Transmodulation of Epidermal Growth Factor Receptor Function by Cyclic AMP-dependent Protein Kinase

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Binding of epidermal growth factor (EGF) to its receptor (EGFR) augments the tyrosine kinase activity of the receptor and autophosphorylation. Exposure of some tissues and cells to EGF also stimulates adenylyl cyclase activity and results in an increase in cyclic AMP (cAMP) levels. Because cAMP activates the cAMP-dependent protein kinase A (PKA), we investigated the effect of PKA on the EGFR. The purified catalytic subunit of PKA (PKAc) stoichiometrically phosphorylated the purified full-length wild type (WT) and kinase negative (K721M) forms of the EGFR. PKAc phosphorylated both WT-EGFR as well as a mutant truncated form of EGFR (Δ1022–1186) exclusively on serine residues. Moreover, PKAc also phosphorylated the cytosolic domain of the EGFR (EGFRKD). Phosphorylation of the purified WT as well as EGFRΔ1022–1186 and EGFRKD was accompanied by decreased autophosphorylation and diminished tyrosine kinase activity. Pretreatment of REF-52 cells with the nonhydrolyzable cAMP analog, 8-(4-chlorophenylthio)-cAMP, decreased EGF-induced tyrosine phosphorylation of cellular proteins as well as activation of the WT-EGFR. Similar effects were also observed in B82L cells transfected to express the Δ1022–1186 form of EGFR. Furthermore, activation of PKAc in intact cells resulted in serine phosphorylation of the EGFR. The decreased phosphorylation of cellular proteins and diminished activation of the EGFR in cells treated with the cAMP analog was not the result of altered binding of EGF to its receptors or changes in receptor internalization. Therefore, we conclude that PKA phosphorylates the EGFR on Ser residues and decreases its tyrosine kinase activity and signal transduction both in vitro and in vivo.

Epidermal Growth Factor (EGF) is responsible for a variety of biological effects ranging from mitogenesis (1) to influences on glucose metabolism (2). Upon binding of EGF to its receptor, the epidermal growth factor receptor (EGFR) undergoes dimerization and displays tyrosine kinase activity. This leads to autophosphorylation of the EGFR as well as phosphorylation of intracellular substrates (3). In addition, we have shown that, in the heart, EGF increases contractility and heart rate by augmenting cAMP accumulation (4), a result of stimulation of adenylyl cyclase activity via activation of Gαs (4–6). Additionally, we have also demonstrated that EGF-elicited stimulation of adenylyl cyclase activity requires the tyrosine kinase activity of the EGFR and may involve phosphorylation of Gαs (7, 8). The cyclic AMP formed in response to EGF can activate the heterotrimeric cAMP-dependent protein kinase A (PKA) by binding to its two regulatory subunits, thereby dissociating them from the catalytic subunits (9). The catalytic subunit of PKA (PKAc) can then phosphorylate a variety of intracellular proteins (9).

The EGFR is a 170-kDa glycoprotein with a single transmembrane domain. Its intracellular domain is susceptible to phosphorylation on various residues. Thus, the five autophosphorylation sites in the cytosolic domain of EGFR are located at tyrosine residues 992, 1068, 1086, 1148, and 1173 (10–12). Furthermore, the EGFR is subject to regulation by other kinases. Hence, protein kinase C (PKC) phosphorylates the EGFR at Thr-654, thereby decreasing the intrinsic tyrosine kinase activity of the EGFR (13, 14). Phosphorylation of Ser-1002 by pp60src (15) and of Ser-1046/1047 on the EGFR by cdc2 (16) is likewise associated with inhibition of tyrosine kinase activity. The EGFR can also be phosphorylated on Thr-669 by mitogen-associated protein kinase (MAPK; Refs. 17 and 18). In addition, in vivo pp60src phosphorylates the EGFR on three tyrosines (Tyr-845, -891, and -820) that are not the autophosphorylation sites (19, 20). These novel phosphotyrosines may provide docking sites for SH2 domain-containing proteins, which would explain the enhancement of the mitogenic response to EGF observed in pp60src-overexpressing cells (20).

Previous in vitro studies have shown that the EGFR is a substrate for phosphorylation by PKA (21, 22). However, to date, the functional significance of the phosphorylation has not been described. Since we and others have previously shown that EGF can activate adenylyl cyclase and increase cAMP accumulation in several tissues (4, 23–25), in the present study we have investigated the possibility that PKA may modulate
EGFR tyrosine kinase activity. Our data show that phosphorylation of the EGFR by PKA on serine residues leads to decreased tyrosine kinase activity and diminished autophosphorylation of the EGFR both in vitro and in vivo.

MATERIALS AND METHODS

Purification of Wild Type (WT), K721M, and Δ1022–1186 Forms of EGFR—Using the procedures described previously (26) wild type EGFR was purified from A431 cells. Likewise, using the same method (26), the EGFR K721M and EGFR Δ1022–1186 were purified from B82L cells transfected to express these forms of the receptor. The final step in this method involved elution of the EGFR with EGFR from an EGFR antibody affinity column.

Phosphorylation of EGFR by PKA—The catalytic subunit of PKA (PKAc; Sigma) was dissolved in a buffer containing 20 mM MES, pH 6.5, 100 mM NaCl, 100 μM EDTA, 20 mM β-mercaptoethanol, and 50% ethylene glycol (8000 units/ml). The various forms of purified EGFR WT, EGFRΔ1022–1186, K721M, and EGFRKD (amino acids 644–1186) were incubated with 480 units/ml PKAc in 10 μl of phosphorylation buffer containing 20 mM Heps (pH 7.4), 5 mM MgSO₄, 2 mM MnCl₂, 1 mM NaF, 1 mM dithiothreitol, 10 μM g-malonaldehyde, 20 μg/ml leupeptin, 10 μM AT, 100 μM benzamidine, and 10 mM EGFR (Upstate Biotechnology Inc. or Intergen Co.) for the indicated times at 30 °C. The reaction was terminated by solubilizing in 2% Laemmli sample medium and boiling at 100 °C for 5 min. Proteins were separated on 7.5% SDS-PAGE gels. The samples for autoradiography and phosphoamino acid analyses contained 5 μl (6000 Ci/mmol) [γ-³²P]ATP (NEN Life Science Products) and were electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories).

Phosphoamino Acid Analysis of EGFR—Bands corresponding to the ³²P-labeled proteins were excised from polyvinylidene difluoride membranes and subjected to phosphoamino acid analyses as described previously (8).

Stoichiometry of EGFR Phosphorylation by PKA—This was achieved by pursuing two approaches. Both approaches involved decreasing the autophosphorylation of the EGFR. First, we employed the purified, kinase negative form of the EGFR (EGFR K721M; Ref. 27). This receptor (10 ng) was phosphorylated as described above. In the second approach, the tyrosine kinase activity of the wild type EGFR (100 ng) was inactivated by incubation with 1 mM N-ethylmaleimide (28) for 15 min in the phosphorylation buffer described above except that vanadate and dithiotreitol were absent. After addition of dithiothreitol (2 mM) to block further modifications of sulfhydryl groups, PKA (480 units/ml) and [γ-³²P]ATP (10 μCi) were added and the reaction was terminated by boiling at 100 °C for 5 min. The samples were separated on 7.5% SDS-PAGE gels. The samples for autoradiography and phosphoamino acid analyses contained 5 μl (6000 Ci/mmol) [γ-³²P]ATP (NEN Life Science Products) and were electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories).

Detection of Tyrosine Phosphorylated Cellular Proteins and Active EGFR in Cell Lysates—B82L cells expressing EGFRΔ1022–1186 (12) and REF-52 cells were plated at a density of 2 × 10⁵ cells per 35-mm dish and allowed to grow for 24 h. The cells were then serum starved overnight and treated with 100 μM 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP, Sigma) for the indicated times before addition of 100 nM EGF. Cells were harvested in 2 × 1-ml sample medium and the samples were boiled at 100 °C for 5 min. An aliquot (20 μl) of the proteins in the Laemmli sample medium was diluted with water (80 μl) and mixed with 400 μl of 0.1 m sodium phosphate, pH 7.2, to precipitate the SDS. Standards of bovine serum albumin were similarly treated. The supernatant from this mixture was subjected to SDS-PAGE and Western analyses with anti-phosphotyrosine antibody (clone A49, Alexis Corp., 1:250 dilution) according to the manufacturer instructions. The blot was reprobed with anti-EGFR antibody (Santa Cruz) to ensure that equal amounts of EGFR were immunoprecipitated from equal amounts of cell lysates (400 μg of protein) with anti-EGFR antibody (Transduction Laboratories).

Serine Phosphorylation of the EGFR by PKA in Intact Cells—B82L cells (4 × 10⁶ cells per 35-mm dish) expressing the K721M form of the EGFR were treated with and without 8-CPT-cAMP for 30 min. The EGFR was then immunoprecipitated from equal amounts of cell lysates (400 μg of protein) with anti-EGFR antibody (Transduction Laboratories) as described before (7). Following separation of proteins in the immunoprecipitate by SDS-PAGE, Western analysis was performed with anti-phosphoserine antibody (clone A49, Alexis Corp., 1:250 dilution) according to the manufacturer instructions. The blot was reprobed with anti-EGFR antibody (Santa Cruz) to ensure that equal amounts of EGFR were immunoprecipitated from equal amounts of cell lysates (400 μg of protein).

Tyrosine Kinase Assays—Purified WT, EGFRΔ1022–1186, or EGFRKD was preincubated with the indicated amounts of PKAc in phosphorylation buffer described above for 30 min at 30 °C. Thereafter, 1 μM Val-5-angiotensin II (Novabiochem) and 1.5 μCi of [γ-³²P]ATP were added and phosphorylation was allowed to proceed for 10 min at room temperature in the presence of 25 μl of 20% trichloroacetic acid. After centrifugation, the supernatants were spotted onto P81 Whatman filter disks, rinsed five times for 2 min each in 75 mM phosphoric acid, dried, and counted for ³²P incorporation into Val-5-angiotensin II.

Ligand Binding and Internalization of EGFR—B82L cells expressing either the WT or Δ1022–1186 forms of EGFR were plated in 24-well plates (30,000 cells/well) and serum starved overnight. The cells were washed with 0.5 ml of ice-cold Krebs-Henseleit buffer modified to contain 20 mM Heps, pH 7.4, and incubated on ice for 25 min with the same buffer containing 0.5 mg/ml bovine serum albumin. Thereafter, 125I-EGF (50 μM) was added, and incubation was continued on ice for a further 2 h. Cells were then washed three times with 0.5 ml of ice-cold Krebs-Henseleit buffer modified to contain 20 mM Heps and 100 mM NaF. Non-specific binding was determined in the presence of excess (1 μM) unlabelled EGF. To monitor internalization of the EGFR, the method of Honeggar et al. (29) was pursued. Essentially, following binding of 125I-EGF to cells as described above, the cells were incubated at 37 °C for 30 min and then washed with Krebs-Henseleit buffer as described above. Cell surface-associated 125I-EGF was removed and counted with 0.5 ml ice-cold 0.5 M acetic acid and 150 mM NaCl. Thereafter, cells were washed once with Krebs Henseleit buffer and lysed at 37 °C in 0.5 ml of 1 N NaOH to determine the amount of internalized 125I-EGF.

RESULTS AND DISCUSSION

Because EGF can increase cAMP accumulation in the heart and other tissues (4–6, 23–25), we postulated that akin to the negative regulation of the EGFR by protein kinase C (14), calmodulin-dependent protein kinase II (16) and p34cdc2 (15), PKA may alter the functional activity of EGFR. To address this hypothesis, initially, we determined the effect of pure PKAc on autophosphorylation of the purified wild type and Δ1022–1186 forms of EGFR in the Δ1022–1186 form of EGFR, four of the five autophosphorylation sites were deleted. Fig. 1A shows that PKAc decreases the incorporation of ³²P label from [γ-³²P]ATP into the WT-EGFR, whereas ³²P incorporation into EGFRΔ1022–1186 remained unchanged in the presence of PKAc. Similar results, i.e. decreases in ³²P incorporation, were obtained when the WT-EGFR was incubated with the PKA holoenzyme activated by 8-CPT-cAMP (not shown). This is in contrast with the reports of Rackoff et al. (21) and Ghosh-Dastidar et al. (22) who showed an increase in ³²P incorporation from [γ-³²P]ATP into the purified EGFR after addition of PKA. Possible explanations for this difference are as follows. First, Rackoff et al. (21) incubated the EGFR with PKA for just 30–60 s, which may be too short to reach steady state phosphorylation of the EGFR on tyrosine and serine/threonine residues. Second, in the experiments of Rackoff et al. (21) and Ghosh-Dastidar and Fox (22), vanadate, an inhibitor of phosphotyrosine phosphatases was absent. Under these conditions, tyrosine phosphatases, which may co-purify with the EGFR, would decrease tyrosine phosphorylation of the EGFR, thereby allowing the net increase in PKA-mediated phosphorylation to be monitored. In contrast, in our experiments that employed a longer incubation time, vanadate was present and tyrosine phosphatase incorporation would represent the sum of tyrosine and serine/threonine phosphorylation of the EGFR. Therefore, to determine whether PKAc altered tyrosine phosphorylation of the EGFR, experiments similar to those in Fig. 1A were performed with unlabeled ATP. The samples were then subjected to SDS-PAGE and Western analyses with anti-phosphotyrosine anti-
body. As demonstrated by data in Fig. 1B, incubation of the WT-EGFR with PKAc decreased tyrosine phosphorylation of the receptor. Similarly, autophosphorylation of the D1022–1186 form of the EGFR was also decreased in the presence of PKAc. Thus, the decrease in 32P incorporation in WT-EGFR observed in the presence of PKAc (Fig. 1A) represents a decrease in autophosphorylation of the receptor. Likewise, because tyrosine phosphorylation of the one site (Tyr-992) on the EGFRD1022–1186 is decreased (Fig. 1B), in the presence of a serine or threonine phosphorylation of this receptor by PKAc, no net change in 32P incorporation would be evident (Fig. 1A), i.e. phosphorylation of one serine or threonine residue by PKAc on EGFRD1022–1186 would compensate for the loss of phosphorylation at Tyr-992.

To determine whether PKAc phosphorylates serine or threonine residues on the EGFR, phosphoamino acid analyses were performed. Fig. 2A shows that phosphorylation of the WT-EGFR occurs predominantly on serine residues, as also found by Rackoff et al. (21) and Ghosh-Dastidar and Fox (22). Furthermore, Fig. 2A shows that the appearance of phosphoserine in the presence of PKAc is accompanied by a decrease in phosphotyrosine. PKAc also phosphorylates EGFRD1022–1186 on serine residues (Fig. 2B), and this increase in serine phosphorylation is accompanied by the loss of tyrosine phosphorylation. These data support the contention that, in experiments performed with EGFRD1022–1186 and [γ-32P]ATP, no net difference in 32P incorporation into the receptor is observed in the presence of PKAc (Fig. 1A, right panel) because of a gain of serine phosphorylation and concomitant loss of tyrosine phosphorylation. Because both the WT and EGFRD1022–1186 are phosphorylated on serine residues by PKAc, the data in Fig. 2 demonstrate that the PKAc phosphorylation site(s) is(are) located in the region between amino acids 1022 and 1186. In additional experiments, we determined whether the purified, cytosolic region of the EGFR (amino acids 645–1186; EGFRKD) is also phosphorylated by PKAc. As demonstrated by data in Fig. 3A, phosphorylation of the purified EGFRKD by PKAc resulted in a mobility shift of the EGFRKD on polyacrylamide gels. These data coupled with the findings with the wild type and Δ1022–1186 forms of the EGFR (Figs. 1 and 2) demonstrate that the serine residue(s) on the EGFR, which is(are)
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Phosphorylation of the purified cytosolic domain of the EGFR (EGFRKD) and stoichiometrically phosphorylates the kinase negative (K721M) form of the EGFR and kinase-inactivated WT-EGFR. Panel A, purified, cytosolic domain of the EGFR (10 ng) was incubated in the presence of PKAc (480ml) as described under “Materials and Methods.” Proteins were subjected to SDS-PAGE and detected by autoradiography. The phosphorylation of EGFRKD by PKAc alters its migration (indicated by **) on the gel as seen for many phosphoproteins. A representative of three experiments is shown. Panel B, purified EGFR K721M (10 ng) was incubated in the presence of PKAc (480 units/ml, Promega) and [γ-32P]ATP (10 μM) for 10 min at room temperature in the reaction mixture described under “Materials and Methods.” The reactions were terminated by the addition of Laemmli sample medium. Proteins were separated by SDS-PAGE (7.5% gels) and subjected to autoradiography. The bands corresponding to EGFR K721M in the presence and absence of PKAc were excised and counted for 32P content. A representative of three similar experiments is shown. Stoichiometry of phosphorylation was 0.74 mol of P i per mol of EGFR. Panel C, purified EGFR (100 ng) was incubated with N-ethylmaleimide (1 mM) for 15 min as described under “Materials and Methods.” PKAc (480 units/ml) was then added to the incubation containing all ingredients of the phosphorylation mixture. Reactions were terminated after 30 min and proteins separated by SDS-PAGE. Autoradiography was performed to detect the phosphoproteins. The EGFR band was excised from the gel, and the 32P label incorporated was determined by counting. Stoichiometry of phosphorylation was 0.87 mol of P i per mol of EGFR. Note that autophosphorylated PKAc is visible on the autoradiographs.

Because the wild type EGFR is phosphorylated both on Tyr and as well as Ser residues in the presence of PKAc, and because tyrosine phosphorylation of the receptor decreases in the presence of PKAc (Fig. 2, A and B), the stoichiometry of phosphorylation was difficult to monitor. To circumvent this problem, we employed two approaches. First, phosphorylation of the purified, kinase negative form of the EGFR (EGFR K721M) was monitored. Second, the WT-EGFR was incubated with N-ethylmaleimide to inhibit its tyrosine kinase activity (28). Thereafter, the PKAc-mediated phosphorylation was monitored. As demonstrated in Figs. 3, B and C, autophosphorylation of either the K721M form of the EGFR or the wild type receptor in the presence of N-ethylmaleimide was minimal. Under these conditions, PKAc phosphorylated both the kinase negative EGFR (EGFR K721M, Fig. 3B) and WT-EGFR with a stoichiometry of 0.74 and 0.87 mol of P i per mol of EGFR, respectively.

Because incubation of the WT and Δ1022–1186 forms of EGFR in the presence of PKAc decreased tyrosine phosphorylation of both receptors (Figs. 1B and 2), it would appear that PKAc-elicited phosphorylation of EGFR on serine residues modulates the tyrosine kinase activity of the EGFR. Therefore, to directly evaluate the functional significance of phosphorylation of EGFR by PKAc, we studied the influence of PKAc on the tyrosine kinase activity of the WT and Δ1022–1186 forms of EGFR. Fig. 4 demonstrates that PKAc, in a concentration-dependent manner, inhibited tyrosine kinase activity of both types of EGFR. Moreover, as demonstrated in Fig. 4C, PKAc also inhibited the tyrosine kinase activity of the EGFRKD. Together, the data in Figs. 1–4 demonstrate that the stoichiometric serine phosphorylation of the EGFR by PKAc is accompanied by a decrease in tyrosine kinase activity of the receptor. Moreover, the site of serine phosphorylation on the EGFR is located between residues 644–1022.

Next we investigated whether or not activation of PKA in vivo alters EGF-induced tyrosine phosphorylation of cellular proteins and activation of the EGFR. For this purpose, cells were incubated with 8-CPT-cAMP, a nonhydrolyzable cAMP analog, that activates PKA (30). Fig. 5A(i) illustrates that in REF-52 cells, in the absence of 8-CPT-cAMP, the addition of EGF resulted in a marked increase in tyrosine phosphorylation of cellular proteins. The most prominent tyrosine phosphorylation in response to EGF was observed in proteins of molecular masses of ~180 and ~70 kDa (Fig. 5A(ii)). Treatment of REF-52 cells with 8-CPT-cAMP resulted in a decrease in EGF-elicited tyrosine phosphorylation of these proteins (Fig. 5A(ii)). The decrease in tyrosine phosphorylation of cellular proteins in the presence of 8-CPT-cAMP cannot be attributed to differences in protein loading because reprobing the same blot with anti-PKAc antibody showed that the amount of PKAc in each lane was the same (lower panel of Fig. 5A(i)). Moreover, as assessed with the anti-active EGFR antibody, EGFR-elicited activation of the EGFR in REF-52 cells was also markedly attenuated in the presence of 8-CPT-cAMP (Fig. 5A(ii)); immunoprecipitation of the EGFR from REF-52 cells treated with 8-CPT-cAMP also showed a decrease in EGF-elicited autophosphorylation of the immunoprecipitated EGFR (not shown). Similarly, in B82L cells transfected to express EGFRΔ1022–1186, activation of PKA by 8-CPT-cAMP for different times also led to a decrease in tyrosine phosphorylation of proteins (Fig. 5B). Thus, 5 min after treatment of cells with 8-CPT-cAMP, a decrease in EGF-mediated tyrosine phosphorylation of cellular proteins was observed (Fig. 5B). Treatment of these cells with forskolin, which directly activates adenyl cyclase and increases cAMP levels (31), yielded similar results (not shown).

To determine whether or not activation of PKA in intact cells phosphorylates the EGFR on serine residues, the experiment depicted in Fig. 5C was performed. Essentially, B82L cells transfected to express the kinase-negative form of the EGFR (EGFR K721M) were treated with 8-CPT-cAMP for 30 min in the absence of EGF. Thereafter, the cells were lysed, and EGFR was immunoprecipitated. Following separation of proteins in the immunoprecipitate, Western analysis with anti-phosphoserine antibody was performed. As shown in Fig. 5C, treatment of cells with 8-CPT-cAMP resulted in serine phosphorylation of the immunoprecipitated EGFR; the amount of receptor immunoprecipitated from cells treated with and without 8-CPT-
cAMP was the same (Fig. 5C). The data in Fig. 5 demonstrate that activation of PKA in intact cells results in serine phosphorylation of the EGFR, decreased activation of the receptor (Fig. 5A(iii)) and decreased phosphorylation of cellular proteins in response to EGF. Moreover, the data in Fig. 5C demonstrate that activation of PKA in intact cells can phosphorylate the EGFR on serine residues in the absence of EGF. Thus, the EGFR does not have to be activated by its ligand for phosphorylation by PKA.

One possible reason for a decrease in autophosphorylation of EGFR in intact cells in the presence of the PKA activator 8-CPT-cAMP is that PKA-elicited phosphorylation of the EGFR may alter binding of EGF to its receptor or enhance receptor internalization, thereby decreasing the number of cell surface EGF receptors. To address this possibility, we monitored the binding of $^{125}$I-EGF and internalization of the ligand in B82L cells expressing EGFR1022–1186 that had been pretreated with or without 8-CPT-cAMP. These experiments were performed with $^{125}$I-EGF concentrations of 50 pM, the $K_d$ of EGFR for EGF, because any change in binding affinity would be most pronounced at this concentration of the ligand. Essentially, our data demonstrated that, in B82L cells expressing EGFR1022–1186, the binding of EGF was not altered (Table I). Likewise, pretreatment of B82L cells expressing EGFR1022–1186 with 8-CPT-cAMP did not alter receptor internalization (Table I). These data indicate that the decrease in EGF-elicited receptor autophosphorylation observed in cells preincubated with 8-CPT-cAMP is not the result of a modification in either EGF binding to its receptors or a change in cell surface EGFR numbers because of receptor internalization. Thus, the effects of PKA activation in intact cells reflect the in vitro findings which demonstrate that PKA, by phosphorylating EGFR on serine residues, decreases its protein-tyrosine kinase activity.

Regulation of EGFR tyrosine kinase activity by phosphorylation has been described for PKC (13, 14), calmodulin-dependent protein kinase II (16), and p34cdc2 (15). In all of these cases, phosphorylation decreases tyrosine kinase activity. Our data presented here add to the list of protein kinases that regulate EGFR and, for the first time, demonstrate an inhibitory effect of PKA on the early steps of the EGF signaling cascade both in vivo and in vitro. To date, studies of the inhibitory influence of the cAMP-PKA pathway on EGF signaling have focused mainly on events downstream of Ras, more specifically on MAPK. Thus, it is known that activation of the cAMP-PKA pathway can have inhibitory (32–34), stimulatory (35), or no (36) effects on the activation of MAPK by EGF, depending on the cell type used. This variability is probably because of the various Raf isoforms expressed in the different cell types (37).

However, these studies in which intracellular cAMP levels were raised to modulate MAPK activation in response to EGF failed to show an inhibitory effect on WT-EGFR autophosphorylation (32, 33, 38, 39). This is in contrast with our data (Fig. 5) which demonstrate that PKA can interfere with the EGFR signaling cascade at the level of the EGFR itself. One explanation for this discordance may be that the cell lines overexpressing the WT-EGFR, which were used in a number of the studies concerning MAPK activation, may not be suitable for detecting this inhibitory effect of PKA because the amount of EGFR far exceeds the amount of PKA. Indeed, in B82L cells that overexpress the WT-EGFR, we have also not observed any significant decrease in EGF-elicited tyrosine phosphorylation of cellular proteins in the presence of 8-CPT-cAMP (not shown). In any event, our findings, together with those of others (32–34) suggest that, in some cells, PKA can inhibit EGF-mediated activation of MAPK by attenuating both the EGFR kinase as well as interactions of Raf and Ras, and these two mechanisms may act in a mutually reinforcing manner.

Interestingly, the EGFR is not the only receptor tyrosine kinase target for regulation by PKA. Hence, pretreatment of cells with cAMP-elevating agents increases serine/threonine phosphorylation of the insulin receptor and decreases its insulin-dependent tyrosine kinase activity by 50% (40). This decrease in insulin receptor tyrosine kinase activity by cAMP-elevating agents has been confirmed in in vitro experiments which demonstrated that PKA phosphorylates the insulin receptor and decreases its tyrosine kinase activity (41). These findings coupled with our observations would suggest that PKA may play a more generalized role in regulation of receptor protein-tyrosine kinase signaling.

Although, at present, the identity of the serine residue(s) on EGFR which is(are) phosphorylated remains unknown, there are some sites that can be discarded. Thus, because PKA phosphorylates and modulates the activity of the WT-EGFR, EGFR1022–1186, and cytosolic domain (EGFRKD) of the receptor similarly, it would appear that the functionally important serine residue(s) which is(are) phosphorylated and modulates EGFR kinase activity must reside in the cytosolic region, i.e. between amino acids 644 and 1022. Within this region of the EGFR, serine residues 671, 967, 1022, 971, and 1002 have been shown to be phosphorylated (15, 42). Among these, however, only the phosphorylation of Ser-1002 by p34cdc2 has been shown to decrease EGF tyrosine kinase activity (15). The functional significance of phosphorylation of Ser-671,
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B82L cells (30,000 cells per well) expressing the A1022–1186 form of the EGF receptor were serum deprived overnight. The cells were then treated with and without cAMP analog, 8-CPT-cAMP for 30 min. Thereafter the binding of 125I-EGF (50 pM) to the cells was monitored as described under “Materials and Methods.” The means ± S.E.M. of three experiments are presented. In a separate series of experiments, the internalization of the bound 125I-EGF to the cells was monitored as described under “Materials and Methods.” The means ± S.E.M. of three experiments are presented.

-967, and -971 remains to be determined (42). Notably, however, none of these sites including Ser-1002 conforms to the PKA consensus sequence. Nevertheless, the identity of the precise serine residues on EGFR that are phosphorylated by PKA and that alter EGFR kinase activity forms the subject of future investigations.

In conclusion, we have demonstrated that PKA can phosphorylate EGFR on serine residue(s) and that this phosphorylation is accompanied by a decrease in EGFR protein-tyrosine kinase activity both in vitro and in vivo. In view of our previous findings that EGFR kinase activity is required to stimulate adenylyl cyclase activity (7) and increase intracellular cAMP levels (5), the findings presented here would suggest that activation of PKA can, in a feedback regulatory manner, attenuate EGFR tyrosine kinase activity.

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