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Regulation of Raf-1 by Direct Feedback Phosphorylation

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Summary

The Raf-1 kinase is an important signaling molecule, functioning in the Ras pathway to transmit mitogenic, differentiative, and oncogenic signals to the downstream kinases MEK and ERK. Because of its integral role in cell signaling, Raf-1 activity must be precisely controlled. Previous studies have shown that phosphorylation is required for Raf-1 activation, and here, we identify six phosphorylation sites that contribute to the downregulation of Raf-1 after mitogen stimulation. Five of the identified sites are proline-directed targets of activated ERK, and phosphorylation of all six sites requires MEK signaling, indicating a negative feedback mechanism. Hyperphosphorylation of these six sites inhibits the Ras/Raf-1 interaction and desensitizes Raf-1 to additional stimuli. The hyperphosphorylated/desensitized Raf-1 is subsequently dephosphorylated and returned to a signaling-competent state through interactions with the protein phosphatase PP2A and the prolyl isomerase Pin1. These findings elucidate a critical Raf-1 regulatory mechanism that contributes to the sensitive, temporal modulation of Ras signaling.

Introduction

The Ras pathway is a critical signal transduction cascade involved in regulating cellular proliferation, differentiation, survival, and oncogenic transformation. Members of the Raf serine/threonine kinase family are key intermediates in this cascade, functioning to relay signals from activated Ras to the downstream protein kinases MEK and ERK (Marshall, 1996). Three Raf proteins are found in mammalian cells, Raf-1, A-Raf, and B-Raf (Hagemann and Rapp, 1999). Raf-1 is the most widely expressed of these family members with significant protein levels detected in all cell types examined (Storm et al., 1990). In addition, mouse knockout studies have revealed that Raf-1 is required for viability, acting in the transmission of normal growth and developmental cues (Hüser et al., 2001; Mikula et al., 2001; Wojnowski et al., 1998).

As might be expected for a protein so centrally involved in cell signaling, Raf-1 also contributes to oncogenic processes. For example, deregulated or constitutively active Raf-1 proteins can themselves cause cell transformation (Heidecker et al., 1990; Stanton et al., 1989). In addition, mutation or amplification of upstream regulators of Raf-1, such as receptor tyrosine kinases (Holbro et al., 2003; Marmor et al., 2004) and Ras (Bos, 1989; Pruitt and Der, 2001), frequently induces deregulated signaling in tumors through the Raf/MEK/ERK cascade. More recently, Raf-1 has been found to be activated in cells expressing oncogenic B-Raf proteins (Wan et al., 2004). Mutation of B-Raf, is observed in ~67% of malignant melanomas as well as in many colorectal, ovarian, and papillary thyroid carcinomas (Garnett and Marais, 2004). Along with altering B-Raf activity, the vast majority of the oncogenic mutations allow B-Raf to cooperatively activate Raf-1, providing an increased proliferative capacity to cells harboring these mutations.

The regulation of Raf-1 activity is complex, involving inter- and intramolecular protein interactions as well as direct phosphorylation (reviewed in Chong et al. [2003] and Dhillon and Kolch [2002]). In a quiescent cell, Raf-1 exists in an inactive state in the cytosol. The inactive conformation of Raf-1 is maintained by autoinhibitory interactions occurring between the N-terminal regulatory and the C-terminal catalytic domains and by the binding of a 14-3-3 dimer that contacts two phosphorylation sites, S259 and S621. Typically, the activation of Raf-1 is initiated by its interaction with Ras, which leads to the relocalization of cytosolic Raf-1 to the plasma membrane. Ras binding also promotes conformational changes that relieve Raf-1 autoinhibition and facilitate the phoshorylation of activating sites. These activating sites are found in the Raf-1 catalytic domain and include S338, Y341, T491, and S494. Although many details of the Raf-1 activation process have been elucidated, surprisingly little is known regarding how Raf-1 activity is attenuated after signaling events. Uncovering the mechanism(s) responsible for Raf-1 inactivation, however, is an important goal, given that constitutive signaling from Raf-1 results in cell transformation.

In this report, we investigate the molecular events that mediate the downregulation of Raf-1 activity. Here, we identify six residues of Raf-1 (S29, S43, S289, S296, S301, and S642) that become hyperphosphorylated in a manner coincident with Raf-1 inactivation. We find that the hyperphosphorylation of these six sites is dependent on downstream MEK signaling and renders Raf-1 unresponsive to subsequent activational events. Raf-1 proteins mutated at these phosphorylation sites exhibit prolonged membrane localization after mitogen stimulation and have increased enzymatic and biologi-

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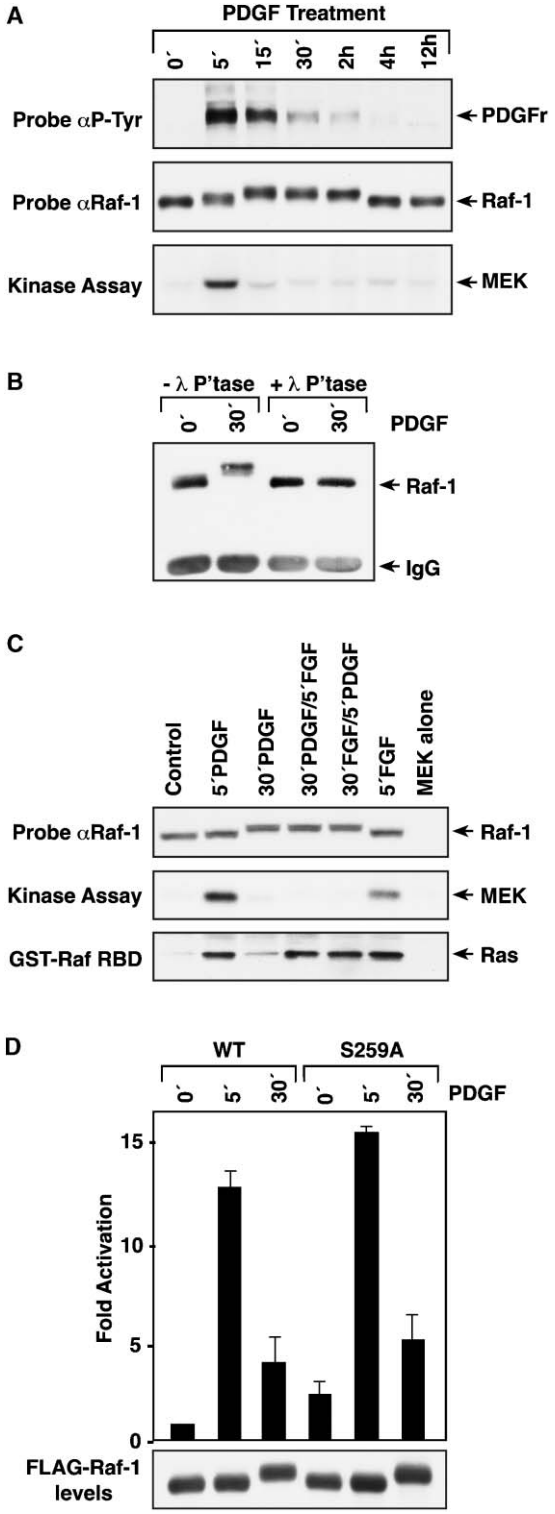

Figure 1. Hyperphosphorylation of Raf-1 after Mitogen Activation

(A) Quiescent NIH3T3 cells were stimulated with PDGF for the indicated times. Cell lysates were prepared and examined by immunoblot analysis for the presence of tyrosine phosphorylated PDGF receptor (PDGFr) (top) and Raf-1 (middle). Raf-1 kinase activity was determined by immune complex kinase assays with recombinant MEK (K79M) as a substrate. Labeled proteins were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography (bottom).

cal activity. Moreover, we find that the hyperphosphorylated/desensitized Raf-1 interacts with the protein phosphatase PP2A and the Pin1 prolyl isomerase, both of which are required for the recycling of Raf-1 to an activation-competent state. Taken together, these findings suggest that Raf-1 is negatively regulated by direct feedback phosphorylation, that the inactive Raf-1 returns to an activation-competent state by a mechanism involving conformational changes and dephosphorylation, and that this regulatory cycle is critical for the temporal modulation of Ras signaling.

Results

Raf-1 Inactivation by Feedback Phosphorylation

Treatment of quiescent NIH3T3 cells with a mitogenic growth factor such as platelet-derived growth factor (PDGF) results in the rapid but transient activation of Raf-1. When the activation state of Raf-1 is measured with MEK as an exogenous substrate, Raf-1 activity peaks at 5 min of PDGF treatment and returns to near basal levels by 15 min (Figure 1A). In addition, mitogen stimulation results in a change in the electrophoretic mobility of Raf-1 with the entire pool of Raf-1 shifting to a slower migrating form by 15 min of treatment (Figure 1A). This shifted form persists for several hours, and the original mobility of Raf-1 is not restored until approximately 4 hr after stimulation. This change in Raf-1 mobility is due to increased phosphorylation, because treatment of the shifted Raf-1 protein with λ phosphatase returns Raf-1 to the faster migrating form (Figure 1B). Significantly, the hyperphosphorylation of Raf-1 occurs at a time coincident with the inactivation of Raf-1, and this hyperphosphorylated Raf-1 appears to be refractory to subsequent stimuli. As shown in Figure 1C, Raf-1 becomes activated when quiescent fibroblasts are treated for 5 min with either PDGF or FGF. However, when cells are treated with one growth factor for 30 min and are then challenged with another growth factor for 5 min, Raf-1 can no longer be activated even though the subsequent treatment causes upstream Ras activation. These findings suggest that Raf-1 may be the target of feedback phosphorylation that inactivates Raf-1 and/or desensitizes it to subsequent stimulatory events.

The phosphorylation of S259 has been previously implicated in the downregulation of Raf-1 mediated by

(B) Raf-1 immunoprecipitates prepared from quiescent and PDGF-stimulated NIH3T3 cells were treated or not with λ phosphatase. The electrophoretic mobility of Raf-1 was then monitored by immunoblot analysis.

(C) Quiescent NIH3T3 cells were stimulated as indicated with PDGF and/or FGF prior to lysis. Raf-1 electrophoretic mobility (αRaf-1) and kinase activity were examined as described in (A). Cell lysates were incubated with agarose beads containing GST-Raf-RBD. Activated Ras proteins binding to the Raf RBD were detected by immunoblot analysis.

(D) Quiescent NIH3T3 cells expressing FLAG-tagged wt or S259A Raf-1 were stimulated with PDGF prior to lysis. Immunoprecipitated FLAG-Raf-1 proteins were examined for kinase activity, protein level, and electrophoretic mobility. Raf-1 kinase activity is expressed as fold activation relative to wt Raf-1 from quiescent cells. Error bars represent the standard deviation.

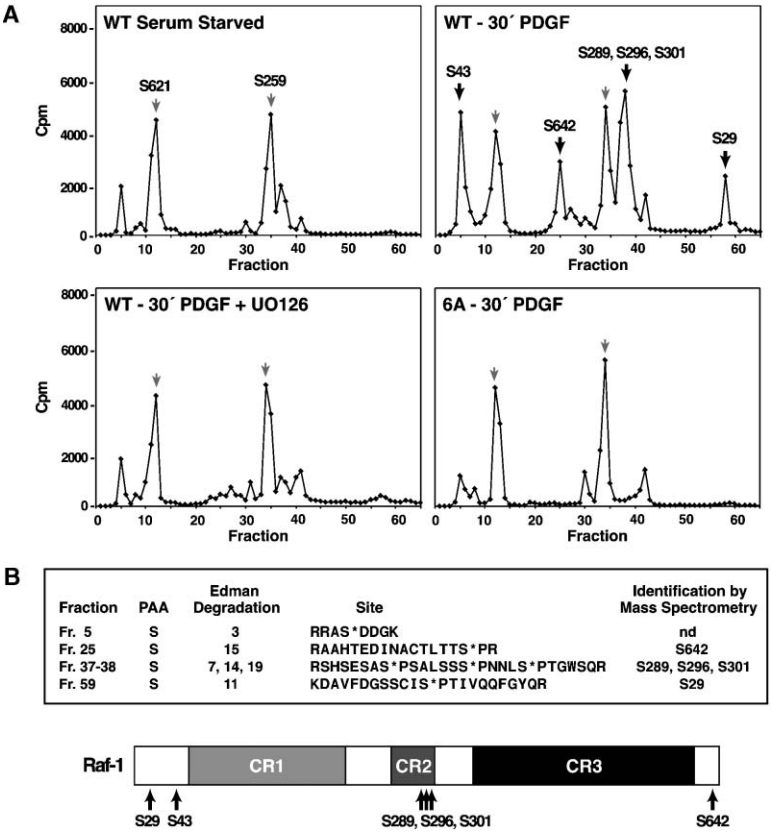

Figure 2. Identification of Raf-1 Phosphorylation Sites

Quiescent NIH3T3 cells expressing FLAG-tagged wt or 6A Raf-1 were metabolically labeled with [^{32}P]orthophosphate and treated with PDGF or PDGF and U0126 prior to lysis. Immunoprecipitated FLAG-Raf-1 proteins were digested with trypsin and the tryptic phosphopeptides were separated by HPLC. (A) Shown are the profiles of radioactivity collected in the HPLC fractions. (B) Phosphopeptides isolated in the HPLC fractions were subjected to phosphoamino acid analysis (PAA), Edman degradation, and mass spectrometry to determine the residue(s) phosphorylated (indicated by the asterisk). The number under the Edman degradation column indicates the cycle in which ^{32}P counts were released. A schematic representation of Raf-1 is shown indicating the location of S29, S43, S289, S296, S301, and S642. The shaded boxes represent the three conserved regions (CR1, CR2, and CR3) of the Raf family members.

protein kinase A (PKA) and AKT (Dhillon et al., 2002; Dumaz and Marais, 2003; Zimmermann and Moelling, 1999). To determine whether S259 phosphorylation contributes to the hyperphosphorylation and inactivation of Raf-1 observed after mitogen stimulation, we examined the activation kinetics and electrophoretic mobility of Raf-1 mutated at the S259 site (S259A). As depicted in Figure 1D, S259A Raf-1 has a higher basal activity than wild-type (wt) Raf-1; however, both proteins become activated and subsequently inactivated with similar kinetics (Figure 1D). In addition, the migration of S259A Raf-1 at the 30 min time point was reduced to a similar extent as wt Raf-1, indicating that the phosphorylation events causing this mobility shift are independent of S259 phosphorylation.

Identification of Raf-1 Phosphorylation Sites

Metabolic labeling experiments were performed with NIH3T3 cells expressing wt Raf-1 to determine which residues are phosphorylated at the time of Raf-1 inactivation. Analysis of Raf-1 tryptic phosphopeptides by high pressure liquid chromatography (HPLC), phosphoamino acid analysis, and Edman degradation revealed that Raf-1 isolated from quiescent cells was highly phosphorylated on two sites, S259 and S621 (HPLC fraction 12 and fraction 34–35 eluates, respectively) (Figure 2A). These sites have been previously characterized, and both function as binding sites for 14-3-3 proteins (Morrison et al., 1993; Muslin et al., 1996). After 30 min of PDGF treatment, phosphorylation of S259 and S621 was still observed; however, numerous other phosphopeptides were also detected (Figure 2A). Eluting from the HPLC column in fraction 5 was a peptide phosphorylated at S43, a site previously demonstrated to be a substrate of PKA (Wu et al., 1993). Although weak phosphorylation of S43 was observed in quiescent cells, its phosphorylation state increased 3- to 7-fold after 30 min of PDGF treatment. Eluting in fractions 25 and 58 were peptides phosphorylated at S642 and S29, respectively, and eluting in fraction 37–38 was a peptide triply phosphorylated on residues S289, S296, and S301. Interestingly, S29, S289, S296, S301, and S642 all represent phosphorylated serine residues followed by a proline (pSP) (Figure 2B). Analysis of the hyperphosphorylated Raf-1 by mass spectrometry confirmed the identity of these five pSP sites (Figure 2B). Moreover, when the S43 site and the five pSP sites were mutated to alanine (6A Raf-1), the hyperphosphorylation of Raf-1 was abolished. Together, these results demonstrate that Raf-1 is phosphorylated on multiple serine sites subsequent to its activation after mitogen treatment.

Raf-1 Is a Substrate of Activated ERK

Because S29, S289, S296, S301, and S642 are likely phosphorylated by a proline-directed kinase and ERK is a proline-directed kinase activated downstream of Raf-1, we investigated whether ERK might mediate the phosphorylation of these specific SP sites. First, using the MEK inhibitor U0126 to prevent the in vivo activation of ERK, we found that the electrophoretic mobility (Figure 3A) and phosphorylation state of Raf-1 (Figure 2A) did not change when cells were stimulated with PDGF

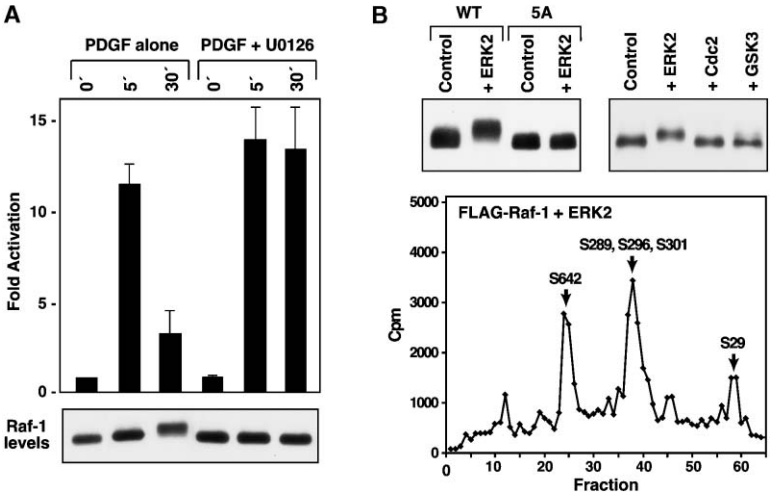

Figure 3. Feedback Phosphorylation of Raf-1 Requires Activation of the MEK/ERK Cascade

(A) Quiescent NIH3T3 cells were stimulated with PDGF or PDGF and UO126 prior to lysis. Raf-1 immunoprecipitates were examined for kinase activity, protein levels, and electrophoretic mobility. Error bars represent the standard deviation.

(B) FLAG-tagged wt and 5A Raf-1 proteins were affinity purified from serum-starved NIH3T3 cells. In vitro kinase assays were performed with the purified FLAG-Raf-1 proteins as substrates and activated ERK2, Cdc2, or GSK3 as kinases. Proteins were separated by SDS-PAGE and the electrophoretic mobility of Raf-1 was monitored by immunoblot analysis. FLAG-Raf-1 phosphorylated by activated ERK2 in the presence of γ[^{32}P]ATP was isolated and digested with trypsin. The tryptic phosphopeptides were separated by HPLC. Shown are the profiles of radioactivity collected in the HPLC fractions and the identities of the residues phosphorylated.

for 30 min in the presence of U0126. Interestingly, U0126 prevented the hyperphosphorylation of not only the five SP sites but also the S43 site. In addition, as previously reported (Alessi et al., 1995), Raf-1 activity was sustained in cells treated with U0126 (Figure 3A). Thus, signaling downstream of MEK is required for the inactivation and hyperphosphorylation of Raf-1 observed after mitogen treatment.

We next examined whether Raf-1 is an in vitro substrate of ERK. As shown in Figure 3B, when purified activated ERK2 was incubated with Raf-1 isolated from quiescent cells, it phosphorylated Raf-1 on sites that induced a shift in Raf-1's electrophoretic mobility. Further analysis revealed that the sites phosphorylated by ERK2 in vitro were S29, S289, S296, S301, and S642, the same pSP sites phosphorylated in vivo (Figure 3B). Mutation of these five pSP sites (5A Raf-1) abolished ERK's ability to shift Raf-1 to its slower migrating form (Figure 3B). Interestingly, when similar in vitro kinase assays were performed with two other proline-directed kinases, Cdc2 and GSK3, neither of these kinases was able to phosphorylate Raf-1 or induce a change in its mobility (Figure 3B). Therefore, these results are consistent with a model whereby ERK kinases mediate the in vivo phosphorylation of Raf-1 on S29, S289, S296, S301, and S642.

Effects of Feedback Phosphorylation on Raf-1 Activity

To address whether the hyperphosphorylation of Raf-1 alters its function, we used the 6A Raf-1 protein in which the five SP sites as well as the S43 site were mutated to alanine. Wt and 6A Raf proteins were expressed in NIH3T3 cells, and their enzymatic activity was determined in response to PDGF treatment. As shown in Figure 4A, both proteins were activated to a similar extent after 5 min of stimulation; however, the 6A protein had a 2-fold higher basal activity than did wt Raf-1 and exhibited sustained kinase activity at the 30 min time point. In addition, the electrophoretic mobility of 6A Raf-1 did not change over the time course of treatment.

To evaluate the biological activity of the 6A mutant, we used the meiotic maturation of *Xenopus* oocytes as an assay system. In this system, expression of activated Ras^{V12} alone causes oocyte maturation; yet, the kinetics of maturation can be accelerated when Ras^{V12} is coexpressed with wt Raf-1. As has been observed for wt Raf-1 (Fabian et al., 1993), expression of 6A Raf-1 alone was insufficient to induce oocyte maturation; nevertheless, when 6A Raf-1 was coexpressed with Ras^{V12}, it accelerated the maturation kinetics to an even greater extent than did wt Raf-1 (Figure 4B). These findings together with the results from the in vitro kinase assays indicate that 6A Raf-1 has increased enzymatic and biological activity.

Hyperphosphorylation of Raf-1 Inhibits the Ras/Raf-1 Interaction

The ability of wt and 6A Raf-1 to associate with various known Raf-1 binding partners was compared to further assess the effect of feedback phosphorylation on Raf-1 function. Coimmunoprecipitation experiments or glutathione-S-transferase (GST) pull-down assays were performed with lysates of wt or 6A-Raf-1 expressing NIH3T3 cells that had been serum starved or treated with PDGF for 30 min. As shown in Figure 4C, equivalent amounts of wt or 6A Raf-1 from quiescent and PDGF-treated cells interacted with a GST-14-3-3 fusion protein. Coimmunoprecipitation experiments confirmed this finding and further indicated that mitogen treatment had no effect on the ability of wt or 6A Raf-1 to associate with hsp90, cdc37, or MEK (data not shown). In contrast, feedback phosphorylation was found to alter the interaction between wt Raf-1 and activated Ras^{V12}. Although equivalent amounts of wt and 6A Raf-1 from quiescent cells interacted with a GST-Ras^{V12} fusion protein, PDGF treatment greatly reduced the amount of wt Raf-1 that associated with GST-Ras^{V12}, whereas it had no effect on the 6A Raf-1/GST-Ras^{V12} interaction (Figure 4C).

One in vivo consequence of the Ras/Raf-1 interaction is the recruitment of Raf-1 to the plasma membrane. Because the above results suggest that the hyperphosphorylation of Raf-1 may inhibit or disrupt the Ras/Raf-1 interaction, the intracellular localization of wt and 6A

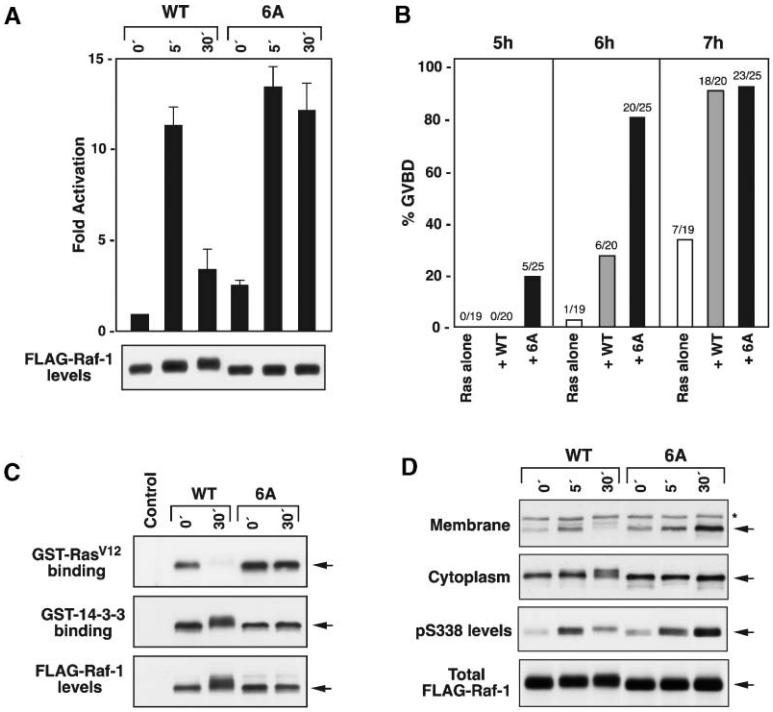

Figure 4. Feedback Phosphorylation Alters Raf-1 Function

(A) Quiescent NIH3T3 cells expressing FLAG-tagged wt or 6A Raf-1 were treated with PDGF prior to lysis. Immunoprecipitated FLAG-Raf-1 proteins were examined for kinase activity, protein level, and electrophoretic mobility. Error bars represent the standard deviation.

(B) Stage VI *Xenopus* oocytes were injected with mRNAs encoding wt and 6A Raf-1. Oocytes were subsequently injected with activated Ras^{V12} mRNA and scored for maturation (as determined by germinal vesicle breakdown, GVBD) 5, 6, and 7 hr after Ras injection.

(C) Cell lysates were prepared from quiescent or PDGF-treated NIH3T3 cells expressing FLAG-tagged wt or 6A Raf-1. The lysates were incubated with agarose beads containing the indicated GST-fusion protein or αFLAG antibody. Complexes were separated by SDS-PAGE and examined by immunoblot analysis with αFLAG antibody.

(D) Membrane and cytoplasmic fractions were prepared from quiescent or PDGF-treated NIH3T3 cells expressing FLAG-tagged wt or 6A Raf-1. Levels of FLAG-Raf-1 in each fraction were determined by immunoblot analysis (top panels). The asterisk denotes a nonspecific protein in membrane fractions that crossreacts with the αFLAG antibody. FLAG-Raf-1 immunoprecipitates from total cell lysates were examined for phospho-S338 (αpS338) and total FLAG-Raf-1 levels (bottom panels).

Raf-1 was examined (Figure 4D). In cell fractionation experiments, wt Raf-1 was detected in the membrane fraction after 5 min of PDGF treatment; however, little protein was observed prior to stimulation or at the 30 min time point. In contrast, significant levels of 6A Raf-1 were present in the membrane fraction prior to PDGF stimulation, and the 6A mutant continued to accumulate at the membrane even after 30 min of treatment.

An activation event that occurs upon the translocation of Raf-1 to the plasma membrane is the phosphorylation of S338. Using antibodies that recognize phospho-S338, we found that wt-Raf-1 was maximally phosphorylated on S338 after 5 min of PDGF treatment (Figure 4D), coincident with its membrane localization and peak kinase activity. However, for 6A Raf-1, S338 phosphorylation persisted at the 30 min time point, correlating with this protein's sustained kinase activity and prolonged membrane localization.

Hyperphosphorylated Raf-1 Is Recycled by PP2A-Mediated Dephosphorylation

Pulse-chase experiments were performed with pooled stable cell lines expressing FLAG-tagged wt or 6A Raf-1 to determine the fate of the hyperphosphorylated Raf-1 protein. The cells were labeled for 2 hr with [^{35}S]methionine, after which they were washed and incubated in chase media containing either U0126 (to prevent Raf-1 hyperphosphorylation) or PDGF (to induce Raf-1 hyperphosphorylation). As shown in Figure 5A, the half-life of both 6A and wt Raf-1 was ~12 hr, and neither U0126 nor PDGF treatment altered the stability of these proteins. In addition, the ^{35}S-labeled wt protein was found to shift to a slower migrating form after 30 min of PDGF treatment but returned to its faster migrating form by 4 hr (Figure 5A).

The above findings indicate that the hyperphosphorylated Raf-1 protein is not degraded but, rather, is recycled as a result of dephosphorylation. A major cellular phosphatase that dephosphorylates p(S/T)P consensus motifs is the protein phosphatase 2A (PP2A). Consistent with previous findings that PP2A interacts with Raf-1 (Ory et al., 2003), all three subunits of the heterotrimeric PP2A enzyme were detected by mass spectrometry in Raf-1 complexes isolated from COS cells treated for 30 min with EGF (Figure 5B). Experiments were performed with the phosphatase inhibitor okadaic acid (OA) to determine if PP2A contributes to the dephosphorylation of Raf-1. Quiescent cells were stimulated for 5 min with PDGF to initiate the signaling cascade, after which OA was added to the cells under conditions previously shown to inhibit PP2A, but not other OA-sensitive phosphatases (Favre et al., 1997). In cells treated with PDGF and OA, the migration of endogenous Raf-1 was still retarded at the 4 hr time point (Figure 5C). Moreover, by using an antibody that recognizes phospho-S642, the phosphorylation of this specific pSP site was strongly detected at the 4 hr time point in cells treated with PDGF and OA, whereas in cells treated with PDGF alone, phosphorylation of this residue peaked at the 30 min time point but returned to baseline levels by 4 hr.

Involvement of the Pin1 Prolyl Isomerase in the Dephosphorylation/Recycling of Raf-1

p(S/T)P peptide bonds can exist in two conformations, *cis* and *trans*; however, PP2A only efficiently dephosphorylates p(S/T)P consensus motifs if the bond is in

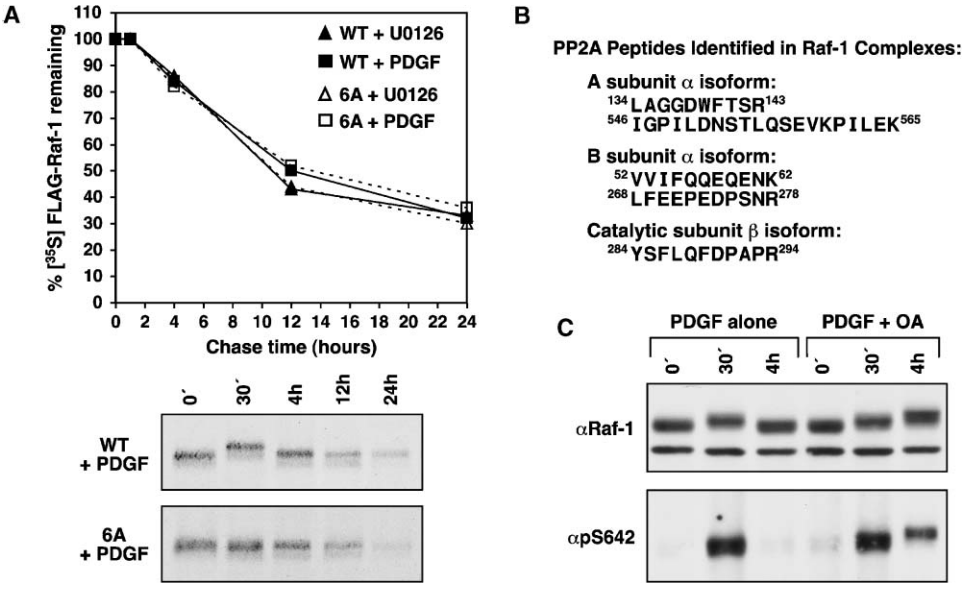

Figure 5. Hyperphosphorylated Raf-1 Is Recycled by PP2A-Mediated Dephosphorylation

(A) NIH3T3 cells stably expressing FLAG-tagged wt or 6A Raf-1 were metabolically labeled for 2 hr with [^{35}S]-methionine. Cells were washed and chased in media containing PDGF or U0126 for the times indicated. Labeled FLAG-Raf-1 proteins were immunoprecipitated from cell lysates, separated by SDS-PAGE, and examined by autoradiography. Radioactivity incorporated into the immunoprecipitated Raf-1 was quantified and normalized to the 0 hr time point.

(B) Raf-1 complexes isolated from COS cells treated with EGF for 30 min were analyzed by mass spectrometry. Shown are sequences of PP2A peptides identified.

(C) Quiescent NIH3T3 cells were stimulated with PDGF for 5 min to initiate the signaling cascade after which the PDGF treatment was continued in the presence or absence of okadaic acid (OA). Raf-1 immunoprecipitates were prepared and examined for electrophoretic mobility (αRaf-1) and phospho-S642 levels (αpS642).

the *trans* conformation (Zhou et al., 2000). Pin1 is the cellular protein that isomerizes p(S/T)P bonds (Yaffe et al., 1997), and Pin1 activity has been shown to be required for the PP2A-mediated dephosphorylation of Tau, Cdc25C, and Myc (Yeh et al., 2004; Zhou et al., 2000). Given our results that PP2A contributes to the dephosphorylation/recycling of Raf-1, we next examined whether Pin1 might also be involved. Interestingly, Pin1 contains a WW domain that interacts specifically with p(S/T)P motifs, recruiting the isomerase to its targets (Lu et al., 1999). Using a GST fusion protein to detect Pin1-interacting molecules, we found that hyperphosphorylated wt Raf-1 from PDGF-treated cells strongly associated with GST-Pin1 (Figure 6A). In contrast, wt Raf-1 from untreated cells or 6A Raf-1 from PDGF-treated cells did not interact with Pin1. Confirming that this interaction occurs in vivo, peptides of Pin1 were detected in mass spectrometry analysis of Raf-1 complexes isolated from COS cells treated for 30 min with EGF (Figure 6B). Moreover, a Pin1 mutant (Pin1 S16A) (Lu et al., 2002) that constitutively associates with its targets interacted with wt, but not 6A Raf-1, when coexpression experiments were performed in COS cells (Figure 6C).

To address the involvement of Pin1 in the dephosphorylation of Raf-1, we next examined the phosphorylation status of endogenous Raf-1 in mouse embryo fibroblasts (MEFs) lacking Pin1 (Figure 6D). In wt MEFs, as was observed in NIH3T3 cells, Raf-1 shifted to a slower migrating form after 30 min of PDGF treatment but then returned to its faster migrating form by 4 hr. As expected, the increased phosphorylation of S642 (one of the five newly identifed pSP sites) correlated with the appearance of the shifted Raf-1 protein. In Pin1$^{-/-}$ MEFs, however, Raf-1 exhibited a reduced mobility prior to mitogen treatment and remained shifted at the 4 hr time point. Moreover, activation of Raf-1 induced by PDGF treatment was severely attenuated in the Pin1$^{-/-}$ MEFs (Figure 6E), consistent with our earlier finding that the hyperphosphorylated/shifted Raf-1 is refractory to activation. Demonstrating the importance of Pin1's isomerase activity to Raf-1 dephosphorylation, overexpression of a catalytically inactive Pin1 mutant (K63A Pin1) resulted in the sustained hyperphosphorylation of Raf-1 after mitogen treatment (Figure 6F). From these results, we conclude that Raf-1 is a substrate of Pin1 and that Pin1 activity is required for the efficient dephosphorylation and recycling of Raf-1 after mitogen activation.

Discussion

Despite its integral role in the Ras pathway, the molecular events regulating Raf-1 activity are still not fully understood. Many years of research have revealed key steps involved in Raf-1 activation; however, comparatively little is known regarding how Raf-1 activity is attenuated after signaling events. In this report, we demonstrate that direct feedback phosphorylation is an important mechanism contributing to the downregulation of Raf-1 after mitogen activation. Moreover, we find that feedback phosphorylation generates a desensitized Raf-1 protein that recycles to a signaling-competent state through dephosphorylation events requiring the activities of both PP2A and Pin1 (Figure 7). Our results

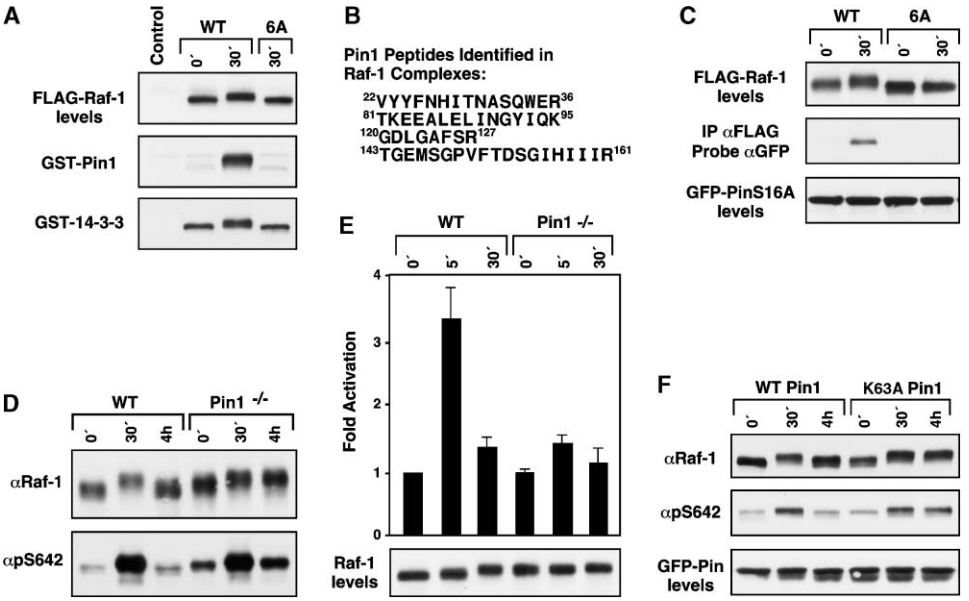

Figure 6. The Prolyl Isomerase Pin1 Facilitates PP2A-Mediated Raf-1 Dephosphorylation

(A) Cell lysates were prepared from quiescent or PDGF-treated NIH3T3 cells expressing FLAG-tagged wt or 6A Raf-1. The lysates were incubated with agarose beads containing the indicated GST-fusion protein or αFLAG antibody. Complexes were separated by SDS-PAGE and examined by immunoblot analysis with αFLAG antibody.

(B) Raf-1 complexes isolated from COS cells treated with EGF for 30 min were analyzed by mass spectrometry. Shown are sequences of Pin1 peptides identified.

(C) COS cells coexpressing GFP-tagged S16A Pin1 and FLAG-tagged wt or 6A Raf-1 were stimulated with EGF and lysed. FLAG-Raf-1 immunoprecipitates were examined for the presence of GFP-Pin1 and lysates were monitored for GFP-Pin1 and FLAG-Raf-1 levels.

(D) Wt or Pin1$^{-/-}$ MEFs were stimulated with PDGF prior to lysis. Raf-1 immunoprecipitates were examined for electrophoretic mobility (αRaf-1) and phospho-S642 (αpS642) levels.

(E) Wt and Pin1$^{-/-}$ MEFs were treated with PDGF prior to lysis. Immunoprecipitated Raf-1 proteins were examined for kinase activity, protein levels, and electrophoretic mobility. Error bars represent the standard deviation.

(F) COS cells expressing GFP-tagged wt or K63A Pin1 were stimulated with EGF and lysed. Raf-1 immunoprecipitates were examined for electrophoretic mobility (αRaf-1) and phospho-S642 (αpS642) levels, and cell lysates were monitored for GFP-Pin1 levels.

indicate that PP2A and Pin1 regulate Raf-1 in a manner similar to their effects on other phosphorylated proteins such as Cdc25C, Tau, and Myc, indicating that this mechanism of conformation-dependent dephosphorylation may be widely utilized in numerous cellular contexts.

Previous observations have suggested that Raf-1 might be a target of negative feedback inhibition. For example, in contrast to its usual transient activation after mitogen stimulation, Raf-1 activation is prolonged when MEK signaling is blocked by pharmacological inhibition (Alessi et al., 1995). In addition, increased Raf-1 activity is observed in cells expressing a dominant-negative KSR protein that prevents ERK activation by sequestering MEK (Therrien et al., 1996). Similarly, Raf-1 activity is elevated in cells overexpressing IMP, an inhibitor of KSR that disrupts the Raf-MEK interaction (Matheny et al., 2004). Together, these findings have implicated the activity of the MEK/ERK cascade in the downregulation of Raf-1; however, they have not elucidated the precise mechanism(s) mediating this effect.

In this study, we identify six Raf-1 residues that become hyperphosphorylated after mitogen activation. Phosphorylation of these sites induces a shift in the electrophoretic mobility of Raf-1 and correlates with the attenuation of Raf-1 kinase activity. Moreover, we find that this hyperphosphorylated/shifted form of Raf-1 is unresponsive to subsequent growth factor treatment and that a Raf-1 protein mutated at these six sites (6A Raf-1) exhibits sustained mitogen-induced kinase activity. Significantly, using the MEK inhibitor U0126, we find that the in vivo phosphorylation of all six sites as well as the mobility shift and downregulation of Raf-1 are dependent on activation of the MEK/ERK pathway.

Of the six phosphorylation sites identified, five represent previously uncharacterized proline-directed serine sites with S43 being the only site previously known. Although numerous studies have found S43 phosphorylation to be induced when cAMP levels and PKA activity are high, phosphorylation of this site has also been shown to increase after mitogen stimulation (Abraham et al., 2000; Dhillon et al., 2002). Our results indicate that the mitogen-induced phosphorylation of S43 is dependent on MEK activation; however, the kinase that phosphorylates S43 under these conditions is currently unknown. In contrast to the S43 site, our data strongly implicate ERK as the enzyme responsible for the phosphorylation of the five pSP sites. ERK is a proline-directed kinase whose activity is directly regulated by MEK, the downstream target of Raf-1. Moreover, we find that activated ERK2 is capable of phosphorylating Raf-1 on these five SP sites in vitro, whereas other proline-directed kinases such as Cdc2 and GSK3 cannot.

The six identified sites of feedback phosphorylation are distributed throughout the Raf-1 protein—S29 and S43 are located in the N-terminal region close to the

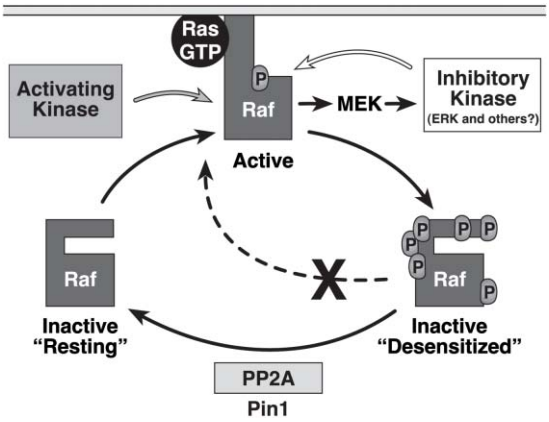

Figure 7. A Model for Raf-1 Regulation by Feedback Phosphorylation

Inactive, resting Raf-1 translocates from the cytosol to the plasma membrane upon mitogen stimulation. Raf-1 interacts with GTP bound Ras, and its catalytic domain is phosphorylated on activating sites. Active Raf-1 then phosphorylates and activates MEK, which, in turn, activates ERK. Through a negative feedback mechanism involving ERK, Raf-1 is phosphorylated on inhibitory sites, generating an inactive, desensitized Raf-1. Desensitized Raf-1 has a greatly reduced affinity for Ras-GTP and does not respond to additional stimulatory events. The Pin1 prolyl isomerase cooperates with the PP2A protein phosphatase to return the desensitized Raf-1 to the resting, activation-competent state.

Ras binding domain (RBD), S642 is found at the Raf-1 C terminus, and S289, S296, and S301 cluster in a region upstream of the kinase domain (Figure 2B). Phosphorylation of these sites might mediate the interaction of Raf-1 with an inhibitor protein, or they may promote or stabilize an inactive conformation of Raf-1 that may be more susceptible to the dephosphorylation of activating sites and/or be altered in its ability to respond to upstream signals. In support of the latter possibility, we find that hyperphosphorylation of Raf-1 inhibits the binding of Raf-1 to activated Ras and that mutation of theses sites to noncharged residues results in increased Ras binding, prolonged membrane association, and sustained phosphorylation of the activating pS338 site after mitogen treatment. Although S43 phosphorylation has been shown to reduce the affinity of Raf-1 for RasGTP in vitro (Wu et al., 1993), we and others (Dhillon et al., 2002) find that S43 phosphorylation alone does not abolish the Ras/Raf interaction or prevent the membrane localization of Raf-1, indicating that other phosphorylation events are required for these effects. The exact mechanism for how each of the feedback phosphorylation sites alters Raf-1 function is beyond the scope of this study; however, preliminary analysis indicates that all six sites contribute to the downregulation of Raf-1.

An important observation from this study is that the total cellular pool of Raf-1 appears to shift to the hyperphosphorylated/desensitized form after mitogen stimulation. Given that only a small fraction of Raf-1 becomes activated in response to mitogen treatment (Hallberg et al., 1994), Raf-1 activation does not appear to be required for the hyperphosphorylation of these sites. In addition, we find that the hyperphosphorylated/desensitized form of Raf-1 persists for several hours. The transitioning of the total pool of Raf to this prolonged desensitized state may have important consequences for the cell. In particular, it may be a key mechanism for preventing the overamplification of a proliferative signal. Furthermore, the generation of a desensitized Raf protein whose binding to Ras is inhibited may be a way to increase the signaling potential of other Ras effector cascades. In addition, the persistence of this desensitized form may protect the cell from transducing multiple, potentially conflicting signals, thus allowing the cell to commit to a particular response (i.e., differentiation or proliferation) before responding to a new signal.

Interestingly, we find that the hyperphosphorylated/desensitized Raf-1 is not degraded but, rather, is recycled to a signaling-competent state. In pulse-chase experiments, we find that the half-life of Raf-1 is approximately 12 hr in NIH3T3 cells and that the stability of Raf-1 is not altered by mutation of the phosphorylation sites or by mitogen treatment. We also find that the ^{35}S-labeled wt Raf-1 returns to its original electrophoretic mobility subsequent to its mitogen-induced shift, indicating that the hyperphosphorylated/shifted Raf-1 is dephosphorylated. An important cellular phosphatase that dephosphorylates p(S/T)P consensus motifs is PP2A, and our findings strongly implicate PP2A in the dephosphorylation of Raf-1. By mass spectrometry analysis, all three subunits of the heterotrimeric PP2A enzyme were detected in complexes containing the hyperphosphorylated/shifted form of Raf-1. Moreover, when PP2A activity was inhibited after signal activation, Raf-1 remained in the hyperphosphorylated state. PP2A has been previously shown to interact with Raf-1 and to function in the Raf-1 activation process by dephosphorylating an inhibitory phosphorylation site that mediates 14-3-3 binding (Ory et al., 2003). Given our findings implicating PP2A in the dephosphorylation of attenuated Raf-1, PP2A emerges as a pivotal regulatory component of this signaling pathway, having dual roles in Raf-1 activation as well as recycling.

Five of the Raf-1 feedback phosphorylation sites are pSP sites, and for PP2A to dephosphorylate these motifs, the p(S/T)P bond must be in the *trans* conformation (Zhou et al., 2000). Pin1 is the cellular protein that isomerizes p(S/T)P bonds and it contains a WW domain that binds specifically to p(S/T)P motifs, thereby targeting Pin1 to its substrates (Lu et al., 1999). Here, we show that Raf-1 interacts with Pin1 but only when the feedback sites are phosphorylated (five of which are pSP sites). We also find that Raf-1 persists in a hyperphosphorylated state in Pin1$^{-/-}$ MEFs and in COS cells overexpressing a catalytically inactive Pin1 mutant and that mitogen activation of Raf-1 is strongly suppressed in Pin1$^{-/-}$ MEFs, indicating that Pin1 activity is needed for the efficient dephosphorylation and recycling of Raf-1 to a signaling-competent state. Interestingly, like PP2A, which preferentially dephosphorylates the *trans* p(S/T)P isomer, proline-directed kinases such as ERK preferentially phosphorylate the *trans* (S/T)P isomer (Weiwad et al., 2000). Therefore, our finding that efficient dephosphorylation of hyperphosphorylated Raf-1 requires Pin1 activity suggests that at least one of the Raf-1 pSP sites transitions to the *cis* conformation after phosphorylation. The *cis* pSP isomer may be generated because of

the action of Pin1, which catalyzes *trans* to *cis* as well as *cis* to *trans* isomerization, or alternatively, phosphorylation may cause local structural constraints in the native protein that promote *cis* isomer formation.

The Ras/RAF/MEK/ERK signaling cascade plays an important role in the development of human malignancies as well as in normal growth processes. In this era of targeted cancer therapeutics, elucidating the precise mechanisms regulating Ras-dependent signal transduction can provide valuable insight for the design of anticancer strategies. The findings presented in this study not only elucidate a key mechanism contributing to the temporal regulation of Ras/Raf signaling, they also identify critical molecules involved in this process. In particular, the identification of Pin1 as a regulator of Raf-1 recycling is intriguing, given that Pin1 is overexpressed in a broad array of human tumors and has been found to potentiate the function of several known oncogenic pathways (reviewed in Lu [2003] and Ryo et al. [2003]). The inhibition of Pin1 activity has also been shown to trigger the apoptosis of tumor cells (Lu et al., 1996; Rippmann et al., 2000; Ryo et al., 2002), and deletion of Pin1 suppresses tumorigenesis induced by oncogenic Ras or Neu in mice (Wulf et al., 2004). Thus, in addition to the core components of the Ras pathway, Pin1 may be an appealing therapeutic target in tumors with constitutive Ras- or Raf-dependent signaling.

Experimental Procedures

Antibodies and Reagents

The phosphotyrosine antibody, λ phosphatase, okadaic acid, PDGF, GST-Raf RBD agarose, ERK2, and GSK3 were purchased from Upstate. FGF and EGF were from Invitrogen, GST-14-3-3 and GST-Ras^{V12} agarose were from Calbiochem, and Cdc2 kinase was from Promega. FLAG antibody was from Sigma, Raf-1 C-12 antibody from Santa Cruz, and the Ras antibody from Roche. U0126 and the Pin1 and Raf-1 phospho-S338 antibodies were obtained from Cell Signaling. The Raf-1 phospho-S642 antibody was generated by PhosphoSolutions, Inc.

Constructs and Cell Culture

The pcDNA3 construct encoding FLAG-wt Raf1 has been previously described (Laird et al., 1999). Point mutations were introduced by site-directed mutagenesis, and all mutations were confirmed by DNA sequencing. GFP-tagged Pin1 S16A is described in Lu et al. (2002). NIH3T3, MEF, and COS cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum at 37°C in 5% CO_2. Plasmid DNAs were transfected into COS and NIH3T3 cells with FuGENE-6, and NIH3T3 cells were infected with recombinant adenovirus as described in Müller et al. (2000). Transfected or infected cells were either incubated for 48 hr in fully supplemented media or were serum starved for 18 hr prior to analysis.

Metabolic Labeling and Phosphorylation Site Mapping

NIH3T3 cells expressing FLAG-tagged Raf-1 constructs were incubated for 4–6 hr at 37°C in phosphate-free DMEM containing 2.5% dialyzed calf serum and [^{32}P]orthophosphate (1 mCi/ml media). Cells were then washed with Tris-buffered saline (TBS) (20 mM Tris [pH 7.4] and 137 mM NaCl) and lysed at 4°C in NP-40 lysis buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 10% glycerol, 1% NP-40, 0.15 U/ml aprotinin, 1 mM PMSF, 20 μM leupeptin, 5 mM sodium vanadate, and 0.1 μM calyculin). Labeled FLAG-Raf-1 proteins were immunoprecipitated from cell lysates, separated by SDS-PAGE, eluted from the gel matrix, digested with trypsin, and analyzed by reverse-phase HPLC, phosphoamino acid analysis, and Edman degradation (Morrison et al., 1993). For pulse-chase experiments, NIH3T3 cells stably expressing FLAG-tagged Raf-1 constructs were starved for 30 min in DMEM lacking L-methionine and L-cysteine, after which they were labeled for 2 hr in the same media containing ^{35}S-methionine/cysteine (250 μCi/ml media). After labeling, cells were washed with chase media (DMEM containing 0.2% FBS, 5 mM L-Methionine, and 3 mM L-cysteine) and then incubated in chase media containing PDGF or U0126 for the indicated times.

In Vitro Kinase Assays

Raf-1 immunoprecipitates were incubated in 40 μl kinase buffer (30 mM Hepes [pH7.4], 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM DTT, 1 mM $NaVO_4$, 15 μM ATP, and 10 mM $MgCl_2$) containing 20 μCi [γ-^{32}P]ATP and 0.1 μg of purified kinase-inactive MEK to quantify Raf-1 kinase activity. After incubation for 30 min at 25°C, the samples were resolved by SDS-PAGE and transferred to nitrocellulose. The radioactivity incorporated into MEK was quantified. In experiments where Raf-1 was used as an in vitro substrate, FLAG-Raf-1 proteins were affinity purified from lysates of serum-starved NIH3T3 cells, incubated in kinase buffer containing the appropriate purified kinase, and examined by immunoblot analysis.

Coimmunoprecipitation and GST Pull-Down Assays and Mass Spectrometry Analysis of Raf-1 Complexes

For GST pull-down assays and coimmunoprecipitation experiments, cells were washed with TBS and lysed at 4°C in NP-40 lysis buffer. Lysates were cleared of insoluble material by centrifugation at 4°C for 20 min at 16,000 × g and then incubated for 2–4 hr at 4°C with agarose beads containing either the indicated GST-fusion protein or αFLAG antibody. After extensive washing in NP-40 lysis buffer, the complexes were examined by immunoblot analysis. Mass spectrometry analysis of Raf-1 complexes was performed as described in Ory et al. (2003) with Raf-1 proteins isolated from serum-starved COS cells that were treated with EGF for 30 min prior to lysis.

Membrane Fractionation and *Xenopus* Oocyte Meiotic Maturation Assay

Fractionation of cells into membrane and cytosolic components was performed as previously described (Stokoe and McCormick, 1997). For the *Xenopus* meiotic maturation assay, mRNA was transcribed with the Message Machine kit (Ambion). Buffer or RNA (15 ng) encoding the Raf-1 constructs was injected into stage VI oocytes. Approximately 12 hr later, the oocytes were injected with Ras^{V12} RNA (15 ng) and were subsequently scored for GVBD.

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