Differential Modulation of Mitogen-activated Protein (MAP) Kinase/Extracellular Signal-related Kinase Kinase and MAP Kinase Activities by a Mutant Epidermal Growth Factor Receptor*

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A paradigm has been established whereby mutant tyrosine kinase receptors such as the v-erbB and v-fms gene products function as oncoproteins in the absence of ligand. A spontaneously occurring deletion mutant of the human epidermal growth factor receptor (EGF-RvIII) has been isolated from astrocytic neoplasms and transforms NIH3T3 cells in the absence of ligand. The EGF-RvIII is constitutively complexed with the majority of cellular GRB2, suggesting a link to the Ras-Mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (MEK) pathway. The presence of EGFRvIII in fibroblasts is associated with downstream activation of mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (MEK) and modest activation of p42 and p44 MAP kinases. The presence of EGFRvIII suppresses activation of p42 and p44 MAP kinases by phorbol 12-myristate 13-acetate (PMA) and serum; however, MEK activation by PMA is not suppressed by EGFRvIII. Basal and PMA-stimulated MAP kinase activity in EGFRvIII-transfected cells is augmented by the tyrosine phosphatase inhibitor sodium vanadate. EGFRvIII is capable of transducing downstream signals through MAP kinase as evidenced by activation of cytoplasmic phospholipase A2 at levels similar to that induced by intact EGFR. Our results suggest that EGFRvIII constitutively activates downstream signal transduction through MAP kinase, and this chronic stimulation of the MAP kinase pathway may represent one means by which mutant EGFR transduces an oncogenic signal.

The epidermal growth factor receptor (EGFR) transduces signals for proliferation and differentiation in neuronal and epithelial tissues. EGFR signal transduction is initiated by ligand binding, receptor dimerization, and inter- and intrareceptor activation of kinase activity, with concomitant phosphorylation of tyrosine residues. Assembly of SH2 containing signal transduction molecules ensues at the receptor with activation of downstream signaling pathways, several of which appear to converge at the MAPK/extracellular signal-related kinase kinase (MEK) with subsequent activation of the p42/p44 MAP kinases, a family of dual specificity serine/threonine kinases. MAP kinases phosphorylate a number of cytoplasmic substrates including cytoplasmic phospholipase A2, as well as the nuclear ternary complex factor Elk-1 and Myc.

Overexpression of intact EGFR in the presence of ligand or truncation of receptor by removal of the extracellular domain induces transformation in fibroblast systems. The overexpression of intact EGFR has been documented in a variety of neuronal and epithelial neoplasms and carries a poor prognosis for patients with these malignancies, suggesting that this overexpression of receptor may confer a proliferative advantage in the presence of limiting amounts of growth factor. The retroviral oncogene v-erbB encodes a form of the EGFR lacking the majority of the extracellular domain and containing mutations in the transmembrane region and carboxyl terminus. Mutational analysis has demonstrated that deletion of the extracellular domain causes downstream activation of MAP kinase and contributes significantly to transformation by v-erbB. In human tumors, various deletions of the extracellular domain of the EGFR have been identified in astrocytic neoplasms. The most common of these alterations, termed the EGFRvIII, contains a deletion of nucleotides 275-1075 of the EGFR cDNA. The mutant gene product is a 145-kDa receptor that undergoes spontaneous homodimerization in transfected NIH3T3 cells and demonstrates increased receptor autophosphorylation.

Expression of this mutant receptor in NIH3T3 cells is transforming, and a large proportion of cellular GRB2 associates with the EGFRvIII. This suggests a link with the Ras-MAP kinase pathway, which we have explored to delineate novel aspects of EGFRvIII signaling.

In this study, we show that in NIH3T3 cells the EGFRvIII stimulates strong downstream activation of MEK, but the degree of p42 and p44 MAP kinase activation is more modest, recapitulating chronic EGF exposure in cells containing full-length human EGFR. Down-regulation of protein kinase C by phorbol esters does not abrogate constitutive activation by EGFRvIII.
mutant receptor. The chronic low level activation of MAP kinase suppresses the ability of other agonists to induce kinase activity. Suppression of MAP kinase response occurs without suppression of MEK, suggesting that aberrant regulation occurs at the level of MAP kinase. Vanadate treatment up-regulates MAP kinase and restores responsiveness to PMA, implicating a tyrosine phosphatase as the regulatory element stimulated by EGFRvIII. EGFRvIII is able to stimulate signaling downstream from MAP kinase, activating constitutive cPLA₂ activity as reflected by increased arachidonate release. EGFRvIII may utilize this pathway to transduce a transforming signal in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP and [3H]arachidonate were from DuPont NEN, [3H]oleic acid was from Moravek, and EGFR was from Peprotech. MAP kinase (Erk-1-NT) antibody and kinase-inactive MAP kinase were from Upstate Biotechnology. Myelin basic protein and all other reagents were from Sigma.

Cell Culture—HC2 200/c (HC2) and CO12 200/cb (CO12) were generated by transfection of NIH3T3 cells with full-length human EGFR (CO12) and with cDNA encoding mutant EGFR derived from a glioblastoma multiforme cell line (HC2). Cells were maintained in DMEM with 10% FCS, 50 μg/ml G418 for up to 10 passages. Cells were plated at 10⁵ cells per 100-mm plate in DMEM, 10% FCS, 200 units/ml penicillin, 200 μg/ml streptomycin, glutamate and grown for 2 days. Monolayers were then washed once with phosphate-buffered saline and serum starved in DMEM, 0.1% fatty acid-free BSA for 24 h.

In-gel Kinase Assay—Kinase activity using myelin basic protein as substrate was assayed as described (13). Cells from 100-mm plates were lysed in 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10 mM dithiothreitol, 10 μg/ml aprotinin, 10 μM sodium orthovanadate, 10 μg/ml leupeptin, 1 mM sodium orthovanadate. Lysates were scraped and centrifuged at 15,000 × g for 15 min. Supernatant was assayed for protein content and placed in 1 × SDS sample buffer. 30 μg of supernatant protein per lane was run on 10% SDS-PAGE containing a final concentration of 0.5 mM myelin basic protein. The gel was washed twice for 30 min in 50 mM Tris (pH 8.0) and 20% 2-propanol, followed by a 1-h wash in buffer A (50 mM Tris-HCl (pH 8.0) and 5 mM 2-mercaptoethanol). The gel was then washed twice for 30 min in 100 ml of 6 M guanidine HCl in buffer A. Renaturation was carried out in 250 ml of buffer A with 0.04% Tween 40 for 24 h at 4°C with frequent buffer changes. This phosphorylation reaction was carried out at room temperature for 1 h in 60 ml of 40 mM HEPES (pH 8.0) containing 2 mM dithiothreitol, 100 μM EGTA, 5 mM MgCl₂, 10 μM cold ATP, and 250 μCi [γ-32P]ATP. This was followed by extensive washing in 5% trichloroacetic acid and 1% sodium pyrophosphate. The gel was then dried and exposed to film. Autoradiograms were then analyzed by laser densitometry.

MEK Assay—The MEK assay was carried out essentially as described previously (14). Aliquots (100 μg) of protein from lysates were incubated for 30 min at 30°C in a reaction mixture containing 40 mM HEPES (pH 8.0) containing 10 mM MgCl₂, 1 mM dithiothreitol, 10 μM ATP, and 10 μCi of [γ-32P]ATP with 3 μg of kinase-inactive p44 (GST-KI-MAPK) (UBI). Beads were washed twice in 20 ml HEPES (8.0), 150 mM NaCl, and the reaction was terminated by addition of 2 × SDS sample buffer. Reaction mixtures were separated on 10% SDS-PAGE, dried, and autoradiographed.

SDS-PAGE—Cell lysates were generated as for the kinase assay above, and protein samples were normalized for protein concentrations and electrophoresed on 30% polyacrylamide gels. Protein was then transferred to nitrocellulose and incubated with phosphate-buffered saline containing 1% polyvinylpyrrolidone-40, 0.05% Tween 20 for 1 h followed by immunoblotting antibody in the same solution for 4 h. Filters were extensively washed in phosphate-buffered saline, 0.05% Tween 20, and bound antibodies were detected by addition of protein G-horseradish peroxidase (Bio-Rad) at 1:5000 for 1 h followed by enhanced chemiluminescence (Amersham) as per the manufacturer's directions.

cPLA₂ Assay—cPLA₂ activity was assayed essentially as described previously (15, 16). Briefly, confluent cell layers were incubated with 0.5 μCi/ml [3H]arachidonate in DMEM, 0.1% fatty acid-free BSA for 24 h. Monolayers were then washed twice and incubated in 37°C medium containing 0.5% BSA to trap released fatty acids and agonist for indicated periods of time. Medium was then collected, and radioactivity was determined by scintillation counting.
from both cell lines demonstrated essentially equivalent amounts of p44 in both cell lines (Fig. 4). Recently, specific negative regulators of p42 and p44 in the form of dual specificity tyrosine/threonine phosphatases have been described, including CL100 in human fibroblasts and its murine homologue MKP-1, as well as PAC-1 in lymphocytes (18–20). These phosphatases are immediate early gene products whose synthesis is induced by activators of MAP kinases and are believed to mediate the subsequent attenuation of kinase activity. Each appears to be vanadate sensitive and okadaic acid insensitive.

We evaluated the possibility that vanadate exposure may restore the ability to activate MAP kinases in cells transformed by mutant receptor. Both cell lines were exposed to 4 mM vanadate for 1 h followed by stimulation with serum and PMA. As shown in Fig. 5, A and B, vanadate increased basal activity in cells transfected with mutant receptor but not in CO12 cells. Subsequent exposure to PMA further activated MAP kinases, demonstrating that vanadate restored the responsiveness of p42 and p44 in cells containing constitutively activated MAP kinase. Okadaic acid alone or in combination with PMA had no effect on MAP kinase activity (data not shown).

The effect of PMA and Vanadate on MEK Activity—Although vanadate restored MAP kinase responsiveness, it is an assumption that the phosphatase responsible is acting directly on MAP kinase. Alternatively, MAP kinase activity may be controlled by suppression of the more proximal aspects of the activation pathway. To investigate the latter hypothesis, we analyzed activation kinetics of the upstream activator of the MAP kinases, MEK.

We analyzed MEK response to PMA and vanadate using a kinase-inactive MAP kinase substrate as described under “Experimental Procedures.” In a representative experiment, exposure of CO12 cells to 25 ng/ml EGF and 100 nM PMA for 15 min induced an 11-fold and 8-fold increase in MEK activity, respectively (Fig. 6). Basal MEK activity in HC2 cells was 4-fold higher than that of basal CO12 activity. PMA exposure doubled MEK activity in HC2, and the presence of vanadate and okadaic acid did not amplify the response to PMA. Therefore, the presence of mutant EGFR increases basal MEK activity approximately 4-fold but does not suppress PMA stimulation of MEK. Vanadate did not modulate the level of MEK activation. These data suggest that EGFRvIII transduces a chronic low level signal through MEK and MAP kinase and also induces a tyrosine phosphatase that acts at the level of MAP kinase.
kinase pathway could conceivably be very small. cPLA$_2$ is a cytoplasmic substrate for MAP kinase, and its activation is induced by EGFR and oncoproteins that transmit signals through the MAP kinase pathway (21–23). We explored the activation of cPLA$_2$, by normal and EGFRvIII by assaying release of $^{[3]H}$arachidonate from prelabeled CO12 and HC2 cells. The prelabeled cells at ~90% confluence were incubated in DMEM with 0.5% fatty acid-free BSA in the presence or absence of 25 ng/ml EGF, and release of radioactivity into the medium was determined after indicated periods of time (Table I). EGF stimulation of CO12 cells induced a 222 ± 4.5% increase over unstimulated CO12 at 30 min compared to 200 ± 8.4% by unstimulated HC2 cells. Our results for activation of cPLA$_2$ induced by EGF are essentially identical to that found by Goldberg et al. (23) in NIH 3T3 cells transfected with full-length EGFR. Cells prelabeled with $^{[3]H}$oleic acid did not show significant differences in fatty acid release (data not shown), establishing specificity for cPLA$_2$. Although the amplitude of MAP kinase activation in cells transfected with EGFRvIII is relatively small, activation of cPLA$_2$ by EGFRvIII is essentially equivalent to that mediated by EGF stimulation of intact EGFR.

**Table I**

| Cells | Agonist | Time | Control |
|-------|---------|------|---------|
| CO12  | EGF     | 15   | 155 ± 6 |
|       |         | 30   | 222 ± 5 |
| HC2   | None    | 15   | 164 ± 5 |
|       |         | 30   | 199 ± 8 |

**DISCUSSION**

Since the discovery that the oncogenic viral gene v-erbB encoded a truncated and transforming homologue of the EGFR, investigators have sought to define a role for the EGFR in human malignancies. Several mutant forms of the EGFR have been discovered in astrocytic neoplasms that contain either amino- and/or carboxyl-terminal deletions. The EGFRvIII analyzed in this study encodes a receptor that does not detectably bind EGF, and the deletion of the EGF binding domain results in spontaneous homodimerization and receptor autophosphorylation. Cells expressing normal EGFR or EGFRvIII have similar MEK activity, but the EGFRvIII results in p42 and p44 MAP kinase activation similar to that seen with chronic EGF exposure. The inability to further stimulate the p42/p44 MAP kinases in cells expressing EGFRvIII is related to induction of a tyrosine phosphatase activity as evidenced by the increase in MAP kinase activity in the presence of vanadate and the restoration of responsiveness to phorbol esters. Because MEK activation by EGF and PMA in cells containing intact receptor and MEK activation by PMA in cells containing mutant receptor appears to be relatively equivalent, the majority of signal suppression occurs at the level of MAP kinase.

Other investigators have demonstrated that suppression of MEK and MAP kinase responsiveness, apparently by phosphatases, is dependent on the cell line being studied and the agonist used. Samuels et al. (14) utilized an estradiol-inducible Raf mutant to show that in rat 1a cells activation of MEK and MAP kinase by the mutant Raf was restored by treatment with the phosphatase inhibitors okadaic acid and vanadate. However, in C7 3T3 cells, there was no evidence for constitutive suppressors of MEK and MAP kinase. In another study utilizing the same estradiol-dependent Raf protein, these authors showed that constitutive activation of Raf inhibited platelet-derived growth factor activation of MAP kinase, which could be restored by pretreatment with vanadate. No evidence for suppression of MEK was found, suggesting inhibition at the level of MAP kinase (24). However, they found no inhibition of MAP kinase activation by serum or PMA. In this study, we also found no constitutive suppression of MEK and MAP kinases in 3T3 cells transfectected with intact EGFR, but the presence of EGFRvIII induced suppression primarily at the level of p42 and p44 MAP kinase. In our hands, serum and PMA signaling through MAP kinase was also abrogated, consistent with a desensitization of MAP kinase to all agonists. We are currently exploring the identity of the MAP kinase phosphatase in these cells.

Because the degree of MAP kinase activation by EGFRvIII is comparatively low, the biological relevance of this finding is of some concern. Both amplitude and duration of activity have been postulated to be important in determining the biological phenotype, which results from MAP kinase activation (25). Transient MAP kinase activation mediates a proliferative response while sustained, low level activation transduces a differentiative response in neuronal PC12 cells (26, 27). It is possible that prolonged, relatively low amplitude MAP kinase activation, such as that mediated by Ras, may be an important determinant of transforming potential (28).

The subcellular localization of activated MAP kinase and activation of nuclear or cytoplasmic substrates may also modulate the phenotypic response. A significant proportion of MAP kinase undergoes nuclear translocation upon activation, and all of the MAP kinase-specific phosphatases defined to date are nuclear (18, 19). EGFRvIII activates CL100 to a level similar to that induced by activated full-length EGFR. The mechanistic basis for this is not readily apparent; however the MKP-1/CL100 MAP kinase phosphatases are primarily nuclear and would not have a significant effect on activated cytoplasmic MAP kinase. If MKP-1/CL100 is the primary suppressor of MAP kinase activity stimulated by mEGFR, then activation of cytoplasmic substrates would be expected to be equivalent. This would suggest that MAP kinase, which is activated but remains cytosolic, is fully capable of activating cytoplasmic substrates. It also suggests that activation of cPLA$_2$ may play a role in the transforming signal initiated by EGFRvIII.

In summary, our data demonstrate that a transforming mutant of human EGFR activates signaling through the MAP kinase pathway, recapitulating the changes seen with chronic stimulation of intact EGFR. Partial suppression of MAP kinase activity by a vanadate-sensitive phosphatase does not inhibit the signal for transformation, although it is not clear that this signal is necessarily mediated through the MAP kinase pathway. Continued investigation of the mechanisms behind signal suppression at each level of the Ras-MAP kinase cascade should provide new insights into how oncogenes that utilize the same signaling pathways transduce different biological phenotypes. Further study of the signaling pathways activated by mutant EGFR may reveal novel means of inhibiting the oncogenic signal either through direct interference with receptor function or by suppressing components of signaling pathways.

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