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Mechanism of Inhibition of Raf-1 by Protein Kinase A

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The cytoplasmic Raf-1 kinase is essential for mitogenic signalling by growth factors, which couple to tyrosine kinases, and by tumor-promoting phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate, which activate protein kinase C (PKC). Signalling by the Raf-1 kinase can be blocked by activation of the cyclic AMP (cAMP)-dependent protein kinase A (PKA). The molecular mechanism of this inhibition is not precisely known but has been suggested to involve attenuation of Raf-1 binding to Ras. Using purified proteins, we show that in addition to weakening the interaction of Raf-1 with Ras, PKA can inhibit Raf-1 function directly via phosphorylation of the Raf-1 kinase domain. Phosphorylation by PKA interferes with the activation of Raf-1 by either PKCα or the tyrosine kinase Lck and even can downregulate the kinase activity of Raf-1 previously activated by PKCα or amino-terminal truncation. This type of inhibition can be dissociated from the ability of Raf-1 to associate with Ras, since (i) the isolated Raf-1 kinase domain, which lacks the Ras binding domain, is still susceptible to inhibition by PKA, (ii) phosphorylation of Raf-1 by PKCα alleviates the PKA-induced reduction of Ras binding but does not prevent the downregulation of Raf-1 kinase activity by PKA and (iii) cAMP agonists antagonize transformation by v-Raf, which is Ras independent.

Raf-1 is a serine/threonine kinase which plays a central role in the transduction of growth factor signals from the cell membrane to the nucleus. Stimulation of cells with various mitogens results in the rapid phosphorylation and activation of the Raf-1 kinase (reviewed in references 16, 25, and 29). At present, at least three modes of Raf-1 stimulation can be distinguished. First, activation can be mediated via tyrosine phosphorylation by membrane-associated Src family tyrosine kinases, such as Lck (10, 39). Second, certain isoforms of protein kinase (PKC) can activate Raf-1 by direct phosphorylation of serine residues in the regulatory as well as the kinase domain (6, 20, 34). Third, via its regulatory domain, Raf-1 specifically associates with the GTP-bound, active form of Ras in vitro (19, 27, 41–43, 48) and in vivo (14). Ras binding does not activate Raf-1 directly but rather serves to recruit Raf-1 from the cytosol to the plasma membrane in response to growth factor stimulation (24, 35). Translocation to the membrane seems to be a prerequisite for efficient interaction of Raf-1 with activators. The activator(s) has not been identified but might include certain PKC isozymes, e.g., PKCα, which associate with the cell membrane upon activation. This model resolves the apparent paradox that PKCα can activate Raf-1 by direct phosphorylation (6, 20, 34), yet that this process is at least partially Ras dependent in some cell types (38, 40, 45, 47). It also explains why membrane-bound Src tyrosine kinases synergize with Ras to activate Raf-1 (44).

Two downstream pathways stimulated by Raf-1 have recently been elucidated. Activated Raf-1 triggers a kinase cascade by phosphorylating Mek, a dual-specificity threonine/tyrosine kinase, which stimulates mitogen-activated protein kinase (MAPK)/Erk (8, 18, 23). Another target of Raf-1 is represented by the cytosolic complex of the NFκB/IκB transcription factor. Raf-1 phosphorylates the NFκB inhibitor IκB, leading to a release of the NFκB subunits, which are now competent for DNA binding and transcriptional activation (26). Thus, Raf-1 seems to function as a cytosolic interface which integrates upstream signals from tyrosine kinases, PKC, and Ras proteins and processes them into the MAPK and NFκB pathways.

A number of recent reports have demonstrated that protein kinase A (PKA) can interfere with the stimulation of the Ras→Raf-1→Mek→MAPK signalling pathway in living cells (5, 7, 12, 17, 31, 37, 46). The block was mapped to occur at the level of Raf-1 activation (5, 7, 31, 46). Its mechanistic basis is not clear but was suggested to comprise the reduced ability of PKA phosphorylated Raf-1 to associate with Ras proteins (46).

We and others have previously shown that PKCα activates Raf-1 by direct phosphorylation (6, 20, 34) and that PKCα synergizes with Raf-1 to transform NIH 3T3 cells (20). In this study, we have examined the interaction of PKA, PKCα, Lck, and Ras with Raf-1. Our results indicate that in addition to weakening the affinity for activated Ras, PKA inhibits PKCα- and Lck-triggered Raf-1 activation by direct phosphorylation of the Raf-1 kinase domain. This type of inhibition is of physiological relevance, since cyclic AMP (cAMP) agonists can revert transformation by v-Raf, which is Ras independent.

MATERIALS AND METHODS

Cells and reagents. NIH 3T3 cells were grown in Dulbecco modified Eagle medium (Serva) supplemented with glutamine and 10% fetal calf serum (Seromed). Cells were made quiescent by a 24-h incubation in Dulbecco modified Eagle medium without serum. To generate v-Raf-transformed cells, NIH 3T3 cells were transfected with 3611-MSV DNA (30) and seeded into soft agar. To exclude clonal variation, 20 soft agar colonies

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were pooled and designated v-Raf-transformed NIH 3T3 cells. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), forskolin, and 8-bromo-cAMP were purchased from Sigma or Biomol. Guanylimidodiphosphate (GMP-PNP) was obtained from Boehringer. Sequencing-grade trypsin (Promega) was used according to the instructions of the manufacturer. The Raf antiserum (crafVI) was raised in chinchilla bastard rabbits immunized with a synthetic peptide corresponding to the 12 carboxy-terminal amino acids of Raf-1. It recognizes both Raf-1 and v-Raf and was used at a 1:750 dilution for immunoprecipitation and 1:2,000 for Western blotting (immunoblotting). The MAPK antibody was kindly provided by Peter Shaw. This antiserum was raised by immunizing chinchilla bastard rabbits with purified Erk-2. It was used at a 1:250 dilution for immunoprecipitation. The Ha-Ras-specific monoclonal antibody LA69 was obtained from Quality Biotech.

Immunoprecipitation, immunocomplex kinase assays, and Western blotting. Cells were treated as indicated in the figure legends, washed with phosphate-buffered saline, and lysed in TBST buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 μg of leupeptin per ml) and phosphatase inhibitors (20 mM β-glycerophosphate, 2 mM sodium fluoride, and 2 mM sodium pyrophosphate). Cell lysates were cleared by centrifugation at 20,000 × *g* for 10 min and incubated with anti-Raf or anti-MAPK antiserum plus protein A-agarose (Boehringer) for 2 h. Immunoprecipitates were washed three times in TBST and once with Raf kinase buffer (20 mM Tris-HCl [pH 7.4], 20 mM NaCl, 10 mM Mg_2Cl, 1 mM dithiothreitol) and split into three aliquots. One aliquot was subjected to immunoblotting with anti-Raf serum; the other two aliquots were used for assaying Raf and MAPK activity, respectively. Raf kinase reaction mixtures contained 100 ng of purified recombinant kinase-negative Mek (11; kindly provided by Manuela Baccarini) or 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA)-inactivated Mek purified from rabbit skeletal muscle (kindly provided by Thomas Sturgill and Paul Dent) and 5 μCi of [γ-^{32}P]ATP. MAPK was assayed with 1 μg of myelin basic protein (MBP; Gibco/BRL) in the presence of 20 μM ATP and 2 μCi of [γ-^{32}P]ATP. After incubation for 30 min at 25°C, reactions were terminated by boiling in sodium dodecyl sulfate (SDS)-gel sample buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and autoradiographed. Although no coprecipitation of PKA of PKC activity was detectable by immunoblotting or autophosphorylation assays, both the Raf and MAPK assays were sometimes carried out in the presence of specific inhibitors for PKA (1 μM PKA-inhibitory peptide; Sigma) and PKC (1 μM GF109203X; Biomol) to eliminate any possible contamination by these kinases. Omission of these inhibitors did not alter the results. Western blotting was carried out as described previously (22) except that the ECL kit (Amersham) was used for detection.

Protein expression and purification. The catalytic subunit of PKA was purchased from Sigma or Boehringer or else purified from bovine heart (36), yielding identical results. *Escherichia coli* expression vectors were made as follows. To construct the GNS expression plasmid, the human Raf-1 sequence encoding amino acids 1 to 258 was inserted in frame with the glutathione *S*-transferase (GST) portion into the pGEX-KG expression vector (13). The GNX expression vector was constructed by cloning the BXB cDNA (15) as an *Nco*I-*Xba*I fragment into pGEX-KG. Recombinant proteins were purified from isopropylthiogalactopyranoside (IPTG)-induced *E. coli* cultures by glutathione-Sepharose affinity chromatography (Pharmacia). Since the kinase activity of the GNX protein produced in *E. coli* varied from batch to batch, GNX was also expressed in the baculovirus-Sf9 cell system, reproducibly yielding active protein. For this purpose, the GST portion including the 5× glycine linker and the thrombin cleavage site of pGEX-KG was inserted into the baculovirus transfer vector pAcC5 upstream of the polylinker, yielding a vector termed pGST/AcC5. Subsequently, the BXB cDNA was cloned into this vector as an *Nco*I-*Xba*I fragment being in frame with GST. After expression in Sf9 cells, the GST-BXB protein was purified by glutathione-sepharose affinity chromatography in the presence of 1% Triton X-100. To construct GST/Raf, a full-length Raf-1 cDNA was cloned in frame into the *Nco*I-*Xba*I sites of pGST/AcC5 and expressed in Sf9 cells. In the Raf/G2 mutant, a consensus motif for PKA phosphorylation was destroyed by changing the sequence Arg-Arg-Ala-Ser-43 to Leu-Glu-Ala-Ser-43 by site-directed mutagenesis. The Raf/G2, kinase-negative Raf301, and wild-type Raf-1 proteins were expressed in the baculovirus-Sf9 system and immunoprecipitated with Raf antiserum crafVI as described above. PKCα was produced in Sf9 cells and purified to homogeneity (20). Purified Lck was purchased from Upstate Biotechnology, Inc. Ha-Ras protein was purified and loaded with the nonhydrolyzable GTP analog GMP-PNP as described previously (9).

Phosphorylation of Raf proteins with PKA. Raf proteins were immobilized on glutathione-Sepharose (Pharmacia) or on anti-Raf antibody bound to protein A-agarose beads (Boehringer). After three washes with TBST, Raf proteins were washed with H_2O once and resuspended in PKA buffer (20 mM Tris-HCl [pH 7.6], 10 mM magnesium acetate, 2.5 mM β-mercaptoethanol) supplemented with 10 μM ATP and 50 μCi of [γ-^{32}P]ATP. Five units of the catalytic subunit of PKA was added, and reactions were allowed to proceed for 30 min at 25°C. After boiling in SDS-gel sample buffer, the proteins were separated on an SDS–10% gel. To determine the stoichiometry of phosphorylation, GST-Raf was purified by glutathione-Sepharose affinity chromatography and phosphorylated by PKA. Different dilutions of purified GST-Raf were compared against a bovine serum albumin dilution curve to determine the GST-Raf concentration. Under the conditions used, PKA phosphorylated Raf-1 to a stoichiometry of 3.4 mol of phosphate per mol of GST-Raf. GST-Raf yielded the same tryptic phosphopeptide pattern as immunoprecipitated Raf-1 phosphorylated by PKA.

Tryptic peptide mapping and phosphoamino acid analysis. NIH 3T3 cells were labelled with 1 mCi of [^{32}P]orthophosphoric acid per ml for 3 h and incubated with 20 μM forskolin for 30 min or left untreated. Raf-1 was immunoprecipitated and separated by SDS-PAGE. In parallel, baculovirus-produced Raf-1 was phosphorylated with PKA in vitro. Raf protein bands were cut from an SDS gel and processed for two-dimensional tryptic peptide mapping as described previously (4, 20), using pH 8.9 buffer for the first dimension. Phosphoamino acid analysis of full-length Raf-1 phosphorylated by PKA was performed as described previously (4). Briefly, tryptic Raf protein digests were hydrolyzed in 6 M HCl at 110°C for 60 min. Phosphoamino acids were resolved by two-dimensional electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension.

In vitro Raf and Mek activation assays. Immobilized Raf proteins (1 to 10 ng per assay) were phosphorylated with PKCα, Lck, or PKA in the presence of 100 μM ATP as indicated in the figure legends. PKA buffer contained 20 mM Tris-HCl (pH 7.6), 10 mM magnesium acetate, and 2.5 mM β-mercaptoethanol. PKC buffer contained 25 mM Tris-HCl (pH 7.5), 1.32 mM $CaCl_2$, 5 mM $MgCl_2$, 1.25 mM EGTA, 1 mM β-mercaptoethanol, 100 μg of phosphatidylserine (Sigma)

per ml, and 100 ng of TPA per ml. Lck buffer contained 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 2 mM $MnCl_2$. Between phosphorylation reactions, immobilized Raf proteins were washed twice with TBST and once with H_2O. To measure substrate phosphorylation, Raf proteins were resuspended in 25 μl of Raf kinase buffer supplemented with 5 μCi of [γ-^{32}P]ATP. One hundred nanograms of kinase-negative purified Mek or purified IκB (kindly provided by Ulrich Siebenlist) was added as indicated. The reaction was allowed to proceed for 20 min at 25°C before 10 μl of 4× SDS-gel sample buffer was added. The activation of the Mek→MAPK→MBP phosphorylation cascade by Raf was tested in a similar fashion except that the kinase assay mixtures contained 20 μM ATP and 2.5 μCi of [γ-^{32}P]ATP as well as 50 ng of purified Mek, 100 ng of MAPK, and 0.5 μg of MBP. Raf proteins were omitted from control reactions. In some cases, specific inhibitors for PKA (1 μM PKA-inhibitory peptide; Sigma) and PKC (1 μM GF109203X; Biomol) were added to the assays to exclude any possible contamination by PKCα or PKA. The presence or absence of inhibitors did not affect the results.

Ras/Raf binding assay. Raf-1 and Raf/G2 proteins were immunoprecipitated from Sf9 cells, washed, and phosphorylated with PKCα, PKA, or PKA followed by PKCα as described above. After three washes with TBST, the immunoprecipitates were incubated with 0.5 μg of GMP-PNP-loaded Ha-Ras (9) in 1 ml of TBST containing 4% bovine serum albumin as a nonspecific competitor, protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 μg of leupeptin per ml), and phosphatase inhibitors (20 mM β-glycerophosphate, 2 mM sodium fluoride, and 2 mM sodium pyrophosphate) for 1 h at 4°C. After three washes with TBST and one wash with Raf kinase buffer, Raf phosphotransferase activity was measured with IκB as the substrate. The reaction mixtures were separated on an SDS–12.5% polyacrylamide gel and blotted. The blot was autoradiographed to display IκB phosphorylation and subsequently stained (ECL kit) with anti-Raf or anti-Ras antibodies to visualize Raf proteins and associated Ras.

RESULTS

PKA interferes with PKC-stimulated activation of Raf-1 in vivo and blocks transformation by v-*raf*. To measure the impact of PKA activation on the PKC-mediated activation of Raf-1, normal or v-Raf-transformed NIH 3T3 cells were serum starved and subsequently treated with TPA or TPA plus forskolin, a drug which elevates cAMP levels by stimulation of adenylate cyclase (32). Cell lysates were prepared and examined for PKC, Raf, and MAPK activity (Fig. 1).

TPA treatment activated PKC, as measured in cell lysates activity with a PKC assay kit (Amersham), while forskolin had no significant effect on total PKC activity in the lysate. Since PKCα represents the by far most abundant PKC isoform in NIH 3T3 cells, purified PKCα was incubated with the PKA catalytic subunit in vitro. PKA did not phosphorylate nor inhibit PKCα activity in vitro (data not shown).

As expected, TPA stimulation enhanced the phosphotransferase activity of Raf-1 immunoprecipitates toward Mek (Fig. 1a). In contrast, Raf immunoprecipitates from TPA-plus-forskolin-treated NIH 3T3 cells were severely compromised in the ability to phosphorylate Mek. Forskolin also downregulated the constitutive as well as the TPA-induced Mek kinase activity of Raf immunoprecipitates prepared from v-Raf-transformed cells (Fig. 1b). In both cell lines, PKA activation interrupted the Mek→MAPK signalling cascade, as evidenced by the failure of MAPK immunoprecipitates from forskolin treated cells to phosphorylate MBP efficiently (Fig. 1).

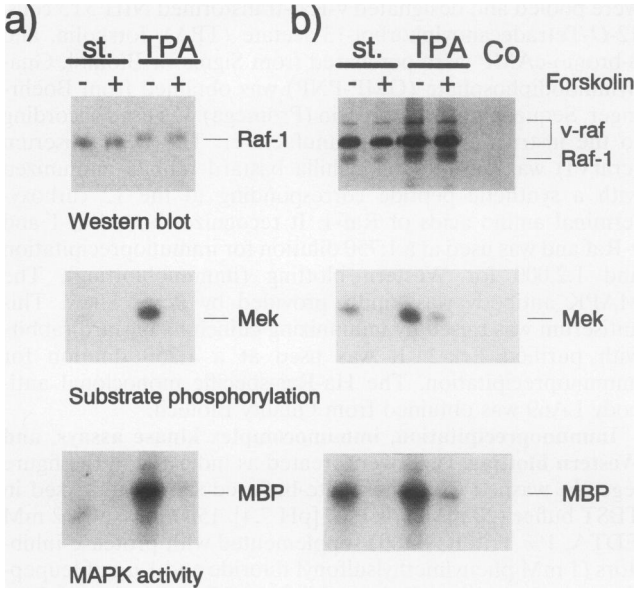

FIG. 1. cAMP agonists antagonize Raf function in normal and v-Raf-transformed NIH 3T3 cells. (A) Elevation of cAMP prevents activation of the Raf-1 kinase by TPA leading to interruption of the Mek→MAPK signalling cascade. Raf proteins were immunoprecipitated from serum-starved (st.) NIH 3T3 cells, which were treated as indicated with TPA (100 ng/ml) or forskolin (20 μM) for 15 min. TPA and forskolin were added simultaneously. Cell lysates were split into three aliquots. One aliquot was immunoprecipitated with antiserum crafVI and immunoblotted with the same antiserum, using the ECL kit (Amersham). Another aliquot was immunoprecipitated with antiserum crafVI and assayed for phosphotransferase activity toward purified kinase-negative Mek. The third aliquot was immunoprecipitated with anti-MAPK antiserum, and MAPK activity was assayed with MBP as the substrate. (B) The same experiment was conducted with v-Raf-transformed NIH 3T3 cells. In the control (Co) lane, Mek or MBP was incubated under kinase conditions without addition of Raf or MAPK immune complexes, respectively.

As previous reports (12, 46) and our own results (1) have shown that neither Mek nor MAPK is a PKA substrate, these results suggest that PKA can directly suppress the kinase activity of both Raf-1 and a deregulated Raf kinase domain represented by v-Raf. To exclude the possibility of unspecific Raf inhibition, forskolin was added to in vitro kinase assays carried out with purified Raf-1 or baculovirus-expressed GST-BXB proteins. It did not alter Raf-1 kinase activity (1).

To test whether PKA activation would be able to antagonize Raf transformation, v-Raf-transformed NIH 3T3 cells were seeded in soft agar and treated with forskolin or 8-bromo-cAMP. Both substances inhibited the growth of soft agar colonies in a dose-dependent manner (Fig. 2), showing that activation of PKA can impede the function of a deregulated Raf kinase domain in vivo and block Raf transformation. At the concentrations used, neither drug was cytotoxic for v-Raf-transformed cells kept in monolayer culture for up to 2 weeks in the presence of drug, although the doubling time increased by approximately 50%. In contrast, cAMP agonists did not affect the capability of simian virus 40 large-T-antigen-transformed NIH cells to grow in soft agar (data not shown).

Raf-1 is a substrate for PKA. Taken together, these observations indicated that PKA might inhibit Raf-1 function directly. We therefore tested whether Raf-1 serves as an in vitro substrate for PKA (Fig. 3). For this purpose, Raf-1 and a

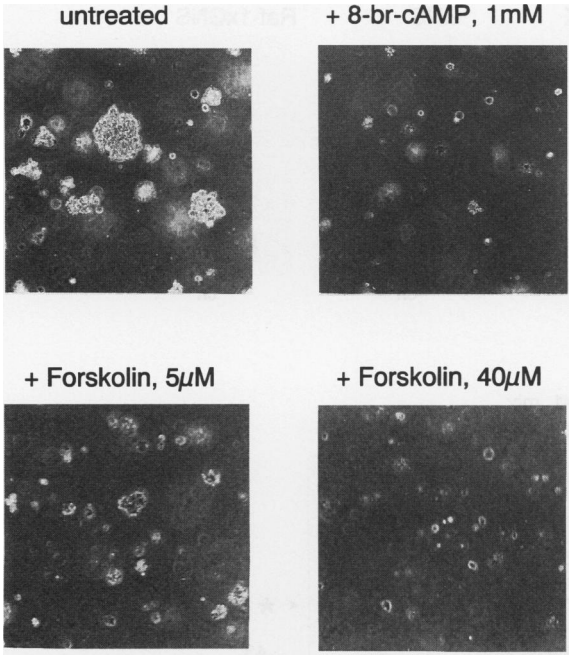

FIG. 2. cAMP agonists inhibit v-Raf transformation. v-Raf-transformed NIH 3T3 cells were seeded into soft agar and overlaid with medium containing forskolin or 8-bromo (br)-cAMP as indicated. The overlay was replenished every third day. Photographs were taken on day 12 at ×100 magnification. The experiments were repeated three times with consistent results.

kinase-negative mutant, Raf301 (16, 22), were expressed in the baculovirus-Sf9 cell system and purified by immunoprecipitation. In addition, amino acids 1 to 258 of the regulatory domain (GNS) and the complete Raf-1 kinase domain (GNX; derived from BXB [15]) were expressed as GST fusion proteins in *E. coli* and purified by glutathione affinity chromatography. Raf proteins were incubated with the catalytic subunit of PKA in the presence of [γ-^{32}P]ATP. Under PKA assay conditions, the autophosphorylation of Raf-1 and GNX was negligible compared with the PKA-mediated phosphorylation. As evidenced by tryptic peptide mapping (Fig. 4A), PKA phosphorylated eight sites in Raf-1 and Raf301 with a mean stoichiometry of 3.4 mol of phosphates incorporated per mol of Raf-1 protein (see Materials and Methods for experimental details). The GST-Raf fusion proteins GNS and GNX exhibited one predominant phosphorylation site each, which corresponded to major sites targeted by PKA in full-length Raf-1, as revealed by mixing experiments (indicated by arrows in Fig. 4A). Upon longer exposure, other phosphorylation sites became visible in the GNS and GNX peptide maps, which each matched a distinct subset of phosphopeptides observed in full-length Raf-1. We do not know at present why PKA preferentially phosphorylated one site in the context of the Raf deletion proteins GNS and GNX. This phenomenon did not seem to be due to grossly improper folding of the *E. coli* expression proteins, since GNS and GNX expressed in the baculovirus-Sf9 system displayed a similar phosphorylation site preference (1).

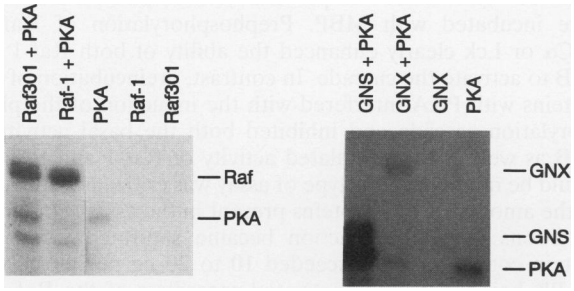

FIG. 3. PKA phosphorylates Raf-1 in vitro. Raf-1 and the kinase-negative point mutant Raf301 were expressed in the baculovirus-Sf9 cell system and purified by immunoprecipitation with serum crafVI. GNS and GNX proteins were expressed in *E. coli* and isolated by glutathione affinity chromatography. GNS encodes amino acids 1 to 258 of the Raf-1 regulatory domain fused to GST. GNX represents the complete Raf-1 kinase domain derived from BXB and tagged with GST. Purified Raf proteins were incubated with the PKA catalytic subunit in PKA buffer plus 10 μM ATP and 20 μCi of [γ-^{32}P]ATP for 30 min at 25°C. Raf-1 autophosphorylation was very minor under these assay conditions. Shown is a 1-h exposure of a representative SDS gel.

To compare whether the in vitro phosphorylation sites correspond to sites phosphorylated in vivo, NIH 3T3 cells were labelled with [^{32}P]orthophosphoric acid and either left untreated or incubated with forskolin for 30 min. Raf-1 was immunoprecipitated, purified by SDS-PAGE, digested with trypsin, and subjected to two-dimensional phosphopeptide mapping (Fig. 4B). The basal phosphopeptide pattern of Raf-1 prepared from cells was remarkably similar to the pattern produced by PKA phosphorylation in vitro. Forskolin treatment did not induce novel phosphorylation sites but augmented phosphorylation of four peptides, which resolved ascending from the origin in the second dimension (Fig. 4B). Three of the PKA sites observed in vitro did not appear in the in vivo maps (shown as open circles in Fig. 4B), and one minor in vivo site (shaded circle in Fig. 4B) was not recognized by PKA in vitro. The other spots, however, comigrated, indicating that a subset of Raf-1 phosphorylation sites targeted by PKA in vitro correspond to sites phosphorylated in vivo. Notably, the two predominant peptides (marked by arrows in Fig. 4B) phosphorylated in the GNS and GNX proteins in vitro matched sites enhanced by forskolin treatment in vivo. By use of a Raf-1 mutant, the major GNS site was shown to be serine 43 (see Fig. 7A), which also has been described as a PKA-inducible site by Wu et al. (46). Mapping of the major GNX site is currently under way in our laboratory. Phosphoamino acid analysis of in vitro phosphorylated Raf-1 revealed serine as the main phosphoamino acid (Fig. 5).

PKA phosphorylation inhibits Raf-1 kinase activity. To measure the impact of PKA phosphorylation on Raf phosphotransferase activity, we used two versions of Raf-1, full-length Raf-1 and an isolated Raf-1 kinase domain, BXB, tagged with GST. The BXB sequence retains phosphorylation sites required for activation by PKCα (20) as well as by tyrosine kinases such as Lck (10). The proteins were expressed in the baculovirus-Sf9 cell system and purified by immunoprecipitation or glutathione-Sepharose affinity chromatography. Immobilized Raf proteins were incubated with PKCα, the tyrosine kinase Lck, which has been shown to phosphorylate and activate Raf-1 (10, 39), the catalytic subunit of PKA or without enzymes in the appropriate buffers supplemented with 100 μM ATP. After washing, Raf phosphotransferase activity was monitored by using kinase-inactive Mek or IκB, both described as physiological Raf-1 substrates (8, 18, 23, 26) (Fig. 6A). No contamination of the Raf pellets with Lck or PKCα could be detected under these conditions. In some experiments, specific inhibitors for PKA (1 μM PKA-inhibitory peptide) and PKC (1 μM GF109203X) were added to the assays to exclude any otherwise undetectable carryover contamination by PKCα or

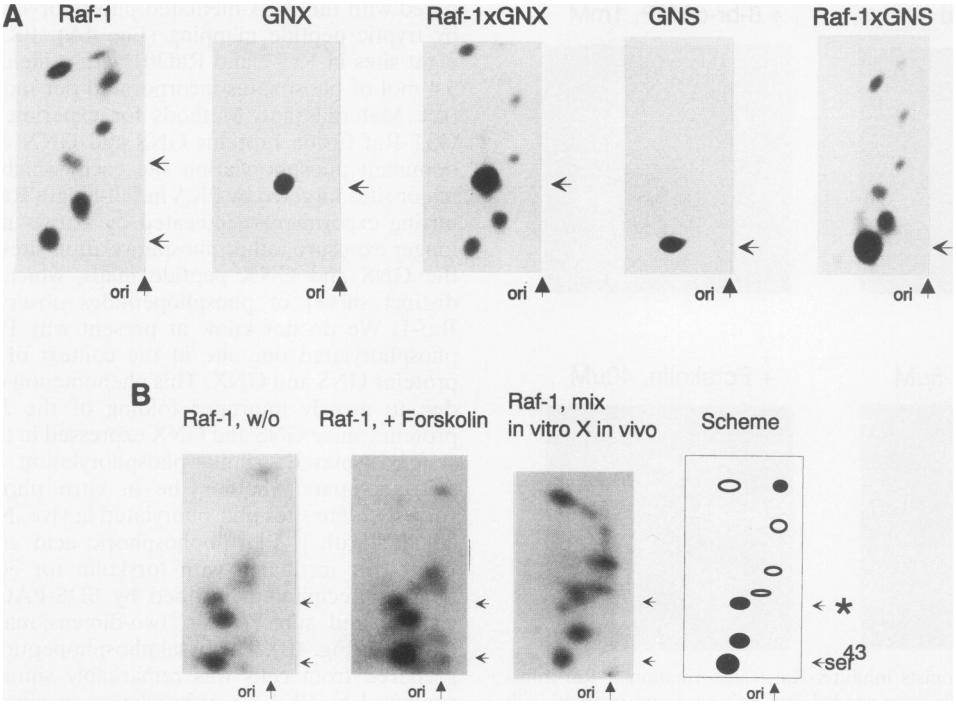

FIG. 4. (A) Tryptic phosphopeptide maps of Raf proteins labelled by PKA in vitro. The origin (ori) is marked by an arrow. Electrophoresis in pH 8.9 buffer was in the horizontal direction with the anode on the left. Thin-layer chromatography in phosphochromatography buffer (4) was in the vertical direction. Major PKA phosphorylation sites in Raf-1 which correspond to the predominant sites phosphorylated in the GNS and GNX proteins are indicated. (B) Tryptic phosphopeptide maps of Raf proteins labelled in vivo. Raf-1 was immunoprecipitated from NIH 3T3 cells, which had been labelled with [^{32}P]orthophosphoric acid for 3 h and incubated with 20 μM forskolin for 30 min or were left untreated. For comparison, the tryptic digest of Raf-1 phosphorylated by PKA in vitro was mixed with Raf-1 prepared from forskolin-treated cells. w/o, without enzymes. To facilitate interpretation, a schematic compilation of the phosphopeptide maps is shown. Phosphopeptides common to in vitro and in vivo maps are displayed as closed circles, spots unique to Raf-1 phosphorylated in vitro are shown as open circles, and one site apparent only in the in vivo map is represented as a shaded circle. The major site phosphorylated in GNX is indicated by an arrow plus asterisk. The major GNS site was identified as serine 43 (see Fig. 7A).

PKA. The presence or absence of inhibitors did not affect the results. Raf protein input was controlled by Western blotting. The basal kinase activity of Raf-1 was low; that of BXB was about threefold higher. Both Raf versions could be significantly activated by pretreatment with PKCα or Lck. In contrast, PKA efficiently suppressed PKCα- or Lck-mediated Raf activation, indicating that PKA can inhibit both full-length Raf-1 as well as a deregulated Raf-1 kinase domain by direct phosphorylation. Comparable results were obtained using either Mek or IκB as a substrate.

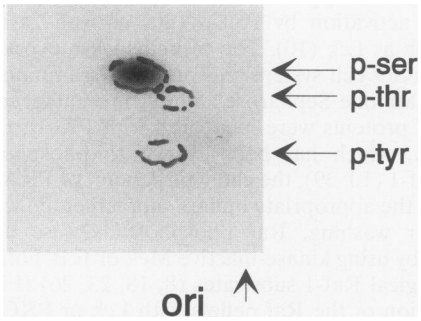

FIG. 5. Phosphoamino acid analysis of Raf-1 phosphorylated by PKA in vitro. Serine is the predominant amino acid phosphorylated by PKA. p-ser, phosphoserine; p-thr, phosphothreonine; p-tyr, phosphotyrosine; ori, origin.

In addition to substrate phosphorylation, we also reconstituted the Raf→Mek→MAPK→MBP phosphorylation cascade in vitro (Fig. 6A). Very little MBP phosphorylation was observed when either Raf proteins or Mek plus MAPK alone were incubated with MBP. Prephosphorylation of Raf by PKCα or Lck clearly enhanced the ability of both Raf-1 and BXB to activate the cascade. In contrast, preincubation of Raf proteins with PKA interfered with the induction of the phosphorylation cascade and inhibited both the basal activity of BXB as well as the stimulated activity of Raf-1 and BXB. It should be noted that this type of assay was critically dependent on the amount of Raf proteins present in the assay. Under the conditions used, the reaction became saturated when Raf protein concentrations exceeded 10 to 20 ng per assay, with MAPK being maximally activated regardless of the Raf pretreatment. These results suggest that PKA reduces but does not totally annihilate Raf activity. This is consistent with the observation that forskolin reverted v-Raf transformation but did not inhibit proliferation as profoundly as ablation of Raf function by expression of Raf antisense RNA or the dominant negative Raf301 mutant did (21).

The finding that PKA could still downregulate constitutively active forms of Raf, such as v-Raf and BXB, prompted us to test whether PKA could deactivate Raf-1 stimulated by PKCα (Fig. 6B). Baculovirus-expressed Raf-1 was immunoprecipi-

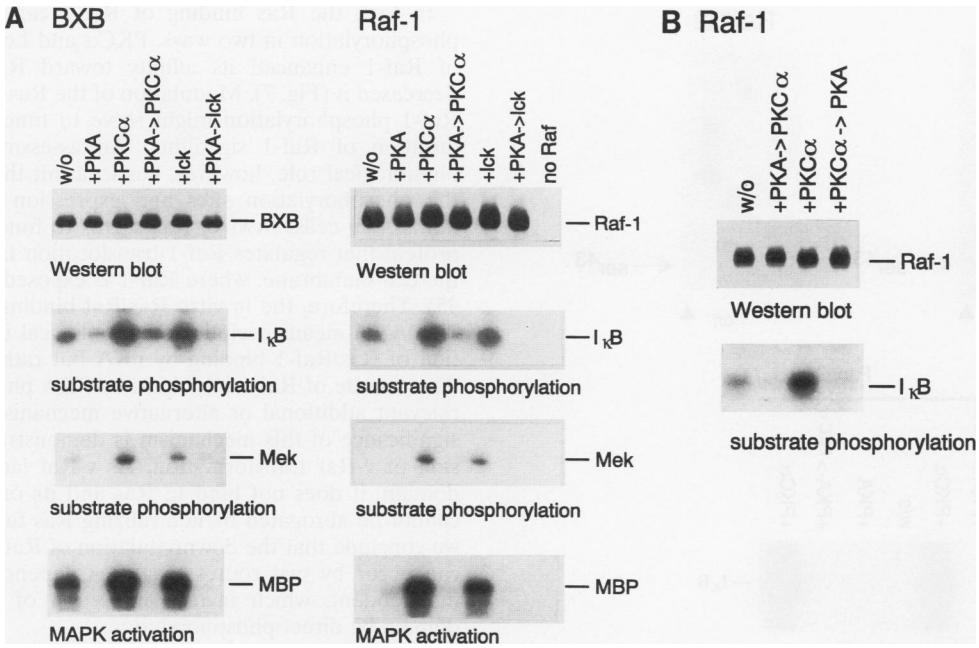

FIG. 6. PKA inhibits the Lck- and PKCα-stimulated phosphotransferase activity of full-length Raf-1 as well of a deregulated Raf-1 kinase domain, BXB. (A) Raf-1 expressed in the baculovirus-Sf9 cell system was immunopurified. The GST-tagged BXB protein encompassing the Raf-1 kinase domain was purified by glutathione-Sepharose affinity chromatography. Raf proteins were prephosphorylated with PKA, PKCα, Lck, or PKA followed by PKCα or Lck. After each phosphorylation step, the immobilized Raf proteins were washed with TBST, H_2O, and the appropriate kinase buffer. No carryover contamination of PKCα or Lck was detectable after this treatment. The input of Raf proteins was controlled by Western blotting of an aliquot of the reaction mixes. Raf phosphotransferase activity was measured with purified IκB or kinase-negative Mek as the substrate. Further, the Raf proteins were used to reconstitute the Mek→MAPK→MBP phosphorylation cascade in vitro (for experimental details, see Materials and Methods). The "no Raf" lane contains control reactions with IκB, kinase-negative Mek, or Mek plus MAPK, respectively. The kinase assays shown were carried out in the presence of specific inhibitors for PKA (1 μM PKA-inhibitory peptide) and PKC (1 μM GF109203X; Biomol) to exclude any possible contamination by PKCα or PKA. The same results were obtained when these inhibitors were omitted. (B) PKA suppresses the kinase activity of PKCα-activated Raf-1. Immunopurified Raf-1 was prephosphorylated with PKCα, PKA followed by PKCα, or PKCα followed by PKA. Raf-1 phosphotransferase activity was assayed with IκB as the substrate. w/o, without enzymes.

tated and sequentially treated with PKA and PKCα or PKCα followed by PKA. The inhibitory effect of PKA was dominant. PKA phosphorylation of Raf-1 did not preclude subsequent phosphorylation of Raf-1 by PKCα or Lck (1), arguing that PKA targets phosphorylation sites with a dominant inhibitory effect. This interpretation is in keeping with findings reported by Russell et al. (31), who showed that activation of PKA in Rat-1a cells can reverse epidermal growth factor-mediated stimulation of Raf-1 kinase activity.

Raf-1 inhibition by PKA is independent of Ras/Raf-1 association. A recent report has shown that PKA-phosphorylated Raf-1 is impaired in its ability to bind activated Ras proteins and suggested that phosphorylation of serine 43 in Raf-1 might be responsible for disruption of Ras/Raf association and downstream signalling (46). Mainly because the isolated Raf-1 kinase domain, which lacks the Ras binding domain, can still be inhibited by PKA phosphorylation, our results point to a direct mechanism of PKA-mediated Raf inhibition exerted by functional downregulation due to phosphorylation by PKA.

To further explore the role of Ras/Raf association, we used a Raf-1 mutant, Raf/G2, in which the serine 43 PKA consensus phosphorylation motif has been destroyed by site-directed mutagenesis. Baculovirus-expressed Raf/G2 was immunopurified and phosphorylated with PKA. Tryptic peptide mapping demonstrated that Raf/G2 lacks a major PKA phosphorylation site, which corresponds to the main site phosphorylated in the GNS protein (Fig. 7A; compare with Fig. 4). Immunopurified Raf-1 or Raf/G2 proteins were phosphorylated with PKCα, PKA, or PKA followed by PKCα in the appropriate buffers plus ATP and then incubated with purified Ha-Ras protein loaded with the nonhydrolyzable GTP analog GMP-PNP. After washing, the Raf phosphotransferase activity was assayed with IκB as the substrate. Raf protein input and bound Ras were visualized by Western blotting (Fig. 7B). PKA treatment diminished the association of both wild-type and mutant Raf-1 with Ras, while Raf phosphorylation by PKCα counteracted this effect. Nevertheless, the PKCα-induced kinase activity of both versions of Raf proteins was still downmodulated by PKA, showing that inactivation of Raf-1 by PKA can occur despite substantial Ras binding.

DISCUSSION

In conclusion, we have shown (i) that Raf-1 is a substrate for PKA, (ii) that PKA directly inhibits Raf-1 activation by PKC in vitro and in vivo, (iii) that PKA reduces the activity of a deregulated Raf kinase domain in vitro, and (iv) that cAMP agonists counteract v-Raf transformation. These results define a mechanistic basis for the negative regulation of Raf-1 by PKA. They also reveal a molecular basis for the PKA-PKC antagonism observed in some signalling systems (2, 3, 33), which pinpoints Raf-1 as the pivotal enzyme integrating both stimulatory and inhibitory signalling pathways.

At present, at least three partially interdigitating mechanisms of Raf-1 activation, involving Ras proteins, Src family tyrosine kinases, and PKC, can be distinguished. Recent data

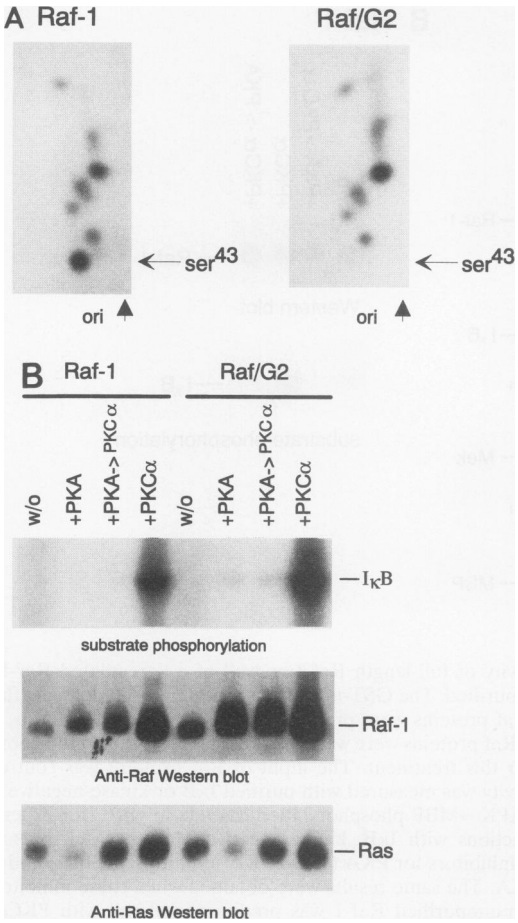

FIG. 7. Inhibition of Raf-1 by PKA is Ras independent. (A) Phosphopeptide maps of wild-type Raf-1 and Raf/G2 phosphorylated by PKA. In Raf/G2, the PKA consensus phosphorylation sequence Arg-Arg-Ala-Ser-43 was changed to Leu-Glu-Ala-Ser-43 by site-directed mutagenesis, resulting in loss of serine 43 phosphorylation. (B) Immunopurified Raf-1 and Raf/G2 proteins were prephosphorylated with PKA, PKCα, PKA followed by PKCα, or without enzymes (w/o). Phosphorylated Raf pellets were incubated with 0.5 μg of GMP-PNP-loaded Ras in TBST substituted with 4% bovine serum albumin, protease, and phosphatase inhibitors for 1 h at 4°C. After three washes with TBST and one wash with Raf kinase buffer, Raf phosphotransferase activity was measured with IκB as the substrate. The reaction mixtures were separated on an SDS–12.5% gel, blotted, and autoradiographed. Raf protein input and associated Ras proteins were visualized by immunoblotting with antibodies specific for Raf-1 (crafVI) and for Ras (LA69; Quality Biotech), respectively. ori, origin.

in conjunction with our results indicate that PKA modulation of Raf-1 activation might be of a similar complexity. Wu et al. (46) found that cAMP agonists prevent stimulation of MAP kinase by the epidermal growth factor receptor and that this blockade is due to inhibition of Raf-1 activation, which interrupts the signalling cascade Raf-1→Mek kinase→MAPK. They further showed that elevation of cAMP levels in the cell induces hyperphosphorylation of Raf-1 at serine 43 and reduces the binding affinity of Raf-1 to Ras. Our results with the Raf/G2 mutant confirm that serine 43 is a PKA target site but illustrate that serine 43 phosphorylation is not solely responsible for controlling Ras/Raf interaction. Since PKA phosphorylation of Raf/G2 still decreases association with Ras, other sites must be involved as well.

In fact, the Ras binding of Raf-1 can be modulated by phosphorylation in two ways. PKCα and Lck phosphorylation of Raf-1 enhanced its affinity toward Ras, whereas PKA decreased it (Fig. 7). Modulation of the Ras/Raf interaction by Raf-1 phosphorylation might serve to tune the strength and duration of Raf-1 signalling. An assessment of the exact physiological role, however, has to await the identification of the phosphorylation sites and expression of corresponding mutants in cells. In vivo, Ras seems to function as a docking protein that regulates Raf-1 translocation from the cytosol to the cell membrane, where Raf-1 is exposed to activators (24, 35). Therefore, the in vitro Ras/Raf binding experiment (Fig. 7B) by no means excludes a physiological impact of attenuation of Ras/Raf-1 binding by PKA but rather establishes the direct mode of Raf-1 inhibition by PKA phosphorylation as a relevant additional or alternative mechanism. The biological significance of this mechanism is demonstrated by the reversion of v-Raf transformation. As v-Raf lacks the regulatory domain, it does not bind to Ras and its oncogenic potential cannot be abrogated by neutralizing Ras function (28). Thus, we conclude that the downregulation of Raf-1 by PKA in cells can occur by two routes, one Ras dependent and one Ras independent, which involve inhibition of the Raf-1 kinase domain by direct phosphorylation.

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The first two authors contributed equally to this work.

ADDENDUM IN PROOF

We have assessed the PKA-mediated inhibition of the Raf→Mek→MAPK signalling pathway in living cells more directly, supporting the results reported in this paper. Raf-1 immunoprecipitates prepared from TPA- or TPA- and forskolin-treated NIH 3T3 cells were tested for Mek activation with a linked Mek→MAPK$^-$ assay to test whether Raf-1-phosphorylated Mek can phosphorylate a kinase-negative MAPK. While Raf-1 immunoprecipitates from TPA-treated cells induced Mek activation, simultaneous administration of forskolin reduced Mek activation by Raf-1 to the basal level.

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