Raf Kinase Inhibitory Protein Regulates Raf–1 but not B-Raf Kinase Activation

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ABSTRACT

Raf Kinase Inhibitory Protein (RKIP, also known as PEBP), is a modulator of the Raf/MAP kinase signaling cascade and a suppressor of metastatic cancer. Here we show that RKIP inhibits MAP kinase by regulating Raf-1 activation; specifically, RKIP acts subsequent to Raf-1 membrane recruitment, prevents association of Raf-1 and Pak, and blocks phosphorylation of the Raf-1 kinase domain by Pak and Src family kinases. Mutation of the Pak and Src phosphorylation sites on Raf-1 to aspartate, a phosphate mimic, prevents RKIP association with or inhibition of Raf-1 signaling. Interestingly, although RKIP can interact with B-Raf, RKIP depletion has no effect on activation of B-Raf. Since c-Raf-1 and B-Raf are both required for maximal MAP kinase stimulation by EGF in neuronal and epithelial cell lines, we determined whether RKIP significantly affects MAP kinase signaling. In fact, RKIP depletion increases not only the amplitude but also the sensitivity of MAP kinase and DNA synthesis to EGF stimulation by up to an order of magnitude. These results indicate that selective modulation of c-Raf-1 but not B-Raf activation by RKIP can limit the dynamic range of the MAP kinase signaling response to growth factors and may play a critical role in growth and development.

INTRODUCTION

Signaling cascades that control fundamental cellular processes need to be tightly regulated. The MAP kinase cascade is an evolutionarily conserved signaling module that is activated by a diverse set of signals and stimulates numerous biological processes including growth and differentiation. The pathway consists of 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)). Extracellular signal regulated kinases (ERKs), the first characterized subfamily of MAP kinases, are activated by growth factors and other stimuli via a cascade involving Ras, Raf 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 membrane association, 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)). Activation of B-Raf, another isoform of Raf that has been implicated in MAP kinase signaling, is less complex since a phosphomimetic residue has replaced the equivalent of the Y341 phosphorylation site of Raf-1. Recently, several inhibitory proteins have been identified that modulate the Ras-Raf-MAP kinase signal either directly or through feedback regulation, among them Sprouty, Spred and Raf Kinase Inhibitory Protein (RKIP or PEBP) (3-5).

RKIP is a ubiquitously expressed and highly conserved protein with homologues in A. thaliana, S. cerevisiae, C. elegans, and D. melanogaster that display remarkable degrees of interspecies sequence and structural similarity (reviewed in (6)). Mammalian RKIP is distinct from other known proteins and its function has remained largely enigmatic. A role for RKIP in signaling cascades was demonstrated when it was shown that RKIP binds to Raf-1 (7). However, RKIP also has a number of other reported functions including inhibition of other kinases such as G protein-coupled Receptor kinase2...
(GRK2) (8), and upstream kinase activators of IKK (9). Recently, RKIP has been identified as a metastasis suppressor gene, and this function correlates with MAP kinase activity (10).

Elucidating the mechanism of RKIP action is important both for a complete understanding of Raf regulation and for generating potential therapeutic reagents. Previous studies have suggested that RKIP binds to and acts downstream of Raf-1 by competitive interference with MEK binding to Raf-1 (11). We previously identified a mechanism for overcoming inhibition of MAP kinase signaling by RKIP (12). Protein kinase C (PKC), activated by either phorbol esters or epidermal growth factor (EGF), phosphorylates RKIP at S153, and this phosphorylation causes the dissociation of RKIP from Raf-1 and subsequent activation of the MAPK pathway. The goal of the present studies was to determine whether RKIP regulates Raf kinase directly.

The work reported here identifies a new mechanism by which RKIP regulates MAP kinase signaling. We demonstrate that RKIP inhibits activation of Raf-1 by blocking phosphorylation of Raf-1 by Pak and Src family kinases. Furthermore, the regulation by RKIP is shown to be specific to Raf-1 and not B-Raf in at least two cell systems, and both the range and dose response of the MAP kinase signal and physiological outputs are affected by RKIP. These results reveal a Raf isoform-specific mechanism for modulation of the Raf-MAP kinase signaling cascade by RKIP.

MATERIALS AND METHODS

Cell culture: The immortalized H19-7 cells were generated from embryonic rat hippocampal cells and grown as described previously (13). H19-7 cells were maintained in 10% fetal bovine serum, 50 units/ml penicillin, 50mg/ml streptomycin, and 200mg/ml G418 at 33°C. Cells were serum-starved at 39°C in DMEM overnight prior to treatment. HeLa, 293, and 293T cells were grown in DMEM with 10% FBS, 50 units/ml penicillin and 50mg/ml streptomycin, and serum starved overnight at 37°C in DMEM prior to treatment.

Stable shRNA cell lines: The following 19bp regions of RKIP were used for the creation of shRNA retroviral vectors: human 5'–GGTGGAGTCCTTCCGCAAG-3' and Rat 5'–GGTGGGCGCTCCTTCCGTAAA-3'. Oligonucleotides were annealed and cloned into the PQY15 vector, a pMCSV puromycin-based vector containing the H1 RNA promoter. Vectors were then used for transient transfections or transfected into the Phoenix-Ampho packaging cell line for virus production. To make stable cell lines, H19-7, HeLa, 293, and 293T cells were exposed 3-5 times to virus containing supernatant, and stable populations were obtained by selection and subsequent maintenance with 2mg/ml puromycin. RKIP Rescue (Rescue) cell lines were made by transducing stable H19-7 shRNA cell lines with retrovirus containing pCLE-HA-RKIP.

To assess RKIP depletion and MAPK activation, cells were serum starved overnight and treated with 100ng/ml EGF (Biomedical Technologies) or left untreated. Cells were lysed in a Triton-X-100 lysis buffer (TLB): 1% Triton-X-100, 1mM EDTA, 150mM NaCl, 50mM NaF, plus a protease inhibitor cocktail (Calbiochem). 25 µg of cell lysate was resolved by SDS-PAGE, transferred to nitrocellulose, analyzed by immunoblotting with anti-αTubulin (Santa Cruz), anti-ERK (Cell Signaling), anti-phospho-ERK (Cell Signaling), anti-phospho-MEK (Cell Signaling) or anti-RKIP antibodies (12). Digital analysis of immunoreactivity was done using an imaging system and software from Alpha Innotech.

Kinase Assays: To assay Raf-1 and B-Raf kinase activity, an in vitro-coupled kinase assay was used as described (14) using either a Raf-1 or B-Raf kinase assay kit (Upstate Biological). Briefly, cells were serum starved overnight, and then either left untreated or treated with 100ng/ml EGF and lysed in TLB. Raf-1 and B-Raf were immunoprecipitated using a polyclonal anti-Raf-1 antibody (Santa Cruz) and a polyclonal anti-B-Raf antibody (Santa Cruz), respectively. Immunoprecipitates were washed and recombinant MEK, ERK, and myelin basic protein (MBP) (Invitrogen) were sequentially added to the kinase reaction. MBP phosphorylation was quantified using a scintillation counter, and normalized to the level of Raf-1 or B-Raf immunoprecipitated, as assessed by Western blotting with either a monoclonal Raf-1 antibody (Transduction Labs) or a polyclonal anti-B-Raf antibody.

To assess p21-activated kinase (Pak) activity, 293T cells were transfected with wild type myc-Pak or myc-Pak 299R, a kinase dead
mutant, either in the presence or absence of transfected RKIP. At 24 hours post-transfection, cells were serum starved overnight and treated with 100ng/ml EGF for 5’ or left untreated. Cells were lysed in TLB, and myc-Pak was immunoprecipitated using a monoclonal anti-myc antibody (9E10, Sigma). Immunoprecipitates were washed and subjected to a kinase assay using MBP as a substrate. Kinase assays were resolved by SDS-PAGE electrophoresis, and MBP phosphorylation was quantified by phosphoimaging and normalized to PAK levels by immunoblotting with a polyclonal PAK antibody (Santa Cruz).

**Raf phosphorylation Assay:** 293T cells were transfected with GST-Raf-1 with or without a constitutively active Pak (myc-Pak TE, a Pak that is mutated at residue 423 from threonine to glutamic acid in the activation loop of the kinase) or Src (v-src) along with increasing amounts of HA-RKIP. At 24 hours post transfection, cells were serum starved overnight, and either left untreated or treated with 100 ng/ml EGF for 2 min, and lysed in TLB. GST-Raf-1 was purified using Glutathione Sepharose (Pharmacia), and the purified protein, as well as whole cell extract, were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-GST (Zymed), anti-phosphoserine 338 Raf-1 (Upstate Biological), anti-phosphoserine 259 Raf-1 (Cell Signaling), anti-phosphotyrosine 340/41 Raf-1 (Sigma), anti-myc, or anti-HA (Roche) antibodies.

**Mutant Raf Assays:** 293T cells were transfected with either rat RKIP shRNA (Control) or human RKIP shRNA along with ΔRaf-1:ER and pBABE-PURO, pCDNA or HA-RKIP. At 24 hours post transfection cells were selected in 2µg/ml puromycin-containing media for 24 hours to obtain pure transfected populations. Cells were serum starved overnight and treated with 100ng/ml EGF for 2 min or 1mM estradiol for 1 hour. Cells were lysed in TLB, and 25 mg whole cell extract was resolved by SDS-PAGE and transferred to nitrocellulose. Vectors expressing myc-Raf-CAAX (15) or myc-Raf S338/Y340/Y341 D/D/D (16) were transfected into 293T cells along with HA-ERK2 or FLAG-RKIP. At 24 hours post-transfection, cells were serum starved overnight and lysed in TLB. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose and probed with antibodies for HA, phospho-ERK and RKIP.

**Co-Immunoprecipitations:** 293T cells were transfected with GST-Raf-1, GST-B-Raf, or myc-Raf-1-DDD with or without HA-RKIP. 24 hours after transfection cells were serum starved overnight and either left untreated or treated with 800nM 12-O-tetradecanoylphorbol-13-acetate TPA for 15 minutes. Cells were lysed in a TAP lysis buffer containing 10mM Hepes, 3mM MgCl, 10mM Kcl, 5% glycerol, 0.1% NP40. GST-Raf-1 and GST-B-Raf were purified using Glutathione Sepharose and myc-Raf-1-DDD was purified using Anti-myc conjugated agarose beads (Profound Kit, Pierce). Purified complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies for GST, myc, and HA.

293T cells were also transfected with GST-Raf-1 and myc-PAK either with or without HA-RKIP. 24 hours after transfection, cells were serum starved overnight and either left untreated or treated with 100ng/ml EGF for 2 minutes. GST-Raf was purified using Glutathione Sepharose and purified complexes were resolved by SDS-PAGE, transferred to nitrocellulose and probed with antibodies for GST and myc. Whole cell extract (25 mg) was also resolved by SDS-PAGE and probed with anti-GST, myc, and HA antibodies to assess the expression levels of the proteins.

**Reduction of Raf-1 or B-Raf by siRNA:** siRNA specific to rat or human Raf-1 and B-Raf were obtained (Dharmacon) and transfected at a concentration of 100nM using either Oligofectamine (Invitrogen) or Nucleofector reagent V followed by electroporation (Amaxa). 24 hours post transfection cells were serum starved, treated with 100 ng/ml EGF or left unstimulated and lysed in TLB. 25µg cell lysate was resolved by SDS-PAGE, transferred to nitrocellulose and probed with antibodies for α-tubulin, Raf-1, B-Raf, and phosphoERK. α-Tubulin rather than ERK was used as a loading control for immunoblots to avoid background arising from stripping and reprobing the nitrocellulose blots.

**DNA synthesis:** H19-7 or 293T cells were seeded at 2 X 10⁴ cells/ml into poly-lysine coated 96 well culture dishes and allowed to adhere and grow for 24 hours. The cells were serum deprived in media containing 0.2% FBS for 3 days and a
BrdU cell proliferation assay kit (Calbiochem) was used to detect DNA synthesis with the following modifications. Cells were treated with EGF (0 to 100 ng/ml) in serum deprived media containing a 1:2000 dilution of the BrdU label for 24 hours. Cells were denatured, fixed and then blocked for 24 hours in 5% BSA/PBS. The remainder of the protocol is as recommended by the manufacturer.

RESULTS
In order to investigate the physiological role of RKIP, we generated cell lines with reduced levels of endogenous RKIP by RNA interference. Human embryonic kidney 293 cells were stably transduced with either a retroviral vector or species-specific shRNA vectors for human or rat RKIP, and cells were stimulated with EGF to confirm that RKIP is an endogenous inhibitor of MAP kinase. Human RKIP shRNA suppressed RKIP levels to approximately 10% of control (Fig. 1A), and suppression of RKIP by human RKIP shRNA caused a 2-fold potentiation of ERK activation in response to EGF similar to previous results using antisense RKIP (7,12). To determine whether RKIP inhibits Raf-1 activation under physiological conditions, we assayed endogenous Raf-1 activity from EGF-stimulated 293 cells stably expressing control vector or human RKIP shRNA. Depletion of RKIP in 293 cells by human RKIP shRNA caused a greater than 2-fold increase in the activity of Raf-1 kinase compared to control (Fig. 1B). Similar results were obtained for endogenous Raf-1 in other cell lines including human HeLa cells (Fig. 1C) and rat H19-7 cells as well as with exogenous Raf-1 (data not shown). These results indicate that RKIP regulates the activation of Raf-1 kinase.

Activation of Raf-1 is a complicated process involving membrane translocation, dephosphorylation at negative regulatory sites, and subsequent phosphorylation at activating sites in the kinase domain (2). To address the first step where RKIP could act, we expressed the Raf-1-CAAX mutant that is constitutively targeted to the membrane. If RKIP inhibits membrane association, RKIP depletion would have no effect on the activity of Raf-1-CAAX. However, in 293T cells transfected with myc-Raf-1-CAAX, HA-ERK2 and either human RKIP shRNA or a control vector, HA-ERK2 phosphorylation was still potentiated by RKIP depletion to a level comparable to that observed with wild type Raf-1 (Fig. 1D). It should be noted that endogenous RKIP regulates myc-Raf-1-CAAX despite the fact that the membrane-associated myc-Raf-1-CAAX is highly overexpressed and constitutively active. These results demonstrate that RKIP is functioning subsequent to Raf-1 membrane association.

Previous studies have shown that the Raf kinase domain is phosphorylated at serine 338 by Pak (17) and tyrosines 340/341 by Src family kinases (18). Both the S338 and Y341 phosphorylations are required for Raf-1 activation by growth factors such as EGF (20). Since RKIP binds to subdomains I and II of the Raf-1 kinase domain, a region that includes S338 and Y340/341 (11), we determined whether RKIP might prevent Raf-1 activation by sterically blocking accessibility of the serine and tyrosine phosphorylation sites to their respective kinases. Initially, rat neuronal H19-7 cells were co-transfected with GST-Raf-1 and either a control vector, HA-RKIP, or HA-RKIP S153V. Similar to endogenous Raf-1, GST-Raf-1 was phosphorylated on S338 following EGF treatment, but high expression of either wild type HA-RKIP or a S153V mutant that cannot be phosphorylated by PKC inhibited this induction (Fig. 2A). Conversely, when 293 cells stably expressing control or human RKIP shRNA were stimulated with EGF, RKIP depletion caused an increase in endogenous S338 Raf-1 phosphorylation (Fig. 2B). Similar results were obtained with exogenously expressed Raf-1; however, Y340/341 phosphorylation could not be detected due to limitations in antibody sensitivity (data not shown). In contrast, phosphorylation of S259, which has been reported to negatively regulate Raf activity, was unchanged by RKIP expression. These results indicate that RKIP modulates endogenous S338 phosphorylation of Raf-1 by PAK.

To determine whether RKIP binding to Raf-1 directly blocks the ability of Pak to bind and phosphorylate Raf-1, we assessed the effect of RKIP on constitutively active Pak in the absence of a growth factor stimulus. In serum-starved 293T cells co-transfected with constitutively activated myc-Pak and GST-Raf-1, myc-Pak was able to phosphorylate GST-Raf-1 on S338; however, expression of HA-RKIP inhibited this phosphorylation in a dose dependent manner (Fig.
To determine whether RKIP affects the activation of Pak directly, we examined the kinase activity of myc-tagged wild type or kinase-dead Pak transfected into 293T cells in the presence or absence of RKIP. RKIP overexpression had no effect on Pak kinase activity (Fig 2D). To test the alternative possibility that RKIP prevents Pak association with Raf-1, 293T cells transfected with Myc-Pak and GST-Raf-1 were incubated in the presence or absence of EGF. Pak association with Raf-1 was enhanced by EGF, but high expression of wild type HA-RKIP or the S153V RKIP mutant inhibited Pak binding to Raf-1 (Fig. 2E). These results indicate that RKIP blocks Pak association with and subsequent phosphorylation of Raf-1 on S338.

If RKIP sterically blocks Pak access to Raf-1, then it might similarly block Src since the Pak (S338) and Src (Y341) phosphorylation sites are proximal. We therefore determined whether RKIP expression blocked Raf phosphorylation by constitutively active Src in the absence of a growth factor stimulus. In serum-starved 293T cells co-transfected with constitutively activated v-Src and GST-Raf-1, phosphorylation of Y341 by Src was detected by immunoblotting with antiphospho-Y341 Raf-1 antibodies. As observed with Pak phosphorylation of S338, expression of HA-RKIP inhibited this phosphorylation in a dose dependent manner (Fig. 2F).

If RKIP inhibits Raf signaling by preventing phosphorylation of S338 and Y340/341, then Raf-1 that is mutated at these sites to phosphomimetic residues such as aspartate should be resistant to inhibition by RKIP. To test this possibility, 293T cells were co-transfected with myc-Raf-1 DDD (S338D/Y340D/Y341D), HA-ERK2, and either human RKIP shRNA or control vector (Fig. 3A). As predicted by this model, depletion of RKIP did not alter MAP kinase activation. Taken together, these results indicate that RKIP inhibits Raf-1 activation by preventing phosphorylation of critical residues within Raf-1 that are targets of Pak and Src family kinases.

Since RKIP association with Raf-1 blocks access of the S338/Y341 domain to activating kinases, prior phosphorylation of these sites might prevent RKIP binding. To determine whether RKIP stably associates with activated Raf-1, we used the myc-Raf-1 DDD phosphomimetic mutant that is constitutively activated in the absence of stimuli. This strategy avoids the problem of RKIP phosphorylation and inactivation. Thus, 293T cells were transfected with myc Raf-1 DDD and HA-RKIP and then serum-starved or stimulated with TPA. In contrast to unstimulated Raf-1 or B-Raf (see Fig. 3D), no HA-RKIP could be detected following immunoprecipitation with anti-myc antibody (Fig. 3B). Thus, mutations that mimic constitutive phosphorylation at the key S338 and Y340/341 activation sites lower RKIP affinity for Raf-1.

If RKIP does not bind effectively to activated Raf-1, then RKIP depletion should not significantly affect signaling to MAP kinase downstream of activated Raf. Since RKIP can bind to the kinase domain of Raf, we tested this possibility further using an inducibly activated Raf consisting of an estrogen receptor/Raf kinase domain fusion protein (ΔRaf-1:ER) (19). ΔRaf-1:ER was expressed in human 293T cells stably transfected with either rat (control) or human RKIP shRNA. RKIP depletion did not change ERK phosphorylation by estradiol-stimulated ΔRaf-1:ER, whereas endogenous Raf-1 activation was enhanced in response to EGF in the same cells (Fig. 3C). This lack of response is not due simply to overexpression of ΔRaf-1:ER since overexpressed Raf-1-CAAX was sensitive to RKIP regulation (see Fig. 1). These results are consistent with the model that RKIP inhibits Raf-1 activation.

Since B-Raf is phosphorylated at S445, a site that corresponds to S338 in Raf-1 (20), we also determined whether RKIP regulates activation of the B-Raf kinase under physiological conditions. Initially, we asked whether RKIP associates in a complex with B-Raf. Therefore, 293T cells were transfected with HA-RKIP and either GST-Raf-1 or GST-B-Raf, and then serum-starved or treated with TPA. Endogenous B-Raf or Raf-1 were immunoprecipitated from cell lysates and the immunoprecipitates analysed by immunoblotting with anti-RKIP antibody. The results clearly show that RKIP can bind to B-Raf in cells and is dissociated upon activation of PKC (Fig. 3D). Interestingly, the interaction of B-Raf with RKIP appears to be more robust than that of Raf-1 with RKIP.

If RKIP binding inhibits B-Raf activation, then RKIP depletion should result in further B-Raf
activation by EGF. To address this question, we then determined whether there was a change in the kinase activity of immunoprecipitated B-Raf from either wild type or RKIP-depleted 293T cells stimulated with EGF. Surprisingly, the results show that RKIP depletion has no effect on EGF-stimulated B-Raf kinase activity in conditionally-immortalized rat hippocampal H19-7 cells (Fig. 4A). To ensure that this result is not just an artifact of one cell line or species, we performed comparable experiments using the human 293T cell line. As shown in Figure 4B, EGF-stimulated B-Raf activity is the same independent of RKIP depletion. These results indicate that, in the same cells, RKIP functions as an inhibitor of Raf-1 but not B-Raf activation.

Since both Raf-1 and B-Raf are general mediators of MAP kinase cascades, we determined the relative contribution of each of these Raf isoforms to EGF signaling in both human epithelial 293T and rat hippocampal H19-7 cells. As shown in Figure 4(C,D), transfection of siRNA for rat Raf-1 into H19-7 cells resulted in the selective depletion of Raf-1 and a >60% reduction in the EGF-stimulated MAP kinase activity of the cells. Similarly, transfection of siRNA for rat B-Raf caused B-Raf depletion and an almost 90% decrease in EGF-stimulated MAP kinase activity. Comparable results were obtained when siRNAs for human Raf-1 or B-Raf were transfected into 293T cells (Fig. 4E,F). These results indicate that both Raf-1 and B-Raf are required for maximal EGF stimulation of MAP kinase in these two different cell lines.

Since RKIP selectively regulates Raf-1 activation and Raf-1 is a key mediator of the MAP kinase cascade, we explored more extensively the effect of RKIP on the kinetics of EGF signaling. Signaling pathways can be modulated by regulating the amplitude, duration and/or sensitivity of the resultant signal. To determine the effect of RKIP, we monitored the effect of RKIP depletion on the time course and dose response of ERK activation in both H19-7 and 293T cells stimulated by EGF. The greatest potentiation of ERK activation by RKIP depletion occurred at the time of maximum activation, and the duration of ERK activation was not significantly affected by RKIP (Fig. 5A,B). Depletion of RKIP also caused an increase in the sensitivity of ERK activation to EGF by 5 fold at the lower, more physiologically relevant EGF concentrations (Fig. 5C). A shift in dose response was also observed for human embryonal kidney 293T cells (Fig.5D). These results indicate that RKIP can influence both the amplitude and dose response of MAP kinase to EGF, consistent with its role in regulating the activation of Raf-1.

To determine whether the effects of RKIP on ERK activation translate into a physiological outcome, we monitored DNA synthesis. Like primary hippocampal neural progenitors in culture, H19-7 cells proliferate in response to EGF. As observed for ERK activation, RKIP depletion in H19-7 cells shifted the dose response for EGF-induced DNA synthesis by up to an order of magnitude (Fig. 6A). Thus, in RKIP-depleted cells, 1 nM EGF stimulates DNA synthesis at a rate approaching the maximal rate of DNA synthesis attained at EGF concentrations of 10 nM or higher in the parent H19-7 cells. Again, similar results were observed for human 293T cells Fig. 6B). Taken together, these studies reveal an isoform-specific mechanism by which RKIP can modulate the sensitivity and dynamic range of the Raf-1/MAP kinase signaling cascade and its biological output in response to specific growth factors.

**DISCUSSION**
In this paper we demonstrate that RKIP inhibits MAP kinase signaling at the level of Raf-1 but not B-Raf activation. We have used shRNA to stably suppress RKIP expression and show that RKIP depletion potentiates Raf-1 and MAP kinase activity. This potentiation is due to the ability of RKIP to suppress association with and phosphorylation of Raf-1 on S338 by Pak and probably Y340/341 by Src family kinases, critical steps in the activation of Raf-1. Finally, in two different human and rat cell lines where both Raf-1 and B-Raf are required for ERK activation, RKIP depletion potentiates not only ERK activity but also DNA synthesis in response to EGF in two different cell lines. These results indicate that RKIP limits the permissible range of EGF signaling both in terms of amplitude and dose response. A model summarizing the regulation of Raf-1 kinase by the EGF signaling cascade is shown in Figure 7.

We have employed multiple approaches to demonstrate that, under physiological conditions,
RKIP acts at a specific step in the Raf-1 activation process. Activation of Raf-1 by EGF is a complex process that involves association with Ras, translocation to the membrane, dephosphorylation of S259 and other inhibitory residues to release 14-3-3, and phosphorylation at S338 and Y341 by the Pak and Src family kinases, respectively, as well as phosphorylation of the activation loop of Raf-1 (2). Phosphorylation of S338 was modulated by both over and underexpression of RKIP, suggesting that phosphorylation of this site is the target of RKIP action. Dose response studies with constitutively activated Pak or Src kinases in the absence of an external growth factor stimulus verified that RKIP blocks both association with and phosphorylation of Raf-1 by activating kinase(s). Finally, expression of constitutively activated Raf kinase mutants, including mutants with phosphomimetic aspartic acid residues at S338 and Y341, indicated that endogenous RKIP did not stably associate with and had no effect downstream of activated Raf-1. These results support a model of RKIP regulation at the level of Raf activation.

The selective inhibition of Raf-1 but not B-Raf activation by RKIP is consistent with their respective mechanisms of regulation. Activation of Raf-1 is dependent upon phosphorylation at S338 and Y341 by Ras/Pak and Src, respectively, and maximal activity requires phosphorylation at both sites (20). Thus, inhibition of one or both of these phosphorylations would be sufficient to prevent Raf-1 activation. In contrast, neither of these phosphorylation events is required for B-Raf activation. The S445 site in B-Raf corresponds to the S338 site in Raf-1, but the Y341 equivalent in B-Raf has been substituted by an aspartate residue. Furthermore, S445 in B-Raf is constitutively phosphorylated and is not further stimulated by Ras. Thus, the phosphorylation sites targeted by RKIP in Raf-1 are effectively constitutively active in B-Raf. Given that RKIP does not bind to Raf-1 that has been mutated to activating residues at these sites, it is not surprising that RKIP does not inhibit B-Raf activation.

Although B-Raf activation is not regulated by RKIP, there are at least two mechanisms by which RKIP could influence B-Raf signaling to ERK. First, our results indicate that both B-Raf and Raf-1 are required for ERK activation in two cell systems, indicating that these two isoforms act in concert rather than in an additive fashion. There is precedent for this type of interaction, since an inactive B-Raf mutant in melanoma cells has been shown to activate ERK via Raf-1 (22). Thus RKIP could influence the activity of Raf-1:B-Raf heterodimers or other Raf-1 interacting complexes. Second, previous studies based on exogenous RKIP have suggested that overexpressed RKIP can bind activated Raf-1 and prevent binding of MEK as a substrate (11). Similarly, a recent publication demonstrated that RKIP binds to B-Raf, and exogenous RKIP overexpression inhibits B-Raf in melanoma cells (23). We have also found that overexpression of exogenous RKIP expression can inhibit B-Raf signaling stimulated by NGF in PC12 cells (data not shown). Our observation that HA-RKIP can associate with endogenous B-Raf in 293T cells is consistent with this possibility. Thus, the extent that RKIP can regulate endogenous B-Raf signaling presumably reflects the amount of RKIP and/or Raf-1 present and may vary dependent upon the cell or tissue in question.

Consistent with its role as a modulator, RKIP functions to limit the dynamic range of the EGF signal, even at saturating EGF concentrations, and this inhibition is only relieved by RKIP depletion. Thus, the rate-limiting step appears to be the ability of EGF to activate sufficient PKC to release all of the RKIP from Raf-1. In fact, previous studies from our lab have shown that EGF can further potentiate Raf-1 activity if PKCζ is co-expressed with Raf-1 in cells (21). Inactivation of RKIP by phosphorylation also provides a mechanism for crosstalk with other growth factors and environmental stimuli that activate PKC and can thereby potentiate the EGF response.

Since EGF induces a transient activation of MAP kinase in many cells, the mechanism of inhibition by RKIP is uniquely suited to its role as a regulator of EGF signaling. RKIP targets the initiation of the Raf-1 signal rather than its sustained activation or deactivation, leading to a change primarily in the amplitude of MAP kinase activation with relatively little effect on duration of signal. The transient MAP kinase activation elicited by EGF is in contrast to that of other growth factors, such as FGF or PDGF, that induce a more sustained MAP kinase signal. Although the mechanisms leading to a sustained versus
transient activation are not entirely clear, some suggested hypotheses include differences in adapter molecules associating with distinct receptor tyrosine kinases, such as FRS2 or Gab 1/2, or the engagement of negative feedback regulators such as the MAP kinase phosphatases, MKPs (24,25). Recently a number of phosphorylation sites on Raf-1 have been identified that result from a negative feedback loop and lead to temporary desensitization to further stimuli (26). Whether RKIP has a different affinity for this hyperphosphorylated form of Raf-1 is not known.

The regulation of EGF signaling by RKIP has implications for its physiological role during growth and development. The effect of RKIP depletion on MAP kinase activation in response to EGF is an approximate 2-fold potentiation consistent with that of other recently described MAP kinase signaling modulators such as Spred, Sprouty and IMP (3-5). However, small fluctuations in growth factor concentration and MAP kinase activity can cause significant differences in transcription factor stability and induction of immediate early genes (27), and RKIP has been shown to regulate AP-1-dependent transcription (7). Interestingly, the change in signal amplitude caused by removal of RKIP translates to an even larger shift in dose response leading to increased environmental sensitivity to selective factors. Thus, as shown here, depletion of RKIP either through RNA interference targeting, mutation or potentially other PKC-activating stimuli can induce robust DNA synthesis at EGF concentrations that are normally rate-limiting or subthreshold. Similarly, the IMP protein, an inhibitor of the KSR scaffold protein, can modulate the NGF dose needed for MAP kinase activation and PC12 cell differentiation (4). Finally, other MAP kinase modulators such as Sprouty have been implicated in development where concentration gradients of key factors can determine final outcome (4,28-30). Thus, RKIP, as a growth factor specific inhibitor of the Raf-1/MAP kinase signaling cascade, plays a key role in maintaining cellular equilibrium, suppressing subthreshold signals, and modulating the range of cellular responses to selected environmental stimuli.

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REFERENCES

1. Pearson, G., Robinson, F., Beers Gibson, T., Xu, B., Karandikar, M., Berman, K., and Cobb, M. H. (2001) Endocr Rev 22, 153-183.
2. Kolch, W. (2000) Biochem J 351 Pt 2, 289-305
3. Sasaki, A., Taketomi, T., Kato, R., Saeki, K., Nonami, A., Sasaki, M., Kuriyama, M., Saito, N., Shibuya, M., and Yoshimura, A. (2003) Nat Cell Biol 5, 427-432
4. Matheny, S. A., Chen, C., Kortum, R. L., Razidlo, G. L., Lewis, R. E., and White, M. A. (2004) Nature 427, 256-260
5. Wakioka, T., Sasaki, A., Kato, R., Shouda, T., Matsumoto, A., Miyoshi, K., Tsuneoka, M., Komiya, S., Baron, R., and Yoshimura, A. (2001) Nature 412, 647-651
6. Trakul, N., and Rosner, M. R. (2005) *Cell Res* **15**, 19-23
7. Yeung, K., Seitz, T., Li, S., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K. D., Rose, D. W., Mischak, H., Sedivy, J. M., and Kolch, W. (1999) *Nature* **401**, 173-177
8. Lorenz, K., Lohse, M. J., and Quitterer, U. (2003) *Nature* **426**, 574-579
9. Yeung, K. C., Rose, D. W., Dhillon, A. S., Yaros, D., Gustafsson, M., Chatterjee, D., McFerran, B., Wyche, J., Kolch, W., and Sedivy, J. M. (2001) *Mol Cell Biol* **21**, 7207-7217
10. Fu, Z., Smith, P. C., Zhang, L., Rubin, M. A., Dunn, R. L., Yao, Z., and Keller, E. T. (2003) *J Natl Cancer Inst* **95**, 878-889
11. Yeung, K., Janosch, P., McFerran, B., Rose, D. W., Mischak, H., Sedivy, J. M., and Kolch, W. (2000) *Mol Cell Biol* **20**, 3079-3085
12. Corbit, K. C., Trakul, N., Eves, E. M., Diaz, B., Marshall, M., and Rosner, M. R. (2003) *J Biol Chem* **278**, 13061-13068
13. Eves, E. M., Tucker, M. S., Roback, J. D., Downen, M., Rosner, M. R., and Wainer, B. H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4373-4377
14. Mograbi, B., Bocciardi, R., Bourget, I., Busca, R., Rochet, N., Farahi-Far, D., Juhel, T., and Rossi, B. (2001) *J Biol Chem* **276**, 45307-45319
15. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) *Cell* **84**, 889-897
16. Yip-Schneider, M. T., Miao, W., Lin, A., Barnard, D. S., Tzivion, G., and Marshall, M. S. (2000) *Biochem J* **351**, 151-159
17. King, A. J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S., and Marshall, M. S. (1998) *Nature* **396**, 180-183
18. Morrison, D. K., Heidecker, G., Rapp, U. R., and Copeland, T. D. (1993) *J Biol Chem* **268**, 17309-17316
19. Kuo, W.-L., Abe, M., Rhee, J., Eves, E. M., McCarthy, S. A., Yan, M., Templeton, D. J., McMahon, M., and Rosner, M. R. (1996) *Mol. Cell. Biol.* **16**, 1458-1470
20. Mason, C. S., Springer, C. J., Cooper, R. G., Superti-Furga, G., Marshall, C. J., and Marais, R. (1999) *Embo J* **18**, 2137-2148
21. Corbit, K. C., Soh, J. W., Yoshida, K., Eves, E. M., Weinstein, I. B., and Rosner, M. R. (2000) *Mol Cell Biol* **20**, 5392-5403
22. Wan, P. T., Garnett, M. J., Roe, S. M., Lee, S., Niculescu-Duvaz, D., Good, V. M., Jones, C. M., Marshall, C. J., Springer, C. J., Barford, D., and Marais, R. (2004) *Cell* **116**, 855-867
23. Park, S., Yeung, M. L., Beach, S., Shields, J. M., and Yeung, K. C. (2005) *Oncogene*
24. Brightman, F. A., and Fell, D. A. (2000) *FEBS Lett* **482**, 169-174
25. Yamada, S., Taketomi, T., and Yoshimura, A. (2004) *Biochem Biophys Res Commun* **314**, 1113-1120
26. Dougherty, M. K., Muller, J., Ritt, D. A., Zhou, M., Zhou, X. Z., Copeland, T. D., Conrads, T. P., Veenstra, T. D., Lu, K. P., and Morrison, D. K. (2005) *Mol Cell* **17**, 215-224
27. Murphy, L. O., MacKeigan, J. P., and Blenis, J. (2004) *Mol Cell Biol* **24**, 144-153
28. Kim, H. J., and Bar-Sagi, D. (2004) *Nat Rev Mol Cell Biol* **5**, 441-450
29. Gross, I., Morrison, D. J., Hynick, D. P., Georgas, K., English, M. A., Mericskay, M., Hosono, S., Sassoon, D., Wilson, P. D., Little, M., and Licht, J. D. (2003) *J Biol Chem* **278**, 41420-41430
30. Basson, M. A., Akhult, S., Watson-Johnson, J., Simon, R., Carroll, T. J., Shakya, R., Gross, I., Martin, G. R., Lufkin, T., McMahon, A. P., Wilson, P. D., Costantini, F. D., Mason, I. J., and Licht, J. D. (2005) *Dev Cell* **8**, 229-239

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Figure 1. RKIP shRNA enhances ERK and Raf-1 activity in response to EGF but does not affect DRaf-1:ER or Raf-1-CAAX Activity. (A) 293 cells stably expressing either human RKIP shRNA (shRNA) or a rat RKIP shRNA control vector (CON) were serum starved and treated with 100ng/ml EGF for 2 minutes. Whole cell extracts were probed with anti-phospho-ERK, anti-ERK, or anti-RKIP antibodies where indicated. ERK phosphorylation and RKIP expression were assessed by normalizing phospho-ERK or RKIP levels to ERK, and all samples were then normalized to the untreated control. The result shown is representative of 4 independent experiments. (B) 293 cells stably expressing a rat RKIP shRNA control (CON) or human RKIP shRNA (shRNA) vector were serum starved and treated with 100ng/ml EGF for 2 minutes where indicated (EGF), or left untreated (UT). Raf-1 was immunoprecipitated from whole cell extracts and subjected to an in vitro kinase assay using recombinant MEK, ERK, and myelin basic protein (MBP). MBP phosphorylation was normalized to the quantity of Raf-1 present. The graph depicts the range and mean of the fold increase in Raf-1 activity in response to EGF from two independent experiments. (C) HeLa cells stably expressing rat RKIP shRNA (CON) or human RKIP shRNA (shRNA) were serum-starved and either treated with 100 ng/ml EGF for two minutes or left untreated. Endogenous Raf-1, immunoprecipitated from whole cell lysates, was assayed as described in Methods. MBP phosphorylation was normalized to the quantity of Raf-1. The graph depicts the range and mean of the fold increase in Raf-1 activity in response to EGF from two independent experiments. (D) 293T control (CON) or human RKIP shRNA cell lines (shRNA) were transfected with 1µg HA-ERK2 and 2µg myc-Raf-1-CAAX or pcDNA where indicated. At 24 hours post transfection, cells were serum starved overnight. 25 µg whole cell extract was probed with anti-HA, anti-myc, anti-RKIP or anti-phospho-ERK antibodies where indicated. ERK phosphorylation was assessed by normalizing phospho-ERK levels to HA. The result shown is representative of three independent experiments.

Figure 2. RKIP inhibits phosphorylation of wild type Raf-1 on S338 by Pak and Y340/341 by Src but has no effect on constitutively active Raf-1. (A) 293T cells were transfected with 2mg GST-Raf-1, and either 8mg pcDNA (CON), FLAG-RKIP (WT), or FLAG-RKIP S153V (MUT) as indicated. At 24 hours after transfection, cells were serum starved overnight, treated with 100ng/ml EGF for 5’ or left untreated (UT). Purified GST-Raf was probed with anti-phosphoserine 338 Raf-1, anti-phosphoserine 259, or anti-GST antibodies where indicated. 25 µg whole cell extract was probed with anti-RKIP antibody to verify the presence of FLAG-RKIP. The result shown is representative of three independent experiments. (B) 293T cells stably expressing a control (CON) or human RKIP shRNA (shRNA) vector were serum starved and treated with 100ng/ml EGF for 5 minutes or left untreated (UT). 50µg whole cell extract was probed with anti-phosphoserine338 Raf-1, anti-phosphoserine 259 Raf-1, anti-tubulin, or anti-RKIP antibodies as indicated. The result shown is representative of three independent experiments. (C) 293T cells were transfected with 2µg GST-Raf-1, 0.5 µg of myc-Pak (TE) and increasing amounts of FLAG-RKIP as indicated. At 24 hours after transfection, cells were serum starved overnight. Purified GST-Raf was probed with anti-phosphoserine 338 Raf-1 or anti-GST antibodies where indicated. 25 µg whole cell extract was probed with anti-RKIP and anti-myc antibodies (IB) to verify the presence of FLAG-RKIP and myc-Pak. The result shown is representative of three independent experiments. (D) 293T cells were transfected with myc-Pak (WT) or myc-Pak 299R (MUT), a kinase dead mutant and either pcDNA3 (CON) or FLAG-RKIP (RKIP). At 24 hours post-transfection, cells were serum starved overnight and treated with 100ng/ml EGF for 5 minutes or left untreated. Immunoprecipitated Myc-PAK kinase activity was normalized to Pak levels. The graph shown is a plot of mean values ± S.D. from three independent experiments. (E) 293T cells were transfected with 1µg GST-Raf-1, 2µg myc-Pak,
and 7μg of either pcDNA3 (CON), FLAG-RKIP (WT), or FLAG-RKIP S153V (MUT). At 24 hours post transfection, cells were serum starved overnight and treated with 100ng/ml EGF for 5′ or left untreated (UT) where indicated. Purified GST-Raf-1 was probed with anti-myc or anti-GST antibodies where indicated. 25μg whole cell extract was used for immunoblotting (IB) with anti-myc or anti-RKIP antibodies where indicated. The result shown is representative of three independent experiments. (F) 293T cells were transfected with 2μg GST-Raf-1, 1 μg of v-Src and increasing amounts of FLAG-RKIP as indicated. At 24 hours after transfection, cells were serum starved overnight. Purified GST-Raf was probed with anti-phosphotyrosine 340/41 Raf-1 or anti-GST antibodies where indicated. 25 μg whole cell extract was probed with anti-RKIP and anti-Src antibodies (IB) to verify the presence of FLAG-RKIP and v-Src. v-Src induced an average 70% increase in the amount of tyrosine-phosphorylated 340/41 Raf-1. The result shown is representative of three independent experiments.

Figure 3. B-Raf and Raf-1 but not activated Raf-1-DDD stably associate with RKIP. (A) 293T control (CON) or human RKIP shRNA cell lines were transfected with 1μg HA-ERK2, 2μg myc-Raf-1-S338D/Y340D/Y341D (DDD) with 7μg FLAG-RKIP or pcDNA where indicated. At 24 hours post transfection, cells were serum starved overnight. 25 μg whole cell extract was probed with anti-HA, anti-myc, anti-RKIP or anti-phospho-ERK antibodies where indicated. ERK phosphorylation was assessed by normalizing phospho-ERK levels to HA-ERK. The result shown is representative of three independent experiments. (B) 293T cells were transfected with 2μg myc-Raf-1-S338D/Y340D/Y341D (DDD) and 8μg HA-RKIP or pcDNA. At 24 hours post transfection, cells were serum starved overnight. Cells were untreated or treated with 800nM TPA for 15 minutes and lysed with TAP lysis buffer. Raf-1-DDD was immunoprecipitated using anti-myc conjugated agarose beads. Immunoprecipitations and whole cells lysates (25μg) were probed with anti-myc or anti-HA antibodies. The result shown is representative of three independent experiments. (C) 293T cells were transfected with 5μg rat (-) or human RKIP shRNA (+) along with 1μg ΔRaf-1:ER or pcDNA and 1μg pBABE-PURO. At 24 hours post transfection, cells were selected for 24 hours in 2μg/ml puromycin-containing media to enrich the transfected populations. Cells were serum starved overnight and treated with 100ng/ml EGF for 2 minutes or 1μM estradiol for 1 hour. 25 μg whole cell extract was probed with anti-RKIP, anti-Raf-1, anti-tubulin, or anti-phospho-ERK antibodies where indicated. ERK phosphorylation was assessed by normalizing phospho-ERK levels to tubulin. The result shown is representative of three independent experiments. (D) 293T cells were transfected with either pcDNA or 2μg GST-Raf-1 and 8μg HA-RKIP or 2μg GST-B-Raf and 8μg HA-RKIP. At 24 hours post transfection, cells were serum starved overnight. Cells were untreated or treated with 800nM TPA for 15 minutes and lysed with TAP lysis buffer. Raf-1 or B-Raf were immunoprecipitated using Glutathione Sepharose. Immunoprecipitations were probed with anti-GST or anti-HA antibodies. The result shown is representative of at least three independent experiments.

Figure 4. EGF activates B-Raf kinase and depletion of Raf-1 and B-Raf decrease EGF stimulation of MAPK. (A) H19-7 cells stably expressing a human RKIP shRNA (control) or rat RKIP shRNA (shRKIP) vector were serum starved overnight and treated with 100ng/ml EGF for 5 minutes where indicated (EGF), or left untreated. B-Raf was immunoprecipitated from whole cell extracts and subjected to an in vitro kinase assay using recombinant MEK, ERK, and myelin basic protein (MBP). MBP phosphorylation was normalized to the amount of B-Raf present. The graph shown is a plot of mean data ± range from two independent experiments with duplicate samples. (B) 293T cells stably expressing a rat RKIP shRNA (control) or human RKIP shRNA (shRKIP) vector were serum starved overnight and treated with 100ng/ml EGF for 5 minutes where indicated (EGF), or left untreated. B-Raf was immunoprecipitated from whole cell extracts and subjected to an in vitro kinase assay using recombinant MEK, ERK, and myelin basic protein.
MBP phosphorylation was normalized to the amount of B-Raf present. The graph shown is a plot of mean data ± range from two independent experiments with duplicate samples. (C) H19-7 cells were mock transfected (control) or transfected with rat B-Raf siRNA, serum starved overnight and treated with 100ng/ml EGF for 5 minutes where indicated (EGF), or left untreated. Whole cell extracts (25 µg) were probed with anti-phospho-ERK, anti-B-Raf, or anti-tubulin antibodies where indicated. ERK phosphorylation was assessed by normalizing phospho-ERK to tubulin, and all samples were then normalized to the untreated control. The graph shown is a plot of mean data ± SD from three independent experiments. (D) H19-7 cells were mock transfected (control) or transfected with rat Raf-1 siRNA, serum starved overnight and treated with 100ng/ml EGF for 5 minutes where indicated (EGF), or left untreated. Whole cell extracts (25 µg) were probed with anti-phospho-ERK, anti-Raf-1, or anti-tubulin antibodies where indicated. ERK phosphorylation was assessed by normalizing phospho-ERK to tubulin, and all samples were then normalized to the untreated control. The graph shown is a plot of mean data ± SD from three independent experiments. (E) 293T cells were mock transfected (control) or transfected with human B-Raf siRNA, serum starved overnight and treated with 100ng/ml EGF for 5 minutes where indicated (EGF), or left untreated. Whole cell extracts (25 µg) were probed with anti-phospho-ERK, anti-B-Raf, or anti-tubulin antibodies where indicated. ERK phosphorylation was assessed by normalizing phospho-ERK to tubulin, and all samples were then normalized to the untreated control. The graph shown is a plot of mean data ± range from two independent experiments. (F) 293T cells were mock transfected (control) or transfected with human Raf-1 siRNA, serum starved overnight and treated with 100ng/ml EGF for 5 minutes where indicated (EGF), or left untreated. Whole cell extracts (25 µg) were probed with anti-phospho-ERK, anti-Raf-1, or anti-tubulin antibodies where indicated. ERK phosphorylation was assessed by normalizing phospho-ERK to tubulin, and all samples were then normalized to the untreated control. The graph shown is a plot of mean data ± range from two independent experiments.

Figure 5. RKIP depletion alters the amplitude and dose response but not duration of ERK stimulation in response to EGF. (A) H19-7 cells stably expressing Rat RKIP shRNA (Rat shRKIP) or Human RKIP shRNA (CON) were serum starved overnight and treated for the times indicated with 100ng/ml EGF. 25µg whole cell extract was immunoblotted with anti-tubulin, anti-phospho-ERK, or anti-RKIP antibodies where indicated. ERK phosphorylation was assessed by normalizing phospho-ERK levels to tubulin as shown in the graph. The result shown is representative of two independent experiments. (B) 293T cells stably expressing Rat RKIP shRNA (CON) or Human RKIP shRNA (shRNA) were serum starved overnight and treated for the times indicated with 100ng/ml EGF. 25µg whole cell extract was immunoblotted with anti-tubulin, anti-phospho-ERK, or anti-RKIP antibodies where indicated. ERK phosphorylation was assessed by normalizing phospho-ERK levels to tubulin as shown in the graph. The result shown is representative of three independent experiments. (C) H19-7 cells stably expressing Human RKIP shRNA (CON) or Rat RKIP shRNA (Rat shRKIP) were serum starved overnight and treated for 5 minutes with EGF at the doses indicated. 25µg whole cell extract was immunoblotted with anti-tubulin, anti-phospho-ERK, or anti-RKIP antibodies. ERK phosphorylation was assessed by normalizing phospho-ERK levels to tubulin as shown in the graph. The result shown is representative of two independent experiments. (D) 293T cells stably expressing Human RKIP shRNA (Human shRKIP) or Rat RKIP shRNA (Control) were serum starved overnight and treated for 5 minutes with EGF at the doses indicated. 25µg whole cell extract was immunoblotted with anti-tubulin, anti-phospho-ERK, or anti-RKIP antibodies. ERK phosphorylation was assessed by normalizing phospho-ERK levels to tubulin as shown in the graph. The result shown is representative of three independent experiments.
Figure 6. RKIP depletion alters the dose response for DNA synthesis in response to EGF. (A) H19-7 cells stably expressing Human RKIP shRNA (CON) or Rat RKIP shRNA (Rat shRKIP) were serum deprived in 0.2% FBS for 3 days and treated overnight with EGF and BrdU label. Cells were fixed, denatured and then blocked overnight in 5% BSA/PBS. The Calbiochem cell proliferation assay kit was used to detect DNA synthesis. B) 293T cells stably expressing Human RKIP shRNA (Human shRKIP) or Rat RKIP shRNA (Control) were serum deprived in 0.2% FBS for 3 days and treated for overnight with EGF and BrdU label. Cells were treated as in A.

Figure 7. Scheme depicting RKIP regulation of EGF-Stimulated Raf-1. RKIP regulates EGF-stimulated Raf-1 activation by binding to Raf-1 and inhibiting phosphorylation by Pak and Src family kinases. In unstimulated cells, RKIP is bound to Raf-1 and blocks access to key activating kinases. Upon stimulation of cells by EGF, RKIP/Raf-1 is recruited to the membrane and phosphorylated by PKC at S153, resulting in release of RKIP from Raf-1. The activating Pak and Src family kinases can now associate with Raf-1, resulting in the phosphorylation of Raf-1 at the key residues S338 and Y341.
Raf kinase inhibitory protein regulates Raf-1 but not B-Raf kinase activation
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