Phorbol 12-Myristate 13-Acetate Induces Epidermal Growth Factor Receptor Transactivation via Protein Kinase Cε/c-Src Pathways in Glioblastoma Cells*

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Both the epidermal growth factor receptor (EGFR) and protein kinase C (PKC) play important roles in glioblastoma invasive growth; however, the interaction between the EGFR and PKC is not well characterized in glioblastomas. Treatment with EGFR stimulated global phosphorylation of the EGFR at Tyr845, Tyr992, Tyr1068 and Tyr1045 in glioblastoma cell lines (U-1242 MG and U-87 MG). Interestingly, phorbol 12-myristate 13-acetate (PMA) stimulated phosphorylation of the EGFR only at Tyr1068 in the two glioblastoma cell lines. Phosphorylation of the EGFR at Tyr1068 was not detected in normal human astrocytes treated with the phorbol ester. PMA-induced phosphorylation of the EGFR at Tyr1068 was blocked by bisindolylmaleimide (BIM), a PKC inhibitor, and rottlerin, a PKCδ-specific inhibitor. In contrast, Gö 6976, an inhibitor of classical PKC isoforms, had no effect on PMA-induced EGFR phosphorylation. Furthermore, gene silencing with PKC8 small interfering RNA (siRNA), siRNA against ε-Src, and mutant ε-Src(S12C/S48A) and treatment with a c-Src inhibitor (4-amino-5-(4-chlorophenyl)-7-(4-buty1pyrazolo[3,4-d]pyrimidine) abrogated PMA-induced EGFR phosphorylation at Tyr1068. PMA induced serine/threonine phosphorylation of Src, which was blocked by both BIM and rottlerin. Inhibition of the EGFR with AG 1478 did not significantly alter PMA-induced EGFR Tyr1068 phosphorylation, but completely blocked EGF-induced phosphorylation of the EGFR. The effects of PMA on MAPK phosphorylation and glioblastoma cell proliferation were reduced by BIM, rottlerin, the MEK inhibitor U0126, and PKCδ and c-Src siRNAs. Taken together, our data demonstrate that PMA transactivates the EGFR and increases cell proliferation by activating the PKCδ/c-Src pathway in glioblastomas.

Glioblastoma multiforme (GBM)1 as a biologically aggressive neoplasm has an elevated, often aberrant, proliferative capacity with a diffuse pattern of brain invasion. It is the most malignant astrocytic tumor, composed of poorly differentiated neoplastic astrocytes (1–3). 50–60 and 40% of GBMs have overexpression and amplification of the epidermal growth factor receptor (EGFR), respectively (4–7). The overexpression and amplification of the EGFR contribute to the malignant phenotype of human glioblastomas (6, 8). In addition, malignant glioma cells have higher levels of protein kinase C (PKC) than non-neoplastic astrocytes (9–12). This suggests that excessive PKC activity may significantly contribute to astroglial tumorigenicity.

The EGFR or ErbB family belongs to subclass I of the superfamily of the receptor tyrosine kinases. Receptor tyrosine kinases represent an important subclass of these transmembrane proteins, with the EGFR being the most prominent representative. The EGFR controls a wide variety of biological processes such as cell proliferation, differentiation, and migration and modulation of apoptosis (13). There are four members of the ErbB family: the EGFR (also termed ErbB1/HER1), ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. Members of the ErbB family are characterized by a modular structure consisting of an extracellular ligand-binding domain, a single hydrophobic transmembrane region, and the intracellular part harboring the highly conserved tyrosine kinase domain (14). The ErbB receptors are activated by a number of ligands known as EGF-related peptide growth factors (15). These ligands include EGF, amphiregulin, and transforming growth factor-α, which bind specifically to ErbB1, betacellulin, heparin-binding EGF, and epiregulin, which exhibit dual specificity in that they bind ErbB1 and ErbB4. Neuregulin-1 and -2 both bind to ErbB3 and ErbB4 (16), whereas neuregulin-3 and -4 bind to ErbB4 and not ErbB3 (17, 18). The EGF-related peptide growth factors bind the extracellular domain of the ErbB receptor, leading to formation of homo- and heterodimers. Dimerization consequently stimulates the intrinsic tyrosine kinase activity of the receptors and triggers autophosphorylation of specific residues within the cytoplasmic domain. These phosphorylated residues serve as docking sites for signaling molecules involved in the regulation of intracellular cascades (19). Activation of the EGFR in a large percentage of glioblastomas suggests an important role for this receptor in astrocytic tumor development. EGFR activation may therefore contribute to tumor malignancy by promoting invasion, proliferation, and metastasis (20).

PKC represents a major cellular receptor for tumor-promoting phorbol esters (21–23). PKC comprises a family of phospholipid-dependent serine/threonine kinases that play important roles in signal transduction associated with a variety of cellular responses, including cell growth and differentiation, gene expression, hormone secretion, and membrane function (24–27). Activation of PKC leads to the phosphorylation of proteins that

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1 The abbreviations used are: GBM, glioblastoma multiforme; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; BIM, bisindolylmaleimide; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; siRNA, small interfering RNA; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase.
are involved in the regulation of cell growth, differentiation, and apoptosis (28–30). PKC consists of at least 11 isoforms that show diversity in their structures, cellular distribution, and biological functions and that have been divided into three groups based on their structures and cofactor requirements (31). The conventional PKC isoforms α, βI, βII, and γ require diacylglycerol, phosphatidylserine, and Ca2+ for activity. The novel PKC isoforms δ, ε, η, and θ do not require Ca2+ as a cofactor, but do require diacylglycerol and phosphatidylserine. The atypical PKC isoforms ζ and η do not require Ca2+ and are not activated by phorbol 12-myristate 13-acetate (PMA) or diacylglycerol, but do bind to phosphatidylserine when active (24, 28, 29, 32).

The EGFR, a protein-tyrosine kinase, is phosphorylated by PKC at Thr541 in A431 cells (33–35). Moro et al. (36) have reported that integrins induce phosphorylation of the EGFR at Tyr1068, Tyr845, and Tyr1173 by complexing with the p130Cas adaptor protein. Furthermore, Samet et al. (37) have shown that Zn2+, a particular matter, induces phosphorylation of the EGFR at Tyr1068 in addition to Tyr1068 and Tyr1173. The mechanism through which PKC transactivates the EGFR is not fully understood. In this study, we examined the relationship between PKC and EGFR expression in glioblastoma cell lines to identify the PKC isoform and the intermediate downstream target involved in activation of the EGFR. The data suggest that PKCζ and c-Src are involved in transactivation of the EGFR and the glioblastoma mitogenic response to PMA. The existence of cross-talk between PKC activation and the receptor tyrosine kinase EGFR, which is overexpressed in >50% of primary GBMs, provides a novel signaling pathway that is altered in astrocytic tumors and that may provide a useful therapeutic target.

**EXPERIMENTAL PROCEDURES**

**Materials**—PMA, EGF, and anti-tubulin antibody (DMA1) were purchased from Sigma. The phospho-specific antibodies directed against the EGFR at Tyr945, Tyr1068, and Tyr1086 and anti-EGFR antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Phospho-specific antibodies directed against the EGFR at Tyr1068 were purchased from BIOSOURCE (Camarillo, CA) and Cell Signaling Technology. The PKC-specific inhibitors Go 6976, rotterlin, PP2 (4-amino-5-(4-chlorophenyl)-1-t-butyl)pyrazolo[3,4-d]pyrimidine, PP3 (4-amino-7-phenylpyrazolo[3,4-d]pyrimidine), and the EGFR kinase inhibitor AG 1478 are products of Calbiochem. Ingenol was purchased from RBI (Natick, MA).

**Cell Cultures**—The human U-1242 MG cell line was kindly supplied by Dr. A. J. Yates (Ohio State University), and the U-87 MG cell line was obtained from American Type Culture Collection (Manassas, VA). Normal human astrocytes were obtained from Clonetics (San Diego). The cell lines were originally isolated from astrocytic tumors that were described previously by Hussaini et al. (38). Cell lines were regularly determined to be free of mycoplasma with reagent from Gen-Probe Inc. (San Diego). Cells were grown in modified α-minimal essential medium with 10% defined fetal bovine serum (Hyclone, Logan, UT) and 20 μg/ml bovine zinc insulin (25.7 IU/mg; Sigma). The cells were cultured to 100% confluence, passaged every 4–5 days from an initial concentration of 6-well flasks or 6 or 24-well plates, and cultured in astrocyte growth medium 5% fetal bovine serum at 37 °C in 5% CO2 and 90% relative humidity. Prior to assays, cultures that were 80–100% confluent were washed three times with serum-free medium before exposure to PMA (100 nM). In studies involving the pharmacological inhibitors, the inhibitors were added to the cells for 60 min before PMA. After the treatments, the medium was removed, and the cells were examined by Western blot analysis.

**Western Blot Analysis**—After the different treatments, the cells were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na2HPO4, 2.7 mM KCl, and 1.5 mM KH2PO4, pH 7.4) containing 0.2 mM sodium orthovanadate and extracted with 1% Triton X-100 containing 0.2 mM sodium orthovanadate and extracted with 1% Triton X-100 and c-Src small interfering RNAs (siRNAs) were purchased from Dharmacon, Inc. (Lafayette, CO). Mutant c-Src/S12C/S48A was made in our laboratory (S. J. P.).

**siRNA Transfections**—PKCζ and c-Src siRNAs were synthesized and purified by Dharmacon, Inc. PKCζ (200 nm) and c-Src (400 nm) siRNAs
were transfected separately into both U-1242 MG and U-87 MG cells using the Amaxa Nucleofector™ (Amaxa, Gaithersburg, MD). Briefly, confluent cells were trypsinized and resuspended in Amaxa Nucleofector solution V at a density of $2 \times 10^6$ cells/ml of solution, and either 200 nm PKCδ siRNA or 400 nm c-Src siRNA was added. Cells were transfected using the A23 pulsing program. Immediately after electroporation, cells were suspended in 4.9 ml of α-minimal essential medium plus 10% fetal bovine serum and incubated at 37 °C (39). Similarly, 5 μg of mutant c-Src(S12C/S48A) was transfected into astrocytic tumor cells following a similar protocol. 48 h later, the cells were lysed as described above. The cell lysates (200 μg of protein/lane) were separated by SDS-PAGE on 8% polyacrylamide gels. Proteins were then electro-

**FIG. 2.** PMA induces phosphorylation of the EGFR at Tyr1068. Serum-starved U-1242 MG and U-87 MG cells were treated with PMA (100 nM) at different time points (0, 10, 30, and 60 min). Triton-solubilized astrocytic tumor cell lysates (200 μg/lane) were separated using 8% polyacrylamide gels and electroblotted onto nitrocellulose. The blots were probed with antibodies against phospho-EGFR Tyr1068 and total EGFR.

**FIG. 3.** PMA stimulates phosphorylation of the EGFR at Tyr1068. Serum-starved U-1242 MG and U-87 MG cells were stimulated with PMA for the indicated time periods. These cells were lysed using 1% Triton X-100 lysis buffer after washing with ice-cold PBS. Cell lysates were immunoprecipitated (I.P.) with anti-EGFR antibody and then Western-blotted (W.B.) with antibody against phospho-EGFR Tyr1068 or total EGFR. N.C, negative antibody control.
blotted onto nitrocellulose and reacted with anti-PKC\(\theta\) monoclonal antibody, anti-PKC\(\theta\) polyclonal antibody, and anti-phospho-EGFR Tyr\(^{1068}\) antibody. The antibodies were detected with peroxidase-conjugated anti-mouse or anti-rabbit antibody, and final detection was carried out using ECL reagents as described by the manufacturer.

**Immunoprecipitation**—Cells were allowed to grow to 70–90% confluence. After starvation for 24 h in α-minimal essential medium containing no fetal bovine serum, cells were treated with EGF (25 ng/ml) or PMA (100 nM) for 10 and 30 min, respectively, at 37 °C. Other cultures were treated with the PKC inhibitor BIM (1 μM) and the PKC\(\theta\) inhibitor rottlerin (5 μM) for 60 min before the addition of PMA. Cells were washed twice with cold PBS and extracted with 1% Triton X-100 and 0.2% Nonidet P-40 in the presence of 2 μM EDTA, 100 μM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 1 μg/ml aprotinin. Cell lysates were then subjected to centrifugation at 14,000 \(\times g\) for 1 min at 4 °C. After normalization of supernatants derived from untreated and treated cells by protein concentration (Bio-Rad), 1 mg of each cell lysate was incubated with anti-c-Src-(2–17) monoclonal antibody overnight at 4 °C. Immune complexes were collected with protein G beads and washed five times with PBS. This was followed by 10-min washes with 10% sodium acetate buffer, pH 5.0, 500 mM NaCl, 0.1% SDS, and 1% Nonidet P-40. The resulting immunoprecipitate was then subjected to 8% SDS-PAGE and transferred onto nitrocellulose membranes, followed by Western blot analysis with anti-phosphoserine/phosphothreonine and anti-c-Src-(2–17) antibodies using the ECL detection system.

**Incorporation of \(^{3}H\)Thymidine into DNA**—Relative rates of DNA synthesis were assessed by determination of \(^{3}H\)thymidine incorporation. U-87 MG cells (4.5 \(\times 10^4\)) were seeded onto a 24-well plate. Upon serum deprivation for 24 h, cells were subjected to a 60-min preincubation with BIM (1 μM), rottlerin (5 μM), PP2 (5 μM), AG 1478 (100 nM), or U0126 (10 μM), followed by treatment with PMA (100 nM) or EGF (25 ng/ml) for an additional 18 h. In another set of experiments, cells were transfected with either siRNA directed against PKC\(\theta\) or c-Src were also seeded onto a 24-well plate at a density of 4.5 \(\times 10^4\). Upon serum starvation, the transfected cells were treated with either PMA or EGF for 18 h. Cells were pulsed with 0.5 μCi of \(^{3}H\)thymidine for 6 h and washed with PBS. This was followed by 10-min washes with 10% trichloroacetic acid, first at 4 °C and then at room temperature. Cells were dissolved overnight in 1 N NaOH, neutralized with an equal volume of 2 N HCl, and placed in scintillation fluid. \(^{3}H\)Thymidine incorporation was determined in a Beckman scintillation counter.

**Statistical Analysis**—Data are expressed as means ± S.E. Differences between two or multiple groups were tested using analysis of variance, followed by a calculation of Fisher’s least significant difference. Differences with a p value of <0.05 were considered statistically significant.

### RESULTS

**PMA and EGF Induce EGFR Tyrosine Phosphorylation in Glioblastoma Cells**—Two well characterized glioblastoma cell lines (U-1242 MG and U-87 MG) were used for phosphorylation studies using EGF and PMA. The U-1242 MG cells have mutant p53 (40), whereas the U-87 MG cells express wild-type p53 and mutant PTEN (41). Treatment with EGF (25 ng/ml) induced EGFR phosphorylation at all tyrosine residues tested: Tyr\(^{1068}\), Tyr\(^{1045}\), Tyr\(^{992}\), and Tyr\(^{845}\). The phosphorylation induced by EGF was time-dependent (Fig. 1). The two cell lines showed detectable levels of total EGFR, which was not altered by transient treatment with EGF for 10–60 min. Unlike EGF, treatment of the cells with PMA (100 nM) did not induce global phosphorylation of all the tyrosine residues. Only one of the major autophosphorylation sites of the EGFR (Tyr\(^{1068}\)) in

![Normal Human Astrocyte](https://example.com/image1.png)

**FIG. 4.** Effect of PMA and EGF on normal human astrocytes. Normal human astrocytes cells grown to 80–100% confluence were serum-starved overnight. The cells were then treated with PMA (100 nM) for 30 min and EGF (25 ng/ml) for 10 min. Protein lysates (200 μg/lane) were fractionated using 8% SDS-polyacrylamide gels and electrophoresed onto nitrocellulose. The blots were probed with antibody to EGFR Tyr\(^{1068}\). The membrane was stripped and reprobed for total EGFR.

![BIM PKC inhibitor, rottlerin PKC\(\theta\) inhibitor, PP2 (Src inhibitor), and Gö 6976 on EGFR Tyr\(^{1068}\) activation by PMA](https://example.com/image2.png)

**FIG. 5.** Effect of BIM (PKC inhibitor), rottlerin (PKC\(\theta\) inhibitor), PP2 (Src inhibitor), and Gö 6976 on EGFR Tyr\(^{1068}\) activation by PMA. Serum-starved U-1242 MG (A) and U-87 MG (B) cells were incubated with inhibitors for 60 min and then treated with PMA (100 nM) for 30 min. After washing twice with ice-cold PBS, cells were solubilized in 1% Triton X-100 lysis buffer, analyzed, and immunoblotted for EGFR Tyr\(^{1068}\). Blots were stripped and reprobed for total EGFR. Gö, Gö 6976; Rott, rottlerin.

**TABLE 1.** Summary of results from Figs. 4 and 5

| Compound   | Normal Human Astrocyte | U-1242 MG | U-87 MG |
|------------|------------------------|-----------|---------|
| PMA        | -                      | -         | -       |
| BIM        | +                      | +         | -       |
| Go         | -                      | -         | +       |
| Rott       | -                      | -         | +       |
| PP2        | -                      | -         | +       |
| PP3        | -                      | -         | +       |
| Total EGFR| -                      | +         | +       |
U-1242 MG and U-87 MG cells was phosphorylated (Fig. 2). Similarly, PMA did not change the level of total EGFR protein. When the cell lysates were immunoprecipitated with anti-EGFR antibody and immunoblotted with anti-phospho-EGFR Tyr1068 antibody, both PMA and EGF induced EGFR phosphorylation at Tyr1068 (Fig. 3). The specificity of the antibody against EGFR Tyr1068 was confirmed in NIH3T3 cells expressing a Tyr1068 mutant in which the tyrosine has been replaced with phenylalanine (data not shown). In contrast, treatment of normal human astrocytes with PMA (100 nM) for 30 min did not induce EGFR phosphorylation at Tyr1068, even though the cells express the receptor (Fig. 4).

Effect of Pharmacological Inhibitors on PMA-induced EGFR Phosphorylation—Since PMA interacts with and activates both classical (α, βI, βII, and γ) and novel (η, δ, ε, and θ) PKC isozymes, we designed experiments to identify the PKC isozyme(s) mediating PMA-induced phosphorylation of the EGFR at Tyr1068. Studies from our laboratory and others have shown that BIM inhibits activation of both novel and classical PKCs, whereas Gö 6976 blocks the activities of classical PKCs only. To determine whether classical or novel PKC isozymes are involved in PMA-induced Tyr1068 phosphorylation, we pre-treated the cells with either BIM (1 μM) or Gö 6976 (10 μM) for 60 min prior to PMA addition to the cultures. BIM attenuated PMA-induced EGFR phosphorylation in both U-87 MG and U-1242 MG, whereas Gö 6976 had no effect on PMA-induced phosphorylation of Tyr1068 (Fig. 5).

Since BIM but not Gö 6976 inhibited PMA-induced EGFR phosphorylation, we targeted the novel PKC isozymes to demonstrate the specificity of PKCδ in mediating the response to PMA. To determine whether classical or novel PKC isozymes are involved in PMA-induced Tyr1068 phosphorylation, we pre-treated the cells with either BIM (1 μM) or Gö 6976 (10 μM) for 60 min prior to PMA addition to the cultures. BIM attenuated PMA-induced EGFR phosphorylation in both U-87 MG and U-1242 MG, whereas Gö 6976 had no effect on PMA-induced phosphorylation of Tyr1068 (Fig. 5).

The non-receptor tyrosine kinase c-Src has been shown to phosphorylate the EGFR at Tyr845 in the C3H10T1/2 murine fibroblast cell line and the MDA468 breast cancer cell line (43) and at Tyr1068 in A431 human epidermoid cells (37). We therefore generated mutants of c-Src by replacing Ser12 and Ser48, which are the phosphorylation site for PKCs, with Cys and Ala (c-Src(S12C/S48A)), respectively. Correct cloning and mutagenesis of all mutants were verified by sequencing. The c-Src mutant was transiently transfected into our glioblastomas cell line expressing EGFR (U-1242 MG and U-87 MG) and subjected to siRNA interference with siRNA transfection performed as described under “Experimental Procedures.” 24 h after transfection, cells were quiesced for 24 h. U-1242 MG (A) and U-87 MG (B) cells were transiently transfected with siRNA PKCδ and treated with or without PMA. Cells were then lysed, and cell lysates with equal concentrations of proteins (200 μg/lane) were separated on 8% SDS-polyacrylamide gels and immunoblotted for phospho-EGFR Tyr1068, total PKCδ, PKCα, and tubulin.

PKCδ mediates PMA-induced EGFR Tyr1068 phosphorylation. RNA interference with siRNA transfection was performed as described under “Experimental Procedures.” 24 h after transfection, cells were quiesced for 24 h. U-1242 MG (A) and U-87 MG (B) cells were transiently transfected with siRNA PKCδ and treated with or without PMA. Cells were then lysed, and cell lysates with equal concentrations of proteins (200 μg/lane) were separated on 8% SDS-polyacrylamide gels and immunoblotted for phospho-EGFR Tyr1068, total PKCδ, PKCα, and tubulin.

At Tyr1068 by 90%, but the inactive isomer did not attenuate this effect (Fig. 5). To confirm our data on the role of c-Src in mediating the transactivation of EGFR Tyr1068 phosphorylation by PMA, we used siRNA against c-Src. c-Src siRNA (400 nm) was transfected into astrocytic tumor cells as described under “Experimental Procedures.” The results show that the siRNA knocked down the expression of c-Src and attenuated phosphorylation of the EGFR at Tyr1068 by PMA (Fig. 7, A and B). These data suggest that PMA-induced tyrosine phosphorylation of the EGFR is mediated directly or indirectly by Src kinase.

**PKCδ and Src Mediate EGFR Transactivation**

**A**

| PKCδ | PKCα | Tubulin |
|------|------|---------|
| 170 KDa | - | + |
| 80 KDa | + | - |
| 50 KDa | + | + |

**B**

| PKCδ | PKCα | Tubulin |
|------|------|---------|
| 170 KDa | - | + |
| 80 KDa | + | - |
| 50 KDa | + | + |

**Additional Notes:**

The data presented above suggest that PMA-induced EGFR Tyr1068 phosphorylation is mediated by PKCδ. However, PKCα is a serine/threonine kinase and not a tyrosine kinase. What then is the intermediate kinase between PKCδ and the EGFR? The non-receptor tyrosine kinase c-Src has been shown to phosphorylate the EGFR at Tyr413 in the C3H10T1/2 murine fibroblast cell line and the MDA468 breast cancer cell line (43) and at Tyr1068 in A431 human epidermoid cells (37). We therefore determined the effect of the c-Src kinase-specific inhibitor PP2 and the inactive isomer PP3 on EGFR phosphorylation in glioblastoma cell lines exposed to PMA (100 nm). As expected, the addition of PMA caused phosphorylation of the EGFR at Tyr1068. Pretreatment of glioblastoma cell lines for 60 min with PP2 (5 μM) blocked PMA-induced phosphorylation of the EGFR...
lines, and we determined its inhibitory effect on PMA-induced EGFR phosphorylation at Tyr1068. The c-Src(S12C/S48A) mutant abrogated phosphorylation of the EGFR at Tyr1068 by ∼75% (Fig. 7D).

To further explore the possibility that PKCα may associate with c-Src in our cell lines, co-immunoprecipitation experiments were performed. When U-1242 MG and U-87 MG cell lysates were immunoprecipitated with anti-c-Src-(2–17) antibody and immunoblotted with anti-PKCα antibody, PKCα was detected in the c-Src immunoprecipitates. Transient treatment with PMA (30 min) did not increase the level of PKCα pulled down with anti-c-Src antibody (data not shown). These data demonstrate that PKCα associates with c-Src in glioblastoma cell lines, suggesting that PKCα activation may result in direct c-Src activation and subsequent phosphorylation of the EGFR at Tyr1068. The results argue that PMA first interacts with PKCα to activate or phosphorylate c-Src, which then acts as a co-transducer of EGFR Tyr1068 phosphorylation.

Inhibition of EGFR Kinase Activity Does Not Block PMA-induced Phosphorylation of the EGFR at Tyr1068—To evaluate whether PMA activates EGFR kinase activity first before inducing phosphorylation of the EGFR at Tyr1068, we investigated the effect of AG 1478, a potent inhibitor of EGFR kinase activity, on PMA-induced cell proliferation. We found that pretreatment of the glioblastoma cell lines with BIM, rottlerin, and AG 1478 for 60 min, before the addition of PMA (100 nM) for 30 min, partially depressed ERK1/ERK2 activation induced by PMA. The remaining MAPK phosphorylation may be as a result of EGFR-independent activation of the Ras/Raf/MEK/MAPK pathway by PMA. Furthermore, pharmacological inhibition of c-Src with PP2 also decreased PMA-induced activation of EGFR kinase activity, AG 1478 effectively attenuated the robust EGFR phosphorylation at Tyr1068 following EGF treatment. In contrast, the effective inhibition of EGFR kinase activity by AG 1478 had no discernable effect on the phosphorylation of Tyr1068 in cells activated with PMA (Fig. 8, A and B). These results indicate that PMA induced phosphorylation of the EGFR at Tyr1068 first, which then lead to an increase in EGFR kinase activity and activation of the Ras/Raf/MEK/MAPK pathway as shown below.

Pharmacological Inhibition of PMA-induced ERK Activation—Activation of the ERK/MAPK pathway is a key step in the regulation of important cellular responses such as cell proliferation. In many cell types, the MAPK pathway has been implicated in both EGF- and PMA-induced growth stimulatory responses. Our laboratory had earlier shown that PMA (100 nM) and EGF (25 ng/ml) induce activation of ERK1/ERK2 in glioblastoma cell lines and that inhibition of MEK with U0126 blocks PMA-induced cell proliferation (38). In the present study, we investigated the role of EGFR transactivation in PMA-induced mitogenic signaling. We found that pretreatment of the glioblastoma cell lines with BIM, rottlerin, and AG 1478 for 60 min, before the addition of PMA (100 nM) for 30 min, partially depressed ERK1/ERK2 activation induced by PMA. The remaining MAPK phosphorylation may be as a result of EGFR-independent activation of the Ras/Raf/MEK/MAPK pathway by PMA. Furthermore, pharmacological inhibition of c-Src with PP2 also decreased PMA-induced activation of EGFR kinase activity, AG 1478 effectively attenuated the robust EGFR phosphorylation at Tyr1068 following EGF treatment. In contrast, the effective inhibition of EGFR kinase activity by AG 1478 had no discernable effect on the phosphorylation of Tyr1068 in cells activated with PMA (Fig. 8, A and B). These results indicate that PMA induced phosphorylation of the EGFR at Tyr1068 first, which then lead to an increase in EGFR kinase activity and activation of the Ras/Raf/MEK/MAPK pathway as shown below.
Inhibition of MEK with U0126 (10 μM) completely abrogated both EGFR-dependent and -independent MAPK phosphorylation by PMA (Fig. 9, A and B).

Effect of PMA on [3H]Thymidine Incorporation—To determine the biological function of PMA-induced EGFR transactivation in astrocytic tumor cells, thymidine incorporation was used as a measure of DNA synthesis and cell proliferation. We investigated the roles of PKC and c-Src in cell proliferation after stimulation with either PMA or EGF. As shown in Fig. 10A, PMA increased [3H]thymidine uptake in U-87 MG cells by 1.6-fold. Pretreatment of the cells with BIM (PKC inhibitor), rottlerin (PKC- specific inhibitor), PP2 (Src inhibitor), AG 1478 (EGFR kinase inhibitor), or U0126 (MEK inhibitor) abrogated the PMA effect on [3H]thymidine uptake. Similarly, EGF (25 ng/ml) increased [3H]thymidine uptake in U-87 MG cells by 1.5-fold, which was attenuated by these inhibitors (Fig. 10C).

To further investigate the role of PKC and c-Src in PMA-induced cell proliferation, U-87 MG cells were transfected with the same concentration of PKC5-specific siRNA (200 nM) or c-Src siRNA (400 nM) that completely blocked PKC5 or c-Src expression, respectively, and the effect of PMA on thymidine incorporation was investigated. PKC5 and c-Src siRNAs reduced PMA-induced [3H]thymidine uptake by ~87 and 60%, respectively (Fig. 10B). Similarly, EGF-induced increases in thymidine incorporation were attenuated by PKC5 and c-Src siRNAs by 30 and 50%, respectively (Fig. 10D).

**DISCUSSION**

Malignant gliomas are the most common adult brain tumors, are refractory to classical chemotherapy and radiotherapy, and have poor prognosis (45). The EGFR is overexpressed in 50–60% of GBMs and amplified in 40% of the tumors (7), which contribute to the malignant phenotype of human glioblastomas (4, 6, 8). In the last 10 years, the molecular mechanisms underlying astrocytic neoplastic transformation have been widely studied, and a number of signaling pathways, including that of PKC, are altered in GBM (46, 47). The expression and activity of PKC isozymes are highly elevated in gliomas and glioma cell lines compared with normal astrocytes (12), and PKC inhibitors markedly reduce glioma cell proliferation (38, 48).

Both EGFR expression and PKC activity play a significant role in astrocytic tumor biology. Our data provide evidence for the first time that treatment of glioblastoma cell lines with PMA results in EGFR phosphorylation at Tyr1068, but not at other tyrosine residues, and that this phosphorylation is mediated by a PKC5-specific-dependent pathway. PMA-induced phosphorylation of Tyr1068 was blocked by BIM, an inhibitor of classical as well as novel PKC isozymes, but not by Gö 6976, an inhibitor of classical PKC isozymes. Tyr1068 is a major Grb2-
binding and autophosphorylation site of the EGFR (49). In contrast, EGF induced phosphorylation of the EGFR at multiple sites, including Tyr992, Tyr845, Tyr1045, and Tyr1068. The mechanism of EGFR transactivation is not well characterized. Some studies have shown that the EGFR is involved in signaling networks activated by a number of stimuli that do not interact directly with this receptor (50). These stimuli include the G protein-coupled receptor agonists thrombin and lysophosphatidic acid (51), calcium (52), and UV irradiation (53). In our study, prior incubation with the pharmacological PKC/H9254 inhibitor rottlerin, attenuated PMA-induced [3H]thymidine incorporation in U-87 MG cells. Quiescent U-87 MG cells were preincubated with BIM (1 µM), rottlerin (ROTT, 5 µM), or U0126 (10 µM) for 60 min. Cells were then pulse-labeled with [3H]thymidine for 6 h, and thymidine incorporation was measured using a Beckman scintillation counter. Results are from the quantitative analysis of three independent experiments (means ± S.E.). Cell proliferation was calculated as percent of control. The data are means ± S.E. of at least two independent experiments, with each experiment performed in quadruplicate.

Our immunoprecipitation data reveal an association between PKCδ and c-Src in astrocytic tumor cells, as reported in other cell types. PKCδ is a widely expressed member of the novel PKC family, and this isoform has been associated with cell proliferation in a number of cell types, including NIH3T3 fibroblasts, smooth muscle cells, and human keratinocytes (54), and has also been shown to phosphorylate c-Src and to interact with c-Src (55–58).

Since PKC isozymes do not directly phosphorylate proteins at tyrosine residues, we examined the role of c-Src as an intermediate kinase between PKCδ and the EGFR. Our immunoprecipitation data reveal an association between PKCδ and c-Src in astrocytic tumor cells, as reported in other cell types. PKCδ is a widely expressed member of the novel PKC family, and this isoform has been associated with cell proliferation in a number of cell types, including NIH3T3 fibroblasts, smooth muscle cells, and human keratinocytes (54), and has also been shown to phosphorylate c-Src and to interact with c-Src (55–58).

Since PKCδ and c-Src inhibition abrogates PMA-induced cell proliferation, we examined the role of PKCδ and c-Src in mediating EGFR phosphorylation at Tyr1068 (Fig. 6, A and B).
ported in lung, breast, and brain tumors (43). Evidence derived from experimental models indicates that Src activation potentiates EGF-induced mitogenesis and transformation (43). Our study has shown that the c-Src inhibitor PP2 inhibited PMA-induced EGF phosphorylation (Fig. 5) and mitogenic response (Fig. 9, A and B), whereas the inactive form (PP3) had no effect on phosphorylation of the receptor. To further confirm the role of c-Src in mediating PMA-induced EGFR transactivation, we used siRNA directed against c-Src to knock down the expression of c-Src. Gene silencing using c-Src siRNA abrogated PMA-induced EGFR phosphorylation at Tyr1068 (Fig. 7, A and B). These data imply that PMA interacts with PKCβ, which in turn phosphorylates Src to activate the EGFR. Src structurally has a poorly conserved unique domain that contains serine/threonine residues (44). The unique domain of c-Src, which is the least conserved region among Src family members, mediates protein interaction and is phosphorylated by protein kinase A, PKC, and the Cdc2-cyclin complex (59–61). To test the hypothesis that PMA may indirectly activate Src through PKCβ, we used mutant c-Src (S12C/S48A). Transient transfection of this mutant into astrocytic tumor cells abrogated phosphorylation of the EGFR at Tyr1068 induced by PMA (Fig. 7D).

The EGFR kinase inhibitor AG 1478 did not affect PMA-induced EGFR phosphorylation at Tyr1068, but completely abrogated EGF-induced phosphorylation of the EGFR. This suggests that PMA-induced EGFR Tyr1068 phosphorylation through PKCβ and c-Src is upstream of EGFR kinase activity. This result agrees with similar studies that used H2O2 to transactivate the EGFR (62).

Activation of the ERK/MAPK pathway is a key step in the regulation of important cellular responses such as cell proliferation (63). ERK1 and ERK2 are 44- and 42-kDa members of the MAPK family and are involved in the regulation of gene expression, protein synthesis, cell growth and proliferation, and, in some cases, cell differentiation and secretion (64). ERK phosphorylation is initially observed after ligand activation of such receptor tyrosine kinases as the EGFR, but many Gαq, Gαi, and Gα12-coupled receptors also initiate the ERK cascade through transactivation of the EGFR (65, 66). ERK activation often involves sequential steps that include transactivation of the receptor tyrosine kinases (EGFR) in a c-Src kinase-dependent manner (51). In our study, we found that BIM (PKC inhibitor), rottlerin (PKCβ-specific inhibitor), and PP2 (Src inhibitor) significantly inhibited ERK/MAPK activation by PMA (Fig. 9). Similarly, AG 1478, which did not block EGFR Tyr1068 phosphorylation, and the MEK inhibitor U0126 inhibited PMA-induced ERK phosphorylation (Fig. 9). These data further suggest that PMA-induced Tyr1068 phosphorylation could lead to an increase in EGFR kinase activity (blocked by AG 1478) to activate the Ras/Raf/MEK/MAPK pathway in glioblastoma cells.

Activation of PKC leads to the phosphorylation of several proteins that are involved in the regulation of cell growth, differentiation, and apoptosis (28–30). Pretreatment of GBM cells with BIM or rottlerin blocked PMA-induced as well as EGF-induced increases in [3H]thymidine uptake, suggesting that PKC isoforms play a critical role in glioblastoma proliferation (38). Gene silencing of PKCβ and c-Src with siRNA and pharmacological inhibition with PP2 and rottlerin also abrogated PMA-induced cell proliferation in U-87 MG cells. It is well established that, following binding to the EGFR, EGF increases PKC activity in a variety of cells (67, 68). In addition, PKCβ has been shown to increase transformation and metastatic progression of a number of tumors (69), and activation of PKCβ has been shown to be involved in proliferation of epithelial breast cells (70). In conclusion, our data provide evidence for the first time that PMA phosphorylates the EGFR at Tyr1068 through a PKCβ-specific-Src-dependent pathway to activate MAPK and to increase cell proliferation in glioblastoma cells (Fig. 11). Furthermore, our study reveals a novel pathway that may contribute to glioblastoma invasive growth.

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REFERENCES

1. Collins, V. P. (1998) Cancer Surv. 32, 37–51
2. Rashheed, B. K., Wilshire, R. N., Bigner, S. H., and Bigner, D. D. (1999) Curr. Opin. Oncol. 11, 162–167
3. Kielhues, P., Burger, P. C., Collins, V. P., Newcomb, E. W., Ohgaki, H., and Cavenese, W. K. (2000) in Glioblastoma: Tumors of the Nervous System (Kielhues, P., and Cavenese, W. K., eds) pp. 29–39, IARC Press, Lyon, France
4. Libermann, T. A., Nusbaum, H. R., Razon, N., Kris, R., Lax, I., Soreq, H., Whitle, N., Waterfield, M. D., Ullrich, A., and Schlessinger, J. (1985) Nature 313, 144–147
5. Ekstrand, A. J., Sugawa, N., James, C. D., and Collins, V. P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1169–1174
6. Wang, J., Bigner, S. H., Bigner, D. D., Kinzler, K. W., Hamilton, S. R., and Vogelstein, B. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6899–6903
7. Ohgaki, H., Schabule, B., Zar, H., von Ammon, K., and Kielhues, P. (1995) Virchows Arch. 427, 113–118
8. Ekstrand, A. J., James, C. D., Cavenee, W. K., Seliger, B., Peterson, H. F., and Collins, V. P. (1991) Cancer Res. 51, 2164–2172
9. Todo, T., Shiitara, N., Nakamura, H., Takakura, K., and Ikeda, K. (1991) Neurosurgery (Baltim.) 29, 880–887
10. Benzil, D. L., Finkelstein, S. D., Epstein, M. H., and Finch, P. W. (1992) Cancer Res. 52, 2952–2956
11. Coulwdell, W. T., Uhm, J. H., Antel, J. P., and Yong, W. Y. (1991) Neurosurgery (Baltim.) 29, 880–887
12. Coulwdell, W. T., Uhm, J. H., Antel, J. P., and Yong, W. Y. (1992) Neurosurgery (Baltim.) 31, 717–724
13. van der Geer, P., Hunter, T., and Linberg, R. A. (1994) Annu. Rev. Cell Biol. 10, 251–337
14. Prenzel, N., Fischer, O. M., Streit, S., Hart, S., and Ulrich, A. (1991) Endocr. Relat. Cancer 8, 11–31
15. Riese, D. J., Il, and Stern, D. F. (1998) BioEssays 20, 41–48
16. Chang, H., Riese, D. J., Il, Gilbert, W., Stern, D. F., and McMahan, U. J. (1997) Nature 387, 509–512
17. Zhang, D., Slivkowska, M. X., Mark, M., Frantz, G., Akita, R., Sun, Y., Hillan, K., Crowley, C., Brush, J., and Godowski, P. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9562–9567
18. Harari, D., Trazar, E., Romano, J., Shelly, M., Pierce, J. H., Andrews, G. C., and Ullrich, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7277–7281
19. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, V., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851
20. Nishikawa, R., Ji, X. D., Harman, R. C., Lazar, C. S., Gill, G. N., Cavenee, W. K., and Huang, H. J. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7277–7281
21. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, V., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851
22. Niedel, J. E., Kuhn, L. J., and Vanderbark, G. R. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 36–40
23. Leach, K. L., James, M. L., and Blumberg, P. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4208–4212
24. Nishizuka, Y. (1984) Nature 308, 693–698
25. Nishizuka, Y. (1988) Nature 334, 661–665
26. Basu, A. (1995) Pharmacol. Ther. 59, 257–280
27. Toker, A. (1998) Proc. Natl. Acad. S. A. 95, 11134–11147
