Constitutive Activation of c-Jun N-terminal Kinase by a Mutant Epidermal Growth Factor Receptor*  

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Epidermal growth factor receptor (EGF) variant type III (EGFRvIII) is a constitutively active, naturally occurring mutation of the EGF receptor that is found in many types of human tumors. When overexpressed in NIH3T3 fibroblasts, EGFRvIII induces transformation by enhancing cell growth and reducing apoptosis. Analysis of downstream signaling pathways has revealed that extracellular signal-regulated kinase activity is down-regulated, raising doubt as to the significance of this pathway in promoting transformation. We investigated whether the c-Jun N-terminal kinase (JNK) pathway was affected by EGFRvIII. NIH3T3 cells expressing EGFRvIII exhibited a high basal level of JNK activity, which was not present in cells overexpressing the normal EGF receptor. Treatment of cells overexpressing EGFRvIII with inhibitors of the EGF receptor or phosphatidylinositol 3-kinase resulted in the down-regulation of JNK activity. Furthermore, the down-regulation of JNK activity was associated with a loss of properties related to transformation, and there was no evidence for JNK activity in the promotion of apoptosis in these cells. These findings implicate constitutive activation of the JNK pathway in transformation by EGFRvIII.

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¶ The abbreviations used are: EGF, epidermal growth factor; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; EGFRvIII, EGF receptor variant type III; PI, phosphatidylinositol; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; MEK, MAP kinase.

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a phosphatase. Down-regulation of the ERK MAP kinase pathway in other types of transformed cells has also been reported (24, 25).

Recently, we found that phosphatidylinositol (PI) 3-kinase is constitutively active in EGFRvIII-transformed cells and that this activity is required for colony formation in soft agar (26). Interestingly, PI 3-kinase has been implicated in the activation of the JNK pathway (27, 28). We have investigated JNK activity in NIH3T3 cells expressing EGFRvIII and have found a high basal activity. Inhibition of EGFRvIII or PI 3-kinase activity down-regulated JNK activity, which was correlated with a loss of transformed morphology. Despite this chronic high level of JNK activity and the low level of ERK activity, these cells have a low rate of apoptosis.

**EXPERIMENTAL PROCEDURES**

*Materials—*Cell culture media and recombinant human EGF were from Life Technologies, Inc. Typhostin AG 1478 (Calbiochem) and LY 294002 (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) were dissolved in Me_SO to a concentration of 20 μM and stored at –80 °C. [γ-32P]ATP, 125I-labeled goat anti-mouse IgG, and 125I-labeled goat anti-rabbit IgG were from NEN Life Science Products. Unlabeled ATP was from Pharmacia Biotech Inc. The anti-phosphotyrosine monoclonal antibody PY20 and the anti-pan-ERK monoclonal antibody were from Transduction Laboratories (Lexington, KY). The anti-JNK1 (C-17) polyclonal antibody was from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA), and the anti-phospho-p44/p42 MAP kinase monoclonal antibody was from New England Biolabs Inc. (Beverly, MA). All other materials were from Fisher unless otherwise indicated.

*Cell Culture—*CO12 20c2/b, HC2 2042/c, and LTR b2 clones were generated by transfection of NIH3T3 cells with the cDNA encoding the normal human EGF receptor, the EGFRvIII cDNA, and vector only, respectively (17). The NIH3T3 transfectants were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin, and 350 μg/ml G418. Cells were rinsed with phosphate-buffered saline and then lysed with 1 ml of cell lysis buffer (10 mM Na2HPO4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2% NaH2PO4, 0.004% Na3, 1 mM NaN3, 25 mM β-glycerolphosphoric acid, 100 μM phenylmethylsulfonyl fluoride, and 1 μg/ml each aprotinin and leupeptin, pH 7.5). Lysates were clarified by centrifugation at 12,000 × g for 10 min at 4 °C. Protein concentrations were determined using the Bio-Rad DC protein assay.

*Western Blot Analysis—*Total cell lysate (30 μg) of each sample was subjected to SDS-polyacrylamide gel electrophoresis on 8.5% acrylamide gels for resolution of MAP kinases and 4–20% Tris/glycine gels for resolution of the EGF receptor. Protein was transferred to nitrocellulose filters and blocked for 2 h at room temperature in 100 mM Tris, pH 7.5, 0.9% NaCl, and 0.1% Tween 20 with 5% nonfat dry milk. The anti-phospho-MAP kinase monoclonal antibody was used at a 1:10,000 dilution, whereas the anti-phosphotyrosine monoclonal antibody, the anti-pan-ERK monoclonal antibody, and the anti-JNK1 polyclonal antibody were used at 1 μg/ml in the same blocking solution. 125I-labeled goat anti-mouse IgG (5 × 108 cpm/ml) was used to detect the monoclonal antibodies, and 125I-labeled goat anti-rabbit IgG was used to detect the polyclonal antibodies. Quantitation of signals was performed on a PhosphorImager utilizing GST-c-Jun (amino acids 1–79) as a substrate for JNK a high affinity for this substrate, so a solid-phase kinase assay was performed. An autoradiogram of the kinase assay is shown. Western blot analysis of the same whole cell lysates was performed, and blots were probed with the anti-phosphotyrosine antibody (αTyr), anti-JNK antibody (αJNK), anti-ERK MAP kinase antibody (αActive MAPK), or anti-pan-ERK antibody (αERK). The positions of the activated forms of the mutant and normal EGF receptors and of activated ERK (p-ERK1/2) are noted.

*Assay for Apoptosis—*Cells were seeded on coverslips in 6-well dishes and grown in complete medium for 5 days. The cells were then incubated in serum-free DMEM containing either 2 μM tyrphostin AG 1478 or 5 μM LY 294002 for 1 or 3 days and fixed and stained with 4,6-diamidino-2-phenylindole (2 μg/ml) for viewing by fluorescence microscopy. Apoptotic cells were identified by condensed nuclei.

**RESULTS**

*EGFRvIII Constitutively Activates JNK—*Previous work on the signaling effects of EGFRvIII in NIH3T3 fibroblasts showed that EGF receptor activity was down-regulated (17, 18), suggesting that signaling through the Ras/ERK pathway was not required for transformation. We wished to investigate whether an additional MAP kinase pathway was affected by the signaling of EGFRvIII. We chose to study the effect of the mutant receptor on the JNK pathway. JNKs are defined in general by their ability to phosphorylate two serine residues in the amino-terminal domain of c-Jun in vitro (29, 30). These enzymes have a high affinity for this substrate, so a solid-phase kinase assay utilizing GST-c-Jun (amino acids 1–79) as a substrate for JNK allows measurement of the kinase activity associated with a cell lysate. We employed this technique to compare JNK activity in NIH3T3 cells transfected with an empty expression vector (LTR b2); the normal human EGF receptor (CO12 20c2/b); and the mutant EGF receptor, EGFRvIII (HC2 20d2/c). Unstimulated LTR b2 and CO12 20c2/b cells exhibited very low levels of JNK activity, whereas the mutant and normal EGF receptors and of activated ERK (p-ERK1/2) are noted.

![Fig. 1. Analysis of JNK and ERK activation in LTR b2, CO12 20c2/b, and HC2 20d2/c cells in response to different stimuli.](http://www.jbc.org/)

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*Fig. 1.* Analysis of JNK and ERK activation in LTR b2, CO12 20c2/b, and HC2 20d2/c cells in response to different stimuli. NIH3T3 transfectants overexpressing EGFRvIII (HC2), the normal EGF receptor (CO12), or vector only (LTR) were serum-starved for 10 h and then treated with serum-free DMEM (−), 100 ng/ml EGF (E), or 500 mM sorbitol in DMEM (S) for 10 min and lysed. To determine JNK activity, an *in vitro* c-Jun kinase assay was performed on whole cell lysates. An autoradiogram of the kinase assay is shown. Western blot analysis of the same whole cell lysates was performed, and blots were probed with the anti-phosphotyrosine antibody (αTyr), anti-JNK antibody (αJNK), anti-ERK MAP kinase antibody (αActive MAPK), or anti-pan-ERK antibody (αERK). The positions of the activated forms of the mutant and normal EGF receptors and of activated ERK (p-ERK1/2) are noted.
JNK activity is not due to the up-regulation of protein expression.

Osmotic stress is known to potently induce JNK activity and can be used to measure the maximum potential in vivo activity of this kinase. Osmotic stress was induced by incubation of cell lines for 15 min in DMEM containing 500 mM sorbitol. This potently increased JNK activity in the transfected cell lines relative to EGF stimulation (Fig. 1). Sorbitol-treated HC2 20d2/c cells achieved the highest level of JNK activation, indicating that the signaling pathway leading to JNK activation was also enhanced in these cells.

ERK activities were determined for LTR b2, CO12 20c2/b, and HC2 20d2/c cell lines under the same conditions used to analyze JNK activity. ERK activity was measured using an antibody that specifically recognizes the phosphorylated forms of ERK1/2. Consistent with earlier findings, EGF stimulation of CO12 20c2/b cells activated ERK, whereas unstimulated or EGF-treated HC2 20d2/c cells had undetectable levels of ERK activity (Fig. 1, aActive MAPK despite equivalent protein expression (Fig. 1, aERK). The anti-ERK antibody also revealed a slower mobility form of ERK in this blot (Fig. 1, aERK) that corresponds to the active form of ERK, but the anti-active MAP kinase antibody appeared to be more sensitive to detecting activated ERK. Incubation of cells with sorbitol had no detectable effect on ERK activation in HC2 20d2/c cells and showed only a slight increase in LTR b2 cells (Fig. 1, aActive MAPK). However, sorbitol-treated CO12 20c2/b cells did show substantial ERK activation. The exaggerated JNK and ERK activation observed in sorbitol-treated CO12 20c2/b cells relative to LTR b2 cells suggests that the EGFR receptor may enhance the response of NIH3T3 cells to osmotic stress. It has been reported that sorbitol treatment of HeLa cells can result in EGFR receptor phosphorylation (13). We tested if the increases in ERK and JNK activities induced by osmotic shock in our cell lines were due to receptor activation. Anti-phosphotyrosine blots revealed that EGFR receptor activity in sorbitol-treated CO12 20c2/b and LTR b2 cells was not elevated above basal levels (Fig. 1, aPyr), whereas EGF stimulation of CO12 20c2/b and LTR b2 resulted in EGFR receptor phosphorylation. Taken together, these results indicate that overexpression of the EGFR receptor indirectly enhances JNK and ERK responses to stress and further demonstrate the differences in JNK versus ERK activation in cells overexpressing the mutant receptor.

Continuous Stimulation of the Normal EGF Receptor Does Not Result in Elevated Levels of JNK Activity—Since the constitutive activity of EGFRvIII resulted in a high basal level of JNK activity, we wanted to determine if the normal EGFR receptor could produce the same effect when continuously stimulated with ligand. Quiescent CO12 20c2/b cells were stimulated with EGF (100 nM) and assayed at certain intervals for the activation of the EGFR receptor, JNK, and ERK. CO12 20c2/b cells stimulated with EGF showed a maximal activation of the EGFR receptor at 5 min, which slowly declined to basal levels by 24 h of stimulation (Fig. 2, aPyr). EGF induction coincided with EGFR receptor activation, which was maximal at 5 min of ligand stimulation and declined to undetectable levels by 24 h (Fig. 2, aActive MAPK). Maximal JNK activity was detected after 5 min of EGF stimulation, but was greatly reduced by 1 h (Fig. 2, JNK Assay). JNK activity was above basal levels up to 48 h of EGF stimulation, but was still well below that seen in HC2 20d2/c cells (Figs. 1 and 2).

Inhibition of EGFRvIII Down-regulates JNK, and Receptor Reactivation Leads to Transient ERK but Sustained JNK Activation—Other studies (11, 12) and our data have shown that the normal EGFR receptor does not strongly activate JNK. We wished to determine if the constitutive JNK activity associated with the HC2 20d2/c cell line was an effect of signaling from the mutant receptor. To examine this, we utilized a specific inhibitor of the EGFR receptor, tyrphostin AG 1478. Previous work has shown that HC2 20d2/c cells maintained in 2 μM tyrphostin AG 1478 for 3 days and then serum-starved for 10 h showed a nearly complete loss of tyrosine phosphorylation of EGFRvIII (26). Tyrphostin AG 1478 inhibited ERK activation of EGF-treated, but not platelet-derived growth factor-treated, CO12 20c2/b cells, indicating the specificity of the inhibitor for the EGFR receptor (26). When HC2 20d2/c cells were treated with tyrphostin AG 1478, there was a reduction in JNK activity in association with inhibition of receptor activity (Fig. 3A, aPyr and JNK Assay, 0 time point).

To study responses after receptor reactivation, cells were then switched to serum-free DMEM. A transient activation of ERK occurred with the initial reactivation of the mutant receptor. Interestingly, ERK activity was maximal 5 min after release from tyrphostin AG 1478, was dramatically reduced 6 h after release, and declined to undetectable levels within 24 h (Fig. 3A, aActive MAPK). In contrast to ERKs, JNK activity remained at levels similar to those in HC2 20d2/c cells that had not been released from tyrphostin AG 1478 until after 6 h (Fig. 3A, JNK Assay). Increased levels of JNK activity became evident 12 h after release and reached levels that were equivalent to those in untreated HC2 20d2/c cells by 24 h of release. The increased JNK activity following release from tyrphostin AG 1478 correlated with the reestablishment of maximal activity of EGFRvIII (Fig. 3A, aPyr).

Transformed Morphology of HC2 20d2/c Cells Parallels JNK Activity—We examined the morphology of these cells after tyrphostin AG 1478 treatment and then following release. Cells treated as described above showed a reversion to the normal planar appearance of fibroblasts. Following release from tyrphostin AG 1478, HC2 20d2/c cells displayed a similar morphology for the first 12 h of release (Fig. 3B). However, between 12 and 24 h of release, these cells regained their transformed appearance and resembled untreated HC2 20d2/c cells, which coincided with the reactivation of JNK.

HC2 20d2/c Cells Treated with Tyrophostin AG 1478 Lose the Ability to Grow in Soft Agar—The reversion of the transformed morphology of HC2 20d2/c cells treated with tyrphostin AG 1478 suggests a reduction or loss of transformation potential. To further analyze this, anchorage-independent growth studies were performed on HC2 20d2/c and CO12 20c2/b cells treated with tyrphostin AG 1478. Both cell lines formed colonies in soft agar.
antibody (was performed, and blots were probed with the anti-phosphotyrosine antibody (pTyr) and the anti-active MAP kinase antibody (Active MAPK). The positions of activated ERK are indicated (P-ERK1/2). A, cell morphology changes of HC2 20d2/c cells treated or not treated with LY 294002. Photographs were taken just prior to the collection of lysates in A.

Constitutive JNK Activity Is Dependent on PI 3-Kinase in HC2 20d2/c Cells—Our laboratory has recently found that one of the effects of chronic signaling from EGFRvIII is constitutive PI 3-kinase activity (26). Recently, it was shown that constitutively active forms of PI 3-kinase activate the JNK pathway (27, 28). This led us to ask if the high level of PI 3-kinase activity was responsible for the high basal level of JNK activity in HC2 20d2/c cells. HC2 20d2/c and CO12 20c2/b clones were treated with LY 294002, a specific inhibitor of PI 3-kinase activity, in the same manner as described for tyrphostin AG 1478. The inhibition of PI 3-kinase activity in HC2 20d2/c cells resulted in a corresponding reduction in JNK activity without affecting ERK activity (Fig. 4A). The addition of LY 294002 to HC2 20d2/c cells resulted in a partial morphological reversion and growth inhibition (Fig. 4B). In a previous publication (26), we also found that treatment of HC2 20d2/c cells with 2 and 5 μM LY 294002 caused 71 and 99% reductions in soft agar colony efficiency. Interestingly, the reduction of JNK activity induced in LY 294002-treated HC2 20d2/c cells was nearly equivalent to that seen in tyrphostin AG 1478-treated HC2 20d2/c cells, indicating that the high basal level of JNK activity was dependent on the up-regulated PI 3-kinase activity. JNK and ERK activation levels were also determined in LY 294002-treated CO12 20c2/b cells. EGF-stimulated CO12 20c2/b cells showed similar levels of ERK activation regardless of treatment with the PI 3-kinase inhibitor (Fig. 4A, Active MAPK). However, EGF-stimulated CO12 20c2/b cells showed a decrease in JNK activation when compared with cells treated with LY 294002 (Fig. 4A, Kinase Assay).

JNK Activity Does Not Correlate with the Induction of Apoptosis in Cells Expressing EGFRvIII—In PC12 cells, prolonged JNK activity and a loss of ERK activity correlate with the induction of apoptosis (8). HC2 20d2/c cells possess the same enzymatic profile, but, in contrast, are actively growing. Since this is in the presence of serum, we examined HC2 20d2/c cells following 1 and 3 days of serum starvation for the induction of apoptosis.
apoptosis and compared the results with LTR b2 cells. Cells were assayed for the presence of nuclear condensation and JNK activity. A small percentage of apoptotic cells was noted in the LTR b2 cell line following 1 day of serum starvation, whereas there was negligible apoptosis in the HC2 20d2/c cell line (Fig. 5, bar graphs). The low basal JNK activity in LTR b2 cells was further decreased after 1 day of serum starvation, whereas the robust activity in HC2 20d2/c cells remained unaltered (Fig. 5, Kinase Assay). After 3 days of serum starvation, there was a large increase in the number of apoptotic cells in the LTR b2 cell line, but only a slight increase in the HC2 20d2/c cell line. JNK activity in LTR b2 cells remained extremely low, whereas there was actually a 77% decrease in HC2 20d2/c cells. Since EGFRvIII and PI 3-kinase contribute to the enhanced growth rate in HC2 20d2/c cells, we examined the effect of tyrphostin AG 1478 and LY 294002 on apoptosis. After 1 day of treatment, tyrphostin AG 1478 showed a small effect on LTR b2 cells, but by 3 days, there was a high percentage of apoptotic cells in both the LTR b2 and HC2 20d2/c cell lines (Fig. 5, bar graphs). LY 294002 also increased the incidence of apoptosis in both cell lines, although not to the same extent. There was no JNK activity in LTR b2 cells in the presence of either inhibitor, whereas there was a 60% decrease in HC2 20d2/c cells (Fig. 5, Kinase Assay). Taken together, these data suggest that EGFRvIII and PI 3-kinase contribute to cell survival, but that JNK activity does not contribute to cell death.

**DISCUSSION**

Expression of mutant forms of the EGF receptor has been implicated in the progression of many types of human tumors (20, 21). EGFRvIII is the most frequently occurring natural alteration associated with this receptor (20–22). We studied NIH3T3 fibroblasts transfected with EGFRvIII to elucidate the signaling mechanisms responsible for transformation in these cells. Work in this and other laboratories showed that activation of the normal EGF receptor resulted in a transient but potent ERK induction, whereas EGFRvIII generated minimal ERK induction (17, 18). This low level of ERK activity raised questions as to the significance of the ERK pathway in promoting transformation. Analysis of the JNK pathway showed that EGFRvIII-transfected cells displayed a high constitutive level of JNK activity, which was not present in cells overexpressing the normal EGF receptor. Although stimulation of the EGF receptor can transiently activate JNK, the constitutive activation of JNK seen in EGFRvIII-transfected NIH3T3 cells may define a novel mechanism associated with transformation by this receptor.

We have further defined the pathway by identifying PI 3-kinase as the upstream activator of JNK. Interestingly, EGFRvIII was recently shown to constitutively activate PI 3-kinase, and this activity was essential for the transforming potential of the mutant receptor (26). Inhibition of PI 3-kinase activity associated with HC2 20d2/c cells resulted in a reduction in JNK activity and partial morphological reversion of the transformed appearance of HC2 20d2/c cells. The ability of PI 3-kinase to influence both cell morphology and JNK activity could be explained by its interaction with members of the Rho family of proteins (31, 32), which are known to regulate the actin cytoskeleton (33, 34) and to activate the JNK pathway (35–38). Taken together, we speculate that the constitutive PI 3-kinase activity induced by EGFRvIII expression is responsible for the high basal level of JNK activity and that this contributes to the transformed phenotype induced by EGFRvIII.
Constitutive Activation of JNK by EGFRvIII

The low level of ERK activation exhibited by cells expressing the mutant receptor has been suggested to be regulated by expression of a phosphatase (17). Interestingly, in NIH3T3 fibroblasts, expression of MAP kinase phosphatase-1, a phosphatase specific for ERKs, is induced by activation of the JNK pathway (39). The constitutive JNK activity associated with EGFRvIII-transfected cells may induce constant expression of MAP kinase phosphatase-1 resulting in the low basal ERK activity exhibited in these cells. We are currently determining if this is the mechanism responsible for down-regulating ERK activation in HC2 2042/c cells and if such down-regulation is essential for the transformation potential of the mutant receptor.

In support of our data implicating continuous JNK activity with a growth advantage in EGFRvIII transfectants, recent studies found that the JNK pathway was required for transformation by both the Trp-Met (40) and Bcr-Abl (41) oncoproteins. Like EGFRvIII, the Trp-Met oncoprotein is a constitutively active receptor tyrosine kinase that is not influenced by ligand (42). Dominant-negatives forms of Grb2 transformed into cells expressing the Trp-Met protein resulted in a reverted morphology that was associated with both down-regulated PI 3-kinase and JNK activities (40). It is interesting to speculate that preferential activation of the JNK pathway may be a characteristic of unregulated kinases with high transformation potential, such as seen in fibroblasts expressing EGFRvIII, Trp-Met, or Bcr-Abl.

Analysis of EGFRvIII in NIH3T3 cells has provided a model in which the transforming effects of this mutant receptor can be elucidated. We have shown that the transformed state of EGFRvIII-expressing cells is associated with the preferential and constitutive activation of the JNK pathway. These findings also demonstrate that cells are capable of sustained JNK activity and low ERK activity without the induction of apoptosis. In light of our findings and recent reports by others (40, 41) implicating the JNK pathway in the promotion of transformation, it is becoming clear that certain oncoproteins and/or cell types actually utilize JNK and not ERK for transformation.

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