

Forced Dimerization Increases the Activity of Δ EGFR/EGFRvIII and Enhances Its Oncogenicity

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Abstract

Delta epidermal growth factor receptor (Δ EGFR), an in-frame deletion mutant of the extracellular ligand-binding domain, which occurs in about 30% of glioblastoma, is a potent oncogene that promotes tumor growth and progression. The signaling of Δ EGFR is ligand-independent and low intensity, allowing it to evade the normal mechanisms of internalization and degradation by the endocytic machinery and hence is persistent. The basis of the oncogenic potential of Δ EGFR remains incompletely understood, including whether dimerization plays an important role in its signal and whether its oncogenic potential is dependent on its relatively low intensity, when compared with the acutely activated wild-type receptor. To examine these two important questions, we have generated a chimeric Δ EGFR that allows forced dimerization via domains derived from variants of the FKBP12 protein that are brought together by FK506 derivatives. Forced dimerization of chimeric Δ EGFR significantly increased the intensity of its signal, as measured by receptor phosphorylation levels, suggesting that the naturally occurring Δ EGFR does not form strong or stable dimers as part of its low level signal. Interestingly, the increased activity of dimerized, chimeric Δ EGFR did not promote receptor internalization, implying that reduced rate of endocytic downregulation of Δ EGFR is an inherent characteristic. Significantly, forced dimerization enhanced the oncogenic signal of the receptor, implying that the Δ EGFR is a potent oncogene despite, not because of its low intensity. *Mol Cancer Res*; 9(9); 1199–208. ©2011 AACR.

Introduction

Aberrant receptor tyrosine kinase signaling is a major contributor to cancer, including glioma, in which *EGFR* gene amplification is frequently accompanied by gene rearrangements, with the most common mutation, delta epidermal growth factor receptor (Δ EGFR), leading to deletion of exon 2 to 7 (1, 2). This deletion results in the loss of 267 amino acids from the extracellular domain and renders the Δ EGFR unable to bind ligand (3). Δ EGFR is tumor specific and associated with advanced disease and resistance to therapy (4, 5). Δ EGFR occurs in glioma in the context of an overexpressed EGFR (6), and so presumably high levels of EGFR signaling, but nevertheless makes an important contribution to glioblastoma growth. Patients with Δ EGFR-expressing tumors have a shorter interval to clinical relapse and poorer survival than patients with Δ EGFR-negative tumors. For glioblastoma multiforme

patients who survive 1 year or longer after diagnosis, the expression of Δ EGFR is also an independent negative prognostic indicator of survival (7, 8). In xenograft models, Δ EGFR is also capable of enhancing the tumorigenicity of glioma cells, lending greater take and growth rates (9, 10), which it does by reducing apoptosis and increasing proliferation (11). Astrocytes or neural stem cells from *INK4A/Arf*-deficient mice can be transformed by Δ EGFR and induce high-grade glioma when implanted (12). Therefore, Δ EGFR is a potent glioma oncogene and attenuating its signal is important.

Δ EGFR differs from EGFR in a number of ways. The signaling of Δ EGFR is ligand independent and, as a result, is low intensity and also evades the normal mechanisms of internalization and degradation by the endocytic machinery, and hence its signaling is persistent (13, 14). The markedly different contributors of Δ EGFR and EGFR to glioma formation suggested that their signals are different (15, 16). Analysis of downstream targets has led to the finding that certain elements in the EGFR pathway are activated to a greater degree or in a more sustained fashion by Δ EGFR. Early studies showed that Δ EGFR associates strongly with adaptor proteins Shc1 and Grb2 (17, 18). The pronounced and preferential activation of phosphatidylinositol 3-kinase (PI3K) in Δ EGFR-expressing cells (19) was recently substantiated by a broad proteomics screen (20). In addition, *c-Met* and *STAT* pathways have been identified as an important characteristic of Δ EGFR signaling. The activating phosphorylation site on the *c-Met* receptor was

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found to be highly responsive to Δ EGFR levels, indicating cross-activation of c-Met by Δ EGFR (21). Our recent phosphoproteomic screen also found this strong connection to c-Met signaling and identified STAT5 as a novel downstream target of Δ EGFR (22).

The lack of interaction with ligand, as well as the lack of internalization, suggests that Δ EGFR may not dimerize efficiently, a hypothesis that has however not been tested experimentally before. Observations of Δ EGFR in cell lines have not found strong evidence for dimerized Δ EGFR by crosslinking and Western blot analysis in transiently transfected fibroblasts or in glioma cells (13, 23). To examine this question experimentally, we have generated a chimeric Δ EGFR that allows forced dimerization via domains derived from variants of the FKBP12 that are brought together by FK506 derivatives (24, 25). This approach has been used to induce dimerization of EGFR/ErbB1 and ErbB2/Her2/Neu in which it resulted in signaling indistinguishable from that caused by physiologic ligand and also for the study of other signaling interactions, such as those of JNK2, Raf-1, and receptors for insulin, platelet-derived growth factor and granulocyte colony stimulating factor (26–29). Whereas chimeric wild-type EGFR showed similar levels of activity, when dimerized by epidermal growth factor (EGF) or the dimerization agent, forcing the dimerization of chimeric Δ EGFR significantly increased the intensity of its signal, as measured by autophosphorylation. Forced dimerization of Δ EGFR did not noticeably alter the signal generated by the receptor, either at the level of downstream targets or at the level of cellular responses. Interestingly, increasing the activity of Δ EGFR did not enhance its internalization and downregulation, but it did increase the oncogenic impact of the receptor.

Materials and Methods

Cell lines and constructs

Glioma cell lines, U87, LN428, and LN238, were a kind gift from Dr. W.K. Alfred Yung (UT MD Anderson Cancer Center) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Cells were proven to be *Mycoplasma* free by routine testing, authenticated by short tandem repeat fingerprinting.

Chimeric EGFR constructs were made by modification of pCLEGFRv2E (kind gift from Dr. Victor Rivera, Ariad Pharmaceuticals), which encodes a C-terminal fusion of 2 FKBP36V domains, termed Fv2 (25). Then fusion cDNA was recloned into pLRNL containing either wild-type EGFR or Δ EGFR to generate 2 plasmids pL(EGFv2)RNL encoding the chimeric wild-type EGFR and pL(Δ EGFv2)RNL encoding chimeric Δ EGFR.

Immunoprecipitation and Western blot

Cells were washed with ice-cold PBS and were lysed in Triton-X-100 lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1% Triton-X100, 1 mmol/L EDTA, 1 mmol/L EGTA, 20 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μ g/mL of aprotinin and leupeptin, and a

phosphatase inhibitor cocktail]. For the immunoprecipitation, lysates were incubated with anti-EGFR at 4°C overnight. Ab–protein complexes were precipitated with protein A/G-Agarose, washed, and analyzed by Western blotting. For Western blotting, samples were separated by SDS-PAGE by using NuPAGE Bis–Tris gels (Invitrogen), blotted to polyvinylidene difluoride and incubated with primary Ab for overnight. Ab complex was visualized by chemiluminescence. All antibodies for signaling study were purchased from Cell Signaling.

BrdU incorporation

Five thousand cells were seeded in 96-well culture plates. After 24-hour serum starvation, cells were incubated with 20 ng/mL EGF (Sigma) or 50 nmol/L AP20187 in the presence of 100 μ mol/L bromodeoxyuridine (BrdU) for 24 hours. After labeling, BrdU incorporation was measured by colorimetric immunoassay by using a commercially available cell proliferation ELISA kit (Roche).

Cell surface biotinylation

All the biotinylation procedures were conducted on ice. The cells were serum starved, were incubated with 0.25 mg/mL EZ-Link NHS-SS-biotin (Pierce) for 1 hour, and unreacted biotin was quenched with 20 mmol/L glycine for 10 minutes. After washing, cells were either subjected to 15-minute poststimulation with 20 ng/mL EGF or 50 nmol/L AP20187 at 37°C (+ internalization) or proceeded to the next step immediately (– internalization). One dish of cells was treated with cleavage buffer (50 mmol/L glutathione, 90 mmol/L NaCl, 1 mmol/L MgCl₂, 0.1 mmol/L CaCl₂, 60 mmol/L NaOH, and 0.2% bovine serum albumin, pH 8.6) for 20 minutes twice. Another dish of cells was left untreated with cleavage buffer and directly processed for extraction to define total biotinylated proteins. Cells were lysed and the cleared lysates were incubated with streptavidin-conjugated beads (Amersham Biosciences) for overnight to isolate biotinylated proteins, and precipitated proteins were analyzed by Western blot with FKBP antibody (Abcam).

Wound-healing assay

The cells were plated onto 6-well dishes and were allowed to grow to complete confluence. After serum starvation, wounds were then created by using pipette tip, and EGF or AP20187 were added to each well. The pictures of same area were taken for each wound every day until EGF/AP20187 wells had healed by using an inverted microscope at 5 \times magnification; photos were taken immediately after a wound was inflicted to the cell monolayer, the distances between the edges of the wound were measured. The degree of motility is expressed as percent of wound closure as compared with the zero time point.

Animal experiments

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of MD Anderson Cancer Center. Four groups of 10 animals each

in glioma cells, which have endogenous EGFR, the chEGFR is readily distinguishable as it migrates at a higher position in the gel. However, the ch Δ EGFR migrates very close to the endogenous EGFR, requiring detection with anti-FKBP antibody (Fig. 1C). Expression of the chimeric receptors was lower than the endogenous EGFR in glioma cells, as shown by the relative intensity of the endogenous and chEGFR bands in the EGFR Western blot and the comparable level of expression of chEGFR and ch Δ EGFR in the FKBP12 blot (Fig. 1C). The simplest model in which to examine the relative activity of these receptors are cells that lack endogenous EGFR and so we first tested their activation in Chinese hamster ovary (CHO) cells. EGFR and chEGFR (Fig. 1D), but not Δ EGFR or ch Δ EGFR, responded to EGF stimulation with increased levels of phosphorylation, as measured by both pan-phosphotyrosine and site-specific antibodies. Furthermore, as expected chEGFR was activated by AP20187, but neither of the nonchimeric receptors were. Interestingly, ch Δ EGFR was strongly stimulated by AP20187 treatment, to levels comparable with AP20187-stimulated chEGFR. The dimerization induced by AP20187 in the chimeric receptors could be detected on Western blots as a band that migrated well above EGFR monomers with anti-EGFR antibodies. Similar, but fainter bands can be seen in EGF-stimulated EGFR and chEGFR, but not in any of the other samples in which Δ EGFR was expressed. These data show that forced dimerization of ch Δ EGFR significantly enhances the phosphorylation level of this receptor and does so in association with the apparent formation of dimers.

Forced dimerization increases but does not fundamentally alter downstream signaling of EGFR receptors

To examine the signaling of the chimeric receptors in glioma cells, we measured phosphorylation of downstream targets in the PI3K and mitogen-activated protein kinase (MAPK) pathways that are commonly associated with EGFR (Fig. 2). It is important to stress that in these cells, EGF stimulation activates the endogenous EGFR as well as chEGFR when present but not ch Δ EGFR; AP20187 activates the 2 chimeric receptors selectively. EGF stimulation of cells expressing ch Δ EGFR can therefore be used to compare signaling of the endogenous EGFR with elevated ch Δ EGFR via stimulation with AP20187. In the PTEN-negative cell line U87, we observed elevated levels of pAkt under all conditions, likely because of lack of regulation in the PI3K pathway. In LNZ308 cells, which also lack PTEN, an increase in pAkt was observed in cells in which endogenous EGFR, chEGFR, or ch Δ EGFR was stimulated when compared with serum-starved cells. In LN428 cells, which express wild-type PTEN, pAkt levels were elevated by stimulation of endogenous EGFR by EGF but not when both chimeric receptors were stimulated with AP20187. We also observed elevated pS6 under all conditions in all 3 of cell lines. A slightly different pattern emerged with pS6K, which showed lower basal levels in serum-starved cells, including the lines that lack PTEN. For this node, a positive response was registered whenever endogenous EGFR, chEGFR, or ch Δ EGFR was stimulated. Upon PI3K activation, S6K is phosphorylated at T389 site by one of key

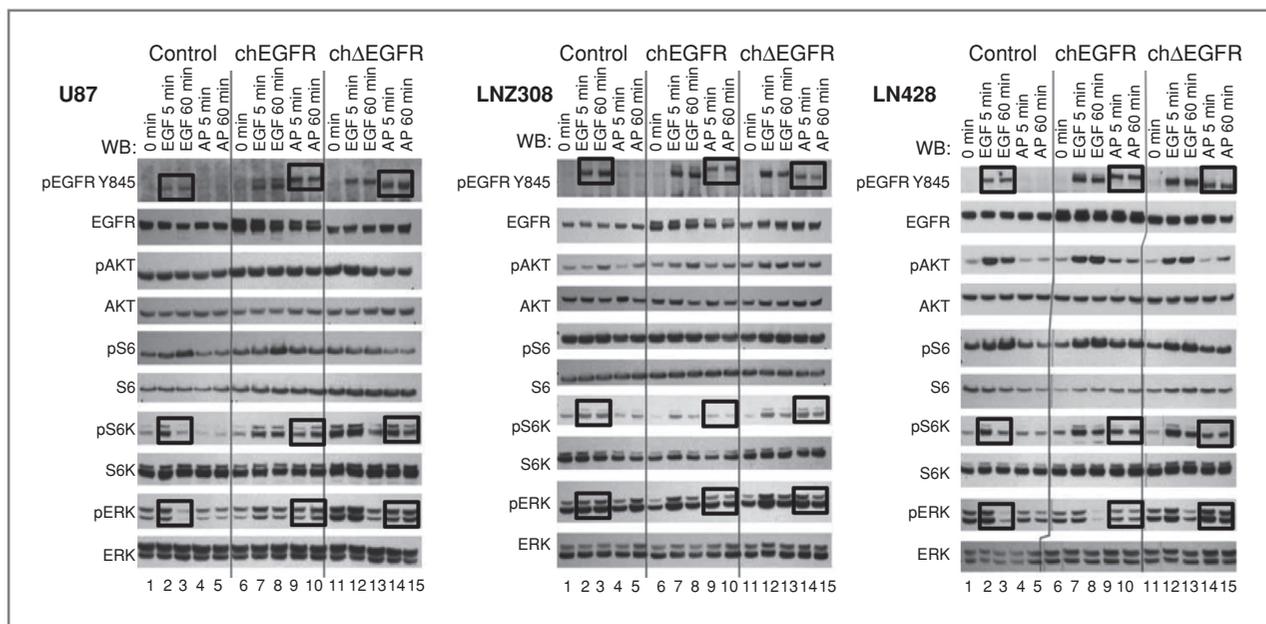


Figure 2. Signaling by chEGFR and ch Δ EGFR receptors. Glioma cells U87, LNZ308, and LN428 stably expressing control vector, chEGFR or ch Δ EGFR were serum starved and then stimulated with 20 ng/mL EGF or 50 nmol/L AP20187 and analyzed at 2 time points, 5 and 60 minutes. EGFR downstream signaling was analyzed by immunoblotting with indicated pan- and phospho antibodies; pEGFR(Tyr 845), pAKT(Ser473), pS6K(Thr389), pS6(Ser235/236), and pERK1/2(Thr202/Tyr204).

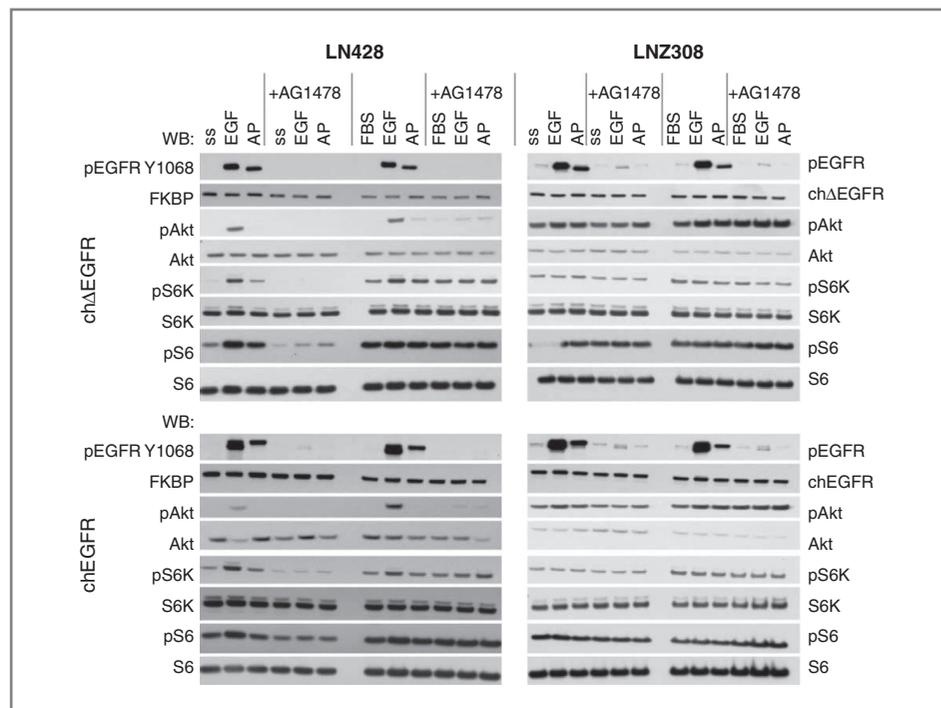
effectors PI3K pathway, PDK1. Here we observed prolonged phosphorylation of S6K at T398 site, suggesting that forced dimerization is able to increase the signal received by pS6K. The pERK signals, in general, responded rapidly to EGF stimulation, with elevations at 5 minutes and a return to lower levels at 60 minutes. Elevations of pERK after AP20187 treatment were less pronounced than those caused by EGF (compare lanes 7 and 9 and lanes 12 and 14 in all 3 panels in Fig. 2) but also more sustained (compare lanes 8 and 10 and lanes 13 and 15 in all 3 panels in Fig. 2). In summary, these data show that chimeric receptors activate the same downstream targets in the PI3K and MAPK pathways as nonchimeric receptors, with somewhat slower and more sustained kinetics. Importantly, they show that chemically induced dimerization of ch Δ EGFR causes an increase in downstream signaling to levels resembling those seen with acutely stimulated EGFR and significantly higher than those observed in cells expressing ch Δ EGFR alone.

EGFR and Δ EGFR are both sensitive to inhibition by active site inhibitors, such as AG1478/tyrphostin. To confirm that the chimeric receptors were also susceptible to inhibition by this drug, we treated cells with AG1478 before stimulating them with EGF or AP20187 and assessed activity in EGFRs and downstream targets. At the level of the receptor, endogenous EGFR and chEGFR stimulated by EGF, or chEGFR and ch Δ EGFR stimulated by AP20187 were inhibited by AG1478, regardless of whether they were cultured in the presence of serum or not and in both LN428 and LN308 cells (Fig. 3). In terms of

downstream pathways, cells in serum showed activation of Akt, S6, and S6K even when AG1478 was present, suggesting EGFR-independent pathways were active as expected. Interestingly, one exception to this was levels of pAkt in the PTEN wild-type LN428 cells stimulated by EGF, which were lowered by AG1478 even in the presence of serum, though not to baseline, suggesting that Akt is more closely linked to EGFR in these cells but that other serum ligands can stimulate pS6 and pS6K by other pathways. As before, stimulation of chEGFR or ch Δ EGFR with AP20187 did not lead to an increase in pAkt levels. In the absence of serum, AG1478 was able to suppress the activation of Akt, S6, and S6K in LN428 cells but had no impact in LN308 cells. Importantly, the effect of AG1478 on EGF-stimulated and AP20187-stimulated cells was similar, suggesting that chimeric receptors responded to the inhibitor in a manner similar to the endogenous EGFR and so that chemically induced dimerization did not fundamentally alter the mechanism of action of the receptor.

STAT5 was identified as an important target of Δ EGFR and so we measured STAT5 phosphorylation status along with that of the signaling proteins GAB1, SHP2, SRC, and STAT3 (Supplementary Fig. S1). Even in the absence of stimulation, the cells displayed a high level of phosphorylation of these signaling proteins, making it difficult to detect increases, although the level of pSTAT5 was higher in ch Δ EGFR cells than cells expressing chEGFR, consistent with our previous work (22). We did see an increase in phosphorylated GAB1 when ch Δ EGFR-expressing cells were stimulated with AP20187 or when chEGFR cells were

Figure 3. Chimeric receptors are inhibited by AG1478. LN428 and LN308 glioma cells expressing either chEGFR or ch Δ EGFR were incubated in media containing no serum (serum starved, ss) or with regular growth medium containing 10% FBS, as indicated. They were left untreated or treated with 10 μ mol/L AG1478 for 2 hours before stimulation with 20 ng/mL EGF or 50 nmol/L AP20187 for another 15 minutes. Signaling was analyzed by immunoblotting of lysates with the indicated antibodies.



stimulated with EGF, with the chemically induced dimers generating a more sustained signal as before. These data further support the conclusion that increasing the activity of Δ EGFR does not lead to the engagement of fundamentally different pathways.

A more comprehensive and open approach to comparing signaling patterns emanating from ch Δ EGFR is to use phosphotyrosine directed shotgun proteomics. We employed an immunoaffinity-based method to enrich phosphotyrosine containing peptides and analyzed them on an ion-trap mass spectrometer. We identified 89 tyrosine-phosphorylated peptides and quantified 75 of those peptides (Supplementary Fig. S4 and Table S1). Similar to the observations made with specific signaling nodes by Western blot, we did not detect many peptides with difference in the phosphorylation intensity when we compared AP20187-stimulated ch Δ EGFR with AP20187-stimulated chEGFR, suggesting that when both receptors are maximally active that they resemble each other more in the overall downstream signal. Nevertheless, some specific differences remained, including SHC1, CDC2, and paxillin which were phosphorylated at a higher level in AP20187-treated cells expressing ch Δ EGFR than chEGFR and are known downstream targets of EGFR (31, 32). We did observe a larger group of peptides, including one derived from EGFR, which show increased phosphorylation upon stimulation with AP20187 in ch Δ EGFR cells, as was expected from the finding presented above.

The increased activity of Δ EGFR has no effect on its internalization

One characteristic of Δ EGFR is a reduced rate of internalization when compared with acutely stimulated EGFR, which we attributed to its relatively low level of activity (14). To determine whether increasing the activity level of ch Δ EGFR increased its rate of internalization, we labeled extracellular proteins with biotin, stimulated the cells with EGF or AP20187 at 37°C for 15 minutes, and then cleaved the remaining surface-exposed biotin. Recovery of proteins with streptavidin and Western blotting for FKBP selective revealed chimeric EGFRs that had been internalized (Fig. 4A). When cells were kept at 0°C throughout the experiment, very little receptor was detected after cleavage, as expected, because little membrane trafficking occurs at this temperature, effectively preventing internalization. The simple elevation of cells to 37°C for 15 minutes leads to a basal level of signal, as membranes resume normal turnover. In the case of chEGFR stimulation with either EGF or AP20187 further increased the level of internalized receptor, in accordance with the activity-dependent internalization that this receptor undergoes. The ch Δ EGFR did not respond to EGF as expected, but it also showed no increase in internalization in response to AP20187 (all these data are quantified across 3 experiments in Figure 4B). This suggests that reduced internalization rate of Δ EGFR is not solely due to its inherently lower level of activity as previously proposed but may reflect an inherent difference in how this receptor interacts with the endocytosis machinery. These

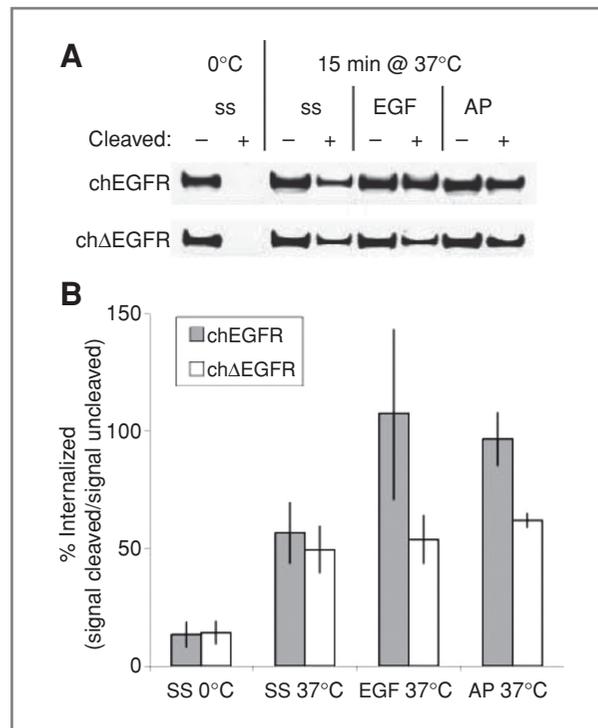


Figure 4. Chemically induced dimerization does not promote ch Δ EGFR internalization. A, cells were cooled to 4°C on ice and surface proteins were biotinylated, before being subjected to a variety of conditions, and then exposed to a biotin cleavage agent incapable of crossing the membrane, or left uncleaved. Biotinylated proteins were recovered with streptavidin-conjugated beads, and the resulting precipitates immunoblotted with anti-FKBP. Conditions prior to cleavage included leaving the cells on ice, incubation with prewarmed media containing no serum (ss), 20 ng/mL EGF or 50 nmol/L AP20187 for 15 minutes at 37°C. As internalization protects proteins from cleavage, the degree of internalization can be determined by the signal after cleavage divided by the signal in the absence of cleavage. A typical experiment is shown. B, graphical representation of quantification of Western blots as in (A) and showing the average percent of internalized receptors from 3 independent experiments.

data do suggest that the increase in signal we obtain with chemically induced dimerization of ch Δ EGFR is not able to overcome this aspect of the behavior of mutant receptor.

Cell motility is not dependent on Δ EGFR activity

At the cellular level we examined 2 behaviors, DNA synthesis in S-phase and motility. Cells were serum starved, grown in FBS or stimulated with EGF or AP20187, and BrdU incorporation determined (Fig. 5). As expected, cells expressing chEGFR or ch Δ EGFR responded with increased BrdU incorporation when stimulated with any of these agents (Fig. 5, left hand panels). In the case of ch Δ EGFR-expressing cells, this shows that increasing the signal from the mutant receptor has impact at the cellular level. In LN428 cells, we also observed higher basal BrdU incorporation in the absence of stimulation, suggesting that the ligand-independent activity of ch Δ EGFR was also manifest at this level. In contrast to DNA synthesis, motility as measured by a scratch assay was not enhanced by

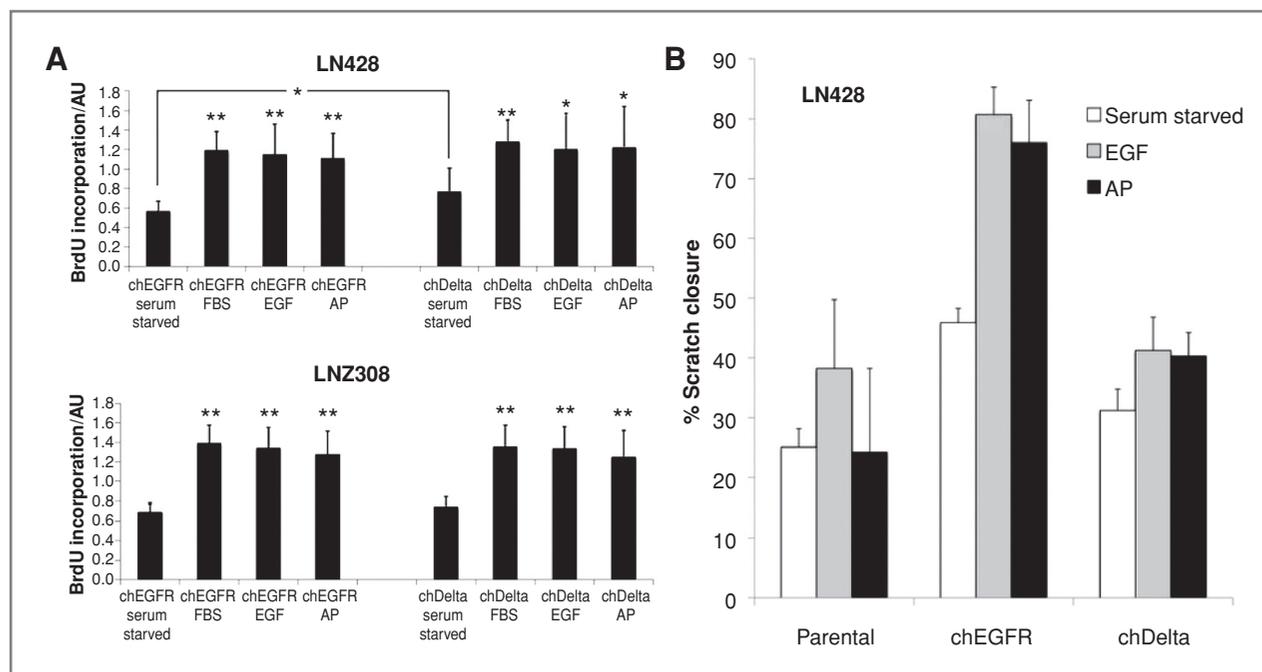


Figure 5. Dimerization of ch Δ EGFR induces cell division but not migration. A, LN428 or LNZ308 glioma cells were serum starved and stimulated with 20 ng/mL EGF or 50 nmol/L AP20187 in the presence of 100 μ M BrdU for 24 hours. BrdU incorporation was measured by a colorimetric immunoassay. Data are from 3 independent experiments (*, $P < 0.05$; **, $P < 0.01$; t test). B, a scratch was made in a confluent cell culture of LN428 cells that were grown in the absence of serum supplemented with 20 ng/mL EGF or 50 nmol/L AP20187 as indicated, and the degree of scratch closure was measured at 24 hours by using a digital photograph and XCAP-Lite software. Graph shows the average \pm SD from 4 independent experiments.

ch Δ EGFR, even when it was stimulated by AP20187 (Fig. 5, right hand panel). LN428 cells expressing ch Δ EGFR showed migration rates similar to the parental cells, whereas chEGFR-expressing cells showed elevated motility in the presence of either EGF or AP20187. This implies that elevated activity chEGFR, but not ch Δ EGFR, could accelerate cell movement, consistent with a role of EGFR in cell motility shown but none for Δ EGFR. These data therefore also support that chemically induced dimerization enhanced the ch Δ EGFR signal but did not alter it from that of Δ EGFR at the level of cell behavior.

Increased activity of Δ EGFR shortens survival time in a xenograft model

To evaluate whether increasing the signal from ch Δ EGFR strengthens its tumorigenic signal, chimeric receptor expressing U87 cells were intracranially implanted into nude mice and the tumors treated with AP20187 or vehicle for 2 weeks by using Osmotic pumps. We confirmed expression of chimeric receptors in tumors and observed that the chimeric receptors were signaling *in vivo* as they had in cultured cells by immunoblotting (Supplementary Fig. S2). AP20187 stimulation of cells expressing ch Δ EGFR shortened median survival by 1 day, from 24 to 23 days ($P = 0.0145$, Wilcoxon test), when compared with vehicle-treated mice (Fig. 6). Furthermore, histologic examination of H&E-stained tumor sections showed that AP20187 and vehicle tumors had a similar appearance, typical of U87 intracranial

tumors, suggesting that the shortening of survival was not related to changes in tumor morphology such as invasion caused by AP20187 (Supplementary Fig. S3). Therefore, increasing the intensity of the ch Δ EGFR signal also enhances its already potent oncogenic signal *in vivo*.

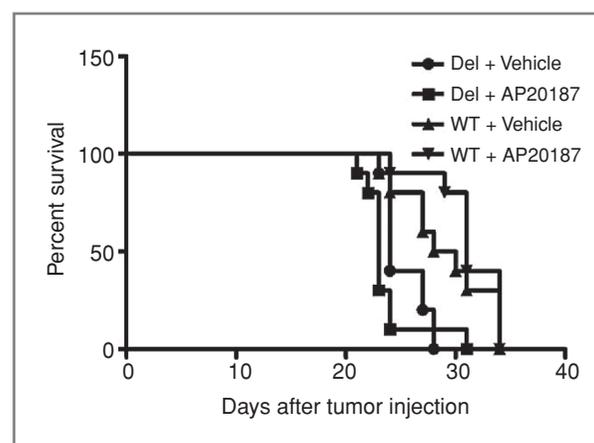


Figure 6. Comparison of the survival curves. AP20187 treatment caused early death in ch Δ EGFR-implanted mice. Data were analyzed by using Gehan-Breslow-Wilcoxon tests of the statistical package Prism version 5.0 (GraphPad Software Inc.). Values of $P < 0.05$ were taken as statistically significant.

Discussion

The absence of an intact ligand-binding domain in Δ EGFR, as well as the lack of response to EGF stimulation, suggested that it is defective in dimerization either as a homodimer or with family members capable of being activated by EGF (33, 34). Nevertheless, under some experimental circumstances, dimers have been reported, and it has been suggested that dimerization is required for Δ EGFR function (23). Here we report for the first time, the experimentally induced dimerization of a chimeric version of Δ EGFR and observed a very significant enhancement of its phosphorylation levels. It is worth noting that the strategy that we used to force dimerization of EGFRs, first established by Dr. David Spencer and colleagues, did not result in abnormally high levels of phosphorylation of chEGFR when compared with EGF stimulation, suggesting that at least the initial impact of dimerization was similar, regardless of whether it was mediated by the natural, extracellular domain and ligand or an artificial, intracellular domain and ligand. We conclude that if ch Δ EGFR was dimerized to any significant degree in terms of duration and stability in the absence of AP20187, the addition of this compound would not have been able to induce such a significant increase in phosphorylation and hence, propose that naturally occurring Δ EGFR does not dimerize to the extent of the ligand-activated wild-type receptor. Interestingly, a recent report showed that a small subpopulation of Δ EGFR is dimerized, via a disulfide bond, and that the dimerized receptors are the most active as judged by tyrosine phosphorylation (35). Taking these data together allows us to suggest that forced dimerization increases the activity of the Δ EGFR pool overall by bringing more receptors into this state and stabilizing them there.

That Δ EGFR generates a signal that is different in some aspect from its wild-type counterpart is likely, as it is a much more potent oncogene in many model systems and is associated with worse outcomes for patients with glioblastoma. Our previous analysis comparing the signal of Δ EGFR and EGFR by using phosphotyrosine directed shotgun phosphoproteomics suggested that there are no absolute differences, in that we were not able to identify any downstream signaling components that were exclusive to mutant or wild-type receptor. However, we did identify several phosphorylated tyrosines in target proteins that showed a stronger association with Δ EGFR, with signals reaching levels similar to what was observed when wild-type EGFR was acutely stimulated in serum-starved cells, a likely non-physiologic maximization of its signal. One of these targets was Y699 on pSTAT5b (22), and we saw a similar preference for phosphorylation of this residue here, in that the signal in ch Δ EGFR cells was higher than in serum-starved chEGFR cells (Supplementary Fig. S1). However, the level of pSTAT5 was not increased further by addition of AP20187 to cells expressing ch Δ EGFR, suggesting that it may be maximal, and not limited by the low level signal of unstimulated ch Δ EGFR. In other words, Δ EGFR may signal at an overall lower level, but reach near-maximal levels

with a few select downstream targets, including STAT5. Other signaling components, such as GAB1 may still be preferentially connected (18, 22) but not maximally so (Supplementary Fig. S1). Comparison of overall patterns of phosphorylation of a spectrum of tyrosine residues by an open approach (Supplementary Fig. S4 and Table S1) did not reveal any profound differences, further suggesting that changing signal strength does not noticeably change signal content, and so that the two are not tightly coupled. Indeed, several of phosphorylations that we did observe to increase with forced dimerization of ch Δ EGFR, such as those on paxillin, might be related to signaling through established downstream targets, in this case c-Met. This conclusion is supported by the analysis of cell behaviors. Increasing the signal of ch Δ EGFR by forced dimerization enhances the existing stimulation of mitosis but does not convert a nonmigration/invasion-inducing Δ EGFR signal to a migration/invasion-inducing EGFR signal. Although the close connection of c-Met and Δ EGFR observed by others and us might suggest that Δ EGFR should promote cell motility, a behavior-associated mesenchymal transition of epithelial tumors, we do not observe it in glioma cells.

Δ EGFR signaling is constitutive with little receptor internalization. We previously hypothesized that the lack of Δ EGFR downregulation is due to the inability of the weakly active Δ EGFR to recruit the CIN85-Cbl complex that initiates endocytosis in EGFR (36). This hypothesis would predict that enhancing the signal by forced dimerization would reestablish the active downregulation of the receptor, but we did not observe this for ch Δ EGFR, although the controls for chEGFR behaved as expected (Fig. 4). Our results strongly suggest that increased Δ EGFR activity is not sufficient to reestablish Δ EGFR downregulation and so we conclude that the impaired downregulation of Δ EGFR is not due to its lower level of activity or lack of dimerization.

In summary, our data provide the first direct, experimental test of Δ EGFR dimerization and imply that it does not form strong, durable dimers under normal circumstances, which may be related to its low level of activity. Increasing the Δ EGFR activity by forced dimerization of ch Δ EGFR also provided the first opportunity to compare signals of Δ EGFR and EGFR at similar amplitudes by an open phosphoproteomic approach, which revealed no major qualitative differences. This in turn suggests that the differences that were observed by others and us previously (21, 22) related to the signals that the naturally occurring, overall low-activity Δ EGFR maintains at an elevated, near-maximal level; they stood out because they were high by comparison with the bulk of EGFR targets. Increasing the ch Δ EGFR signal did increase its ability to stimulate entry into S-phase and tumor growth rate as measured by survival of tumor-bearing mice. Interestingly, however, the lack of downregulation of ch Δ EGFR was not altered by enhancing its activity, suggesting that this may be a fundamental characteristic of this mutant. The picture of Δ EGFR that emerges is of a receptor whose oncogenicity relies on a sustained signal, which although overall of low intensity, is capable of focusing on several select

downstream nodes which are significantly activated. Raising its activity enhances the signal overall profoundly, but only to a modest degree on some of the already very active partners. The key to oncogenicity of Δ EGFR may therefore very well be its inability to be downregulated, and this in turn suggests that altering this behavior may be a key to targeting it therapeutically.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, et al. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature* 1985;313:144-7.
- Wong AJ, Bigner SH, Bigner DD, Kinzler KW, Hamilton SR, Vogelstein B. Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. *Proc Natl Acad Sci U S A* 1987;84:6899-903.
- Ekstrand AJ, Sugawa N, James CD, Collins VP. Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. *Proc Natl Acad Sci U S A* 1992;89:4309-13.
- Moscatello DK, Holgado-Madruga M, Godwin AK, Ramirez G, Gunn G, Zoltick PW, et al. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res* 1995;55:5536-9.
- Tang CK, Gong XQ, Moscatello DK, Wong AJ, Lippman ME. Epidermal growth factor receptor VIII enhances tumorigenicity in human breast cancer. *Cancer Res* 2000;60:3081-7.
- Shinojima N, Tada K, Shiraiishi S, Kamiyo T, Kochi M, Nakamura H, et al. Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme. *Cancer Res* 2003;63:6962-70.
- Heimberger AB, Hlatky R, Suki D, Yang D, Weinberg J, Gilbert M, et al. Prognostic effect of epidermal growth factor receptor and EGFRVIII in glioblastoma multiforme patients. *Clin Cancer Res* 2005;11:1462-6.
- Pelloski CE, Ballman KV, Furth AF, Zhang L, Lin E, Sulman EP, et al. Epidermal growth factor receptor variant III status defines clinically distinct subtypes of glioblastoma. *J Clin Oncol* 2007;25:2288-94.
- Nishikawa R, Ji XD, Harmon RC, Lazar CS, Gill GN, Cavenee WK, et al. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc Natl Acad Sci U S A* 1994;91:7727-31.
- Lal A, Glazer CA, Martinson HM, Friedman HS, Archer GE, Sampson JH, et al. Mutant epidermal growth factor receptor up-regulates molecular effectors of tumor invasion. *Cancer Res* 2002;62:3335-9.
- Nagane M, Coufal F, Lin H, Bogler O, Cavenee WK, Huang HJ. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. *Cancer Res* 1996;56:5079-86.
- Bachoo RM, Maher EA, Ligon KL, Sharpless NE, Chan SS, You MJ, et al. Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis. *Cancer Cell* 2002;1:269-77.
- Huang HS, Nagane M, Klingbeil CK, Lin H, Nishikawa R, Ji XD, et al. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J Biol Chem* 1997;272:2927-35.
- Schmidt MH, Furnari FB, Cavenee WK, Bogler O. Epidermal growth factor receptor signaling intensity determines intracellular protein interactions, ubiquitination, and internalization. *Proc Natl Acad Sci U S A* 2003;100:6505-10.
- Pawson T, Hunter T. Signal transduction and growth control in normal and cancer cells. *Curr Opin Genet Dev* 1994;4:1-4.
- Moscatello DK, Montgomery RB, Sundareshan P, McDanel H, Wong MY, Wong AJ. Transformational and altered signal transduction by a naturally occurring mutant EGF receptor. *Oncogene* 1996;13:85-96.
- Prigent SA, Nagane M, Lin H, Huvar I, Boss GR, Feramisco JR, et al. Enhanced tumorigenic behavior of glioblastoma cells expressing a truncated epidermal growth factor receptor is mediated through the Ras-Shc-Grb2 pathway. *J Biol Chem* 1996;271:25639-45.
- Holgado-Madruga M, Wong AJ. Role of the Grb2-associated binder 1/ SHP-2 interaction in cell growth and transformation. *Cancer Res* 2004;64:2007-15.
- Moscatello DK, Holgado-Madruga M, Emlet DR, Montgomery RB, Wong AJ. Constitutive activation of phosphatidylinositol 3-kinase by a naturally occurring mutant epidermal growth factor receptor. *J Biol Chem* 1998;273:200-6.
- Huang PH, Cavenee WK, Furnari FB, White FM. Uncovering therapeutic targets for glioblastoma: a systems biology approach. *Cell Cycle* 2007;6:2750-4.
- Huang PH, Mukasa A, Bonavia R, Flynn RA, Brewer ZE, Cavenee WK, et al. Quantitative analysis of EGFRVIII cellular signaling networks reveals a combinatorial therapeutic strategy for glioblastoma. *Proc Natl Acad Sci U S A* 2007;104:12867-72.
- Chumbalkar V, Latha K, Hwang Y, Maywald R, Hawley L, Sawaya R, et al. Analysis of phosphotyrosine signaling in glioblastoma identifies STAT5 as a novel downstream target of DeltaEGFR. *J Proteome Res* 2011;10:1343-52.
- Chu CT, Everiss KD, Wikstrand CJ, Batra SK, Kung HJ, Bigner DD. Receptor dimerization is not a factor in the signalling activity of a transforming variant epidermal growth factor receptor (EGFRVIII). *Biochem J* 1997;324:855-61.
- Spencer DM, Wandless TJ, Schreiber SL, Crabtree GR. Controlling signal transduction with synthetic ligands. *Science* 1993;262:1019-24.
- Clackson T, Yang W, Rozamus LW, Hatada M, Amara JF, Rollins CT, et al. Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity. *Proc Natl Acad Sci U S A* 1998;95:10437-42.
- Farrar MA, Alberol-Ila J, Perlmutter RM. Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. *Nature* 1996;383:178-81.

27. Yang J, Symes K, Mercola M, Schreiber SL. Small-molecule control of insulin and PDGF receptor signaling and the role of membrane attachment. *Curr Biol* 1998;8:11–8.
28. Mohi MG, Arai K, Watanabe S. Activation and functional analysis of Janus kinase 2 in BA/F3 cells using the coumermycin/gyrase B system. *Mol Biol Cell* 1998;9:3299–308.
29. Kume A, Ito K, Ueda Y, Hasegawa M, Urabe M, Mano H, et al. A G-CSF receptor-tyrosine kinase fusion gene: A new type of molecular switch for expansion of genetically modified hematopoietic cells. *Biochem Biophys Res Commun* 1999;260:9–12.
30. Muthuswamy SK, Gilman M, Brugge JS. Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. *Mol Cell Biol* 1999;19:6845–57.
31. Sturla LM, Amorino G, Alexander MS, Mikkelsen RB, Valerie K, Schmidt-Ullrich RK. Requirement of Tyr-992 and Tyr-1173 in phosphorylation of the epidermal growth factor receptor by ionizing radiation and modulation by SHP2. *J Biol Chem* 2005;280:14597–604.
32. Liang X, Fonnum G, Hajivandi M, Stene T, Kjus NH, Ragnhildstveit E, et al. Quantitative comparison of IMAC and TiO₂ surfaces used in the study of regulated, dynamic protein phosphorylation. *J Am Soc Mass Spectrom* 2007;18:1932–44.
33. Chen WS, Lazar CS, Poenie M, Tsien RY, Gill GN, Rosenfeld MG. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. *Nature* 1987;328:820–3.
34. Schlessinger J. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. *Cell* 2002;110:669–72.
35. Ymer SI, Greenall SA, Cvrljevic A, Cao DX, Donoghue JF, Epa VC, et al. Glioma specific extracellular missense mutations in the first cysteine rich region of epidermal growth factor receptor (EGFR) initiate ligand independent activation. *Cancers* 2011;3:2032–49.
36. Schmidt MH, Hoeller D, Yu J, Furnari FB, Cavenee WK, Dikic I, et al. Alix/AIP1 antagonizes epidermal growth factor receptor downregulation by the Cbl-SETA/CIN85 complex. *Mol Cell Biol* 2004;24:8981–93.

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