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Phosphorylation of Serine 43 Is Not Required for Inhibition of c-Raf Kinase by the cAMP-dependent Protein Kinase*

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The activity of the serine/threonine kinase c-Raf (Raf) is inhibited by increased intracellular cAMP. This is believed to require phosphorylation with the cAMP-dependent protein kinase (PKA), although the mechanism by which PKA inhibits Raf is controversial. We investigated the requirement for PKA phosphorylation using Raf mutants expressed in HEK293 or NIH 3T3 cells. Phosphopeptide mapping of ^{32}P-labeled Raf (WT) or a mutant lacking a putative PKA phosphorylation site (serine to alanine, S43A) confirmed that serine 43 (Ser^{43}) was the major cAMP (forskolin)-stimulated phosphorylation site *in vivo*. Interestingly, the EGF-stimulated Raf kinase activity of the S43A mutant was inhibited by forskolin equivalently to that of the WT Raf. Forskolin also inhibited the activation of an N-terminal deletion mutant Δ5–50 Raf completely lacking this phosphorylation site. Although WT Raf was phosphorylated by PKA, phosphorylation did not inhibit Raf catalytic activity *in vitro*, nor did forskolin treatment inhibit the activity of an N-terminally truncated Raf protein (Raf 22W) or a full-length Raf protein (Raf-CAAX) expressed in NIH 3T3 cells. In contrast, forskolin inhibited the EGF-dependent activation of a Raf isoform (B-Raf), lacking an analogous phosphorylation site to Ser^{43}. Thus, these results demonstrate that PKA exerts its inhibitory effects independently of direct Raf phosphorylation and suggests instead that PKA prevents an event required for the EGF-dependent activation of Raf.

The mitogen-activated protein kinase (MAPK)[1] cascade is a conserved phosphorylation pathway involved in the transduction of growth and differentiation signals in both simple and complex organisms (reviewed in Ref. 1). In the now established paradigm, peptide growth factors bind to their receptor tyrosine kinases, increasing intracellular protein tyrosine phosphorylation and stimulating the exchange of GDP for GTP in small GTP-binding proteins such as Ras (reviewed in Ref. 2). Raf, a 74-kDa serine/threonine kinase, binds to Ras-GTP at the plasma membrane, thus providing an integral link between the receptor-mediated events and activation of the MAPK cascade (3, 4). The activation of Raf is a complex process involving membrane recruitment, interactions with the proteins 14-3-3 (5), hsp90 (6), and regulation by Src family tyrosine kinases (7), protein kinase Cα (8), the kinase suppressor of Ras (Ksr) (9), and most recently p21-activated kinase (PAK) (10) (for reviews, see Refs. 11 and 12). In addition, Raf activation may also be mediated by cytoskeletal factors that lead to the enhancement Raf but not Ras activity in cells (13). Once activated, Raf phosphorylates and activates MAPK kinase (14, 15) (MAPKK or MEK (16)), which in turn phosphorylates MAPK (17, 18) as part of the sequence of events leading to the activation of MAPK in mammalian cells (1).

Compounds that increase intracellular cAMP (*e.g.* glucagon, epinephrine, prostaglandins) are known to oppose the activation of the MAPK cascade in many cell types (for a review, see Ref. 19). This inhibitory effect requires activation of the cAMP-dependent protein kinase (PKA) (20), although the actual mechanism by which this is achieved is controversial. Neither MAPK kinase nor MAPK are phosphorylated by PKA, and elevating cAMP does not inhibit receptor tyrosine phosphorylation, phosphatidylinositol turnover, or activation of Ras-GTP in response to growth factor treatment (19–23). Instead, considerable effort has focused on the serine/threonine kinase c-Raf-1 (c-Raf) as the target of inhibition, and c-Raf is consistently inhibited in cells after elevation of cAMP. PKA phosphorylates substrates containing tandem basic residues upstream of a phosphorylatable serine or threonine residue (24, 25), and c-Raf contains a consensus PKA phosphorylation site at serine 43 (RRAS). Serine 43 has been shown to be phosphorylated *in vivo* (22, 26), and although the phosphorylation of this amino acid does not inhibit the catalytic activity of c-Raf (26), it has been proposed to disrupt normal Ras/Raf association and c-Raf activation *in vivo* (22).

Although this model has been widely accepted, the requirement for Ser^{43} phosphorylation has not been directly tested *in vivo*, and alternative models have been proposed. The studies of Kolch and co-workers (27, 28) suggest that the catalytic activity of c-Raf is directly inhibited by PKA and propose that phosphorylation of Ser^{621} within the catalytic domain of c-Raf is responsible for this effect. However, this hypothesis has been difficult to test because the autophosphorylation of Ser^{621} is required for Raf kinase activity (5), and increased phosphorylation of Ser^{621} in response to cAMP has not been demonstrated.

The effect of cAMP on other isoforms of c-Raf has also been examined. However, in comparison with the consistent inhibition found with c-Raf, the influence of cAMP on the related

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; cAMP, cyclic adenosine monophosphate; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PKA, cyclic AMP-dependent protein kinase; Raf, Raf protein kinase; PAK, p21-activated kinase; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; WT, wild type; PAGE, polyacrylamide gel electrophoresis.

enzyme B-Raf is more complex. There are examples of inhibition (29, 30), activation (31), or no effect (32), of cAMP on B-RAF which may depend on the stimulus, the cell type, or the culture conditions (33, 34). B-Raf lacks an analogous Ser^{43} phosphorylation as found in c-Raf but does contain a similar Ser^{621} phosphorylation site (35). In contrast to some of the studies on c-Raf (27, 28), B-Raf does not appear to be directly inhibited by PKA phosphorylation (32). The third Raf isoform, A-Raf, is not widely expressed in cells and may be resistant to the inhibitory effects of cAMP (36).

The fact that no single consensus model appears sufficient to explain the regulation of Raf (c- or B-Raf) has led to the search for other targets. The Rap proteins (Rap 1a/b) are phosphorylated by PKA (37), can bind Raf, and block Ras signaling when overexpressed in cells (38, 39). Thus, these proteins were originally proposed to be candidates for mediating the cAMP-dependent inhibition of Raf. However, recent studies suggest that Rap is not required for inhibition of MAPK signaling (40) and demonstrate that Rap 1b is activated by cAMP (41). This appears to occur independently of the action of PKA (42) and may play a mitogenic role (43) through the cAMP-dependent activation of B-Raf (31).

Hence, the studies described herein were designed to resolve some of the remaining questions concerning Raf regulation by cAMP. Importantly, we find that deletion of the major PKA phosphorylation site (Ser^{43}) does not impede the cAMP-dependent inhibition of Raf *in vivo*. Furthermore, we find no evidence for a cAMP-stimulated increase in the phosphorylation of Ser^{621} *in vivo* or a direct inhibitory effect of PKA on Raf either *in vitro* or on constitutively activated Raf kinases (Raf 22W, Raf-CAAX) expressed *in vivo*. Finally, examination of an alternative isoform (B-Raf) lacking an analogous Ser^{43} phosphorylation site demonstrated that forskolin prevented the EGF-dependent activation of this enzyme, consistent with PKA interrupting an event required for Raf activation rather than inhibiting Raf itself.

EXPERIMENTAL PROCEDURES

Materials—EGF, LipofectAMINE, and Opti-MEM I reduced serum media were purchased from Life Technologies, Inc. Forskolin was purchased from Biomol (Plymouth Meeting, PA) and dissolved in Me_2SO. [γ-^{32}P]ATP was obtained from NEN Life Science Products. [^{32}P]Orthophosphate was purchased from ICN (Costa Mesa, CA). Protein A-agarose beads were purchased from Bio-Rad and washed three times with PBS prior to use. The purified active catalytic subunit of PKA was purchased from New England Biolabs. Anti-FLAG M2 antibody was purchased from Eastman Kodak Co. Anti-c-Raf-1 was purchased from Transduction Laboratories (Lexington, KY) for use in immunoblots. In other experiments, antibodies specific for A-Raf (C-20), B-Raf (C-19), and c-Raf (C-20) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-HA antibody was purchased from Berkeley Antibody Company (Berkeley, CA) for use in immunoprecipitations. FLAG epitope-tagged Raf and S43A Raf (pCDNA3) and the baculovirus expressing Raf and Src were provided by Dr. Deborah Morrison (NCI, National Institutes of Health, Frederick MD). The FLAG epitope (DYKDDDDK) was added by deleting the last eight amino acids of Raf and replacing them with the FLAG tag. HA epitope-tagged Raf, Raf-CAAX (pCGN), NIH 3T3 cells expressing Raf 22W, and recombinant MEK and Erk-2 were provided by Dr. Channing Der (University of North Carolina, Chapel Hill, NC). The QuikChange site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). ECL reagents were purchased from NEN Life Science Products. Immobilon polyvinylidene difluoride membrane was purchased from Millipore Corp.

Cell Cultures—Human embryonic kidney cells (HEK 293) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin/streptomycin on 60- or 150-mm plates in a humidified, 5% CO_2 atmosphere. 24 h prior to the experiments, the medium was removed and replaced with DMEM with 0.1% FBS and penicillin/streptomycin. The rat liver epithelial cells (GN4) were grown and harvested as described earlier (44). Sf9 cells were grown and infected with baculovirus as described in Ref. 7.

Transient Transfection of Cells—Transient transfection of the cells was performed using LipofectAMINE according to the manufacturer's suggested protocol (Life Technologies, Inc.). Briefly, 2 μg of plasmid and 2 μg of carrier DNA, usually empty pCDNA3 were transfected with 8 μl of LipofectAMINE in serum-free DMEM into cells grown on 60-mm cell culture plates for 48 h. For transient transfection of 100-mm plates, 4 μg of plasmid and carrier DNA was used. For transient transfection of 150-mm plates, 8 μg of plasmid and 8 μg of carrier DNA in 100 μl of LipofectAMINE were used. Cells grown to 50% confluence were washed once with 1× PBS and once with serum-free DMEM. DNA/LipofectAMINE/DMEM was placed on cells and incubated for 5 h at 37 °C. DMEM with 20% FBS and penicillin/streptomycin was then added to the plates and incubated for an additional 24 h. Cells were serum-starved for 24 h with DMEM with 0.1% FBS and penicillin/streptomycin prior to the experiment.

Cell Lysate Preparation—Following drug treatment, cells were washed twice with cold PBS and harvested in 1 ml of ice-cold lysis buffer (20 mM Tris, pH 7.5, 1% Triton, 10% (v/v) glycerol, 137 mM NaCl, 2 mM EDTA, 5 μg/ml leupeptin, 10 nM calyculin A, 250 μM phenylmethylsulfonyl fluoride, and 150 μM sodium orthovanadate). The cell lysates were centrifuged at 15,000 rpm for 10 min, and the resultant supernatants were transferred to a new microcentrifuge tube. Protein concentration of the cell lysates was determined by Coomassie protein assay reagent (Pierce). The same procedure was used to isolate Raf from baculovirus.

Measurement of c-Raf-1 Kinase Activity—2 μg of anti-FLAG antibody was added to 1 mg of clarified cell lysate. Samples were rotated for 1.5 h at 4 °C. 20 μl of protein A beads in PBS were then added to the supernatants and rotated end over end for an additional 40 min at 4 °C. The immune complexes were placed on ice and washed twice in cold immunoprecipitation buffer and twice in cold PBS. The remaining supernatant was removed from the protein A beads with a Hamilton syringe. Raf activity was measured by using a coupled enzyme assay containing MEK and extracellular signal-regulated kinase-2 (Erk-2) and quantitating the incorporation of radioactive phosphate into myelin basic protein as described below. The immunocomplexes were incubated in 10 μl of a cold ATP mixture (20 mM Hepes, pH 7.3, 10 mM β-glycerophosphate, 1.33 mM EGTA, 0.15 mM Na_3VO_4, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 0.1 mM ATP, and 0.1 mg/ml bovine serum albumin) and 0.5 μg of recombinant MEK for 10 min at 30 °C. 1.25 μg of recombinant extracellular signal-regulated kinase-2 was added to the samples and incubated for an additional 10 min at 30 °C. Finally, 20 μl of a radioactive ATP mixture (2 μCi of [γ-^{32}P]ATP, 10 μg of myelin basic protein, 20 mM Hepes, pH 7.3, 10 mM β-glycerophosphate, 1.33 mM EGTA, 0.15 mM Na_3VO_4, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin) was added and incubated for 10 min at 30 °C. The reaction was terminated with the addition of 20 μl of 100 mM EDTA. Samples were centrifuged at 12,000 × *g* for 1 min to pellet the beads, and 40 μl of the reaction mixture was spotted onto squares (2 × 2 cm) of P-81 paper. P-81 papers were washed in 75 mM phosphoric acid five times (5–10 min), washed once briefly in 100% ethanol, and air-dried. The P-81 papers were counted in a Beckman LS 6500 scintillation counter in 0.5 ml of Scintisafe (Fisher) scintillation fluid. Using these conditions, the assay of Raf activity was determined to be linear with respect to time and Raf concentration.

Phosphopeptide Mapping of c-Raf-1 Kinase—293 cells were transiently transfected with WT or S43A Raf-FLAG and grown to confluence in 150-mm plates. Prior to the addition of [^{32}P]orthophosphate, cells were incubated in 12 ml of phosphate-free, 0.1% FBS DMEM for 30 min. 3.0 mCi of [^{32}P]orthophosphate was then added to each plate for 2.5 h. Following labeling, plates were treated with 25 μM forskolin in Me_2SO for 15 min or left untreated. Cells were harvested and Raf-FLAG was immunoprecipitated with 10 μg of FLAG antibody and 50 μl of protein A beads in PBS. Beads were washed as above. Raf-FLAG was removed from the beads by heating twice in 100 μl of sample buffer. Phosphorylated Raf-FLAG was applied to 10% SDS-PAGE, and the gel was fixed in a 25% isopropyl alcohol, 10% acetic acid solution. The gel was then washed three times in water and once in 0.1 M NH_4HCO_3, pH 8.5. The gel was dried, and autoradiography was performed to identify radioactive bands. The band corresponding to Raf (74 kDa) was excised from the gel and swelled in a solution containing 100 μl of 0.1 M NH_4HCO_3, pH 8.5, and 20 μg of trypsin and incubated at 37 °C overnight. The supernatant was removed and used for two-dimensional phosphopeptide mapping as described previously (45). Peptides were resolved electrophoretically (100 V/14 min) using pH 1.9 buffer in one dimension and chromatographically with isobutyric acid buffer in the second dimension. Autoradiography was performed to visualize the radioactive peptides. In other experiments, synthetic

peptides containing the Ser[43] (GYQRRASDDGKLTD) or Ser[621] (LPKINRSASEPSLHRAA) phosphorylation sites were synthesized by Dr. David G. Klapper (University of North Carolina, Chapel Hill). These peptides were phosphorylated with the purified PKA catalytic subunit (New England Biolabs) in a buffer containing 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 25 μM ATP (2 μCi of [γ-^{32}P]ATP). Following phosphorylation, the peptides were digested with trypsin (0.2 μg) in 0.1 M NH_4HCO_3, pH 8.5, and acidified with trifluoroacetic acid, and the radioactive peptides were purified by reverse high pressure liquid chromatography on a Vydac C18 (4.6 × 100-mm) column with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. The amount of Raf peptide phosphorylation was determined by volume analysis of densitometric scans (ImageQuant, Molecular Dynamics, Inc., Sunnyvale, CA).

Immunoprecipitations and Immunoblotting—For immunoprecipitation, 1 mg of cell lysate was incubated with antibody (α-FLAG or α-Raf) for 2 h at 4 °C. 20 μl of protein A/G-agarose beads were then added and incubated for an additional 1 h at 4 °C. Immune complexes were pelleted by centrifugation (12,000 × *g* for 1 min), washed three times in lysis buffer, and boiled in SDS-PAGE sample buffer. The resulting supernatants were separated by SDS-PAGE, transferred to Immobilon membrane (Millipore Corp.), and incubated with appropriate antibodies. Immunoblots were visualized by ECL according to the manufacturer's instructions (NEN Life Science Products).

Phosphorylation of Raf with PKA in Vitro—Raf was immunoprecipitated and incubated with the purified, active catalytic subunit of PKA (New England Biolabs) in the presence of [γ-^{32}P]ATP and Mg^{2+} as described above. Immunoprecipitates were washed with 1× PBS, and Raf kinase was then assayed for activity as described above or analyzed for incorporation of ^{32}P by SDS-PAGE, autoradiography, and scintillation counting of the radioactive Raf protein. When immunoprecipitated from baculovirus, the amount of Raf protein was estimated by Coomassie staining and comparison with known amounts of bovine serum albumin (0–25 μg) applied to the same SDS-PAGE.

RESULTS

Expression and Phosphorylation of Wild Type and Mutant Raf Kinases in HEK 293 Cells—c-Raf (Raf) contains an optimal consensus PKA phosphorylation site (RRAS) at Ser[43] and is phosphorylated on this amino acid *in vivo* (22, 26). To investigate the importance of Ser[43] phosphorylation in mediating the inhibition of Raf by cAMP, the phosphorylation and regulation of a Raf mutant protein containing an alanine substitution at this position (S43A) was examined. HEK 293 (HEK) cells were transiently transfected with the cDNA for WT or S43A Raf containing a FLAG epitope tag. The WT and S43A Raf proteins were expressed at similar levels as determined by Western blotting of cell lysates for the FLAG epitope tag (data not shown). HEK cells were metabolically labeled with [^{32}P]orthophosphate, and the phosphorylation of the WT and S43A Raf proteins was examined by two-dimensional tryptic phosphopeptide mapping (45). From untreated WT or S43A Raf cells, two major phosphopeptides (I, II) were obtained after tryptic digestion of Raf (Fig. 1, *A–D*). One of these peptides (*II*), co-migrated with a synthetic peptide corresponding to the tryptic peptide containing the Ser[621] autophosphorylation site as described earlier (26) (5) (see *upper right-hand corner* of Fig. 1*E*). Treatment of HEK cells with forskolin to increase intracellular cAMP resulted in the appearance of an additional phosphorylated peptide (*III*) in the WT Raf protein (Fig. 1*B*). This phosphopeptide (*III*) co-migrated with a synthetic tryptic peptide corresponding to the serine 43 phosphorylation site (RA*S*DDGK) (Fig. 1*E*) and was not observed in tryptic digests of untreated WT Raf (Fig. 1*A*). This phosphopeptide was also absent from tryptic digests of the S43A Raf after forskolin treatment, thus demonstrating the absence of this phosphorylation site *in vivo* (Fig. 1, *C* and *D*). Relative to the peptide (*I*) that was constitutively phosphorylated (see legend to Fig. 1), forskolin did not increase the phosphorylation of the Ser[621] peptide (*II*) or significantly change the phosphorylation of other peptides in Raf (Fig. 1, *B* and *D*). Thus, these results confirm

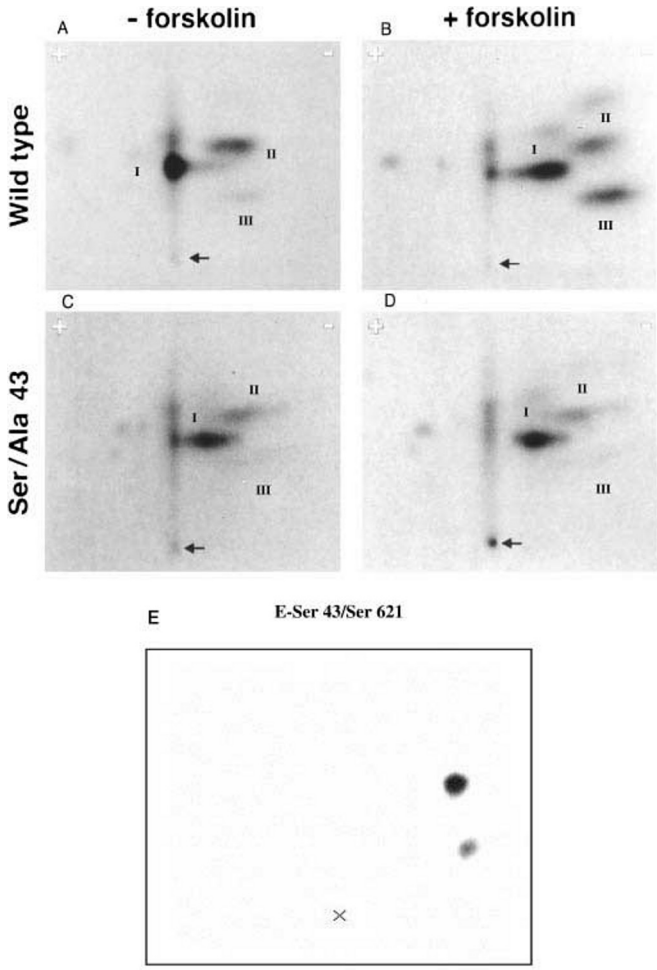

FIG. 1. **Phosphopeptide mapping of WT and S43A Raf kinases.** HEK 293 cells expressing WT Raf (*A* and *B*) or S43A Raf (*C* and *D*) were labeled with [^{32}P]orthophosphate and incubated with carrier (*A* and *C*) or with 25 μM forskolin (*B* and *D*), and the Raf proteins were immunoprecipitated using anti-FLAG antibody. After SDS-PAGE isolation of the phosphorylated Raf, "in gel" trypsin digestion was performed, and the Raf peptides were analyzed by two-dimensional phosphopeptide analysis and autoradiography. *E*, synthetic peptides containing the serine 43 and serine 621 residues were phosphorylated with PKA and [γ-^{32}P]ATP as described under "Experimental Procedures," digested with trypsin, and similarly analyzed. The *x* axis represents separation by electrophoresis; the *y* axis represents separation by chromatography as described earlier (45). The amount of peptide phosphorylation was compared by volume analysis of densitometric scans. *Peptide I*, WT without forskolin, not determined; WT plus forskolin, 111.7; S43A without forskolin, 90.5; S43A plus forskolin, 94.5. *Peptide II*, WT without forskolin, 85.7; WT plus forskolin, 78.9; S43A without forskolin, 66.5; S43A plus forskolin, 65.5. *Peptide III*, WT without forskolin, 14.3; WT plus forskolin, 198.9; S43A without forskolin, 16.2; S43A plus forskolin, 12.1.

that Ser[43] is the major forskolin-stimulated phosphorylation site in Raf.

Regulation of Wild Type and S43A Raf by EGF and cAMP—The regulation of the WT and S43A Raf kinases expressed in HEK cells was compared. HEK cells expressing either the WT or S43A Raf were serum-deprived, and the WT or S43 Raf proteins were immunoprecipitated with antibodies to the epitope tag (FLAG) and immunoblotted (Fig. 2*A*) or assayed for Raf kinase activity (Fig. 2*B*) as described under "Experimental Procedures." As shown in Fig. 2*A*, similar amounts of both WT and S43A Raf were immunoprecipitated from equivalent amounts of HEK cell lysates. The addition of EGF to serum-starved HEK cells resulted in approximately a 10–15-fold increase in both the WT Raf or S43A Raf kinase activities (Fig.

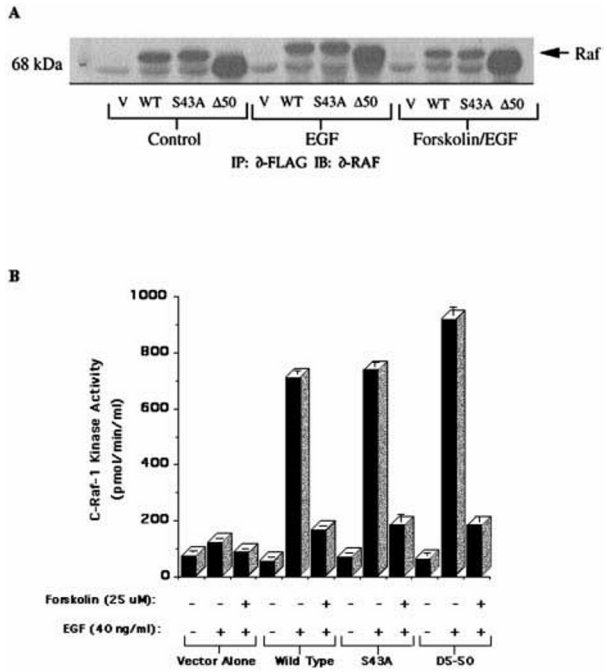

FIG. 2. **Regulation of the WT, S43A, and Δ5–50 Raf by EGF and forskolin in HEK cells.** HEK 293 cells expressing vector alone, WT Raf, S43A Raf, and Δ5–50 Raf were serum-starved, incubated with 40 ng/ml EGF (5 min), or pretreated with 25 μM forskolin (10 min) prior to EGF addition. The cells were lysed, and the Raf immunoprecipitates (anti-FLAG) were immunoblotted for Raf (Santa Cruz C12) (*A*) or assayed for Raf activity as described under "Experimental Procedures" (*B*). In the immunoblotting experiments, the upper band represented Raf (marked with an *arrow*), whereas the lower band was a contaminant found in all samples and was independent of Raf transfection. Raf activity is reported in pmol/min/ml, and the results represent the mean ± S.E. of duplicate samples. Shown is a representative figure of three experiments.

2*B*). In parallel with an increase in Raf activity, the migration of both the WT and S43A Raf proteins decreased slightly on SDS-PAGE (Fig. 2*A*). The time course and extent of activation of the WT and S43A Raf kinases by EGF was indistinguishable; the peak of Raf activity typically occurred after 5 min of EGF addition and rapidly declined to basal levels thereafter (data not shown).

The effect of increasing intracellular cAMP on the activity of the WT and S43A Raf kinases was determined. Acute forskolin treatment (5–10 min) resulted in greater than 90% inhibition of the EGF-stimulated Raf kinase activity in HEK cells expressing WT Raf (Fig. 2*B*). Forskolin treatment also inhibited the activation of the S43A Raf kinase to an equivalent extent. Concomitant with the inhibition of Raf activity, the EGF-stimulated shift on SDS-PAGE was abolished (Fig. 2*B*). The influence of forskolin was independent of the length of exposure to EGF; hence, forskolin treatment did not simply delay the activation of Raf (data not shown). These results indicated that phosphorylation of Ser^{43} was unnecessary for inhibition by cAMP. However, to further confirm this, we compared the regulation of a Raf kinase mutant lacking amino acids 5–50 (Δ5–50 Raf) and the entire consensus PKA phosphorylation site. The Δ5–50 Raf was expressed in HEK cells and treated with EGF as described above. Immunoprecipitation and immunoblotting of lysates for this protein demonstrated that the Δ5–50 Raf protein migrated faster on SDS-PAGE, consistent with its lower molecular weight (Fig. 2*A*). EGF treatment of HEK cells resulted in approximately a 10–15-fold activation of the Δ5–50 Raf kinase, similar to that observed with the WT or the S43A Raf kinases (Fig. 2*B*). Like the results obtained with the WT and S43A Raf, forskolin treatment inhibited the EGF-dependent activation of the Δ5–50 Raf kinase and the EGF-stimulated shift in migration on SDS-PAGE (Fig. 2*A*). Thus, these results unequivocally demonstrate that phosphorylation of Ser^{43} is not required for the inhibition of Raf by cAMP *in vivo*.

PKA Phosphorylation Does Not Inhibit c-Raf Kinase Activity in Vitro—Earlier studies proposed that Raf could be directly inhibited by PKA phosphorylation *in vitro* (27, 28). Since our studies suggested that phosphorylation of Ser^{43} was unnecessary for inhibition of Raf *in vivo*, we tested whether phosphorylation by PKA resulted in the inhibition of Raf kinase activity *in vitro*. Active Raf kinase was obtained from Sf9 cells coinfected with Raf and Src baculovirus as shown earlier (7). Incubation of active Raf with the catalytic subunit of PKA and Mg-ATP resulted in a time-dependent increase in the phosphorylation of this protein (Fig. 3*A*). However, despite stoichiometric phosphorylation (~0.5–1.0 mol/mol) of Raf by PKA *in vitro*, no detectable loss of Raf kinase activity was observed in these samples (Fig. 3*B*). Because the Src-activated Raf kinase may be mechanistically different from Raf obtained from EGF-treated cells, we compared the ability of PKA to inhibit the activity of either the WT or the S43A Raf kinases immunoprecipitated from HEK cells. Incubation of Raf from serum-starved or EGF-stimulated cells with PKA resulted in phosphorylation of the WT enzyme as expected (data not shown). Again, neither the WT nor S43A Raf kinase activity was inhibited by incubation with PKA under these conditions (Fig. 3, *C* and *D*). Hence, these results indicate that PKA does not directly phosphorylate and inhibit the catalytic activity of Raf *in vitro*.

PKA Does Not Inhibit the Activity of the Catalytic Domain of Raf Expressed in NIH 3T3 Cells (Raf 22W) or Raf-CAAX—To further determine the effect of PKA on Raf, we examined the regulation of the catalytic domain of Raf kinase expressed *in vivo*. Raf kinase was immunoprecipitated from NIH 3T3 cells stably transfected with vector (pZip) or a cDNA encoding a constitutively activated Raf protein (Raf 22W, amino acids 305–648). This constitutive activity is due to relief of N-terminal autoinhibition (12), and the Raf 22W cells contained a high level of Raf activity in the absence of serum as reported earlier (14, 46). Incubation of the serum-starved Raf 22W cells with forskolin only slightly inhibited the Raf kinase activity in these cells (Fig. 4). In contrast, forskolin completely inhibited the EGF-dependent activation of the endogenous Raf in the vector control NIH 3T3 (pZip) cells as expected (data not shown). Therefore, the catalytic subunit of Raf itself appears to be resistant to inhibition by cAMP *in vivo*.

Because earlier studies suggested that PKA phosphorylation of Raf on Ser^{43} decreased Raf/Ras association (22), we determined the influence of PKA on a Raf mutant that did not require Ras binding for activity. The addition of a farnesyl group to Raf (Raf-CAAX) artificially targets Raf to the plasma membrane; this has been proposed to mimic Ras binding and results in a constitutively active Raf kinase, ultimately resulting in increased cell transformation (47, 48). HEK cells were transiently transfected with a hemagglutinin (HA)-tagged Raf-CAAX or wild type HA-tagged Raf, and the Raf kinases were immunoprecipitated (α-HA tag) and assayed for Raf kinase activity. As expected, Raf-CAAX was constitutively active in the absence of EGF treatment (Fig. 5). EGF treatment did not increase the activity of this kinase, suggesting that Raf-CAAX was already fully activated in these cells. However, a HA-tagged WT Raf was activated by EGF to a similar extent as that found with the FLAG-tagged Raf kinases as described above (compare with Fig. 2). The effect of forskolin treatment on the Raf kinases in these cells was compared. As shown in Fig. 5, forskolin resulted in almost a complete inhibition of the WT Raf

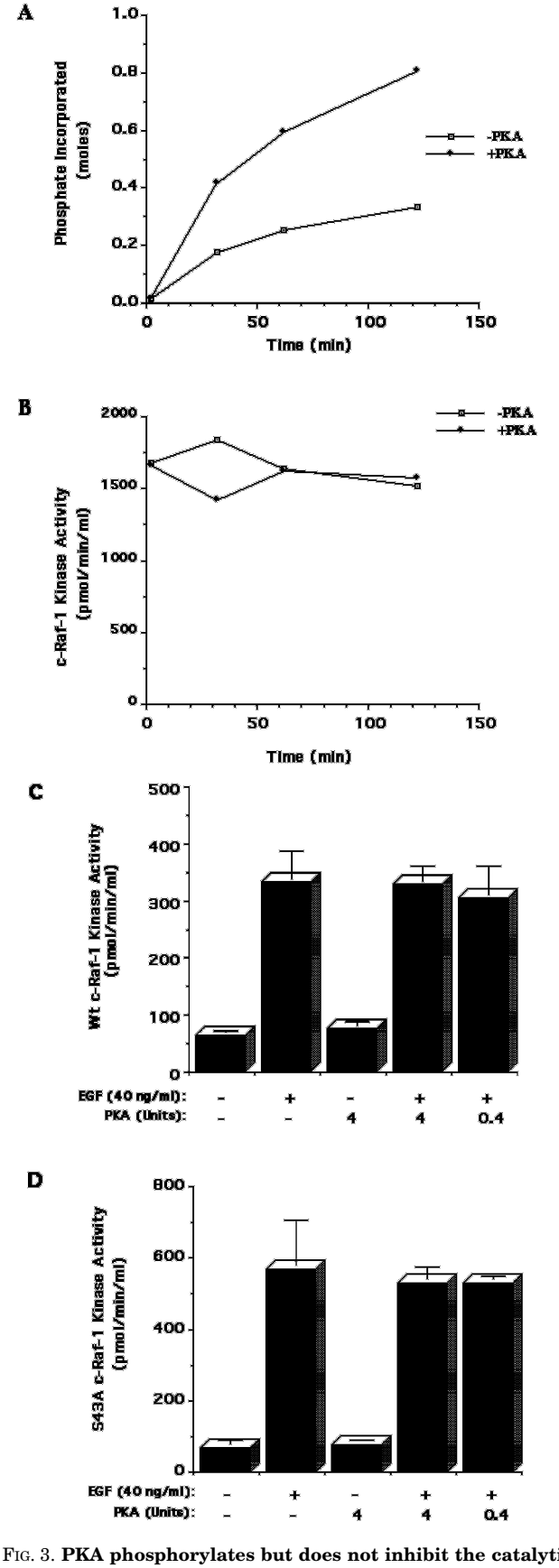

FIG. 3. **PKA phosphorylates but does not inhibit the catalytic activity of Raf *in vitro*.** *A*, Raf immunoprecipitated from Sf9 cell lysates was incubated in a buffer containing Mg^{2+} and [γ-^{32}P] ATP with or without the addition of the purified catalytic subunit of PKA for the amount of time indicated. The incubated Raf samples were divided in half and assayed for phosphorylation (*A*) or Raf kinase activity (*B*). For phosphorylation analysis, Raf was applied to SDS-PAGE, the phosphorylated Raf protein was identified by autoradiography and excised, and the amount of radioactivity was quantitated in a liquid scintillation counter. *B*, for Raf kinase activity, PKA phosphorylation was first terminated with the addition of 100 μM PKI inhibitor peptide, and Raf kinase activity was then measured using the coupled enzyme assay described under "Experimental Procedures." Results are reported as pmol/min/ml. *C* and *D*, HEK 293 cells expressing WT (*C*) or S43A (*D*) Raf were serum-starved and left untreated as controls or incubated with 40 ng/ml of EGF for 5 min. Cells were harvested, and Raf was immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were incubated with 4 or 0.4 units of PKA activity and analyzed for activity as described in *B*. Raf activity is reported as pmol/min/ml and represents the mean ± S.E. of duplicate samples. Shown are representative results of three experiments.

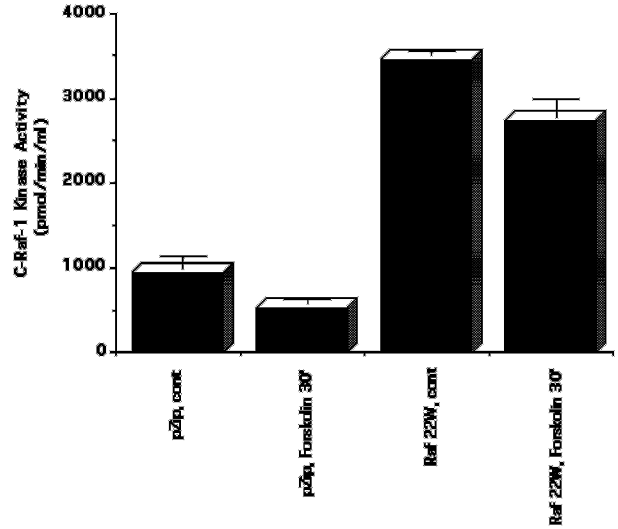

FIG. 4. **The catalytic domain of Raf expressed in NIH 3T3 cells is not inhibited by cAMP.** NIH 3T3 cells stably transfected with vector (pZip) or Raf 22W were serum-starved and treated with or without forskolin (25 μM, 30 min), Raf was immunoprecipitated from cell lysates and assayed for kinase activity. The activity is plotted as pmol/min/ml and represents the mean ± S.E. of duplicate samples.

but was without effect on the activity of the Raf-CAAX kinase.

Regulation of B- and c-Raf by cAMP in Liver Epithelial Cells—Since our results suggested that PKA was not inhibiting c-Raf directly, we compared the effects of cAMP on a related Raf isoform, B-Raf. Although highly homologous to c-Raf throughout the catalytic domain, B-Raf has a longer N-terminal region and lacks an analogous phosphorylation site to serine 43 in c-Raf (35). Rat liver epithelial cells (GN4) express both B- and c-Raf as demonstrated by immunoblotting for these enzymes (data not shown). Using polyclonal antibodies specific for each isoform, we immunoprecipitated B- and c-Raf from serum-starved or EGF-treated GN4 cells and measured Raf kinase activity as described above. In serum-starved cells, B-Raf had considerably higher basal kinase activity than that found with c-Raf (Fig. 6). As expected, EGF stimulated an increase in the kinase activity of both enzymes; however, c-Raf activity was increased to a greater extent than B-Raf. Treatment of GN4 cells with forskolin almost completely inhibited the EGF-stimulated c-Raf kinase and B-Raf kinase activity. In contrast, forskolin did not effect the basal activity of either c- or B-Raf, suggesting that only the EGF-dependent activation was subject to regulation by this treatment (Fig. 6). Forskolin treatment alone did not increase the activity of B-Raf in these cells (data not shown). Thus, these results demonstrate that cAMP prevents the activation of two distinct Raf isoforms in GN4 cells.

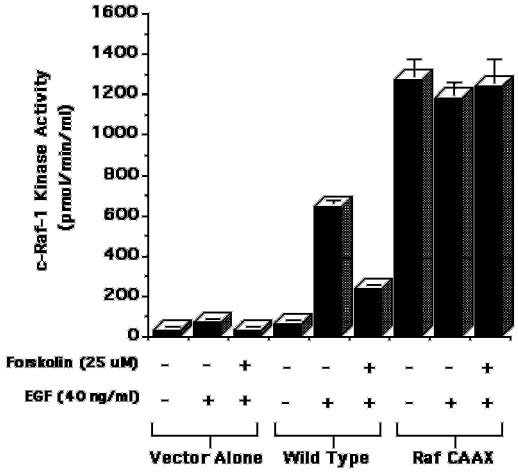

FIG. 5. **Forskolin treatment does not affect Raf-CAAX activity.** HEK 293 cells expressing vector, HA-tagged WT Raf, or HA-tagged Raf-CAAX were serum-starved for 24 h. Cells were then left untreated as controls, treated with EGF (40 ng/ml, 5 min), or treated with forskolin (25 μM, 10 min) followed by EGF (5 min). Cells were lysed, Raf was immunoprecipitated with anti-HA antibody, and the Raf activity was determined. Raf activity is reported as pmol/min/ml and represents the mean ± S.E. of duplicate samples. Shown is a representative figure of three experiments.

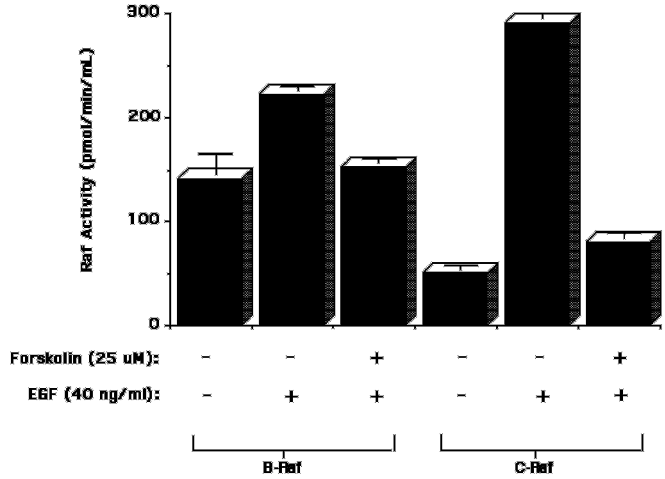

FIG. 6. **Forskolin prevents the activation of B- and c-Raf in GN4 epithelial cells.** Rat liver GN4 epithelial cells were serum-starved (18 h) and incubated with carrier (Me_2SO) or forskolin (25 μM, 10 min), prior to treatment with EGF (40 ng/ml, 5 min). The cells were collected in cell lysis buffer, and the B- and c-Raf kinases were immunoprecipitated and assayed for Raf kinase activity as described earlier. The results represent the mean ± S.E. of duplicate samples. Shown is a representative figure of two experiments.

DISCUSSION

Inhibition of the MAPK cascade by cAMP and the consequential activation of PKA is a widely observed phenomenon in many different cell types (19). However, despite this observation, the mechanism by which this occurs is poorly understood and remains controversial. Of particular interest is whether phosphorylation of Raf is required to explain the inhibition of this kinase *in vivo*. Previous studies have suggested that phosphorylation of Ser^{43} is required for Raf inhibition (22, 49); however, a direct test of this hypothesis has not been performed *in vivo*. By examining two different mutants of Raf (S43A or Δ5–50 Raf), our studies now clearly demonstrate that deletion of this region of Raf is dispensable for normal activation by EGF or for inhibition by cAMP. This is consistent with studies showing that the primary Ras binding domain of Raf includes amino acids 51–131 (see Ref. 12 and references therein) and would imply that Ser^{43} is outside the region required for Raf/Ras binding *in vivo*. These conclusions are further supported by our findings with B-Raf showing that cAMP inhibits activation of a related isoform lacking an analogous Ser^{43} phosphorylation site. Although our phosphopeptide mapping results confirm that Ser^{43} is the major site of cAMP-stimulated phosphorylation in c-Raf *in vivo* (26), the finding that removing Ser^{43} does not affect Raf regulation indicates that phosphorylation of this site remains an enigma.

Previously it was suggested that phosphorylation of Raf on Ser^{621} was responsible for inhibition of c-Raf kinase *in vivo* (28). Although the authors demonstrated that PKA phosphorylated Raf on Ser^{621} *in vitro*, evidence for the forskolin or cAMP-stimulated phosphorylation of Ser^{621} *in vivo* was not presented. In our phosphopeptide mapping studies, we observed two constitutively phosphorylated peptides in Raf, one of which co-migrated with the synthetic Ser^{621} tryptic peptide, whereas the other most likely corresponds to the Ser^{259} autophosphorylation peptide as shown earlier (26). We found no evidence for increased phosphorylation of Ser^{621} in response to forskolin treatment of cells. Instead, this peptide was phosphorylated equally in both untreated and forskolin-treated cells, consistent with this amino acid being a major autophosphorylation site *in vivo* (26) and arguing against Ser^{621} as a valid PKA phosphorylation site *in vivo*. In addition, compared with the Ser^{43} peptide, the Ser^{621} peptide lacks the optimal pair of basic residues (RSAS) and is a poor substrate for PKA *in vitro*. Phosphorylation of the Ser^{621} peptide occurred at a rate approximately 100 times lower than that observed with the Ser^{43} peptide *in vitro*.[2] Instead, Ser^{621} is efficiently phosphorylated by the AMP-activated protein kinase (50). In other studies, phosphorylation of Ser^{621} has been shown to be required for both 14-3-3 binding and Raf activity (5). Hence, it seems unlikely that a phosphorylation site that is essential for Raf activity would also be required for inhibition as suggested by Kolch and co-workers (28). These observations combined with our *in vivo* phosphopeptide mapping studies strongly suggest that phosphorylation of Ser^{621} does not mediate the cAMP-dependent inhibition of Raf kinase *in vivo*.

Although our data demonstrated that phosphorylation of Ser^{43} was not required for the inhibition of Raf kinase *in vivo*, PKA might still prevent Raf/Ras association at the plasma membrane as suggested earlier (22). Our experiments with Raf-CAAX were designed to test this hypothesis, and, consistent with this model, we find that this protein is completely resistant to inhibition by cAMP. Although these results may argue that the primary effect of PKA is to disrupt Ras/Raf association, there are other potential interpretations of these results. For instance, once activated, Raf may be resistant to the effects of PKA. Our results with the PKA phosphorylation of active Raf *in vitro* or forskolin treatment of the Raf 22W cells would support this model. Using either the baculovirus-expressed Raf, the EGF-activated WT, or S43A Raf from HEK cells, the truncated and active form of Raf expressed in NIH 3T3 cells or Raf-CAAX, we found no evidence for the direct inhibition of Raf kinase by PKA either *in vitro* or *in vivo*. In this regard, our results are in agreement with the studies of Whitehurst *et al*. (51), suggesting that the catalytic subunit of c-Raf is not directly inhibited by PKA.

Instead, our findings would be consistent with cAMP inhibiting an event required for the activation of both B- and c-Raf. This hypothesis would be in agreement with earlier studies showing that cAMP inhibited the Ras-dependent activation of B-Raf (29, 30) and would be supported by our results demon-

[2] M. Sidovar, unpublished observations.

strating that forskolin prevents the EGF-dependent activation of both B- and c-Raf in GN4 cells. The lack of effect of forskolin on the basal activity of either B- or c-Raf in GN4 cells reiterates our findings showing that PKA does not directly inhibit the catalytic activity of Raf. Because the catalytic regions of both B-Raf and c-Raf are highly homologous (35) and because other recent studies have found that B-Raf is resistant to inhibition by PKA (32), we believe argues against a direct effect of PKA on the catalytic domain of Raf. Although these authors (32) did see a partial inhibitory effect of PKA on c-Raf (less than 50%), we do not believe that this incomplete effect accounts for the greater than 90% inhibition of c-Raf activity that we observed *in vivo*.

Additional targets that were not investigated in this study include the Rap proteins. Although these proteins are phosphorylated by PKA *in vivo* (37) and can physically antagonize the binding of c-Raf to Ras (52), the most recent studies suggest that PKA phosphorylation may diminish the inhibitory potential of these proteins (53). Furthermore, considerable evidence suggests that cAMP-dependent activation of Rap results in the activation of B-Raf (43) (31). Although we did not observe a stimulatory effect of cAMP on B-Raf in the GN4 cells,[3] this may be explained by cell type differences in the expression of Rap as described by others (34). Thus, these findings, combined with the recent studies showing that Rap is not required for the inhibition of MAPK signaling (40), suggest that Rap proteins do not mediate the inhibition of Raf observed in our studies.

So what might explain the inhibitory effects of cAMP on Raf? Recent studies have shown that PAK3 can function as a Raf kinase, thus contributing to the activation of Raf by growth factors and other stimuli (10, 54). Interestingly, studies from Howe *et al*.[4] show that PAK contains a consensus PKA phosphorylation site and find that PAK is phosphorylated and inhibited in response to forskolin treatment of cells. This observation would be consistent with our findings suggesting that PKA phosphorylation of Raf itself is insufficient to explain the inhibition observed *in vivo*; however, whether or not the phosphorylation of PAK by PKA is required to account for this regulation remains to be established.

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REFERENCES

1. Lewis, T. S., Shapiro, P. S. & Ahn, N. G. (1998) *Adv. Cancer Res.* **74,** 49–139
2. Vojtek, A. B. & Der, C. J. (1998) *J. Biol. Chem.* **273,** 19925–19928
3. Van Aelst, L., Barr, M., Marcus, S., Polverino, A. & Wigler, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6213–6217
4. Vojtek, A. B., Hollenberg, S. M. & Cooper, J. A. (1993) *Cell* **74,** 205–214
5. Thorson, J. A., Yu, L. W., Hsu, A. L., Shih, N. Y., Graves, P. R., Tanner, J. W., Allen, P. M., Piwnica-Worms, H. & Shaw, A. S. (1998) *Mol. Cell. Biol.* **18,** 5229–5238
6. Stancato, L. F., Chow, Y. H., Hutchison, K. A., Perdew, G. H., Jove, R. & Pratt, W. B. (1993) *J. Biol. Chem.* **268,** 21711–21716
7. Fabian, J. R., Daar, I. O. & Morrison, D. K. (1993) *Mol. Cell. Biol.* **13,** 7170–7179
8. Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D. & Rapp, U. R. (1993) *Nature* **364,** 249–252
9. Therrien, M., Chang, H. C., Solomon, N. M., Karim, F. D., Wassarman, D. A. & Rubin, G. M. (1995) *Cell* **83,** 879–888
10. King, A. J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S. & Marshall, M. S. (1998) *Nature* **396,** 180–183
11. Daum, G., Eisenmann, T. I., Fries, H. W., Troppmair, J. & Rapp, U. R. (1994) *Trends Biochem. Sci.* **19,** 474–480
12. Morrison, D. K. & Cutler, R. E. (1997) *Curr. Opin. Cell Biol.* **9,** 174–179
13. Chen, Q., Lin, T. H., Der, C. J. & Juliano, R. L. (1996) *J. Biol. Chem.* **271,** 18122–18127
14. Dent, P., Haser, W., Haystead, T. A., Vincent, L. A., Roberts, T. M. & Sturgill, T. W. (1992) *Science* **257,** 1404–1407
15. Kyriakis, J. M., App, H., Zhang, X. F., Banerjee, P., Brautigan, D. L., Rapp, U. R. & Avruch, J. (1992) *Nature* **358,** 417–421
16. Crews, C. M., Alessandrini, A. & Erikson, R. L. (1992) *Science* **258,** 478–480
17. Ray, L. B. & Sturgill, T. W. (1988) *J. Biol. Chem.* **263,** 12721–12727
18. Ahn, N. G., Seger, R., Bratlien, R. L., Diltz, C. D., Tonks, N. K. & Krebs, E. G. (1991) *J. Biol. Chem.* **266,** 4220–4227
19. Graves, L. M. & Lawrence, J. C. J. (1996) *Trends Endocrinol.* **7,** 43–50
20. Sevetson, B. R., Kong, X. & Lawrence, J. C. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10305–10309
21. Burgering, B. M., Pronk, G. J., Medema, J. P., van der Voorn, L., de Vries Smits, A. M., van Weeren, P. C. & Bos, J. L. (1993) *Biochem. Soc. Trans.* **21,** 888–894
22. Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J. & Sturgill, T. W. (1993) *Science* **262,** 1065–1069
23. Cook, S. J. & McCormick, F. (1993) *Science* **262,** 1069–1072
24. Kennelly, P. J. & Krebs, E. G. (1991) *J. Biol. Chem.* **266,** 15555–15558
25. Songyang, Z., Lu, K. P., Kwon, Y. T., Tsai, L. H., Filhol, O., Cochet, C., Brickey, D. A., Soderling, T. R., Bartleson, C., Graves, D. J., DeMaggio, A. J., Hoekstra, M. F., Blenis, J., Hunter, T. & Cantley, L. C. (1996) *Mol. Cell. Biol.* **16,** 6486–6493
26. Morrison, D. K., Heidecker, G., Rapp, U. R. & Copeland, T. D. (1993) *J. Biol. Chem.* **268,** 17309–17316
27. Hafner, S., Adler, H. S., Mischak, H., Janosch, P., Heidecker, G., Wolfman, A., Pippig, S., Lohse, M., Ueffing, M. & Kolch, W. (1994) *Mol. Cell. Biol.* **14,** 6696–6703
28. Mischak, H., Seitz, T., Janosch, P., Eulitz, M., Steen, H., Schellerer, M., Philipp, A. & Kolch, W. (1996) *Mol. Cell. Biol.* **16,** 5409–5418
29. Vaillancourt, R. R., Gardner, A. M. & Johnson, G. L. (1994) *Mol. Cell. Biol.* **14,** 6522–6530
30. Peraldi, P., Frodin, M., Barnier, J. V., Calleja, V., Scimeca, J. C., Filloux, C., Calothy, G. & Van Obberghen, E. (1995) *FEBS Lett.* **357,** 290–296
31. Vossler, M. R., Yao, H., York, R. D., Pan, M. G., Rim, C. S. & Stork, P. J. (1997) *Cell* **89,** 73–82
32. MacNicol, M. C. & MacNicol, A. M. (1999) *J. Biol. Chem.* **274,** 13193–13197
33. Erhardt, P., Troppmair, J., Rapp, U. R. & Cooper, G. M. (1995) *Mol. Cell. Biol.* **15,** 5524–5530
34. Dugan, L. L., Kim, J. S., Zhang, Y., Bart, R. D., Sun, Y., Holtzman, D. M. & Gutmann, D. H. (1999) *J. Biol. Chem.* **274,** 25842–25848
35. Sithanandam, G., Kolch, W., Duh, F. M. & Rapp, U. R. (1990) *Oncogene* **5,** 1775–1780
36. Bogoyevitch, M. A., Marshall, C. J. & Sugden, P. H. (1995) *J. Biol. Chem.* **270,** 26303–26310
37. Siess, W., Winegar, D. A. & Lapetina, E. G. (1990) *Biochem. Biophys. Res. Commun.* **170,** 944–950
38. Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y. & Noda, M. (1989) *Cell* **56,** 77–84
39. Hu, C. D., Kariya, K., Kotani, G., Shirouzu, M., Yokoyama, S. & Kataoka, T. (1997) *J. Biol. Chem.* **272,** 11702–11705
40. Zwartkruis, F. J., Wolthuis, R. M., Nabben, N. M., Franke, B. & Bos, J. L. (1998) *EMBO J.* **17,** 5905–5912
41. Altschuler, D. L., Peterson, S. N., Ostrowski, M. C. & Lapetina, E. G. (1995) *J. Biol. Chem.* **270,** 10373–10376
42. Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E. & Graybiel, A. M. (1998) *Science* **282,** 2275–2279
43. Altschuler, D. L. & Ribeiro-Neto, F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7475–7479
44. Li, X., Yu, H., Graves, L. M. & Earp, H. S. (1997) *J. Biol. Chem.* **272,** 14996–15002
45. van der Geer, P. & Hunter, T. (1994) *Electrophoresis* **15,** 544–554
46. Oldham, S. M., Clark, G. J., Gangarosa, L. M., Coffey, R. J., Jr. & Der, C. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6924–6928
47. Leevers, S. J., Paterson, H. F. & Marshall, C. J. (1994) *Nature* **369,** 411–414
48. Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M. & Hancock, J. F. (1994) *Science* **264,** 1463–1467
49. Chuang, E., Barnard, D., Hettich, L., Zhang, X. F., Avruch, J. & Marshall, M. S. (1994) *Mol. Cell. Biol.* **14,** 5318–5325
50. Sprenkle, A. B., Davies, S. P., Carling, D., Hardie, D. G. & Sturgill, T. W. (1997) *FEBS Lett.* **403,** 254–258
51. Whitehurst, C. E., Owaki, H., Bruder, J. T., Rapp, U. R. & Geppert, T. D. (1995) *J. Biol. Chem.* **270,** 5594–5599
52. Zhang, X. F., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R. & Avruch, J. (1993) *Nature* **364,** 308–313
53. Hu, C. D., Kariya, K., Okada, T., Qi, X., Song, C. & Kataoka, T. (1999) *J. Biol. Chem.* **274,** 48–51
54. Diaz, B., Barnard, D., Filson, A., Macdonald, S., King, A. & Marshall, M. (1997) *Mol. Cell. Biol.* **17,** 4509–4516

[3] J. W. Lee, unpublished observations.

[4] Howe, A. K., and Juliano, R. L. (2000) *Nat. Cell Biol.*, in press.