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

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Running title: PKC regulation of Raf signaling

Abbreviations: RKIP, Raf Kinase Inhibitory Protein; PEBP, phosphatidylethanolamine binding protein
SUMMARY

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. Classical and atypical but not novel PKC isoforms phosphorylate RKIP at serine 153 (S153). RKIP S153 phosphorylation by PKC either in vitro or in response to TPA or EGF causes release of RKIP from Raf-1, whereas mutant RKIP (S153V or S153E) remains bound. Increased expression of PKC can rescue inhibition of the 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.
INTRODUCTION

The MAP 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 kinase that phosphorylates and activates a MAP kinase kinase, which in turn phosphorylates the TXY activation domain of MAP kinase (reviewed in (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 (S338) and tyrosine 341 (Y341) (reviewed in (2)). A variety of studies have shown that protein kinase C (PKC) isozymes 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 (7)). There are three major classes of PKCs that are distinguished by their physiological activators. The classical PKCs (I, II, and III) require both Ca\(^{2+}\) and diacylglycerol (DAG) for activation whereas the novel PKCs (I, II, and III) are Ca\(^{2+}\)-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, \(\delta\) and \(\phi\), are Ca\(^{2+}\), 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. While the phosphorylation of MEK by PKCs is controversial (6), Raf-1 is phosphorylated by PKCs at multiple sites. For example, PKC\(\beta\) phosphorylates Raf-1 at serine 499 (13) but mutation of this residue did not impede activation of Raf-1 by the physiological stimulators Ras and Lck. 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, while 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. In both the conditionally-immortalized hippocampal cell line H19-7 (15) and primary E16 rat hippocampal cells, two different PKC isoforms, PKC\(\alpha\) and PKC\(\delta\), mediate ERK activation by epidermal growth factor (EGF) and fibroblast growth factor (FGF), 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. While these studies suggested that PKC\(\alpha\) activates Raf-1 in response to EGF, phosphorylation of neither Raf-1 nor MEK appeared to be responsible ((3,6,16) and data not shown). Furthermore, phosphatase 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.

RKIP, the Raf-1 kinase inhibitor protein, was recently identified by yeast two-hybrid cloning utilizing the kinase domain of Raf-1 as bait (17). RKIP was found to be a member of the phosphatidylethanolamine-binding protein (PEBP) family, a ubiquitously expressed protein with homologues in A. thaliana, S. cerevisiae, C. elegans, and D. melanogaster that display high degrees of interspecies sequence similarity (18). There are at least three RKIP-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 recently shown to inhibit thrombin (21). 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 phorbol esters and PKCs have been well established as activators of MAP kinases, the targets for PKCs in this pathway are surprisingly unknown. Since phorbol esters 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 polymerase chain reaction (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 gcc gac atc agc cag tgg 3’; (R) 5’ ctt ccc agc cag ctg atc gtt cag 3’. For the pCR-HA- and FLAG-RKIP constructs the forward primers were as follows: (F-HA) 5’ gcc tcc atc atg tac cca tat gac gtt cca gac tac gct gcc gcc gcc gac atc agc cag tgg 3’; (F-FLAG) 5’ ggc tcc atc atg gac tac aag gac gac gac gac aag gcc gcc gcc gccgcc gac atc agc cag tgg 3’. The reverse primer (R-stop) was: 5’ cta ctt ccc agc cag ctg atc gtt 3’. The PCR products were ligated into the pCR3.1 vector (Invitrogen) and sequenced in both directions to confirm fidelity of the reactions. To make pGEX-RKIP, the ORF of RKIP was excised from pCR-RKIP by EcoRI
digestion and ligated into EcoRI-digested pGEX-2T (Amersham-Pharmacia). Site-directed mutagenesis of S153 to valine was done utilizing pGEX-RKIP as template and the U.S.E. kit (Amersham-Pharmacia). The following mutagenic primer was used: 5’ gta ctt ctt tcg aaa gag ctc cac ctt gaa ctt 3’. This primer binds to the negative strand of pGEX-RKIP and introduces a silent mutation resulting in the creation of a unique BstBI site and conversion of S153 to valine. The NarI/NheI selection primer was from Amersham/Pharmacia. The mutation was confirmed by sequencing in both directions. The S153 to glutamic acid (S153E) mutagenesis was done utilizing pGEX-RKIP as template and the QuickChange kit (Stratagene). The mutagenic primer was as follows: 5’ ggc aag ttc aag gtg gag gag ttg cga aag aag 3’. This primer introduces a silent mutation, resulting in the creation of a unique BstBI site and conversion of S153 to glutamic acid. The mutation was confirmed by sequencing in both directions. The HA- and FLAG-tagged RKIP(S153V) and (S153E) plasmids were constructed by PCR utilizing the appropriate pGEX-RKIP plasmid as template. The F-HA and F-FLAG forward primers were used along with the R-stop reverse primer and ligated into the pCR3.1 vector to make pCR-HA- and FLAG-RKIP(S153V) and (S153E).

**In vitro PKC kinase assays**

1 µg of GST-RKIP or GST-RKIP(S153V) was combined with 200 ng baculovirus-derived PKC (Panvera) in 50 µl of kinase buffer. For PKCγ, δ, ι, and ϵ the buffer used was 20 mM HEPES, pH 7.4, 100 mM CaCl₂, 10 mM MgCl₂, 100 µg/ml L-α-phosphatidylserine and 20 µg/ml diacylglycerol. The buffer used for novel PKCs δ, ϵ, and ι was 25 mM Tris, pH 7.5, 5 mM MgCl₂, 0.5 mM EGTA, 200 µg/ml L-α-phosphatidylerine and 20 µg/ml diacylglycerol. The buffer for PKCγ was 25 mM Tris, pH 7.5, 5 mM MgCl₂, 0.5 mM EGTA, 1 mM DTT, 100 µg/ml L-α-phosphatidyserine. Samples were aliquoted from premixed cocktails to ensure that the amounts of CBP-CR3 or PKCγ were the same in different tubes. The reactions were started by the addition of 5 µCi ³²P-γ-ATP, carried out at 30°C for 30 minutes, and then stopped by the
addition of 6X sample buffer and heating at 100°C for 3 minutes. The samples were loaded onto a 10% acrylamide gel, transferred to nitrocellulose, and exposed to film.

Cell culture

The immortalized H19-7 cells were generated from embryonic rat hippocampal cells and grown as previously described (15). H19-7 cells were maintained in 10% fetal bovine serum (FBS), 50u/ml penicillin-50 μg/ml streptomycin, and 200 μg/ml G418 at 33°C. Cells were serum starved at 39°C in N2 medium or DMEM overnight prior to treatment. COS-7 cells were grown in DMEM with 10% FBS and 50u/ml penicillin-50 μg/ml streptomycin.

In vitro HA-ERK2 activation assays

H19-7 cells were transfected with 10 μg of DNA per 10 cm plate using TransIT-LTI (Panvera). DNA in all transfections was added to equal amounts by addition of empty vector where necessary. 24 hours following transfection, cells were starved overnight followed by treatment with 800 nM tetradecanoyl phorbol acetate (TPA) or 100 ng/ml EGF. HA-ERK2 kinase assays were performed in vitro as described previously with either GST-Elk-1 or MBP as substrates (11). The amount of HA-ERK2 in each sample was determined by immunoblot analysis with an anti-HA monoclonal antibody (3F10, Roche). Relative kinase activity was measured with a phosphoimager and normalized to the amount of HA-ERK2 in each sample. For down-regulation of endogenous RKIP, H19-7 cells were transfected with empty vector, HA-ERK2, or HA-ERK2 plus the RKIP antisense construct AS-C143 and pHACT (17). 24 hours later, cells were starved overnight and activation of HA-ERK2 was assayed as above. In some experiments, ERK activation was determined by immunoblotting with antiphosphoMAPK 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 (NGS) followed by
incubation with 2 μg of GST or GST-RKIP. Coupled GST fusion proteins were then incubated with 100 ng PKCζ with 10% bovine serum albumin as non-specific competitor for 1 hour 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% NP-40, 2.5% sucrose). Bound proteins were resolved in a 12.5% acrylamide gel, transferred to nitrocellulose, and immunoblotted with anti-PKCζ antibodies (Santa Cruz). For in vitro CBP-CR3 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 hours. After extensive washing in TENNS buffer, 100 ng PKCζ was added and kinase assays 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 previously described procedure (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 hours 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 IP myc-Raf-1, 1 μg of anti-myc monoclonal antibody (9E10, Upstate Biotechnology) was added to 250 μg of cell lysate proteins and rotated at 4°C followed by addition of 25 μl of Protein G-sepharose (1:1 slurry). The complex was washed 3 times with cold PBS and boiled in 3X PAGE sample buffer. The proteins were separated in 12.5% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-HA antibodies (12CA5-HRP, Roche) to detect HA-RKIP. The blot was then stripped and re-probed with anti-Raf-1 antibodies (Santa Cruz) to document amounts of myc-Raf-1 in all samples.

**Antibody production**
Anti-RKIP antisera were made by immunization of rabbits with purified GST-RKIP (RKIP). Anti-phospho-S153-RKIP antisera (pS153-RKIP) were made by immunization of rabbits with a peptide (United Biochemicals) corresponding to residues 146-157 of the rat RKIP sequence conjugated to ovalbumin: NH – RGKFKVES’SFRKK-COOH, where the S’ indicates a phosphoserine residue. pS153-RKIP was affinity purified by passing the crude antisera through a column containing the immunogenic peptide followed by Protein A-Sepharose chromatography for concentration.
RESULTS

Endogenous RKIP is a physiological inhibitor of EGF- and TPA-induced ERK activation.

In order to determine if RKIP is able to inhibit ERK activation by EGF or tetradecanoyl phorbol acetate (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 previously shown to suppress endogenous RKIP (17), 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 directly interact. An analysis of the RKIP protein sequence for PKC consensus phosphorylation sites (24) identified serine 153 (S153) 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 *in vitro* kinase assays. As shown in Fig. 1C, RKIP is a substrate for PKCα, βI, βII, γ and δ, and substitution of S153 prevents most of this phosphorylation. Overexposure of the blot reveals some additional phosphorylation by PKCs β and δ but the amount is low compared to the phosphorylation of S153. These data demonstrate that S153 is the major site of RKIP phosphorylation by PKC. Interestingly, the novel PKCs, including, PKCδ, ε and h, are not RKIP kinases *in vitro*, suggesting that RKIP phosphorylation is not a feature of all PKC isozymes.

Since PKCα is able to phosphorylate RKIP, we determined whether PKCα and RKIP could associate *in vitro* and *in vivo*. We focused on the PKCα isoform based on our previous work indicating that PKCα mediates Raf-1 activation by EGF in H19-7 cells (11); however, since classical PKCs can phosphorylate RKIP *in vitro*, we expect that they would also physically associate and mediate the action of TPA as well as specific growth factors dependent upon the particular cell type. As shown in Fig. 1D, recombinant PKCα binds to GST-RKIP but not GST. These results indicate that RKIP and PKCα are capable of specifically interacting. 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.**

Since PKCα potentiates Raf activity (11), we determined whether direct phosphorylation of RKIP by PKCα 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 PKCα 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 S153 on RKIP by PKCα 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 previously published results (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 TPA-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 an HRP-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 S153.

**Endogenous RKIP is phosphorylated at S153 in vivo.**

Since the above phosphorylation studies used exogenous RKIP, we determined whether endogenous cellular RKIP is similarly phosphorylated at S153 in response to PKC activation. Therefore, we generated antibodies against phosphorylated S153 RKIP ([\-pS153-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 [-pS153-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 S153.

Since phorbol esters are the most robust and specific activators of PKCs, we initially examined the state of RKIP S153 phosphorylation in cells following TPA stimulation utilizing the [-pS153-RKIP antibody. Western blot analysis of H19-7 cell lysates revealed minimal pS153-RKIP in starved cells (Fig. 3B). Stimulation with TPA resulted in the induction of a 23 kDa band corresponding in size to RKIP. Pre-incubation of cells with the PKC inhibitor bisindolylmaleimide I (BIM) blocked S153 phosphorylation, demonstrating that PKC is indeed mediating this phosphorylation. Inclusion of the immunizing peptide in the buffer inhibited antibody recognition, verifying the specificity of the recognition site (data not shown). This data demonstrates that RKIP is phosphorylated on S153 in vivo in a PKC-dependent manner.

To determine if the physiological activator EGF can induce phosphorylation of RKIP by PKC, two approaches were utilized. In one set of experiments, H19-7 cells were transfected with FLAG-tagged RKIP or (S153V)RKIP. Following treatment of cells with 100 ng/ml nM EGF for 2-15 minutes, FLAG-RKIP was isolated from cell lysates using an anti-FLAG affinity column and eluted with FLAG peptide. After separation by SDS PAGE, the induction of phosphorylated RKIP was determined by immunoblotting with the [-pS153-RKIP antibody. As shown in Fig. 3C, EGF stimulated phosphorylation of RKIP, and the peak of phosphorylation occurred at 2 minutes. This time course corresponds to those of Raf and ERK activation which also peak at 2 minutes in response to EGF in H19-7 cells (data not shown). In an alternative approach involving endogenous RKIP, H19-7 cells were incubated with 100 ng/ml EGF for 2 minutes. Phospho(S153)RKIP was measured by immunoblotting cell lysates with [-
pS153-RKIP antibody and then normalizing the results to tubulin. As shown in Fig. 3D, EGF treatment caused an increase in phosphoRKIP of approximately 2-fold in H19-7 cells. These results indicate that RKIP is phosphorylated at S153 in response to cell stimulation by the physiological activator EGF.

**Glutamate does not act as a phosphomimetic when substituted at residue S153.**

Since 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 S153 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 (S153) 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, S153 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 S153 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 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).

Since 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 phosphoRKIP to bind. To determine whether an RKIP mutant with glutamic acid replacing S153 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. 4C,4D). Similar results were observed with H19-7 cells (data not shown). Taken together, these data demonstrate that S153 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 S153 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-PKC 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 PKC 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 S153.

**Model for Regulation of RKIP by PKC**

The results described here can be summarized in a relatively simple model that explains how PKC can activate the Raf-1 signaling cascade in response to EGF (Fig. 6).
Dephosphorylation of Raf-1 at residue S259 upon association with EGF-activated Ras results in release of 14-3-3 (2). PKC 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 PKC can also associate with RKIP and phosphorylate residue S153 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. Since RKIP prevents MEK phosphorylation, release of RKIP from Raf-1 also enhances downstream signaling to ERKs.

**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, S153, which results in the displacement of RKIP from Raf-1. This phosphorylation of RKIP at S153 has been observed in vivo in response to both TPA and EGF. A mutant RKIP that has S153 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 since the
interactions *in vivo*, particularly with MEK or ERK, could be indirect. Other scaffold proteins for the MAP kinases such as KSR and JIP have been reported to be inhibitors or potentiators dependent upon whether they act to sequester or bring together different components of the MAP kinase cascade (28-31). To date, RKIP has been characterized as a competitive inhibitor of the substrate MEK. Our results showing that phosphorylation of RKIP by the activator PKC causes release from Raf-1 and upregulation of ERK are also therefore consistent with a role for RKIP as an inhibitor of the Raf-1 signaling cascade. However, we cannot rule out the possibility that RKIP will also be found to function as an activator under other circumstances.

Although our results implicate RKIP as a key regulator of ERK activation by PKCs, removal of RKIP is not sufficient for ERK activation, indicating that other events must also occur. Activation of Raf-1 by growth factors requires interaction with Ras (reviewed in (2)). In several cell types, expression of a dominant negative N17Ras does not block PKC activation of ERK. However, Marshall and coworkers (32) demonstrated that PKC activation of Raf-1 is Ras-dependent. They further showed that a mutant of Raf-1 that cannot associate with Ras (R89L) can be activated by TPA if it is membrane-bound via a CAAX box, indicating that the primary role of Ras is to translocate Raf-1 to the membrane. Similarly, targeting the R89LRaf-1 to the membrane overcomes the inability of tyrosine kinases to activate Raf (33,34). The association of Raf-1 and Ras in response to TPA requires GTP-activated Ras, but the mechanism by which PKC activates Ras is not clear. Taken together with the results presented here, these data suggest that PKC activates Raf-1 signaling by at least two discrete events, one involving transport to the membrane via Ras, and the second involving the dissociation of RKIP.

Although the activation of ERK by EGF and TPA is comparable, the phosphorylation of RKIP by TPA is significantly higher and of longer duration. As previously shown, addition of exogenous PKCζ increases ERK activation by EGF suggesting that the EGFR-associated PKCζ is rate-limiting. It is likely that the phosphorylation of RKIP by EGF involves a small subset of
the cellular RKIP by PKCζ 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 S153 did not mimic the phosphorylated residue is not surprising in light of the crystal structure. These results are analogous to those seen with the S259 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 S153 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 phosphatidyl ethanolamine binding proteins (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 S153 in PEBP2, another isoform of RKIP (GenBank Accession # AF226629) is an alanine, suggesting a different mode of regulation. While the structure and key residues of the PEB binding domain of RKIP are conserved from bacteria to mammals, the loop containing S153 is more variable, consistent with a role in specificity. The most variable region among the different PEBPs is the N-terminus. Interestingly, residues 2-12 of RKIP correspond to the Hippocampal Cholinergic Neurostimulatory Peptide (HCNP) (36). Recent studies have suggested that HCNP may play important role in the development and/or differentiation of the human hippocampus (37), and some learning and memory deficiencies in the hippocampus have been linked to unusually high
levels of either the HCNP peptide or RKIP mRNA (reviewed in (38)). These and other studies suggest that RKIP is a multifunctional protein, and it is possible that RKIP in either its unphosphorylated or phosphorylated state may have other roles within the cell.

The regulation of Raf signaling by RKIP and PKC occurs at a key step in growth factor signaling cascades. Since amplification of the 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 negative 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 IKK cascade (39-41) combined with recent evidence that RKIP can regulate the IKK cascade (42) suggests that a similar regulatory mechanism might exist for other MAP kinases either involving RKIP or other inhibitory proteins.

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REFERENCES


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FIGURE LEGENDS

Figure 1. Endogenous RKIP is a specific inhibitor of EGF-induced ERK activation and a substrate for PKCs.

(A). Overexpression of RKIP blocks ERK activation. H19-7 cells were transfected with 1 μg of HA-ERK2 plus 4 μg of empty vector or FLAG-RKIP. 24 hours later the cells were switched to 39°C, starved overnight in N2 medium, and either left untreated (CTRL) or stimulated with 100 ng/ml EGF or 800 nM TPA for 5 minutes. HA-ERK2 was immunoprecipitated and assayed as described in Methods. The amount of GST-Elk phosphorylation was determined by phosphoimager. The amount of HA-ERK2 in each 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 4 independent experiments. (B). Endogenous RKIP is a specific inhibitor of EGF-induced ERK activation. H19-7 cells were transfected with 1 μg of HA-ERK2 plus 4 μg of empty vector or 3 μg of pAS-C143 and 1 μg of pHACT (17). 24 hours later the cells were switched to 39°C and starved overnight in N2 medium followed by stimulation with 100 ng/ml EGF or 800 nM TPA for 5 minutes. HA-ERK2 was immunoprecipitated and assayed as described in Methods. The amount of GST-Elk phosphorylation was determined by phosphoimager. The amount of HA-ERK2 in each 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 4 independent experiments. (C). PKCs phosphorylate RKIP S153. 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 in Methods. 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 5 independent experiments. (D). RKIP binds to PKC in vitro. GST or GST-RKIP was coupled to Glutathione-Sepharose and incubated with 100 ng of recombinant PKC or recombinant PKC pre-incubated with 1 μg of
competing \(-PKC\) antibody (Upstate Biotechnology) as described in Methods. Following extensive washing, bound proteins were resolved by 12.5% SDS-PAGE and PKC\(z\) was detected by immunoblot analysis. The amount of GST protein in each sample was determined by probing with an \(-GST\) antibody (Upstate Biotechnology). The results shown are representative of 3 independent experiments.

Figure 2. RKIP S153 mediates Raf-1 binding.

(A). PKC\(z\) phosphorylation of RKIP S153 mediates Raf-1 release in vitro. GST, GST-RKIP or GST–RKIP(S153V) was coupled to Glutathione-Sepharose and incubated with 600 ng of bacterially-expressed Raf-1 kinase domain (CBP-CR3). Following extensive washing, 100 ng of recombinant PKC\(z\) with or without cold ATP was added and kinase assays carried out as described in Methods. Further washings were carried out, bound proteins were separated by 12.5% SDS-PAGE and Raf-1 was detected by immunoblot analysis. The amount of GST protein in each sample was determined by stripping the membrane and re-probing with an \(-GST\) antibody. The results shown are representative of 3 independent experiments. (B). Phosphorylation of RKIP S153 mediates Raf-1 release in vivo. COS-7 cells were transfected with 10 \(\mu\)g of either empty vector or HA-RKIP, HA-RKIP S153V, or Myc-Raf alone or in combination. At 24 hours post transfection the cells were serum starved in DMEM for 16h. Cells were then treated with 800nM TPA for 30 minutes as indicated. Myc-Raf-1 was immunoprecipitated, and the bound proteins were separated by 12.5% SDS-PAGE. Myc-Raf-1 and HA-RKIP were detected by immunoblot analysis. The results shown are representative of at least 4 independent experiments. (C) EGF causes release of endogenous RKIP from Raf. H19-7 cells were starved in DMEM for 16h and then treated with 100ng/ml EGF as indicated. Cells were lysed by sonication in PBS. Raf-1 was immunoprecipitated from 1mg of cell lysate protein with 1\(\mu\)g of anti-Raf-1, and immunoprecipitated proteins were separated by 12.5% SDS-PAGE along with 20\(\mu\)g cell lysate (WCE) and 1\(\mu\)g anti-Raf-1 antibody alone as a control (IgG). RKIP
was detected by Western blotting with an HRP-conjugated anti-RKIP, and Raf-1 levels were assessed by immunoblotting for Raf-1.

**Figure 3. TPA and EGF induce phosphorylation of RKIP S153 in vivo.**

(A). The anti-pS153-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 and kinase assays were carried out with cold ATP as described in Methods. Proteins were separated by 10% SDS-PAGE, and pS153-RKIP was detected by immunoblot analysis (α-pS153-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 3 independent experiments. (B). TPA induces RKIP S153 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 minutes prior to stimulation. 100 µg of protein lysates were separated by 12.5% SDS-PAGE, transferred to nitrocellulose, and the membrane was probed with anti-pS153-RKIP. The membrane was then stripped and re-probed with anti-RKIP. The results shown are representative of 3 independent experiments. (C) EGF induces RKIP phosphorylation in vivo. H19-7 cells were transfected with 10µg FLAG-RKIP. After 24 hours, cells were serum starved in DMEM overnight. Cells were then treated with 100ng/ml EGF for 2-15 minutes as indicated. Cells were lysed and FLAG-RKIP was immunoprecipitated using anti-FLAG (M2, Sigma) antibody. Immuprecipitated proteins were eluted using 100µg/ml FLAG peptide, resolved by SDS-PAGE, and blotted for anti-phospho
RKIP S153, and anti-FLAG-RKIP. (D) H19-7 cells starved in DMEM overnight at 39°C. Triplicate plates were treated with 100ng/ml EGF for 2 minutes or not (CTRL). The cells were lysed and 10µg or 20µg of protein from each lysate were loaded on 12.5% polyacrylamide gels, transferred to membrane, and probed for pRKIP (\(\alpha\)-PRKIP S153). The membranes were then stripped and probed for \(\alpha\)-tubulin (Santa Cruz Biotechnology) or RKIP (\(\alpha\)GST-RKIP) to normalize for protein loading. Samples were quantified by digital analysis. These data are representative of 5 independent experiments.

**Figure 4. Substitution of glutamate for serine at residue S153 does not mimic phosphorylation of S153.**

(A) The RKIP crystal structure. The PEB domain is shown in green, and S153 is blue. Either glutamic acid [RKIP(S153E)] or a phosphate group (RKIP-pS153) was substituted for S153 by using the Swiss-PDB viewer in conjunction with POV-RAY software. (B) RKIP S153E is not a phosphomimetic. 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. The amount of GST protein in each sample was determined by stripping the membrane and re-probing with an anti-GST antibody. The results shown are representative of 3 independent experiments. (C) Titration of RKIP’s ability to block ERK activation. COS-7 cells were transfected with 1.5 µg of HA-ERK2 and no HA-RKIP (Control) or HA-RKIP at the indicated ratios. 24 hours later the cells were starved overnight by washing once in PBS and switching the medium to DMEM. The cells were treated with 100 ng/ml EGF for 3 mins. HA-ERK2 was immunoprecipitated and assayed as described in Methods. The amount of HA-ERK2 in each sample was determined by immunoblot analysis (anti-HA, 3F10 antibody), and quantified by digital analysis. The results shown are
representative of 4 independent experiments. (D) COS-7 cells were transfected with 1.5 µg of HA-ERK2 and 7.5 µg of empty vector (Control), or HA-RKIP, or HA-RKIP (S153V), or HA-RKIP(S153E). 24 hours later the cells were starved overnight by washing once in PBS and switching the medium to DMEM. The cells were treated with 100 ng/ml EGF for 3 mins. HA-ERK2 was immunoprecipitated and assayed as described in Methods. The amount of HA-ERK2 in each sample was determined by immunoblot analysis (anti-HA, 3F10 antibody), and quantified by digital analysis. The results shown are representative of 4 independent experiments.

**Figure 5. PKC phosphorylation rescues inhibition of ERK by RKIP but not by the S153V RKIP mutant.**

H19-7 cells were transfected with 10 µg of empty vector, 2 µg of HA-ERK2 plus 8 µg of empty vector, 4 µg of empty vector and 4 µg of FLAG-RKIP or FLAG–RKIP(S153V), or 4 µg of FLAG-PKC and 4 µg of FLAG-RKIP or FLAG-RKIP(S153V). 24 hours later, the cells were switched to 39°C, starved overnight in N2 medium, and either left untreated or stimulated with 10 ng/ml EGF for 5 mins. HA-ERK2 was immunoprecipitated and assayed as described in Methods. 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 4 independent experiments.

**Figure 6. A Scheme Depicting Regulation of Raf-1 signaling by RKIP and PKC**
A

HA-ERK2

CTRL  EGF  TPA

HA-ERK2+FLAG-RKIP

CTRL  EGF  TPA

32P-GST-Elk

HA-ERK2

ERK Activation

CTRL  EGF  TPA

B

HA-ERK2

CTRL  EGF  TPA

HA-ERK2 +RKIP AS

CTRL  EGF  TPA

32P-GST-Elk

HA-ERK2

ERK Activation

CTRL  EGF  TPA
BEADS: GST GST-RKIP
INPUT: □+Ab □+Ab □
Blot: PKC
Blot: GST
A

RKIP  RKIP(S153E)  RKIP-pS153

B

INPUT  

BEADS

GST  GST-RKIP  GST-RKIP (S153V)  GST-RKIP (S153E)

Blot: Raf-1

Blot: GST

C

ERK activation (% of control)

HA-RKIP : HA-ERK (µg DNA)

(1:1)  (3:1)  (5:1)
D

ERK activation (% of control)

HA-RKIP  HA-S153V  HA-S153E
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**Fold Induction**

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