Activation of Raf-1 Signaling by Protein Kinase C through a Mechanism Involving Raf Kinase Inhibitory Protein*

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From the **Department of Neurobiology, Pharmacology and Physiology, **Committee on Cancer Biology, and **Ben May Institute for Cancer Research, University of Chicago, Chicago, Illinois 60637 and ***Lilly Research Laboratories, DC1453, Indianapolis, Indiana 46285

Protein kinase C (PKC) regulates activation of the Raf-1 signaling cascade by growth factors, but the mechanism by which this occurs has not been elucidated. Here we report that one mechanism involves dissociation of Raf kinase inhibitory protein (RKIP) from Raf-1. Classic and atypical but not novel PKC isoforms phosphorylate RKIP at serine 153 (Ser-153). RKIP Ser-153 phosphorylation by PKC either in vitro or in response to 12-O-tetradecanoylphorbol-13-acetate or epidermal growth factor causes release of RKIP from Raf-1, whereas mutant RKIP (S153V or S153E) remains bound. Increased expression of PKC can rescue inhibition of the mitogen-activated protein (MAP) kinase signaling cascade by wild-type but not mutant S153V RKIP. Taken together, these results constitute the first model showing how phosphorylation by PKC relieves a key inhibitor of the Raf/MAP kinase signaling cascade and may represent a general mechanism for the regulation of MAP kinase pathways.

The MAP1 kinase cascade, an evolutionarily conserved signaling module, stimulates numerous biological processes including growth and differentiation. The known elements of the pathway include a MAP kinase kinase that phosphorylates and activates a MAP kinase, which, in turn, phosphorylates the threonine-X-tyrosine (TXY) activation domain of MAP kinase (reviewed in Ref. 1). The first characterized subfamily of MAP kinases, termed extracellular signal-regulated kinases (ERKs), is activated by growth factors and other stimuli via a cascade involving Ras, Raf-1 kinase, and MEK/ERK kinase (MEK). Activation of MAP kinase is under exquisite regulatory control, particularly at the level of Raf-1 activation. The N-terminal regulatory domain of Raf-1 interacts with Ras leading to dephosphorylation at negative regulatory sites, conformational changes to expose the kinase domain, and subsequent phosphorylation at activating sites such as serine 338 (Ser-338) and tyrosine 341 (Tyr-341) (reviewed in Ref. 2). A variety of studies have shown that protein kinase C (PKC) isoforms are also capable of activating Raf-1 (3–5) and/or the downstream MEK (6), but the mechanism has not been elucidated.

The PKC family of serine/threonine kinases are key mediators of several physiological processes including growth, death, differentiation, and transformation (reviewed in Ref. 7). There are three major classes of PKCs that are distinguished by their physiological activators. The classical PKCs (α, βI, βII, and γ) require both Ca²⁺ and diacylglycerol (DAG) for activation whereas the novel PKCs (δ, ε, η, and θ) are Ca²⁺-independent but still require DAG. Both of these classes of PKCs are activated by phorbol esters that mimic the DAG stimulus. In contrast, the atypical PKCs, ζ and η, are Ca²⁺-, DAG-, and phorbol ester-independent. Not only are PKCs able to activate Raf-1, but in a number of cell systems they are required for the activation of ERKs by growth factors (8–11).

Multiple hypotheses have been proposed to explain how PKCs activate the ERK cascade, including direct phosphorylation of either MEK (12) or Raf-1. Although the phosphorylation of MEK by PKCs is controversial (6), Raf-1 is phosphorylated by PKCs at multiple sites. For example, PKCs phosphorylate Raf-1 at serine 499 (13), but mutation of this residue did not impede activation of Raf-1 by the physiological stimulators Ras and Lek. Similarly, both v-Src and phorbol esters were able to activate Raf-1 even though the PKC phosphorylation sites at serine 497 and serine 499 were mutated to alanine (14). Thus, although some PKC phosphorylation sites on Raf-1 have been identified, these sites do not appear to be required for activation of Raf-1.

Recent studies from our laboratory suggest that ERK activation is mediated by specific PKC isoforms in response to different growth factors (10, 11). In both the conditionally immortalized hippocampal cell line H19-7 (15) and primary E16 rat hippocampal cells, two different PKC isoforms, PKCζ and PKCδ, mediate ERK activation by epidermal growth factor (EGF) and fibroblast growth factor, respectively (10, 11). Both PKCs are required for activation of ERKs at a step upstream of MEK and either downstream or at the level of Raf-1. Although these studies suggested that PKCζ activates Raf-1 in response to EGF, phosphorylation of neither Raf-1 nor MEK appeared to be responsible (3, 6, 16) (data not shown). Furthermore, phos-
phatase inhibitors such as okadaic acid did not alleviate the requirement for PKC (data not shown). We therefore considered the possibility that Raf-1 regulatory proteins might be potential targets of PKC.

The Raf-1 kinase inhibitor protein (RKIP), was identified recently (17) by yeast two-hybrid cloning utilizing the kinase domain of Raf-1 as bait. RKIP was found to be a member of the phosphatidylinositol-anchor-binding protein (PEBP) family, a ubiquitously expressed protein with homologues in Saccharomyces cerevisiae, Caenorhabditis elegans, and Drosophila melanogaster that display high degrees of interspecies sequence similarity (18). There are at least three RPK-like PEBPs in rat, two in mice, and one in human (19).

Also, amino acids 2–12 of PEBP are identical to the hippocampal cholinergic neurostimulatory peptide (HCNP) that stimulates acetylcholine synthesis in rat septal nuclei (20). PEBPs are distinct from other known proteins, and their function has remained largely enigmatic. In addition to binding phospholipids, PEBPs bind nucleotides and opioids and were shown recently (21) to inhibit thrombin. A role for PEBP in signaling was demonstrated when it was shown that RKIP binds to Raf-1. RKIP itself is neither a kinase nor a substrate for Raf-1 or MEK (17) but has been reported to be a specific inhibitor of MEK binding to Raf-1 (22), suppressing both Raf-1-induced transformation and AP-1-dependent transcription.

In the present study we demonstrate that one mechanism by which PKCs activate Raf-1 signaling to ERK involves the loss of inhibition by RKIP. Although both phospholipids and PKCs have been well established as activators of MAP kinases, the targets for PKCs in this pathway are surprisingly unknown. Because phospholipids are tumor promoters, and several components of the MAP kinase pathway are mutated in human cancers, the mechanism of PKC-mediated Raf/MAP kinase activation is of paramount importance. These results provide an explanation of how PKCs can physiologically regulate Raf-1 signaling.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction—**All constructs were made by the PCR using pCMV-HA-RKIP as template (a gift from K. Yeung, Brown University). The forward (F) and reverse (R) primers were as follows: F, 5′-atg gcc gac gac aat cag tag-3′; R, 5′-ctc ccc aac cag ctc aat gct-3′. For the pCR-HA-RKIP and FLAG-RKIP constructs the forward primers were as follows: F-HA, 5′-ggc tcc atc atg tac cca tat gac gtt cca gac tac gct gcc-3′; F-FLAG, 5′-ggc tcc atc atg tac cca tat gac gtt cca gac tac gct gcc-3′. The reverse primer (R-stop) was 5′-cata ccc ccc aac cag ctc aat gct-3′. The PCR products were ligated into the pCR3.1 vector to make pCR-HA-RKIP or FLAG-RKIP constructs. The forward primers were pCMV-HA-RKIP as template (a gift from K. Yeung, Brown University).

Directed mutagenesis of Ser-153 to valine was done utilizing pGEX-RKIP and the unique site elimination (U.S.E.) kit (Amerham). The following mutagenic primer was used: 5′/H11032/ggc aag ttc aag gtg gag gag ttt cga aag aag-3′. This primer binds to the negative strand of pGEX-RKIP and introduces a silent mutation resulting in the creation of a unique SacI site. The PCR product was ligated into pGEX-RKIP as template and the unique site elimination (U.S.E.) kit (Amerham). The following mutagenic primer was used: 5′/H11032/ggc aag ttc aag gtg gag gag ttt cga aag aag-3′. This primer introduces a silent mutation, resulting in the creation of a unique EcoRI site and conversion of Ser-153 to glutamic acid (S153E) mutagenesis was done utilizing pGEX-RKIP as template where necessary. 24 h following transfection, cells were starved overnight and subsequently treated as indicated. The amount of HA-RKIP in each sample was determined by immunoblot analysis with an anti-HA monoclonal antibody (3F10, Roche Molecular Biochemicals). Relative kinase activity was measured with a phosphorimager and normalized to the amount of HA-RKIP in each sample. For down-regulation of endogenous RKIP, H19-7 cells were transfected with empty vector, HA-RKIP, or HA-RKIP plus the RKIP antisense construct AS-C143 and pHACT (17). 24 h later, cells were starved overnight, and activation of HA-RKIP was assayed as above. In some experiments, ERK activation was determined by immunoblotting with anti-phospho-MAP kinase antibodies. Samples were quantified by digital analysis (Alpha Innotech).

**In Vitro GST Binding Assays—**In vitro binding assays were carried out as described (23) with modifications. Glutathione-Sepharose 4B was blocked with 10% normal goat serum followed by incubation with 2 μg of GST or GST-RKIP, Coupled GST fusion proteins were then incubated with 100 ng of PKCζ with 10% bovine serum albumin as nonspecific competitor for 1 h at 4 °C followed by extensive washing in TENNS buffer (2.5 mM Tris, pH 7.4, 2.5 mM EDTA, 250 mM NaCl, 1% Nonidet P-40, 2.5% sucrose). Bound proteins were resolved in a 12.5% acrylamide gel, transferred to nitrocellulose, and immunoblotted with anti-PKCζ antisera (Santa Cruz Biotechnology, Inc.). For in vitro binding, GST-RKIP binding assays, GST fusion proteins were coupled to glutathione-Sepharose 4B as above. 600 ng of bacterially expressed Raf-1 kinase domain (CBP-CR3) was added and incubated at 4 °C for 2 h. After extensive washing in TENNS buffer, 100 ng of PKCζ was added, and kinase assays were carried out as above. Bound proteins were resolved in a 12.5% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with anti-Raf-1 antibodies.

**Raf and RKIP Co-immunoprecipitations—**Co-immunoprecipitation of Raf and RKIP were carried out in a modification of a procedure described previously (17). To observe association, immunoprecipitations were done with similarly mild conditions of sonication in phosphate-buffered saline without detergent. 7 μg of Myc-Raf-1 and 3 μg of HA-RKIP or HA-RKIP(S153V) were transfected into COS-7 cells. 24 h later, cells were starved overnight and subsequently treated as indicated in the figures. Cells were lysed by sonication in cold PBS plus protease inhibitors and cleared by centrifugation. To immunoprecipitate myc-Raf-1, 1 μg of anti-Myc monoclonal antibody (9E10; Upstate Biotechnology, Inc.) was added to cell lysate and incubated at 4 °C followed by addition of 25 μl of protein G-Sepharose (1:1 slurry). The complex was washed three times with cold PBS and boiled in 5X PAGE sample buffer. The proteins were separated in 12.5% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-HA antibodies (12CA5-HRP, Roche Molecular Biochemicals) to detect HA-RKIP(S153V) and anti-Raf-1 antibodies (Santa Cruz Biotechnology, Inc.) to document amounts of Myc-Raf-1 in all samples.

**Antibody Production—**Anti-RKIP antibodies were made by immunization of rabbits with purified GST-RKIP (a gift from K. Yeung). Anti-phospho-Ser-

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RESULTS

Endogenous RKIP Is a Physiological Inhibitor of EGF- and TPA-induced ERK Activation—To determine whether RKIP is able to inhibit ERK activation by EGF or TPA in H19-7 cells, exogenous RKIP was introduced into cells. Plasmids expressing HA-ERK2 and FLAG-RKIP were co-transfected into H19-7 cells, and activation of HA-ERK upon cell stimulation was measured by an in vitro kinase assay using Elk-1 as a substrate. As shown in Fig. 1A, overexpression of RKIP is able to block HA-ERK2 activation by EGF and TPA. These results indicate that RKIP is capable of inhibiting the activation of ERK by these stimuli. To determine whether endogenous RKIP normally regulates ERK activity, an antisense RKIP construct, pAS-C143, that was shown previously (17) to suppress endogenous RKIP, was co-transfected, along with HA-ERK2, into H19-7 cells, and HA-ERK2 activation was assayed before and after EGF or TPA stimulation (Fig. 1B). The ability of the antisense RKIP to selectively suppress RKIP expression in H19-7 cells was confirmed by co-transfection of RKIP and antisense RKIP (data not shown). Expression of the antisense RKIP construct significantly increased both EGF- and TPA-stimulated HA-ERK2 activity, suggesting that RKIP physiologically regulates signaling to MAP kinase triggered by EGF, as well as other activators of protein kinase C, in H19-7 cells.

PKCs Are RKIP Kinases—Having established that RKIP regulates the EGF and TPA stimulation of ERK (see Fig. 1A) and previously demonstrated a requirement for PKCζ in EGF-stimulated ERK activation, we investigated whether RKIP and PKCs could interact directly. An analysis of the RKIP protein sequence for PKC consensus phosphorylation sites (24) identified Ser-153 of the rat RKIP sequence as a potential target. This site was mutated to a non-phosphorylatable valine residue (S153V), and the mutant RKIP was cloned into a bacterial expression vector to produce recombinant GST-RKIP(S153V). Purified GST-RKIP and GST-RKIP(S153V) were then used as substrates for PKC in vitro kinase assays. As shown in Fig. 1C, RKIP is a substrate for PKCs α, βI, βII, γ, and ζ, and substitution of Ser-153 prevents most of this phosphorylation. Overexposure of the blot reveals some additional phosphorylation by PKCs α and γ, but the amount is low compared with the phosphorylation of Ser-153. These data demonstrate that Ser-153 is the major site of RKIP phosphorylation by PKC. Interestingly, the novel PKCs, including, PKCs δ, ε, and η, are not sample was determined by immunoblot analysis (anti-HA, 3F10) and quantified by digital analysis. The graph shown is a plot of mean data ± S.D. from four independent experiments. C, PKCs phosphorylate RKIP Ser-153. 1 μg of GST-RKIP or GST-RKIP(S153V) was incubated with vehicle (none) or the indicated recombinant PKC, and kinase assays were carried out as described under “Experimental Procedures.” The amount of GST protein in each sample was determined by stripping the membrane and re-probing with an α-GST antibody. The results shown are representative of five independent experiments. D, RKIP binds to PKCζ in vitro. GST or GST-RKIP was coupled to glutathione-Sepharose and incubated with 100 ng of recombinant PKCζ or recombinant PKCδ pre-incubated with 1 μg of competing α-PKCζ antibody (Upstate Biotechnology) as described under “Experimental Procedures.” Following extensive washing, bound proteins were resolved by 12.5% SDS-PAGE, and PKCζ was detected by immunoblot analysis. The amount of GST protein in each sample was determined by probing with an α-GST antibody (Upstate Biotechnology). The results shown are representative of three independent experiments.
RKIP kinases in vitro, suggesting that RKIP phosphorylation is not a feature of all PKC isozymes.

Because PKCζ is able to phosphorylate RKIP, we determined whether PKCζ and RKIP could associate in vitro and in vivo. We focused on the PKCζ isoform based on our previous work (11) indicating that PKCζ mediates Raf-1 activation by EGF in H19-7 cells; however, because classical PKCs can phosphorylate RKIP in vitro, we expect that they would also associate physically and mediate the action of TPA, as well as specific growth factors dependent upon the particular cell type. As shown in Fig. 1D, recombinant PKCζ binds to GST-RKIP but not GST. These results indicate that RKIP and PKCζ are capable of interacting specifically. However, no stable association was detected in vivo (data not shown), consistent with an enzyme-substrate interaction.

Phosphorylation of RKIP by PKC Causes Release of RKIP from Raf-1 and Increased ERK Activation—Because PKCζ potentiates Raf activity (11), we determined whether direct phosphorylation of RKIP by PKCζ could result in the release of RKIP from Raf-1. To test this possibility in vitro, GST, GST-RKIP, or GST-RKIP(S153V) were pre-bound to glutathione-Sepharose beads and incubated with a bacterially expressed Raf-1 kinase domain (CBP-CR3) (25), the region of Raf that binds to RKIP. The Raf-1 kinase domain bound to both wild-type RKIP and RKIP(S153V) (Fig. 2A). Addition of purified PKCζ and ATP led to the release of the Raf-1 kinase domain from GST-RKIP but not from GST-RKIP(S153V), demonstrating that phosphorylation of Ser-153 on RKIP by PKCζ causes dissociation of the Raf-1 kinase domain and RKIP.

To test the effect of PKC on the interaction of RKIP with full-length Raf-1 in cells, Myc-Raf-1 was co-transfected into COS-7 cells with HA-RKIP or HA-RKIP(S153V), and the association of the two molecules was determined before and after TPA stimulation. In agreement with results published previously (22), we observed that transfected Myc-Raf-1 and HA-RKIP could be co-immunoprecipitated from starved COS-7 cells and that activation of PKC by TPA alone induced the release of HA-RKIP from Myc-Raf-1 (Fig. 2B). Analysis of the cell lysates by immunoblotting indicated that both PKC-treated and untreated cells contain comparable levels of HA-RKIP (data not shown). Treatment of H19-7 cells with physiological PKC activators such as EGF also induced dissociation of endogenous RKIP from endogenous c-Raf-1 by 59%, as shown using a horseradish peroxidase-tagged anti-RKIP antibody for immunoblotting to eliminate background from light chain antibody (Fig. 2C). If the mechanism for RKIP dissociation from Raf-1 involves phosphorylation of RKIP by PKC, then the mutant RKIP(S153V) should remain bound to Raf-1 independent of PKC activation. Consistent with this prediction, HA-RKIP(S153V) co-immunoprecipitated with myc-Raf-1 in both resting and TPA-treated COS-7 cells (Fig. 2B). Similar results were obtained when GST-Raf rather than myc-Raf was co-expressed with RKIP, and the complexes were isolated using glutathione beads (data not shown). These results indicate that PKC can regulate RKIP binding to Raf-1 kinase in vivo by phosphorylating RKIP at residue Ser-153.

Endogenous RKIP Is Phosphorylated at Ser-153 in Vivo—Because the above phosphorylation studies used exogenous RKIP, we determined whether endogenous cellular RKIP is phosphorylated similarly at Ser-153 in response to PKC activation. Therefore, we generated antibodies against phosphorylated Ser-153 RKIP (α-pSer-153-RKIP) by immunizing rabbits with a 12-amino acid peptide containing residues 146–157 of the rat RKIP sequence with a phosphoserine at position 153 and purifying the antibody via peptide affinity chromatography. To test the specificity of the antibody, GST-RKIP and GST-RKIP(S153V) were initially incubated with recombinant PKC and ATP to phosphorylate RKIP. The products of this reaction were then resolved by SDS-PAGE, along with GST, unphosphorylated GST-RKIP, GST-RKIP(S153V), and GST-RKIP(S153E), a mutant of GST-RKIP with glutamic acid substituted at residue 153 to mimic phosphorylation at this site. Immunoblot analysis with the purified α-pSer-153-RKIP antibody strongly detected a single 48-kDa band corresponding to GST-RKIP that was phosphorylated by PKC (Fig. 3A). In contrast, the antibody did not recognize unphosphorylated GST-RKIP, GST, GST-RKIP(S153V), or GST-RKIP(S153E). These data demonstrate that the purified antibody specifically detects RKIP phosphorylated at Ser-153.
RKIP phosphorylated by PKC. To normalize for protein loading, samples were quantified by digital scanning.

Experimental Procedures.

**A.** The anti-pSer-153-RKIP antibody specifically detects RKIP phosphorylated by PKC. 1 μg of GST, GST-RKIP, GST-RKIP(S153V), or GST-RKIP(S153E) was incubated alone or with 100 ng of recombinant PKC α, and kinase assays were carried out with cold ATP as described under “Experimental Procedures.” Proteins were separated by 10% SDS-PAGE, and pSer-153-RKIP was detected by immunoblot analysis (α-pSer-153-RKIP). The amount of GST protein in each sample was determined by stripping the membrane and re-probing with an α-GST antibody. The results shown are representative of three independent experiments.

**B.** TPA induces RKIP Ser-153 phosphorylation in vivo. H19-7 cells were starved overnight in DMEM at 39 °C and then stimulated with 800 nM TPA for the indicated times. In one sample, the cells were pre-incubated with bisindolylmaleimide I (BIM; Calbiochem) for 30 min prior to stimulation. 100 μg of protein lysates were separated by 12.5% SDS-PAGE, and the membrane was probed with anti-pSer-153-RKIP. The membrane was then stripped and re-probed with anti-RKIP. The results shown are representative of three independent experiments.

**C.** EGF induces RKIP phosphorylation in vivo. H19-7 cells were serum-starved overnight in DMEM at 39 °C, then stimulated with 100 ng/ml EGF for 2–15 min as indicated. Cells were lysed, and FLAG-RKIP was immunoprecipitated using anti-FLAG (M2; Sigma) antibody. Immunoprecipitated proteins were eluted using 100 μg/ml FLAG peptide, resolved by SDS-PAGE, and blotted for pSer-153-RKIP. The results shown are representative of three independent experiments.

**D.** EGF causes an increase in phospho-RKIP of ~2-fold in H19-7 cells. These results indicate that RKIP is phosphorylated at Ser-153 in response to cell stimulation by the physiological activator EGF.

**Glutamate Does Not Act as a Phosphomimetic When Substituted at Residue Ser-153**—Because the crystal structure of bovine PEBP (RKIP) has been solved to a resolution of 1.84 Å (26), we used molecular modeling to explore the relationship of Ser-153 to the functional domains of RKIP. Purified RKIP crystallizes as a dimer (26), and the monomer is shown in Fig. 4A, with the evolutionarily conserved phosphatidylethanolamine binding (PEB) domain (residues 64–86) colored in green, and the PKC phosphorylation site (Ser-153) colored in blue. The PEB domain consists of hydrophobic residues that form a β-sheet and mediate the high affinity binding of membrane phosphatidylethanolamine (26). Interestingly, Ser-153 lies on the surface of RKIP in a loop domain that has more variability than other conserved regions of RKIP. To address the structural consequences of phosphorylation, we modeled the RKIP structure with a phosphorylated Ser-153 residue (Fig. 4A). The added phosphate group strikingly extends into the potential Raf-1 binding pocket, supporting our data that phosphorylation at this site mediates the release of Raf-1 from RKIP. Substitution of a glutamic acid residue at this site (S153E) results in a slightly larger group than the original serine residue, but one that is not as bulky as the phosphoserine (Fig. 4A).

Because glutamic acid residues can mimic phosphoserine residues under some circumstances, we determined whether a mutant RKIP with glutamic acid substituted at residue 153 (S153E) would still bind to Raf-1. In fact, GST-RKIP(S153E) bound to the Raf-1 kinase domain (CBP-CR3) at least as well as GST-RKIP or GST-RKIP(S153V) in vitro (Fig. 4B), suggesting that steric hindrance by the phosphate group rather than the negative charge is responsible for the inability of phospho-
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Fig. 4. Substitution of glutamate for serine at residue Ser-153 does not mimic phosphorylation of Ser-153. A, the RKIP crystal structure. The PEB domain is shown in green, and Ser-153 is in blue. Either glutamic acid (RKIP/S153E) or a phosphate group (RKIP-pSer-153) was substituted for Ser-153 by using the Swiss-PDB viewer in conjunction with Pov-Ray software. B, RKIP/S153E is not a phosphoimetic. GST, GST-RKIP, GST-RKIP(S153V), or GST-RKIP(S153E) was coupled to glutathione-Sepharose and incubated with 1 μg of bacterially expressed Raf-1 kinase domain (CBP-CR3). Following extensive washing, bound proteins were separated by 12.5% SDS-PAGE, and Raf-1 was detected by immunoblot analysis. C, titration of the ability of RKIP to bind. To determine whether an RKIP mutant with glutamic acid replacing Ser-153 might also promote binding to full-length Raf-1 in vivo, we transfected COS-7 cells with a vector expressing HA-RKIP, HA-RKIP(S153E), or HA-RKIP(S153V). The use of highly transfectable COS-7 cells enabled us to titrate the amount of transfected RKIP cDNA to maximize inhibition. Consistent with the in vitro binding results, HA-RKIP(S153E) was as effective in blocking EGF-induced ERK activation as HA-RKIP or HA-RKIP(S153V) (Fig. 4, C and D). Similar results were obtained with H19-7 cells (data not shown). Taken together, these data demonstrate that Ser-153 is situated on the surface of RKIP and modulates RKIP binding to Raf-1.

PKC Phosphorylation Rescues Inhibition of ERK by RKIP but Not by the S153V RKIP Mutant—If PKC phosphorylation of Ser-153 causes the physical release of RKIP from Raf-1, then enhanced expression and activation of PKC should be sufficient to overcome the RKIP-mediated inhibition of ERK in cells. However, if the site of PKC phosphorylation is removed, then no significant rescue by PKC should be observed. To test this hypothesis, FLAG-RKIP or FLAG-RKIP(S153V) were co-expressed with HA-ERK2 in H19-7 cells, and activation of HA-ERK2 following EGF stimulation was assayed by in vitro kinase assays. Expression of the mutant FLAG-RKIP, as well as the wild-type FLAG-RKIP, blocks EGF-induced HA-ERK2 activation. Co-transfection of FLAG-PKCα is able to overcome this inhibition in cells expressing FLAG-RKIP but not mutant FLAG-RKIP(S153V), indicating that PKC can mediate activation of ERK in vivo by phosphorylation of RKIP (Fig. 5). Similar results were obtained using COS-7 cells, and similar levels of PKCζ were detected in cell lysates (data not shown). These results demonstrate that Raf-1 signaling to ERK, as well as its physical association with RKIP, is regulated by phosphorylation of RKIP at Ser-153.

Model for Regulation of RKIP by PKC—The results described here can be summarized in a relatively simple model that explains how PKCζ can activate the Raf-1 signaling cascade in response to EGF (Fig. 6). Dephosphorylation of Raf-1 at residue Ser-259 upon association with EGF-activated Ras results in release of 14-3-3 (2). PKCζ can be recruited to Raf-1 by binding to 14-3-3 and subsequently released from Raf-1 by phosphorylating 14-3-3 (27). Our results suggest that PKCζ can also associate with RKIP and phosphorylate residue Ser-153 on RKIP, causing RKIP to be released from Raf-1. However, if a S153V or S153E mutant is expressed, then RKIP inhibition would not be overridden by growth factor signaling via PKC. Because RKIP prevents MEK phosphorylation, release of RKIP from Raf-1 also enhances downstream signaling to ERKs.
and assayed as described under "Experimental Procedures." The amount of HA-ERK2 in each sample was determined by immunoblot analysis, as shown in the lower panel. The results shown are representative of four independent experiments.

**DISCUSSION**

Protein kinase C is a key activator of the Raf/MAP kinase cascade, but the mechanisms by which it promotes Raf-1 signaling either directly or in response to growth factor stimulation have not been clear. Here we demonstrate that RKIP inhibits MAP kinase activation in response to growth factors or PKC activators in neuronal cells and that PKC can regulate Raf-1 signaling through phosphorylation of RKIP. Classical and atypical PKCs phosphorylate RKIP on a serine residue, Ser-153, which results in the displacement of RKIP from Raf-1. This phosphorylation of RKIP at Ser-153 has been observed in vivo in response to both TPA and EGF. A mutant RKIP that has Ser-153 mutated to valine continues to associate with Raf-1 following PKC stimulation. Taken together, these results indicate that PKC can regulate MAP kinase activation under physiological conditions by phosphorylating RKIP and provide a more general model for the regulation of MAP kinase cascades by inhibitory factors.

The fact that RKIP reportedly associates not only with Raf-1 but also weakly with MEK and possibly ERK (17) raises the possibility that RKIP may play a role as a scaffolding protein. However, it should be pointed out that the extremely mild methods for cell disruption required to isolate complexes of RKIP and Raf-1 is not direct evidence for a scaffold function, because the interactions in vivo, particularly with MEK or ERK, could be indirect. Other scaffold proteins for the MAP kinases such as kinase suppressor of Ras (KSR) and JNK-interacting protein (JIP) have been reported to be inhibitors or potent inducers of RKIP by PKC. It is likely that the phosphorylation of RKIP by EGF involves a small subset of the cellular RKIP by PKC that is in close proximity to the EGF receptor and subject to rapid dephosphorylation. ERK activation is limited by the amount of Raf-1 activated in response to EGF. In contrast, TPA would be able to phosphorylate significantly more RKIP by activating both classical and novel PKCs that are widely distributed in the cells; therefore, it is not surprising that TPA is a more potent inducer of RKIP phosphorylation.

The fact that substitution of glutamic acid for Ser-153 did not mimic the phosphorylated residue is not surprising in light of the crystal structure. These results are analogous to those seen with RKIP, Ser-153 did not mimic the phosphorylated residue is not surprising in light of the crystal structure. These results are analogous to those seen with the Ser-259 negative regulatory site of Raf-1. This residue is phosphorylated by protein kinase B/Akt (35) and correlates with Raf-1 inactivation. Mutation of this site to a negatively charged amino acid, however, does not mimic the effects of phosphorylation. Instead, the physical presence of the phosphate group is required, suggesting that such sites are involved sterically in protein-protein interactions (2). The modulation of Raf-1 binding to RKIP by substitution of phosphoserine or glutamate for Ser-153 indicates that this region of RKIP influences the binding interaction, possibly by directly participating as another docking site within the binding pocket.

RKIP is a member of a larger family of PEBPs. In addition to the RKIP-like PEBPs, there is also one more distant family with orthologs in humans and mice (19). Whether all members of the PEBP family act in a similar fashion to regulate kinases is unclear. Interestingly, the residue corresponding to RKIP Ser-153 in PEBP2, another isoform of RKIP (GenBank accession number AF226629) is an alanine, suggesting a different mode of regulation. Although the structure and key residues of...
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by the extent of PKC activation. Thus, this mechanism enables commonly activate Ras and thus relieve inhibition by the neg-
target for regulation. Although two different stimuli might makes sense that this point in the cascade would be a key
step in growth factor signaling cascades. Because ampli-
the cell.

tarylated or phosphorylated state may have other roles within
These and other studies suggest that RKIP is a multifunctional
the hippocampus have been linked to unusually high levels of
either the HCNP peptide or RKIP mRNA (reviewed in Ref. 38).
The regulation of Raf signaling by RKIP and PKC occurs at
a key step in growth factor signaling cascades. Because amplifi-
signal occurs between Raf-1 and MEK (1), it makes sense that this point in the cascade would be a key
target for regulation. Although two different stimuli might commonly activate Ras and thus relieve inhibition by the neg-
ate regulatory domain of Raf-1, the actual amplification of the signal in the case of RKIP-bound Raf-1 could be determined by the extent of PKC activation. Thus, this mechanism enables selective modulation of the signal. Evidence for activation of other MAP kinase cascades by classic or atypical PKCs such as the 1b kinase (IKK) cascade (39–41), combined with recent evidence that RKIP can regulate the IKK cascade (42), sug-
gests that a similar regulatory mechanism might exist for other MAP kinases either involving RKIP or other inhibitory proteins.

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Kevin C. Corbit, Nicholas Trakul, Eva M. Eves, Bruce Diaz, Mark Marshall and Marsha Rich Rosner

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