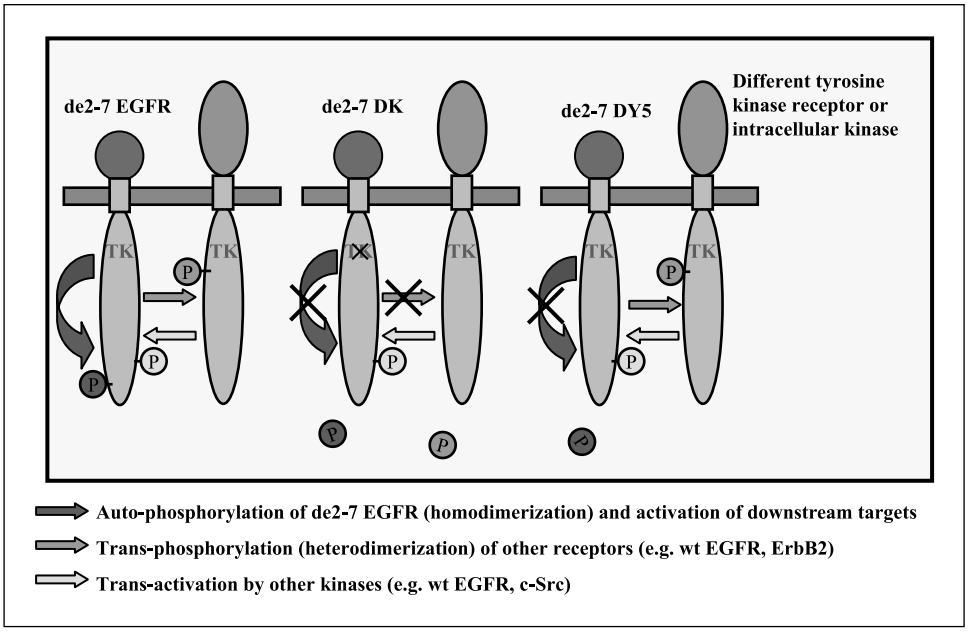

Fig. 11. Schematic representation of the interaction of the de2-7 EGFR variants with other cellular components. The de2-7 EGFR has an active kinase and therefore can autophosphorylate, transphosphorylate, or be the target of phosphorylation by other kinases. In contrast, the DK de2-7 EGFR can only be the target of phosphorylation. Finally, the DY5 construct can be the target of phosphorylation and transphosphorylate other cell targets, such as the wt EGFR. Given that both mAbs 528 and 806 can inhibit U87MG.DY5 xenografts but not U87MG.DK xenografts, it suggests that the ability of these antibodies to prevent the phosphorylation of other cellular components is critical to their antitumor activity.

806 does not target normal tissue, such as the liver, as EGFR activation is extremely low or nondetectable in organs, such as the liver. A myriad of factors can stimulate EGFR activation within tumors (see ref. 31 for a review). We have confirmed that at least three of these, EGFR overexpression (15), mutation (17), and presence of an autocrine loop,[5] can lead to mAb 806 reactivity. The association of wt EGFR overexpression for mAb 806 antitumor activity is intimately related to its unique specificity as overexpression increases the transient, untethered form of the EGFR recognized by mAb 806, through multiple mechanisms, such as ligand-independent activation and alterations in EGFR glycosylation (21). Given that the work described here, along with the clinical data obtained with EGFR-specific TKIs, suggests that EGFR inhibitors are most effective against tumors with an activated EGFR, the unique ability of mAb 806 to specifically recognize activated forms of the EGFR makes it an advantageous therapeutic.

Molecular modeling suggests that mAb 806 binding would prevent the formation of active wt EGFR dimers (14), a hypothesis we have confirmed by solving the crystal structure of mAb 806 in complex with its epitope.[5] Despite this, mAb 806 does not significantly inhibit the phosphorylation of the de2-7 or wt EGFR in xenograft models (16), strongly suggesting that any proposed mechanism of action for mAb 806 includes more than blockade of autophosphorylation. Furthermore, known downstream targets of EGFR signaling, such as Akt and MAPK, are also not inhibited by mAb 806.[4] Consistent with this hypothesis, mAb 806 displayed robust antitumor activity against U87MG.DY2/DY5 xenografts, two models where autophosphorylation is not pertinent. The lack of mAb 806 efficacy against U87MG.DK xenografts emphasizes that the presence of an active kinase and transphosphorylation events (Fig. 11) are critical factors leading to sensitivity. In contrast to mAb 528, mAb 806 was able to inhibit the growth of NR6 cells expressing the de2-7 EGFR in the absence of other ErbB family members. This result indicates that mAb 806 potentially disrupts other targets of de2-7 EGFR transphosphorylation, distinct from the wt EGFR. Interestingly, there was no obvious difference in the internalization and intracellular tracking of mAbs 806 and 528 following binding of either antibody to surface de2-7 EGFR in NR6 cells, suggesting that antibody trafficking did not contribute to the difference in efficacy in this xenograft model.

We report here for the first time that Y845 is phosphorylated on the de2-7 EGFR in a Src-dependent manner. Thus, we examined whether the interaction between the de2-7 EGFR and Src was a potential target of mAb 806 activity. If mAb 806 mediated part of its antitumor activity by inhibiting this interaction, then genetically disrupting this interaction using a DNSrc should have reduced the efficacy of mAb 806. In contrast to this possibility, the presence of a DNSrc dramatically enhanced the antitumor activity of mAb 806. This suggests that Src has a role in limiting the efficacy of EGFR therapeutics and provides a rationale for using Src and EGFR inhibitors in combination.

Conclusion

These studies show the importance of *in vivo* studies for analyzing the sensitivity of cell lines to EGFR therapeutics. Unlike previous studies, we were able to conduct most of our analysis in the same genetic background, making the predominant variable the nature of the EGFR. Using this approach, we conclusively showed the significance of receptor number to efficacy. Although EGFR number is related to EGFR therapeutic susceptibility, this factor alone is not enough as the receptor also needs to contain a functional kinase. Indeed, although somewhat intuitive, this work shows formally that "forcing" a cell line to use EGFR signaling, either by overexpression of the wt EGFR or expression of a constitutive active mutant, can switch it from nonresponsive to responsive. Thus, the EGFR must not only be present at the cell surface but must also be

[5] T.G. Johns et al., in preparation.

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significantly contributing the growth and survival of the cell. Therefore, strategies for selecting patients who will respond to EGFR therapeutics should be directed to identifying tumors highly dependent on the EGFR, not only the presence or absence of receptor protein. This task may be relatively straightforward in some cases, such as when the de2-7 EGFR, *EGFR* gene amplification, or kinase-activating mutants are present, but is clearly more difficult in cases where the wt EGFR is genetically normal. In these cases, the complex interplay of multiple receptor kinases makes it difficult to identify those tumors truly dependent on EGFR signaling. Long-term, detailed expression profiling of yet to be identified target genes unique to each receptor kinase may be the only viable approach to addressing this problem.

Acknowledgments

We thank Rachael Takara and Mark Pypaert for technical assistance.

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