Constitutive Activation of Phosphatidylinositol 3-Kinase by a Naturally Occurring Mutant Epidermal Growth Factor Receptor*

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The most frequently found alteration of the epidermal growth factor receptor (EGFR) in human tumors is a deletion of exons 2–7. This receptor, termed EGFRvIII, can transform NIH 3T3 cells, and the frequent expression of this variant implies that it confers a selective advantage upon tumor cells in vivo. Although EGFRvIII is a constitutively activated tyrosine kinase, there is no increase in Ras-GTP levels and low levels of mitogen-activated protein kinase activity in NIH 3T3 cells expressing this variant. We investigated whether phosphatidylinositol (PI) 3-kinase was an effector in transformation by the EGFRvIII. High levels of PI 3-kinase activity were constitutively present in EGFRvIII-transformed cells and were dependent upon the kinase activity of the receptor. While mitogen-activated protein kinase activity was quickly down-regulated to basal levels after 12 h of continuous EGFR activation, there was a 3-fold increase in PI 3-kinase activity in cells expressing normal EGFR and an 8-fold increase in cells expressing EGFRvIII after 48 h. This increased activity may reflect enhanced binding to EGFRvIII and the presence of novel PI 3-kinase isoforms. Treatment with the PI 3-kinase inhibitors wortmannin and LY294002 blocked both anchorage-independent growth and growth in low serum media and also resulted in morphological rever- sion of EGFRvIII-transformed cells. These results support an essential role for PI 3-kinase in transformation by this EGFR variant.

Overexpression of the EGFR§ has been implicated in the pathogenesis of many human tumors, including those derived from the brain, breast, lung, ovarian, prostate, and skin (1, 2). A number of alterations within the EGFR receptor gene that result in aberrant protein products have also been described, primarily in human glial tumors (3, 4). The most common alteration of the EGFR receptor gene is a deletion encompassing exons 2–7 (3–5) (referred to as EGFRvIII, ΔEGFR, or de2–7EGFR) (6–8). This receptor variant has subsequently been identified in other types of primary human brain tumors as well as breast carcinomas, non-small cell lung carcinomas, and ovarian tumors (6, 7). This deletion results in a receptor with a 267-amino acid deletion in the extracytoplasmic domain near the amino terminus. The frequent expression of this variant in various tumors suggests a strong selective advantage conferred upon tumor cells in vivo (7, 10).

Because the deletion occurs after the signal sequence, the EGFRvIII can be properly targeted to the membrane, and the remaining extracellular portion is glycosylated (11, 12). While the EGFRvIII has been detected on the cell surface of both tumor cells in vivo (13, 14) and a number of different transfectants (12, 14, 15), significant accumulations in the perinuclear area have also been observed, which suggests aberrant traffick- ing of this receptor variant (11, 15). A number of other functional differences between EGFRvIII and normal EGF re- ceptor have been characterized. Although EGFRvIII fails to bind EGF, the receptors can dimerize, and the tyrosine kinase in the intracellular portion of the receptor is constitutively activated (15, 16), so that the receptor undergoes autophosphory- lation as well as phosphorylating substrates such as Shc (15–17). While EGFRvIII can bind Grb2- and Sos complexes, implicating activation of the Ras/Raf/MAP kinase pathway (18, 19), we found no increase in Ras-GTP levels and very low levels of MAP kinase activity (15, 20), so this is unlikely to be the primary proliferative and transforming signal propagated by EGFRvIII. Interestingly, there are two points in the signaling transduction pathway at which MAP kinase activation is down- regulated. Overexpression of EGFRvIII leads to decreased levels of Shc and Grb2, which could reduce Ras activation, and there is an increase in MAP kinase phosphatase activity in these cells as well (15, 20).

The normal EGF receptor is capable of initiating a variety of signaling cascades upon ligand activation. One such effector whose importance in tumorigenesis is becoming increasingly apparent is phosphatidylinositol 3-kinase (PI 3-kinase). PI 3-kinase was first shown to be important in transformation by the observations that it associates with polyoma virus middle T protein upon phosphorylation by c-Src, and that mutants of middle T which fail to recruit PI 3-kinase activity are impaired in their tumorigenic activity (22–24). In addition, PI 3-kinase activation has been shown to be essential for induction of DNA synthesis by EGF (25). We therefore investigated the possible role played by this enzyme in transformation by the EGFRvIII, and we now report that PI 3-kinase is constitutively activated in EGFRvIII-transformed cells and is essential for transformation by this receptor variant.
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**Cell Lines and Materials**—The cell lines which overexpress the normal EGFR receptor (CO12 20c2/b) or EGFRvIII (HC2 20d2/c) and the vector-only control line LTR b2 were derived from NIH 3T3 cells and maintained as described previously (15). Growth experiments in mono-layer and soft agar were performed as described (15). Media, recombiant human EGFR, and PDGF-BB were from Life Technologies, Inc. Tyrothricin AG1478 was from Calbiochem, and the PI 3-kinase inhibitors LY294002 and wortmannin were from Biomol (Plymouth Meeting, PA). Stocks (20 mM) were dissolved in MeSO and stored at −20 °C and were diluted in MeSO so that the same amount of MeSO (0.1%) was present in all conditions. Lipid substrates and standards were from Sigma-\(\gamma\)-32P]ATP, 125I-goat anti-mouse IgG, and anti-rabbit IgG were from NEN Life Science Products. Normal human fibroblast lystate, anti-phosphotyrosine monoclonal antibody P4A20, and anti-panERK monoclonal antibody were from Transduction Laboratories (Lexington, KY), and the polyclonal and monoclonal antibodies to PI 3-kinase p85 were from U.B.I. (Lake Placid, NY). Anti-EGF receptor monoclonal antibody was from Promega (Madison, WI), and anti-GST antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies to the EGFRvIII and Gab1 were produced and affinity-purified as described previously (13, 26). Silica Gel 60 plastic sheets were from EM Science (Gibbstown, NJ), and all other materials were from Fisher.

**Immunoprecipitation and PI 3-Kinase Assay—**Immunoprecipitations and PI 3-kinase assays were carried out as described previously (26, 27) with slight modifications. Cells were washed twice with ice cold phosphate-buffered saline and lysed in Nonidet P-40 lysis buffer (1% Non- idet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM MgCl2, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM leupeptin, 100 \(\mu\)g/ml phenylmethylsulfonyl fluoride). Lysates were centrifuged at 12,000 \(\times\) g for 10 min at 4 °C, and the protein concentration was determined by Bio-Rad DC protein assay. Lysates were adjusted to 1 \(\mu\)g/ml with lysis buffer, and equal amounts were preclared with nonspecific IgG bound to bovine serum albumin-blocked Protein G Plus/Protein A-agarose by rocking at 4 °C overnight, and the immune complexes were washed three times with lysis buffer, twice with 0.5 mM LiCl in 100 mM Tris-HCl (pH 7.5) plus 100 mM sodium orthovanadate, and twice with reaction buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 6.25 mM MgCl2, 0.625 mM dithiothreitol and resuspended in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) buffer, and electrophoresis and immunodetection were as described previously. The PI 3-kinase activities were normalized to that of the receptor.

**RESULTS**

**PI 3-Kinase Is Constitutively Activated in EGFRvIII-transfected Cells**—To study signaling in a cell line expressing EGFRvIII we used the NIH 3T3 transfected clone HC2 20d2/c, and for comparison we used CO12 20c2/b, which overexpresses normal human EGFR receptor (15). PI 3-kinase activity was initially examined in serum-starved cells with or without stimulation with 100 ng/ml EGFR. As an estimate of the maximal PI 3-kinase activity we also stimulated cells with 50 ng/ml PDGF for 5 min, as PDGF caused the highest stimulation of PI 3-kinase in NIH 3T3 cells among several growth factors or combination of growth factors tested (data not shown). Analysis of

**EXPERIMENTAL PROCEDURES**

**PI 3-Kinase Activation (fold):**

| PI 3-Kinase Activity | 1.0 | 2.2 | 37 | 5.7 | 3.0 | 0.9 | 1.3 |
|----------------------|-----|-----|----|-----|-----|-----|-----|
| CO12 20c2/b          | P   | +E  | P  | +E  | P   | +E  | P   |
| HC2 20d2/c           | P   | +E  | P  | +E  | P   | +E  | P   |

**FIG. 1.** PI 3-kinase is constitutively activated in cells expressing EGFRvIII. NIH 3T3 transfectants overexpressing the EGFRvIII (HC2 20d2/c), the normal human EGF receptor (CO12 20c2/b), or vector-only controls (LTR b2) were serum-starved for 1 day and then treated with DMEM only (−), 100 ng/ml EGF (+E), or 50 ng/ml PDGF-BB (+P) for 5 min and lysed. Anti-pTyr immunoprecipitates were analyzed for PI 3-kinase activity as described under “Experimental Procedures.” Autoradiogram of thin layer chromatography plate exposed overnight is shown. The large arrow indicates the position of phosphatidylinositol 3-phosphate, while the small arrow indicates the origin. Fold activation relative to each cell line’s respective unstimulated control was determined by PhosphorImager quantitation of the PI 3-phosphate spot.

**FIG. 2.** The PI 3-kinase activity in cells overexpressing normal EGF receptor or EGFRvIII is dependent upon the kinase activity of the receptor. A, serum-starved cells were treated with DMEM only or DMEM + 2 \(\mu\)M tyrphostin AG1478 (AG1478) for 3 h; CO12 20c2/b cells were then stimulated with EGF for 5 min prior to lysis. Anti-pTyr immunoprecipitates were assayed for PI 3-kinase activity as described previously. The PI 3-kinase activities were normalized to that in the absence of inhibitors for each cell line. B, PI 3-kinase activity of replicate anti-pTyr immunoprecipitates from control HC2 lysate containing the indicated nanomolar concentrations of wortmannin (WORT) in the in vitro PI 3-kinase reactions.

**RESULTS**

**PI 3-Kinase Is Constitutively Activated in EGFRvIII-transfected Cells**—To study signaling in a cell line expressing EGFRvIII we used the NIH 3T3 transfected clone HC2 20d2/c, and for comparison we used CO12 20c2/b, which overexpresses normal human EGFR receptor (15). PI 3-kinase activity was initially examined in serum-starved cells with or without stimulation with 100 ng/ml EGF. As an estimate of the maximal PI 3-kinase activity we also stimulated cells with 50 ng/ml PDGF for 5 min, as PDGF caused the highest stimulation of PI 3-kinase in NIH 3T3 cells among several growth factors or combination of growth factors tested (data not shown). Analysis of
the total phosphotyrosine-associated PI 3-kinase activity revealed a very low basal activity in vector-only control transfectants (LTR b2) and CO12 cells. We observed a much greater stimulation of PI 3-kinase by PDGF than EGF, which was similar in both LTR b2 and CO12 20c2/b cell lines (Fig. 1). In contrast, HC2 20d2/c cells exhibited a high basal level of PI 3-kinase activity which was similar to the maximal activity observed in PDGF-stimulated CO12 20c2/b or LTR b2. As expected, HC2 20d2/c cells showed no response to EGF. There was only a slight increase by PDGF, indicating that the basal activity approached the highest obtainable in these cells (Fig. 1). Preincubation of cells with tyrphostin AG1478, a highly specific inhibitor of the EGF receptor kinase (28), reduced the phosphotyrosine-associated PI 3-kinase activity in cells expressing either receptor (Fig. 2), suggesting that the EGF receptor tyrosine kinase activity is directly involved in PI 3-kinase activation in these cells. Wortmannin, a fungal metabolite, is an irreversible inhibitor of PI 3-kinases, as it binds covalently to the active site of the enzyme (29). Type I PI 3-kinases are especially sensitive to wortmannin at concentrations in the low nanomolar range (21, 29). The IC \textsubscript{50} of the PI 3-kinase activity in phosphotyrosine immunoprecipitates from HC2 20d2/c cells for wortmannin was less than 10 nM (Fig. 2, right), indicating that this activity is due to a type I PI 3-kinase.

**PI 3-Kinase Activity Is Not Down-regulated by the Constitutive Activity of the EGFRvIII**—The fact that MAP kinase activity is down-regulated in HC2 20d2/c cells implies that it plays little role in transformation by EGFRvIII. If PI 3-kinase activity was important for transformation by EGFRvIII, then it should not show evidence of long term down-regulation. Because this receptor is constitutively active we devised a protocol using tyrphostin AG1478 to regulate EGFRvIII. Treatment of HC2 20d2/c cells with 2 \mu M AG1478 resulted in a loss of both EGFRvIII tyrosine phosphorylation and PI 3-kinase activity, but maximum inhibition of PI 3-kinase required serum starvation (Fig. 3A). We found that the daily addition of 2 \mu M AG1478 for 3 days followed by 1 day of serum starvation in the presence of AG1478 resulted in confluent monolayers and a reduction in the level of PI 3-kinase activity to near that of quiescent CO12 20c2/b cells (see below) without a reduction in EGFRvIII levels (Fig. 6B and data not shown). The prolonged AG1478 treatment was required to ensure down-regulation of a MAP kinase phosphatase which would otherwise prevent MAP kinase activation in these cells (20). While Han et al. have reported that the EGFRvIII is more sensitive to AG1478 than the wild-type EGF.
receptor (30), we did not observe such differential sensitivity in the present study (Fig. 3A and data not shown).

We confirmed the specificity of AG1478 for EGF receptor signaling. Treatment of serum-starved CO12 20c2/b cells with either EGF or PDGF resulted in a robust activation of MAP kinase, whereas cells pretreated with AG1478 showed MAP kinase activation only in response to PDGF (Fig. 3B). While neither EGF nor PDGF resulted in MAP kinase activation in serum-starved HC2 20d2/c cells, AG1478-treated HC2 20d2/c exhibited strong MAP kinase activation in response to PDGF (Fig. 3B). Removal of the drug after serum starvation resulted in activation of MAP kinase in HC2 20d2/c concurrent with a recovery of the tyrosine kinase activity of EGFRvIII (Fig. 3B). Tyrphostin removal alone caused no MAP kinase activation after similar treatment of CO12 20c2/b cells, but as expected the cells did recover the ability to respond to EGF (Fig. 3B). These results demonstrate that EGFRvIII can signal through the MAP kinase pathway and that AG1478 does not interfere with other events that are necessary for MAP kinase activation.

We then evaluated the kinetics of PI 3-kinase and MAP kinase activation in these transfectants. EGF stimulation of quiescent CO12 20c2/b resulted in a very rapid activation of PI 3-kinase with the maximum occurring between 5 and 30 min. This was followed by a decline in activity by 12 h to ~3-fold above basal, which remained at this level for at least 48 h (Fig. 3C). MAP kinase activation also peaked between 5 and 30 min but declined to basal activity after 12 h (Fig. 3D). In contrast, when AG1478-treated HC2 20d2/c cells were released from tyrphostin there was a large increase in PI 3-kinase activity, which rose to ~8-fold above basal and persisted for at least 48 h (Fig. 3C). There was only a modest activation of MAP kinase which returned to nearly basal activity within 6 h (Fig. 3D). Taken together, these results indicate that cells expressing EGFRvIII can tolerate a prolonged high level of PI 3-kinase activity, suggesting a role for this enzyme in long term processes. On the other hand, the rapid down-regulation of MAP kinase activity in HC2 20d2/c cells following activation of EGFRvIII further suggests that it has no long term role in neoplastic transformation.

**PI 3-Kinase is Constitutively Associated with the EGFRvIII and Gab1**—We wished to explore how EGFRvIII effected PI 3-kinase activation. PI 3-kinase is activated by the binding of the two SH2 domains of the p85 subunit to pYXXM motifs (21, 27). The five major autophosphorylation sites on the EGF receptor do not fit these motifs, so the direct association of PI 3-kinase with the EGFRvIII further suggests that it has no long term role in neoplastic transformation.

**Fig. 4. PI 3-kinase activity associates with the EGFRvIII and Gab1.** A, serum-starved CO12 20c2/b and HC2 20d2/c cells were lysed without stimulation (-) or after 5 min of stimulation with EGF (+E) or PDGF (+P). Anti-phosphotyrosine (αpTyr), anti-EGFR receptor (αEGFR), or anti-Gab1 (αGab1) immunoprecipitates were analyzed for PI 3-kinase activity as described. Arrows indicate the position of PI 3-phosphate. B, effect of EGF and PDGF on the PI 3-kinase activity in HC2 20d2/c cells associated with phosphotyrosine, the EGFRvIII, and Gab1 relative to the respective unstimulated controls. C, effect of EGF and PDGF on the PI 3-kinase activity in CO12 20c2/b cells associated with phosphotyrosine, the normal EGF receptor, and Gab1 relative to unstimulated controls. Values in B and C are the means ± S.E. of three independent experiments. The absolute amount of phosphotyrosine-associated PI 3-kinase activity in serum-starved CO12 20c2/b and HC2 20d2/c cells was approximately the same as that in PDGF-treated CO12 20c2/b cells.
20d2/c cells had very high levels of PI 3-kinase activity associated with the EGFRvIII and Gab1, and EGF caused no significant change in PI 3-kinase activity in these cells. PDGF addition resulted in only a ~2-fold increase in the total phosphotyrosine-associated activity and actually caused a decrease in the EGFRvIII-associated activity (Fig. 4, A and B). These results indicate that the binding of PI 3-kinase to EGFRvIII, like the normal EGFR, is of lower affinity than the binding to the PDGF receptor, as PI 3-kinase could be recruited away from the EGFRvIII by the two high affinity p85 binding sites on the activated PDGF receptor.

**Direct Association of PI 3-Kinase p85 Is Constitutive and Phosphotyrosine-dependent**—To determine whether the PI 3-kinase activity associated with the EGFRvIII could be accounted for by a direct interaction with p85, we performed precipitations using GST-PI3K-SH2. The resulting blots were probed with anti-phosphotyrosine antibody, confirming that there was an association that was dependent upon tyrosine phosphorylation of the normal and mutant receptors (Fig. 5A). Western blotting with anti-EGF receptor antibodies confirmed the identity of the normal and mutant EGF receptor as the major GST-PI3K-SH2-associated proteins in these experiments (data not shown). Far-Westerns were also performed using GST-PI3K-SH2 fusion protein, also confirming that association with the normal EGF receptor and the PDGF receptor was growth factor-dependent, but the association with the EGFRvIII was independent of EGF.

**Increased Expression of p85-Related Molecules in Cells Overexpressing the EGFRvIII**—Because several isoforms of PI 3-K adapters, including p85α and p85β, have been described, we wondered which form was expressed in these cells. Western blotting of CO12 20c2/b and HC2 20d2/c lysates with an anti-

![Fig. 5](image)

**Fig. 5. Direct association of the p85 subunit of PI 3-kinase with normal EGF receptor and EGFRvIII.** A, association of PI 3-kinase p85 with the EGF receptor is dependent upon tyrosine phosphorylation. Lysates from serum-starved (-), EGF (+E) or PDGF (+P) stimulated cells were used in pulldowns with GST-PI 3-K-SH2 fusion protein as described under “Experimental Procedures,” and the blot was probed with anti-phosphotyrosine (αTyr). The upper arrow indicates the position of the normal EGF receptor and the PDGF receptor which are not resolved on this blot, and the lower arrow indicates the EGFRvIII. B and C, overexpression of EGFRvIII induces elevated expression of molecules immunologically related to p85. B, normal human fibroblast (NHF), CO12 20c2/b (CO12), and HC2 20d2/c (HC2) lysates were separated by SDS-polyacrylamide gel electrophoresis, and the blot was probed with monoclonal antibody (U.B.I. 05–212) specific for the N-SH3 domain of p85α. This monoclonal antibody recognizes the same p85 band in all three cell lines (arrow). C, the same blot was re-probed with a polyclonal antibody to rat p85 (U.B.I. 06–497). This antibody detected 55- and 50-kDa bands (small arrows) as well as p85. Numbers on the left of all blots indicate positions of molecular mass markers (kDa).

![Fig. 6](image)

**Fig. 6. Growth inhibition and morphological reversion of cells expressing EGFRvIII by PI 3-kinase inhibitors.** Shown is growth inhibition by wortmannin (A) or LY294002 (B) in monolayer culture. Cells were plated in 1% CS at 100,000/35-mm well, and the next day the media were changed to 1% CS ± the indicated concentrations of wortmannin or LY294002. Fresh wortmannin was added three times daily, and LY294002 was added once daily, and the cells were trypsinized and counted with a Coulter counter on day 3. Open bars, HC2 20d2/c; gray bars, CO12 20c2/b. C, PI 3-kinase activity in HC2 20d2/c cells correlates with growth inhibition. Cells were treated as above, and PI 3-kinase activity in anti-pTyr immunoprecipitates of day 1 and day 3 lysates was determined. The PI 3-kinase activity relative to the 1% CS + MeSO control (open bars) is shown for cells treated with 100 nM (light gray) or 1 μM (dark gray) wortmannin or with 10 μM LY294002 (black bars). D, morphology of HC2 20d2/c in the absence and presence (E) of 5 μM LY294002 or 2 μM AG1478 (F). Cells were grown as described in B, and phase-contrast photomicrographs of live cells are shown (× 200).
PI 3-Kinase Activity Contributes to Growth Stimulation and Transformation by the EGFRvIII—To determine whether the constitutive PI 3-kinase activity in HC2 20d2/c cells is relevant to transformation by the EGFRvIII, the effects of PI 3-kinase inhibitors on cell growth and morphology were examined. Addition of wortmannin to cells grown in 1% CS resulted in a dose-dependent inhibition of growth HC2 20d2/c cells. A 1 μM dose resulted in about 50% inhibition of HC2 20d2/c cells and nearly completely abolished the growth stimulation of EGF of CO12 20c2/b cells (Fig. 6A). Similar results were also obtained with the PI 3-kinase inhibitor LY294002 (Fig. 6B). The extent of growth inhibition correlated with the degree of inhibition of PI 3-kinase activity by both drugs (Fig. 6C). The PI 3-kinase inhibitors also caused partial morphological reversion of HC2 20d2/c cells (compare Fig. 6, D and E) and abolished EGF-induced changes in the morphology of CO12 20c2/b cells (data not shown). Treatment of cells with AG1478 was more effective than the PI 3-kinase inhibitors in both reducing PI 3-kinase activity and causing morphological reversion (Figs. 3A and 6F, respectively). These results indicate that PI 3-kinase activity contributes to both the growth and morphological transformation induced by EGFRvIII.

Because anchorage-independence is the in vitro characteristic which best correlates with tumorigenicity, we tested the effects of the PI 3-kinase inhibitors on the growth of HC2 20d2/c in soft agar. Wortmannin caused a dose-dependent inhibition of colony formation by HC2 20d2/c cells, which caused a ~40% reduction in the number of colonies at a 1 μM dose, with significant reductions in colony size at concentrations as low as 200 nm (Fig. 7A). LY294002 was even more effective, resulting in complete abolition of colony formation at 5 μM (Fig. 7B). Both PI 3-kinase inhibitors were even more effective in blocking EGF-dependent soft agar colony development by CO12 20c2/b cells than HC2 20d2/c (Fig. 7, A and B). The smaller effect of these inhibitors on HC2 20d2/c cells may reflect the higher endogenous level of PI 3-kinase activity in these cells. These results indicate that PI 3-kinase activity is essential for anchorage-independent growth in EGFRvIII-transformed cells.

DISCUSSION

Work in this and other laboratories has demonstrated that expression of the EGFRvIII results in neoplastic transformation and enhanced tumorigenicity, which is due to its constitutive kinase activity (7, 12, 15). While activation of the normal EGF receptor results in activation of MAP kinase via Ras (15, 18), our studies on EGFRvIII showed only a low level of activation of the Ras-MAP kinase pathway, which was due to decreases in Shc and Grb2 levels and induction of a MAP kinase phosphatase (15, 20). The down-regulation of the MAP kinase pathway has also been observed in NIH 3T3 cells transformed by viral oncoproteins such as v-src and v-ras, and the evidence supports a role for a MAP kinase phosphatase in these cells as well (35).

Because PI 3-kinase is essential for DNA synthesis induced by EGF (25), we studied this enzyme in cells expressing EGFR-vIII for its possible contribution to transformation. We found that EGFRvIII-transformed cells exhibited a high constitutive level of PI 3-kinase activity not shown by cells overexpressing normal EGF receptor. Analysis of the kinetics of PI 3-kinase and MAP kinase activation revealed an important difference in the regulation of these pathways by the EGFRvIII relative to the normal EGF receptor. While the EGF-stimulated PI 3-kinase activity in CO12 20c2/b cells peaked quickly and declined to a moderate level by 12 h, the activity in HC2 20d2/c cells rose more slowly and did not decline throughout the period tested (Fig. 5C). The slower rate of increase in PI 3-kinase activity in the EGFRvIII-expressing cells was most likely due to the gradual decline in tyrphostin activity, but the lack of subsequent down-regulation of PI 3-kinase activity cannot be so explained, because HC2 20d2/c cells which have never been exposed to the drug exhibit high PI 3-kinase activity (Fig. 1). One possibility is that cells achieve down-regulation of EGF receptor-initiated PI 3-kinase activity primarily by down-regulation of the number of receptors. The EGFRvIII does not bind ligand and is not actively down-regulated despite its constitutive activation (15, 16), and this may account for the lack of decrease in PI 3-kinase activity in HC2 20d2/c cells. In contrast, MAP kinase activity...
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declines to barely above basal levels within about 12 h in both cell lines, indicating that regulation of this pathway primarily occurs downstream of the receptor. It further suggests that while prolonged, high level PI 3-kinase activation is compatible with continuous growth, the prolonged, high level activation of MAP kinase is not essential. Thus, our results are in agreement with the recent report that prolonged MAP kinase activation in NIH 3T3 cells results in growth arrest (36).

While we found that p85 can associate with both normal EGFR receptor and EGFRvIII, it is not clear that p85 can account for all PI 3-kinase activity associated with EGFRvIII. Although immunoprecipitation with anti-p85α/β antibody reduced the PI 3-kinase activity in anti-pTyr immunoprecipitates from CO12 20c2/b cells, it did not reduce the pTyr-associated activity in HC2 20d2/c cells (data not shown). Furthermore, we found elevated levels of p85α in HC2 20d2/c, as well as bands with molecular masses of 50 and 55 kDa which cross-react strongly with antibody to rat p85. This suggests that the constitutive activity of the EGFRvIII may influence the expression of PI 3-K adapter subunits. At least five forms of regulatory p85 variants of p85 adapter subunits. At least five forms of regulatory activity detected in EGFRvIII transfectants. There are several mechanisms by which PI 3-kinase may contribute to tumorigenesis. Inhibition of PI 3-kinase activation has been shown to block the EGF-dependent transformation of murine JB6 P+ cells (37). We found that PI 3-kinase inhibitors inhibited both monolayer growth in low serum and anchorage-independent growth of cells expressing normal EGFR receptor and EGFRvIII. These inhibitors also caused a partial reversion of the transformed morphology of HC2 20d2/c and blocked the EGF-induced transformed morphology of CO12 20c2/b. PI 3-kinase activity can influence cell morphology, as it has been shown to affect cytokoskeletal organization (21, 22, 33). For instance, PI 3-kinase interacts with Rac-GTP, a member of the Rho family of small G proteins which regulate the actin cytoskeleton (38). Nagane et al. recently reported that EGFRvIII expression reduces apoptosis of glioblastoma cells both in vitro and in vivo (10). As activation of the Raf/MAP kinase pathway by Ras in the absence of PI 3-kinase activity was recently shown to promote apoptosis in fibroblasts (39), and PI 3-kinase activity has been shown to be essential for survival of a number of cell types (40, 41), these data suggest another mechanism by which constitutive PI 3-kinase activity contributes to tumorigenesis. Thus, because PI 3-kinase can play a central role in growth, morphological transformation, and the inhibition of cell death by the both normal EGFR receptor and EGFRvIII, it seems likely that enhancement of this activity provides an important selective advantage for EGFRvIII-expressing tumor cells in vivo.