Role of Mitogen-activated Protein Kinase Kinase in Regulation of the Epidermal Growth Factor Receptor by Protein Kinase C*

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The epidermal growth factor receptor (EGFR) is regulated by at least two mechanisms involving protein kinase C (PKC), inhibition of EGF binding and inhibition of EGF-stimulated tyrosine kinase activity. In this study we investigated whether mitogen-activated protein kinase (MAPK) mediates the inhibitory effects of PKC on EGFR binding or kinase activity by pretreating NIH3T3 and Chinese hamster ovary cells expressing the EGFR with PD98059, an inhibitor of MAPK/extracellular signal-regulated kinase activity (MEK). We also determined whether substitution of cysteine for threonine at residue 669, the site of MAPK phosphorylation of the EGFR, alters the inhibition of kinase activity by PKC. The results indicate that 1) PKC down-regulates EGFR tyrosine kinase activity by an MEK-dependent mechanism presumably involving MAPK; 2) the inhibition by PKC is not a direct result of phosphorylation of the EGFR by PKC or MAPK; 3) activation of MAPK is not sufficient to regulate EGFR kinase activity; and 4) PKC-mediated down-regulation of EGF binding and EGFR kinase activity occur by different mechanisms. These data are consistent with a model for regulation of the EGFR by other receptors whereby their activation of PKC, in conjunction with MAPK, results in the phosphorylation of a protein(s) that modulates EGFR kinase activity.

Cellular integration of signals transmitted simultaneously by multiple receptor systems involves modulation of one receptor by another and often leads to selective desensitization. Heterologous regulation of receptors can occur through several mechanisms, including modulation of receptor stability, expression, cellular localization, ligand binding, or activity. Understanding how receptor signaling cascades are regulated is fundamental to an understanding of how the process of signal transduction is controlled.

One of the major classes of growth factor receptors is the ligand-stimulated tyrosine kinases, exemplified by the epidermal growth factor receptor (EGFR).1 (1) The EGFR is regulated by at least two mechanisms involving other receptors or agents such as phorbol esters that stimulate protein kinase C (PKC): inhibition of EGF binding and inhibition of EGF-stimulated tyrosine kinase activity. A key issue concerning the mechanism by which this occurs is whether the EGFR is regulated as a result of direct phosphorylation by PKC or whether the EGFR is regulated by phosphorylation of other interacting proteins.

One widely accepted hypothesis is that the receptor is regulated by direct PKC phosphorylation of the EGFR at residue Thr-654. The evidence is based largely on site-directed mutagenesis of Thr-654, which, when mutated to Ala-654, renders the EGFR resistant to regulation by PKC (2, 3). However, an alternative explanation is that the threonine residue itself rather than the phosphothreonine derivative is required for stabilizing interactions between the EGFR and PKC-activated effectors of the receptor. Recent studies have shown that this juxtamembrane region of the EGFR does interact with a number of proteins important for signaling (4–6). Several lines of evidence suggest that direct phosphorylation of the EGFR at Thr-654 by PKC is not sufficient to modulate receptor kinase activity. First, the observation that other agents, such as the calcium-mobilizing tumor promoter thapsigargin, can similarly down-regulate EGFR autophosphorylation independent of PKC activation suggest that another mechanism exists (7). Additionally, the level of phosphorylation of Thr-654 by PKC is well below the near stoichiometric levels expected for such an effect in some responsive cell lines including CHO cells transfected with human EGFR (7, 8). Finally, we have demonstrated that mutant EGFR receptors containing glutamate residues substituted at either Thr-654 or Thr-669, to mimic constitutive phosphorylation, possess EGF binding affinities and EGF-inducible tyrosine kinase activities which are comparable to those of the wild-type receptor (8). Furthermore, the Glu-654 as well as the Glu-669 mutant receptors were still responsive to PKC-mediated down-regulation albeit with slightly diminished sensitivity to phorbol esters. These results indicate that introduction of a negative charge in the juxtamembrane region of the EGFR is not sufficient to modulate EGFR binding or receptor tyrosine kinase activity.

In order to identify an alternative mechanism for regulation of the EGFR receptor, we focused on other kinases that are activated by PKC. The MAP kinase signaling pathway is activated by both phorbol esters and thapsigargin, two tumor promoters that regulate the EGFR kinase in a similar fashion. To test whether MAPK might be responsible for the inhibition of the EGFR kinase activity, we determined the effect of an inhibitor of MAPK/ERK kinase concentration-dependent signal-regulated kinase; MEK, MAP kinase/ERK kinase; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; PDBu, phorbol 12,13-dibutyrate; TBST, Tris-buffered saline and 0.2% Tween 20; MBP, myelin basic protein.

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1 The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, EGF receptor; CHO, Chinese hamster ovary cells; PKC, protein kinase C; MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular signal-regulated kinase; MEK, MAP kinase/ERK kinase; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; PDBu, phorbol 12,13-dibutyrate; TBST, Tris-buffered saline and 0.2% Tween 20; MBP, myelin basic protein.
hibitor of MEK (MAPK kinase), PD98059 (9, 10), on the regulation of the EGFR by PKC. The results indicate that the MAPK signaling pathway is a key step in EGFR regulation, but the effect does not appear to be mediated through direct phosphorylation of the receptor. We have previously shown that MAPK can activate an EGFR-associated phosphatase activity that inhibits the EGFR tyrosine phosphorylation (11). Taken together, these results are consistent with a model whereby PKC and MAPK activate a protein such as a tyrosine phosphatase that inhibits tyrosine phosphorylation by the EGFR.

**EXPERIMENTAL PROCEDURES**

**Generation and Maintenance of Cell Lines** Expressing EGFR Receptor Mutants—CHO cells, maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum, were transfected by the Ca(PO4)2 method (12) with a wild-type human EGFR construct (CHO 2WT) and a Cys-669 EGFR mutant (CHO C669) as described previously. The constructs contained a cytomegalovirus promoter linked to the receptor cDNA, and were co-transfected in a 1:1 ratio with a pWl-Neo (Stratagene, La Jolla, CA) expression vector, which confers resistance to G418. Cells were selected in 200 μg/ml G418, and a fluorescein-activated cell sorter was used to select clones or similarly expressing populations in some cases. For this purpose, the R1 EGFR antibody (α-EGFR) was used in conjunction with a fluorescein-conjugated sheep anti-mouse IgG (Sigma). NIH3T3 cells expressing human EGFR (N24), generously provided by Stuart Decker, were maintained in DMEM supplemented with 5% fetal bovine serum. The MEK inhibitor, PD98059, was described previously (13).

**EGFR Autophosphorylation—** Equivalent protein aliquots from whole cell protein extracts in radiimmune precipitation buffer (20 mM HEPES, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 50 mM NaCl, 25 mM sodium pyrophosphate, 5 mM NaF, 50 mM Na3VO4, 200 μM leupeptin, 5 μM pepstatin, 1 μM phenylmethylsulfonyl fluoride, pH 8.0) were isolated from CHO 2WT cells, CHO C669, and N24 cells expressing wild-type EGFR receptors were resolved by SDS-PAGE. The gels were immunoblotted as described below and probed with a mouse monoclonal anti-phosphotyrosine antibody PY20 (Transduction Laboratories).

MAPK MBP Phosphorylation Assay—N24 cells were serum-starved with 0.5% fetal bovine serum and treated with or without PD98059 for 10 min followed with or without a 4-min 10 μM EGF treatment. Cells were washed in phosphate-buffered saline and lysed with 1% Triton lysis buffer (20 mM Tris, 137 mM NaCl, 5 mM Na2EDTA, 10% glycerol, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 200 μg/ml leupeptin, 1 μg/ml pepstatin, 5 mM NaF, 50 mM Na3VO4, 200 μg/ml aprotinin, 10 mM EGTA, 10 mM Na3VO4, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, pH 7.4) (TLB). Cell lysates were incubated with protein A beads pre-coated to anti-p42 ERK antibody (kindly provided by Martin McMahon) and subsequently washed sequentially in TLB, TLB + 0.5 mM NaCl, and 50 mM HEPES, pH 7.4, 5 mM MgCl2. Kinase reactions were performed at room temperature for 30 min in 30 μl of kinase buffer (50 mM HEPES, pH 7.4, 5 mM MgCl2, 1 mg/ml MBP, and 200 μM ATP (including 5 μCi of [γ-32P]ATP)). Reactions were stopped by addition of Laemmli’s sample buffer and samples were boiled prior to loading onto 12% SDS-PAGE. Gels were transferred to nitrocellulose and the phosphorylated MBP was quantitated by Ambis radioanalytic quantitation.

**MAPK Activation—** Cells were grown to confluence, serum-starved overnight and treated as described in the figure legends. Equivalent protein aliquots from whole cell protein extracts in radiimmune precipitation buffer were separated by 7.5% SDS-PAGE, immunoblotted as described above, and probed with an anti-ERK antibody (Santa Cruz).

**Regulation of EGFR Binding by PD98059—** 24-well plates of confluent serum-starved cells were untreated or preincubated with PD98059 for 10 min and then either left untreated or treated with the indicated concentration of PD98059 for 10 min. Cells were subsequently placed on ice, washed with phosphate-buffered saline, and incubated for 2 h at 4°C in EGF (DuPont NEN) in binding buffer (including 50 mM HEPES, 0.1% bovine serum albumin, pH 7.4). Cells were thoroughly washed with phosphate-buffered saline to remove unbound ligand and then lysed in 1 N NaOH. Radioactivity was quantitated by counting the lysate in an LKB gamma counter.

**Immunoblotting—** Immunoblotting was performed by resolving cell extracts by 6–7.5% SDS-PAGE under reducing conditions and then blotting the proteins onto nitrocellulose with a Fisher semi-dry transfer apparatus according to Fisher’s protocol. The nitrocellulose was blocked with 5% bovine serum albumin in 50 mM Tris, pH 7.5, 150 mM NaCl, 0.2% Tween 20 (TBST), and the blots were then probed with desired antibodies in TBST. Immunoreactivity was localized by horseradish peroxidase-conjugated secondary antibodies (Sigma), visualized by Renaissance chemiluminescence (DuPont NEN), and the autoradiographic films were quantitated by Ambis densitometry.

**RESULTS**

**Inhibition of MAPK Activity in N24 and CHO 2WT Cells by PD98059—** In order to assess the efficacy of the MEK inhibitor, PD98059 was added to either murine NIH3T3 (N24) or Chinese hamster ovary (CHO 2WT) cells that had been stably transfected with human EGFR. Cells were preincubated with or without PD98059 for 10 min and then stimulated with EGF for 4 min. Samples were assayed for the ability of MEK to activate MAPK by two assays: 1) phosphorylation of myelin basic protein (MBP) by immunoprecipitated MAPK, or 2) MAPK gel shift in which activated forms of ERK1 and ERK2 are shifted to a slower mobility on SDS-PAGE. Fig. 1 shows dose-dependent inhibition of EGF-activated MAPK by PD98059. MAPK gel shift analysis demonstrated that this compound can inhibit the activation of ERK1 and ERK2 upon stimulation by EGF in both N24 and CHO 2WT cells (Fig. 1A), although the potency of PD98059 is greater in N24 cells. Whereas 50–100 μM PD98059 resulted in only 50–75% inhibition of MAPK in CHO 2WT cells, quantitative analysis using the MBP phosphorylation assay showed that preincubation of N24 cells with 10–50 μM PD98059 blocked EGF-stimulated MAPK activity by >60–100%. Inhibition of MEK by PD98059 was not limited to EGF-stimulated cells. As shown in Fig. 1B, 50 μM PD98059 also blocked activation of MAPK by 40 nM PDBu in N24 cells.

The incomplete suppression of EGF-stimulated MAPK in CHO 2WT cells by PD98059 could not be enhanced by longer preincubation times. Once added to the cells, the potency of PD98059 diminished gradually over time, but inhibition was still observed following overnight preincubation (Fig. 1C). Analysis of different preincubation times indicated that a 10-min incubation with PD98059 was maximally effective for both cell lines (Fig. 1D; data not shown). The different sensitivities of the two cell lines to PD98059 may be a reflection of differential uptake of the inhibitor or may be attributable to differences in the MEK/MAPK signaling pathways. Since PD98059 completely blocked EGF-stimulated MAPK activation in N24 cells, most of the following studies were done in this cell line.

**Inhibition of MAPK Activity Does Not Alter EGFR Autophosphorylation—** To determine whether PD98059 has any effect on EGFR kinase activity, receptor tyrosine phosphorylation in response to EGF was assessed in N24 cells that were either untreated or pretreated with PD98059. Addition of 50 μM PD98059 to cells had no effect on unstimulated EGFR receptor autophosphorylation (data not shown). Similarly, no effect was observed on EGF-stimulated autophosphorylation with doses of PD98059 up to 100 μM when added to either N24 or CHO 2WT cells (Fig. 2). Finally, pretreatment with 75 μM PD98059 did not change the time course of EGF-stimulated receptor autophosphorylation when EGF was added to NIH3T3 cells for up to 30 min (data not shown). These results confirm that PD98059 has no direct effect on EGFR tyrosine phosphorylation, consistent with the observed specificity of PD98059 for MEK when PD98059 was tested with a number of other serine/threonine and tyrosine kinases including EGFR and PKC (10, 13). Furthermore, the data indicate that EGF-stimulated MAPK does not act as a feedback regulator of EGFR autophosphorylation.

**Inhibition of MEK and MAPK Activity Does Not Alter PKC-mediated Down-regulation of EGF Binding Affinity—** Although activators of PKC have been shown to inhibit high affinity EGF binding, the mechanism by which PKC mediates this effect is unclear (14). One possibility is through PKC activation of the
MAP kinase cascade. To assess whether MAPK plays a role in modulating high affinity EGF binding, down-regulation of EGF binding by the phorbol ester PD98059 was assessed in N24 cells. N24 cells (lanes 1–5) and CHO 2WT cells (lanes 6–10) were treated with or without 10 nM EGF and varying concentrations of PD98059 as indicated. ERK1 and ERK2 activity were analyzed by immunoblotting and gel shift as described under "Experimental Procedures." A, dose response for PD98059 inhibition of EGF-stimulated MAPK in N24 cells. N24 cells (lanes 1–5) and CHO 2WT cells (lanes 6–10) were treated with or without 10 nM EGF and varying concentrations of PD98059 as indicated. ERK1 and ERK2 activity were analyzed by immunoblotting and gel shift as described under "Experimental Procedures." B, dose response for PD98059 inhibition of EGF-stimulated MAPK in CHO 2WT cells. Equivalent numbers of serum-starved CHO 2WT cells were treated with differing amounts of PD98059 for 90 min prior to 4 min with 10 nM EGF. One lane was treated overnight with PD98059 prior to EGF treatment as indicated. ERK1 and ERK2 activity was analyzed by immunoblotting and gel shift as described under "Experimental Procedures." C, dose response for PD98059 inhibition of EGF-stimulated MAPK in CHO 2WT cells. Equivalent numbers of serum-starved CHO 2WT cells were treated with differing amounts of PD98059 for 90 min prior to 4 min with 10 nM EGF. One lane was treated overnight with PD98059 prior to EGF treatment as indicated. ERK1 and ERK2 activity was analyzed by immunoblotting and gel shift as described under "Experimental Procedures." D, inhibition of MAPK activity in response to varying times of exposure of cells to PD98059. Equivalent numbers of serum-starved N24 cells were treated for different times with 75 μM PD98059 prior to 4 min with 10 nM EGF. ERK1 and ERK2 activity was analyzed by immunoblotting and gel shift as described under "Experimental Procedures." The data in this figure represent more than five independent experiments.

MAPK Activity Is Necessary for PKC-mediated Down-regulation of High Affinity EGF Binding. Serum-starved N24 cells were treated with 50 nM PD98059 for 10 min with or without a 10-min preincubation with increasing concentrations of PD98059 prior to incubating at 4°C with 0.1 nM 125I-EGF. The graph depicts percent EGF binding relative to cells that were not treated with PD98059. Error bars represent standard deviations from triplicate wells, and the data are representative of three independent experiments.

Inhibition of MAPK does not alter PDBu down-regulation of high affinity EGF binding. Inhibition of MAPK does not alter EGFR autophosphorylation. Serum-starved N24 cells were treated with 50 nM PD98059 for 10 min with or without 100 nM PDBu, which completely inhibited EGF-stimulated MAPK, was sufficient to prevent down-regulation of EGFR tyrosine phosphorylation by 40 nM PDBu (Fig. 4A). Similarly, preincubation of CHO 2WT cells with 100 μM PD98059, which completely inhibited EGF-stimulated MAPK, was sufficient to prevent down-regulation of EGFR tyrosine phosphorylation by 40 nM PDBu (Fig. 4A). The ability of the PD98059 to block PDBu-mediated down-regulation of the EGFR was dose-dependent, with an IC50 of ~10 μM in N24 cells (Fig. 4C), and correlated with the relative extent of MAPK suppression (Fig. 4D). These results indicate that MEK, probably through activation of MAPK, is required for PKC-mediated down-regulation of EGFR autophosphorylation. Furthermore, these data demonstrate that direct phosphorylation of the EGFR by PKC is insufficient to suppress EGFR-stimulated tyrosine phosphorylation.

Mutating the MAPK Phosphorylation Site on the EGFR to Cysteine Does Not Alter Regulation of the EGFR Kinase by PKC—MAPK has been shown to directly phosphorylate the EGFR at residue Thr-669 (16). To determine whether direct phosphorylation of the EGFR by MAPK is the mechanism by which MEK modulates EGFR kinase activity, CHO cells expressing a mutant Cys-669 EGFR were utilized. These cells in the PKC-mediated inhibition of EGFR tyrosine phosphorylation. Dependent upon the particular cell line and the dose of PDBu, EGFR kinase activity, as measured by EGFR autophosphorylation, could be suppressed up to 90% by PDBu (Fig. 4). Near maximal down-regulation of EGFR tyrosine phosphorylation was achieved in N24 cells with 40 nM PDBu and in CHO 2WT cells with 8 nM PDBu. Preincubation of N24 cells with 100 μM PD98059, which completely inhibited EGF-stimulated MAPK, was sufficient to prevent down-regulation of EGFR tyrosine phosphorylation by 40 nM PDBu (Fig. 4A). Similarly, preincubation of CHO 2WT cells with 100 μM PD98059, which only partially inhibited MAPK, almost completely reversed the inhibitory effects of 8 nM PDBu (Fig. 4B). The ability of the PD98059 to block PDBu-mediated down-regulation of the EGFR was dose-dependent, with an IC50 of ~10 μM in N24 cells (Fig. 4C), and correlated with the relative extent of MAPK suppression (Fig. 4D). These results indicate that MEK, probably through activation of MAPK, is required for PKC-mediated down-regulation of EGFR autophosphorylation. Furthermore, these data demonstrate that direct phosphorylation of the EGFR by PKC is insufficient to suppress EGFR-stimulated tyrosine phosphorylation.
express EGFR with the MAPK phosphorylation site substituted by cysteine, which has a structure similar to that of threonine but cannot be phosphorylated. Analysis of Cys-669 EGFR expression in a population of cells (CHO C669) that had been isolated by fluorescence-activated cell sorting showed comparable expression to that obtained for the wild-type EGFR in the CHO 2WT cells (Fig. 5A). Fig. 5B shows that the Cys-669 EGFR can be autophosphorylated at tyrosine residues in response to EGF treatment to a similar extent as the wild-type EGFR. Furthermore, the kinase activity of the Cys-669 EGFR is sensitive to PDBu down-regulation to the same extent as that of the wild-type receptor, with an IC₅₀ of 5 nM PDBu (Fig. 5C). These results suggest that the ability of MAPK to phosphorylate this site does not play a significant role in the regulation of EGFR tyrosine phosphorylation by PKC.

**DISCUSSION**

It has commonly been assumed that heterologous phosphorylation of the EGFR is both necessary and sufficient for down-regulation of the receptor by PKC. In this study we demonstrate that PD98059, a MEK inhibitor, blocks PKC-mediated down-regulation of EGF-stimulated EGFR kinase activity in two different cell types. In contrast, inhibition of MAPK activation by this compound does not perturb PKC-mediated down-regulation of high affinity EGF binding. These results suggest that the PKC-mediated pathway for down-regulating EGFR kinase activity is MEK-dependent, unlike its pathway for modulating high affinity binding. Furthermore, phosphorylation at the MAPK site is unnecessary, since a nonphosphorylatable Thr-669 mutant receptor (Cys-669) is still sensitive to this down-modulation. These results are consistent with a mechanism for PKC-mediated down-regulation of EGFR kinase activity whereby both PKC and MAPK regulate a modulator of EGFR tyrosine phosphorylation.

Direct phosphorylation of the EGFR by either PKC or MAPK does not appear to be sufficient for regulation of EGFR kinase activity. Substitution of a negatively charged glutamate residue at either Thr-654 or Thr-669 does not block the ability of PKC to down-regulate the EGFR. Furthermore, EGF stimulation of the Glu-654 EGFR kinase is comparable to that of wild type, indicating that MAPK activation, even in conjunction with a negative charge at residue 654, is not sufficient to inhibit the receptor kinase (8). In addition, the present study

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

**Fig. 4.** Inhibition of MAPK blocks PDBu down-regulation of EGFR autophosphorylation. Confluent serum-starved N24 cells (A) and CHO 2WT cells (B) were incubated with (●) or without (○) 100 μM PD98059, followed by 10 min with or without different concentrations of PDBu and then 4 min with 10 nM EGF. Tyrosine-phosphorylated EGFR was analyzed by immunoblotting with anti-phosphotyrosine antibody as described under "Experimental Procedures." Autoradiograms were quantitated by densitometric scanning from anti-phosphotyrosine Western blots. The graphs are plotted as percent tyrosine phosphorylation of EGFR relative to cells treated only with EGF. Error bars represent standard deviations of triplicate samples. C, dose response for effect of PD98059 on EGFR autophosphorylation. Confluent N24 cells were pretreated with varying concentrations of PD98059 and then incubated with 40 nM PDBu for 10 min and 10 nM EGF for 4 min.

Tyrosine-phosphorylated EGFR was analyzed by immunoblotting with anti-phosphotyrosine antibody as described under "Experimental Procedures." The graph is plotted as percent tyrosine phosphorylation of EGFR relative to cells treated with only EGF. Error bars represent deviation from the mean. The results of the PD98059 treatments are representative of more than five independent experiments. D, analysis of MAPK activity in cells pretreated with varying doses of PD98059. Confluent serum-starved CHO 2WT cells (lanes 1–8) and N24 cells (lanes 9–16) were incubated with (lanes 5–8 and 13–16) or without (lanes 1–4 and 9–12) 100 μM PD98059, followed by a 10-min treatment with varying concentrations of PDBu, as indicated, and 10 nM EGF for 4 min. The data in this figure are representative of at least three independent experiments.
ilar regulation of the EGFR has been observed in response to PKC. Second, phosphorylation at Thr-654, the site of PKC phosphorylation, is not required. First, EGFR in several cell lines such as CHO 2WT are not phosphorylated significantly by PKC. This result supports previous studies from our laboratory showing that direct phosphorylation of Thr-669 is not required. This result suggests that direct phosphorylation of Thr-654 by PKC is not required. While MEK is required for regulation by PKC, inhibition of MEK does not alter EGFR tyrosine phosphorylation in the absence of active PKC. This difference between the in vitro and in vivo studies may reflect the absence of key regulatory components in the in vitro system, or may reflect differences in the phosphatases or other proteins expressed in the different cell types. PTP1C is just one member of a large family of both cytoplasmic and transmembrane enzymes, and these enzymes have distinct tissue distributions (27). Thus, a tyrosine phosphatase with a similar ability to negatively regulate EGFR tyrosine phosphorylation in vitro has recently been shown to associate with the EGFR in human epidermoid carcinoma (A431) cells and act as a negative regulator of the receptor upon activation by phosphaacidic acid (26).

We showed previously that purified MAPK can decrease the extent of autophosphorylation of the purified EGFR mutant receptor which was identified in mice (21, 22). Both mutations are in the kinase domain at sites which do not undergo modification in vivo. These studies would seem to indicate that receptor phosphorylation events do not modulate EGFR kinase activity, particularly since the stoichiometry of most of them is too low. These results are consistent with the involvement of another component that can alter EGFR activity such as a tyrosine phosphatase.

Recently, a number of phosphatases that can regulate the EGFR have been described. PTP1D (SH-PTP2, Syp, SH-PTP3, PTP2C), an SH2-containing tyrosine phosphatase, acts as a positive mediator of EGFR signaling (23). A related SH2-containing tyrosine phosphatase which is localized predominantly in hematopoietic cells, PTP1C (SHPTP1, HCP, SHP) (24, 25), has recently been shown to associate with the EGFR in human epidermoid carcinoma (A431) cells and act as a negative regulator of the receptor upon activation by phosphaacidic acid (26).

We showed previously that purified MAPK can decrease the activity of EGFR isolated from A431 cells via stimulation of a tyrosine phosphatase that co-immunoprecipitated with the receptor (11). A similar model may explain the mechanism by which PKC regulates EGFR tyrosine phosphorylation in CHO and NIH3T3 cells in vivo; however, several differences must be noted. While MEK is required for in vivo, regulation by PKC, inhibition of MEK does not alter EGFR tyrosine phosphorylation in the absence of active PKC. This difference between the in vitro and in vivo studies may reflect the absence of key regulatory components in the in vitro system, or it may reflect differences in the phosphatases or other proteins expressed in the different cell types. PTP1C is just one member of a large tyrosine phosphatase family consisting of both cytoplasmic and transmembrane enzymes, and these enzymes have distinct tissue distributions (27). Thus, a tyrosine phosphatase with a similar ability to negatively regulate EGFR tyrosine phosphorylation that is responsive to MAPK and PKC might be the target of PKC in our system.

While the results in this study implicate the MEK signaling pathway in the regulation of EGFR tyrosine phosphorylation by PKC, they clearly show that regulation of EGF binding by calcium-mobilizing agents that do not activate PKC or phosphorylate Thr-654 (7). The studies suggesting that direct EGFR phosphorylation is required are based upon site-directed mutagenesis. Substitution at Thr-654 of either an alanine (2, 3) or cysteine, a closer threonine mimic, completely blocks the modulation of the EGFR kinase by PKC. However, while these studies indicate that this residue is important, they do not establish that phosphorylation is required. The interpretation of these studies is difficult due to the lack of structural information about the EGFR. Taken together, these studies suggest that this region is important in this modulation of the EGFR but that phosphorylation alone cannot account for kinase down-regulation.

To date, no study has demonstrated that a single phosphorylation site on the EGFR is sufficient for modulating EGFR signaling. Numerous studies have altered all known serine and threonine phosphorylation sites alone or in combinations without yielding a receptor that is defective in kinase activity (2, 3, 17, 18). In vitro studies have demonstrated that receptors with mutated autophosphorylation sites have wild-type levels of kinase activity (19). A similar study demonstrated that a decrease in the extent of autophosphorylation of the purified receptor does not alter its kinase activity (20). The only mutants identified to date that have reduced or no kinase activity are the ATP binding site mutant at Lys-721 and the wa-2 mutant receptor which was identified in mice (21, 22). Both mutations are in the kinase domain at sites which do not undergo modification in vivo. These studies would seem to indicate that receptor phosphorylation events do not modulate EGFR kinase activity, particularly since the stoichiometry of most of them is too low. These results are consistent with the involvement of another component that can alter EGFR activity such as a tyrosine phosphatase.

shows that MEK activity is required for regulation by PKC, indicating that direct phosphorylation at Thr-654 by PKC is insufficient. Finally, the Cys-669 mutant, a nonphosphorylatable residue, is indistinguishable from the wild-type EGFR in response to PKC regulation, indicating that MAPK phosphorylation of Thr-669 is not required. This result supports previous studies from our laboratory showing that direct phosphorylation of the EGFR by MAPK in vitro has no effect on EGFR tyrosine phosphorylation (11).

Furthermore, several lines of evidence suggest that phosphorylation at Thr-654, the site of PKC phosphorylation, is not required. First, EGFR in several cell lines such as CHO 2WT (8) are not phosphorylated significantly by PKC. Second, similar regulation of the EGFR has been observed in response to

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2 P. Morrison and M. R. Rosner, unpublished data.
PKC occurs through a different mechanism. Numerous agents have been shown to perturb EGF binding, but no consensus has emerged as to the nature of this modulation. Some agents such as phorbol esters are dependent upon the activation of PKC, while others do so through PKC-independent mechanisms. For example, thapsigargin and A23187 both require extracellular Ca\(^{2+}\) to down-regulate high affinity EGF binding (7). Palytoxin also acts through a PKC-independent mechanism but requires extracellular Na\(^{+}\) to down-regulate total EGF binding (28). These results indicate that several mechanisms must exist for EGF receptor regulation.

The data in this study support an alternative model by which PKC can down-regulate the EGFR kinase. Our results demonstrate that the mechanism requires a PKC-activated cascade involving MEK and presumably MAPK. Taken together with our previous data (8, 11), these results suggest that PKC regulation of the EGFR is dependent upon MAPK phosphorylation of another component, such as a tyrosine phosphatase, that modulates tyrosine phosphorylation by the EGF receptor.

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