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14-3-3 Is Not Essential for Raf-1 Function: Identification of Raf-1 Proteins That Are Biologically Activated in a 14-3-3- and Ras-Independent Manner

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Recent reports have demonstrated the in vivo association of Raf-1 with members of the 14-3-3 protein family. To address the significance of the Raf-1–14-3-3 interaction, we investigated the enzymatic activity and biological function of Raf-1 in the presence and absence of associated 14-3-3. The interaction between these two molecules was disrupted in vivo and in vitro with a combination of molecular and biochemical techniques. Biochemical studies demonstrated that the enzymatic activities of Raf-1 were equivalent in the presence and absence of 14-3-3. Furthermore, mixing of purified Raf-1 and 14-3-3 in vitro was not sufficient to activate Raf-1. With a molecular approach, Cys-165 and Cys-168 as well as Ser-259 were identified as residues of Raf-1 required for the interaction with 14-3-3. Cys-165 and Cys-168 are located within the conserved cysteine-rich region of the CR1 domain, and Ser-259 is a conserved site of serine phosphorylation found within the CR2 domain. Mutation of either Cys-165 and Cys-168 or Ser-259 prevented the stable interaction of Raf-1 with 14-3-3 in vivo. Consistent with the model in which a site of serine phosphorylation is involved in the Raf-1–14-3-3 interaction, dephosphorylated Raf-1 was unable to associate with 14-3-3 in vitro. Phosphorylation may represent a general mechanism mediating 14-3-3 binding, because dephosphorylation of the Bcr kinase (known to interact with 14-3-3) also eliminated its association with 14-3-3. Finally, mutant Raf-1 proteins unable to stably interact with 14-3-3 exhibited enhanced enzymatic activity in human 293 cells and *Xenopus* oocytes and were biologically activated, as demonstrated by their ability to induce meiotic maturation of *Xenopus* oocytes. However, in contrast to wild-type Raf-1, activation of these mutants was independent of Ras. Our results therefore indicate that interaction with 14-3-3 is not essential for Raf-1 function.

The Raf family of serine/threonine kinases are central components involved in the propagation of proliferative and developmental signals from the cell surface to the nucleus. Biochemical and genetic studies have demonstrated that Raf proteins function downstream of activated tyrosine kinases and Ras and upstream of MEK (also known as MKK1) and mitogen-activated protein kinase (MAPK) in many signaling pathways (reviewed in references 28, 32, and 38). In addition, Raf proteins transduce signals in a variety of cell types and developmental systems (29, 35, 38) and in organisms as diverse as mammals, *Xenopus laevis*, *Drosophila melanogaster*, and *Caenorhabditis elegans* (5, 7, 17, 43).

Understanding how Raf proteins receive signals and become activated has been a topic of much interest. Structural studies have indicated that Raf-1 can be divided into two functional domains, the amino-terminal regulatory domain and the carboxy-terminal catalytic domain (19, 40). Present within the amino-terminal domain are two highly conserved regions, CR1, which contains cysteine residue repeats, and CR2, which is rich in serine and threonine residues (18, 36). Deletion of the amino-terminal domain activates the oncogenic potential of Raf-1, suggesting that this domain functions to suppress the catalytic activity of the kinase domain (19, 40). Repression of the catalytic domain must therefore be relieved for Raf-1 to become active in response to signaling events. Because the activation of Raf-1 by mitogens is a transient event, relief of the regulatory domain's suppressive effect would be expected to be mediated by reversible modifications such as phosphorylation or dephosphorylation events or association with one or more regulatory proteins or lipids.

Consistent with a role for phosphorylation in the regulation of Raf-1, the activation of Raf-1 in response to many growth factors is coincident with increased phosphorylation of the molecule on serine, threonine, and, in some cases, tyrosine residues (20, 25, 31). Sites of serine and tyrosine phosphorylation in vivo have been identified that, when mutated, alter Raf-1 activity (6, 23, 31). However, while mutational analysis does suggest that phosphorylation can modulate the catalytic activity of Raf-1, the precise role of phosphorylation in Raf-1 regulation has not been fully elucidated.

Reversible protein interactions may also mediate Raf-1 activation. A prerequisite for Raf-1 activation in many signaling pathways is an association with the Ras proto-oncogene product. The involvement of Ras in Raf-1 function was first demonstrated by studies showing that the activation of Raf-1 in many cases is dependent on Ras activity (39, 42, 49). Subsequently, Raf-1 was shown to interact directly with GTP-bound Ras, both in vitro and in vivo (10, 16, 22, 27, 44–46, 50). The interaction with Ras is mediated by sequences found within the CR1 domain, and mutation of Arg-89 of Raf-1, a residue required for the Ras–Raf-1 association, prevents Ras-dependent activation of Raf-1 (8). Recent studies suggest that the role of Ras in Raf-1 activation is to localize Raf-1 to the plasma membrane (26, 41). However, the event or events that mediate Raf-1 activation at the membrane are not known. Furthermore, several lines of evidence suggest that Raf-1 requires more than recruitment to the plasma membrane for its activity to be maximal. First, in insect cells, coexpression of Raf-1 with both Ras and Src stimulates Raf-1 activity to a higher level

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than does coexpression with either Ras or Src alone (6, 48), and membrane fractions from v-Ras- and v-Src-transformed cells synergistically activate His-tagged Raf-1 in vitro (4). Second, Raf-1 constitutively localized at the plasma membrane can be further activated by treatment with epidermal growth factor (26). Membrane localization alone, therefore, is not sufficient for maximal Raf-1 activity, and the molecules responsible for activating Raf-1 upon localization to the membrane have not been identified.

A protein that has been suggested to be involved in the Ras-dependent activation of Raf-1 is 14-3-3. 14-3-3 represents a highly conserved family of proteins found in a broad range of organisms and tissues (reviewed in reference 1). Numerous biological activities have been attributed to distinct members of this family, and 14-3-3 homologs in yeast have been implicated in cell cycle control (1, 11). Recently, 14-3-3 isoforms have been found to associate with oncogene products such as Raf-1 (9, 12, 21), Bcr-Abl (37), and the polyomavirus middle T antigen (33). The interaction of Raf-1 and 14-3-3 was detected in the yeast two-hybrid interaction system (9, 12, 14, 21), in in vitro association assays (12, 14), and in Raf-1 immunoprecipitates (9, 12, 14). Studies with yeast and *Xenopus* systems further suggested that 14-3-3 isoforms modulate Raf-1 function in vivo and might directly activate Raf-1 (9, 12, 21). However, reported effects of purified 14-3-3 on Raf-1 activity in vitro were conflicting (14, 21). In addition, 14-3-3 was always associated with Raf-1 in vivo, regardless of Raf-1's subcellular location or activation state or whether Raf-1 was bound to Ras (12, 14). Therefore, the precise role that the 14-3-3 proteins play in Raf-1 activation and the functional significance of the Raf-1–14-3-3 interaction are not evident.

In this report, we investigate the biochemical and biological activity of Raf-1 in the presence and absence of associated 14-3-3. Our findings indicate that association with 14-3-3 alone is not sufficient to activate Raf-1 and that Raf-1 mutants unable to stably interact with 14-3-3 are biologically activated in a Ras-independent manner.

MATERIALS AND METHODS

Antibodies. The Raf-1 antibodies used in this study include a rabbit polyclonal antibody generated against the last 12 amino acid residues of Raf-1 (Santa Cruz Biotechnology, Santa Cruz, Calif.) and a mouse monoclonal antibody generated with a 24-kDa protein fragment corresponding to amino acid residues 162 to 378 of human Raf-1 as an immunogen (Transduction Laboratories, Lexington, Ky.). 14-3-3 antibody was a rabbit polyclonal antibody generated against a synthetic peptide corresponding to amino acid residues within the β and ζ isoforms of 14-3-3 (generously provided by D. Pallas, Dana-Farber Cancer Institute [33], or obtained from Upstate Biotechnology Inc. [UBI], Lake Placid, N.Y.). The hsp90 antibody was an affinity-purified polyclonal rabbit antibody (Affinity BioReagents, Neshanic Station, N.J.).

Construction of expression vectors encoding WT and mutant Raf-1 proteins. Raf-1 constructs encoding amino acid point mutations were generated by site-directed mutagenesis as previously described (7) with a human Raf-1 cDNA clone, pKS:cRaf, and the appropriate oligonucleotides to introduce the desired base changes. For the FLAG–Raf-1 construct, sequences encoding the FLAG epitope tag (amino acids DYKDDDDK) were inserted proximal to the amino-terminal methionine of Raf-1 by site-directed mutagenesis of pKS:cRaf with the oligonucleotide 5′-GGATCCATATTATGGACTACAAGGACGACGATGAC AAGGAGCACATACAGGGAGC-3′. The specific base changes in all mutant constructs were confirmed by sequence analysis. cDNA fragments encoding the entire wild-type (WT) and mutant Raf-1 proteins were isolated and inserted into the pVL941 baculoviral transfer vector for expression in Sf9 cells, the pLNCX retroviral vector for expression in 293 cells, and the pSP64T transcription vector for expression in *Xenopus* oocytes (24). Plasmids encoding Raf-1 proteins containing a lysine-to-methionine substitution at amino acid position 375 of the ATP binding site (ATP^M) (31); an arginine-to-leucine substitution at amino acid position 89 (R89L) (8); cysteine-to-serine substitutions at amino acid positions 165 and 168 (CRM) (34); a tyrosine-to-aspartic acid substitution at amino acid position 340 (Y340D) (6); and serine-to-alanine substitutions at amino acid positions 43 (S43A), 259 (S259A), and 621 (S621A) (31) were generated.

Expression of recombinant proteins in Sf9 and 293 cells. Routinely, for recombinant protein production, 2×10^6 Sf9 cells were infected with the desired recombinant baculovirus(es) at a multiplicity of infection of 10 and lysed at 48 h postinfection. Sf9 cells were assayed for the expression of the recombinant proteins by immunoblot analysis. Recombinant baculoviruses encoding WT and mutant Raf-1 proteins, activated Src (supplied by D. Morgan, University of California, San Francisco), Ha-Ras^{V12} (obtained from T. Roberts, Dana-Farber Cancer Institute), and cBcr (provided by A. M. Pendergast, Duke University Medical Center) were used.

For transient expression of Raf-1 proteins in 293 cells, the pLNCX-Raf constructs were transfected into 293 cells by the calcium phosphate method (47). Forty-eight hours after transfection, cells were placed in serum-free medium and cultured for an additional 16 h before lysis.

Preparation of cell lysates and immunoprecipitation assays. Infected Sf9 cells (2×10^6) or transfected 293 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed for 20 min at 4°C in 500 μl of Nonidet P-40 (NP-40) lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 U/ml), 20 μM leupeptin, and 5 mM sodium vanadate. Alternatively, Sf9 cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 U/ml), 20 μM leupeptin, and 5 mM sodium vanadate. Insoluble material was removed by centrifugation at 4°C for 10 min at 16,000 × *g*, and cell lysates were equalized for Raf-1 protein expression by immunoblot analysis. Immunoprecipitation assays were performed by incubating cell lysates with the appropriate antibody for 3 h at 4°C. The antigen-antibody complexes were collected with protein A/G agarose beads (Santa Cruz Biotechnology, Inc.). The immunoprecipitates were then washed four times with cold NP-40 lysis buffer before analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

In vitro protein kinase assay. Raf-1 immunoprecipitates were washed three times with NP-40 lysis buffer containing 1 mM sodium vanadate and once with kinase buffer (30 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4], 10 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5 μM ATP). The complexes were then incubated at 25°C for 15 min in 40 μl of kinase buffer containing 20 μCi of [γ-^{32}P]ATP and 0.1 μg of purified 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA)-treated MKK1 (3). Kinase assays were terminated by the addition of gel loading buffer (4% SDS, 80 mM dithiothreitol, 10% glycerol), the samples were resolved by SDS-PAGE, and phosphoproteins were visualized by autoradiography.

Purification of FLAG epitope-tagged Raf-1. Sf9 cells infected with a recombinant baculovirus encoding FLAG–Raf-1 were lysed 48 h postinfection in RIPA buffer as described above. The FLAG–Raf-1 was then batch purified by incubating the lysates with an anti-FLAG affinity resin for 2 h at 4°C on a rocking platform. After the binding reaction, the resin was washed three times with RIPA buffer and three times with NP-40 lysis buffer. The Raf-1 protein was then eluted from the resin by using a synthetic peptide encoding the FLAG epitope. To accomplish this, the FLAG–Raf-1-containing resin was incubated with the FLAG peptide in NP-40 lysis buffer for 2 h at 4°C on a rocking platform. The resin was then pelleted, and the supernatant containing the eluted FLAG–Raf-1 was collected.

Phosphatase treatment and in vitro binding assays. For phosphatase experiments, 2×10^6 Sf9 cells were lysed in 700 μl of NP-40 lysis buffer lacking sodium vanadate. A 100-μl aliquot of lysate was then taken and diluted with 300 μl of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (pH 6.0) containing 1 mM dithiothreitol, aprotinin (0.15 U/ml), 20 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Three units of type VII potato acid phosphatase (Sigma, St. Louis, Mo.) were added, and the samples were incubated at 30°C for various times. After phosphatase treatment, 0.7 ml of NP-40 lysis buffer was added and the samples were placed on ice. For in vitro binding assays, glutathione *S*-transferase (GST)–14-3-3–Bap-1 fusion protein (37) was expressed in bacteria and affinity purified on glutathione-agarose beads (2). Twenty to 30 μl of agarose beads containing immobilized GST–14-3-3–Bap-1 fusion protein (~10 μg) was then added to the phosphatase-treated lysates, and the samples were incubated with rocking for 2 h at 4°C. The agarose beads were pelleted by centrifugation, washed three times with NP-40 lysis buffer, resuspended in gel loading buffer, and examined by immunoblot analysis.

Oocyte injection and analysis. For protein expression in *Xenopus* oocytes, RNAs encoding the various Raf-1 proteins were transcribed in vitro from the pSP64T-Raf-1 plasmids. The Ha-Ras^{V12} construct used for in vitro transcription was described previously (7). All plasmids were linearized with the appropriate restriction enzyme, and capped RNA transcripts were synthesized as specified by the vendor (Ambion, Austin, Tex.) with SP6 RNA polymerase.

Oocytes were surgically removed from *X. laevis* females (Xenopus I, Ann Arbor, Mich.) and manually defolliculated in modified Barth solution (88 mM NaCl, 1 mM KCl, 2.5 mM $NaHCO_3$, 10 mM HEPES [pH 7.5], 0.82 mM $MgSO_4$, 0.33 mM $[CaNO_3]_2$, 0.4 mM $CaCl_2$). Oocytes were then washed several times in modified Barth solution and cultured in 50% Leibovitz-15 medium. Within 6 h of isolation, oocytes were injected with approximately 30 ng of in vitro-transcribed RNA encoding the various Raf-1 proteins. In some cases, the oocytes were injected 4 h later with 30 ng of Ha-Ras^{V12} RNA. Oocytes were scored for germinal vesicle breakdown (GVBD), as evidenced by the appearance of a white

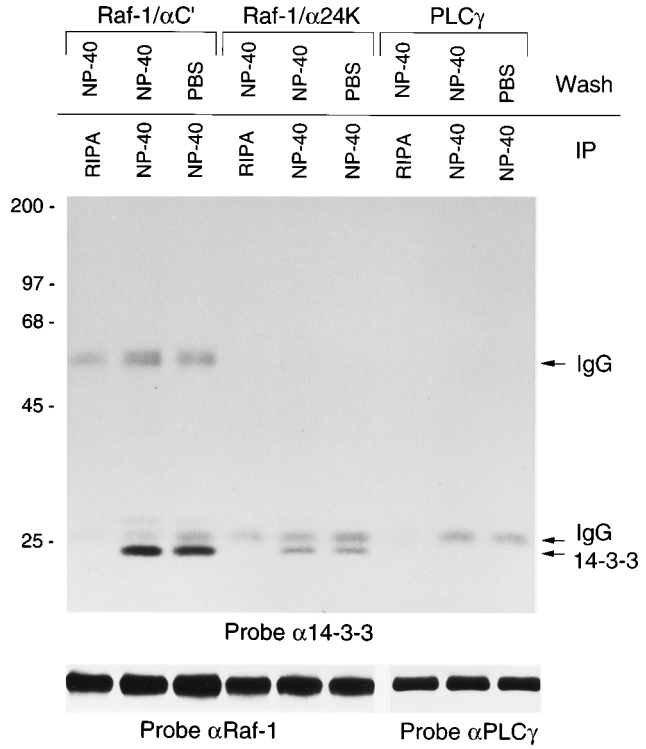

FIG. 1. Coimmunoprecipitation of 14-3-3 with baculovirus-expressed Raf-1. Sf9 cells expressing Raf-1 or PLCγ were lysed in NP-40 or RIPA lysis buffer. PLCγ proteins were immunoprecipitated with antibodies to PLCγ (αPLCγ), and Raf-1 proteins were immunoprecipitated with either of two Raf-1 antibodies (αC′ or α24K). The immunoprecipitates (IP) were washed with NP-40 lysis buffer or PBS, resolved by electrophoresis on an SDS–10% polyacrylamide gel, and examined by immunoblotting with antibodies to 14-3-3. The immunoprecipitated Raf-1 and PLCγ proteins were subsequently detected by immunoblotting with antibodies to Raf-1 (αRaf-1) and PLCγ. Molecular mass markers are shown to the left in kilodaltons. IgG, immunoglobulin G.

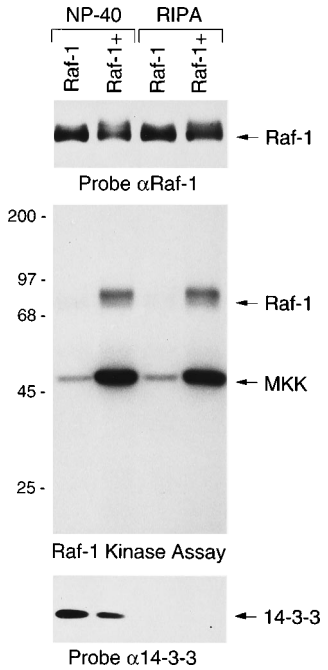

FIG. 2. Comparison of Raf-1 activity in the presence and absence of associated 14-3-3. Sf9 cells expressing Raf-1 alone (Raf-1) or coexpressing Raf-1, Ras, and Src (Raf-1+) were lysed either in NP-40 or in RIPA lysis buffer. The Raf-1 protein was immunoprecipitated from the lysates, and in vitro protein kinase assays were performed. Purified FSBA-treated MKK1 was added as an exogenous substrate. Assays were terminated by the addition of gel loading buffer, the samples were resolved by electrophoresis on SDS–10% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography. The immunoprecipitates were subsequently examined for the presence of Raf-1 and 14-3-3 by immunoblotting with antibodies to Raf-1 (αRaf-1) and 14-3-3 (α14-3-3). Molecular mass markers are shown to the left in kilodaltons.

spot at the animal pole. This observation was verified by manual dissection of oocytes after fixation in 8% trichloroacetic acid. For Raf-1 kinase assays, oocytes were lysed by titration with a pipette tip in NP-40 lysis buffer (10 μl per oocyte). Insoluble material was pelleted by centrifugation at 14,000 × *g* for 5 min at 4°C. Lysates from three to five oocytes were then diluted with 700 μl of RIPA lysis buffer, and the Raf-1 proteins were immunoprecipitated and analyzed with in vitro protein kinase assays.

RESULTS

Association of Raf-1 with 14-3-3 proteins in Sf9 cells. Previous reports have demonstrated that Raf-1 produced in the baculovirus expression system is complexed with endogenous insect 14-3-3 proteins and that an association of these two proteins can be detected in coimmunoprecipitation experiments (12, 14). Consistent with these published reports, we also found that 14-3-3 could be specifically coimmunoprecipitated with baculovirus-expressed Raf-1. However, 14-3-3 was not complexed with all mammalian signaling proteins expressed in this system, because it was not detected in immunoprecipitates of baculovirus-expressed phospholipase C-γ (PLCγ) under any conditions evaluated (Fig. 1). With two different Raf-1 antibodies (C′ and 24K), 14-3-3 was detected in Raf-1 immunoprecipitates prepared from cells lysed in NP-40 lysis buffer (Fig. 1). In contrast, 14-3-3 was not present in Raf-1 immunoprecipitates prepared from cells lysed in RIPA buffer (Fig. 1). This finding suggests that the more stringent lysis of cells in RIPA buffer containing SDS and sodium deoxycholate disrupted the Raf-1–14-3-3 interaction.

The in vitro catalytic activity of Raf-1 is not altered by association with 14-3-3. To evaluate the significance of the Raf-1–14-3-3 interaction, we first examined the effect that 14-3-3 binding has on the enzymatic activity of Raf-1. Because cell lysis conditions which would disrupt the interaction of Raf-1 and 14-3-3 were available, we began our analysis by comparing the in vitro kinase activities of Raf-1 in the presence and absence of associated 14-3-3. Sf9 cells were infected with Raf-1 alone or were coinfected with Raf-1, Ras, and Src, conditions that have been shown to activate Raf-1 (6, 48). Cells were then lysed either in NP-40 lysis buffer or in RIPA lysis buffer. Raf-1 proteins were immunoprecipitated, and their kinase activity was measured with MKK1 as an exogenous substrate. This analysis showed that the catalytic activity of Raf-1 associated with 14-3-3 was the same as that of Raf-1 lacking associated 14-3-3 (Fig. 2).

We next examined whether the addition of purified 14-3-3 could modulate the catalytic activity of Raf-1. For these experiments, Raf-1 containing a FLAG epitope tag was expressed in Sf9 cells either alone or together with activated Ras. The Sf9 cells were then lysed in RIPA buffer, and the Raf-1 protein was purified with an anti-FLAG affinity resin. The soluble Raf-1 protein generated by this protocol was >90% pure by silver staining (Fig. 3B) and did not contain any detectable 14-3-3 protein (Fig. 3A). When either of two preparations of purified 14-3-3 was added to the purified Raf-1 in vitro, no change in kinase activity was observed (Fig. 3C). The presence of exogenously added 14-3-3 did not activate inactive Raf-1 (Raf-1 alone) and did not further stimulate active Raf-1 (Raf-1 plus

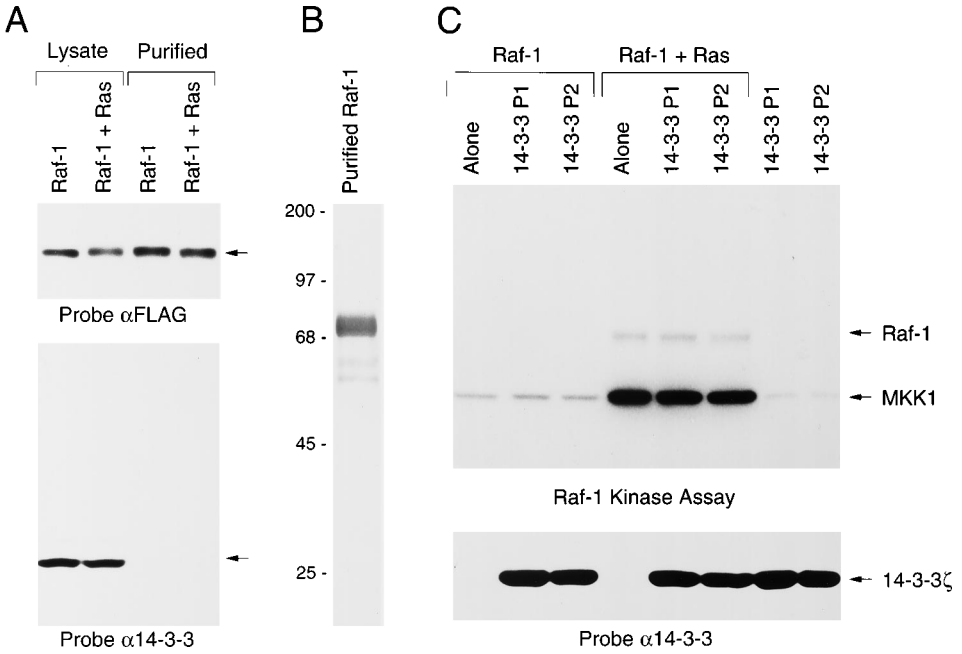

FIG. 3. Analysis of the in vitro kinase activity of purified FLAG–Raf-1 in the presence or absence of purified 14-3-3. Sf9 cells expressing FLAG–Raf-1 alone (Raf-1) or coexpressing FLAG–Raf-1 and Ras (Raf-1 + Ras) were lysed in RIPA buffer, and the FLAG–Raf-1 proteins were purified as described in Materials and Methods. (A) Crude Sf9 cell lysates and purified FLAG–Raf-1 proteins were resolved by electrophoresis on SDS–10% polyacrylamide gels and examined for the presence of Raf-1 and 14-3-3 by immunoblotting with antibodies to FLAG–Raf-1 (αFLAG) and 14-3-3 (α14-3-3). (B) Analysis of purified FLAG–Raf-1 by silver staining. Molecular mass markers are shown to the left in kilodaltons. (C) In vitro protein kinase assay of purified FLAG-Raf-1 proteins incubated in the absence (Alone) or presence of two purified preparations of 14-3-3ζ (14-3-3P1 and 14-3-3P2 [14]). Purified FSBA-treated MKK1 was added as an exogenous substrate. Assays were terminated by the addition of gel loading buffer, the samples were resolved by electrophoresis on SDS–10% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography. 14-3-3ζ added to the assayed samples was detected by immunoblotting with antibodies to 14-3-3.

Ras). However, both purified 14-3-3 batches did have factor activating exoenzyme S activity (13). These results suggest that the mixing of purified Raf-1 and 14-3-3 in vitro is not sufficient to activate Raf-1.

The Raf-1–14-3-3 interaction requires Raf-1 phosphorylation. To further evaluate the association between Raf-1 and 14-3-3, we next performed experiments to characterize the biochemical basis of this interaction. In addition to Raf-1, 14-3-3 interacts with polyomavirus middle T antigen (33), Bcr, and Bcr/Abl (37). Each of these proteins is phosphorylated on serine residues and contains serine-rich regions in their predicted 14-3-3 binding domains (30, 37). Therefore, to determine whether phosphorylation might be involved in the Raf-1–14-3-3 interaction, we examined the ability of phosphatase-treated Raf-1 to interact in vitro with 14-3-3. For these assays, GST–14-3-3 fusion proteins were expressed in bacteria and affinity purified with glutathione-agarose beads (37). The immobilized GST–14-3-3 fusion protein was then incubated with Raf-1 lysates that had been treated for various times with potato acid phosphatase. The association of Raf-1 with GST–14-3-3 was detected by immunoblot analysis. As shown in Fig. 4A, increased phosphatase treatment of Raf-1 reduced and ultimately prevented the interaction with the GST–14-3-3. The decrease in association was not due to proteolysis of Raf-1, because equivalent amounts of Raf-1 were present in all samples after phosphatase treatment (Fig. 4A). GST–14-3-3 has also been shown to interact in vitro with the Bcr protein kinase (37). To determine whether phosphorylation plays a role in the Bcr–14-3-3 interaction, we performed in vitro binding studies with phosphatase-treated Bcr lysates. As was observed for Raf-1, phosphatase treatment eliminated the binding of Bcr to 14-3-3 (Fig. 4B).

Identification of Raf-1 mutant proteins unable to stably interact with 14-3-3. The observation that phosphatase-treated Raf-1 was unable to associate with 14-3-3 in vitro suggests that phosphorylation does play a role in the Raf-1–14-3-3 interaction. Previous studies in our laboratory have identified several

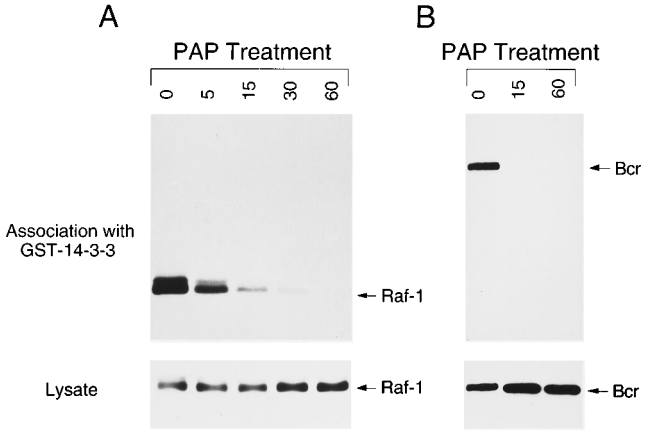

FIG. 4. Phosphatase treatment of Raf-1 and Bcr prevents in vitro association with 14-3-3. (A) Lysates of Sf9 cells expressing Raf-1 were treated with potato acid phosphatase (PAP) for various times (shown in minutes above the lanes) and incubated with GST–14-3-3. Raf-1 protein associated with GST–14-3-3 was detected by immunoblot analysis with antibodies to Raf-1. After phosphatase treatment, an aliquot of each lysate was examined by immunoblotting with antibodies to Raf-1. (B) Same as panel A, except lysates of Sf9 cells expressing Bcr were phosphatase treated and immunoblotting was performed with antibodies to Bcr.

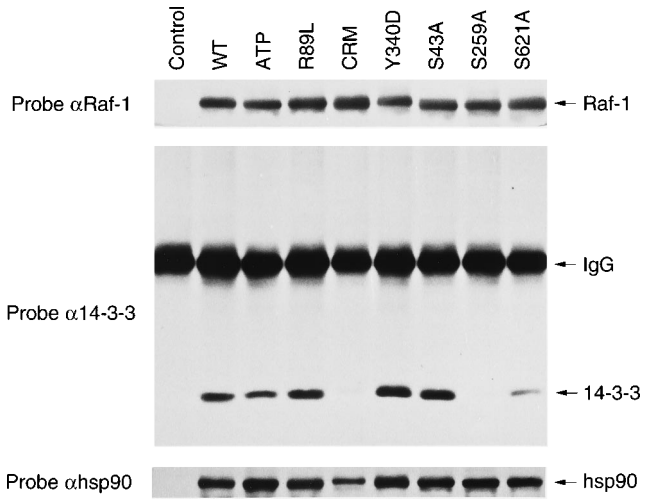

FIG. 5. Association of Raf-1 mutant proteins with 14-3-3 in Sf9 cells. WT and mutant Raf-1 proteins were immunoprecipitated from Sf9 cells lysed in NP-40 lysis buffers. The immunoprecipitates were resolved by electrophoresis on an SDS–10% polyacrylamide gel and examined by immunoblotting with antibodies to Raf-1 (αRaf-1), 14-3-3 (α14-3-3), and hsp90 (αhsp90). Raf-1 proteins ATP^M (ATP) (31), R89L (8), CRM, Y340D (6), and S43A, S259A, and S621A (31) were examined. IgG, immunoglobulin G.

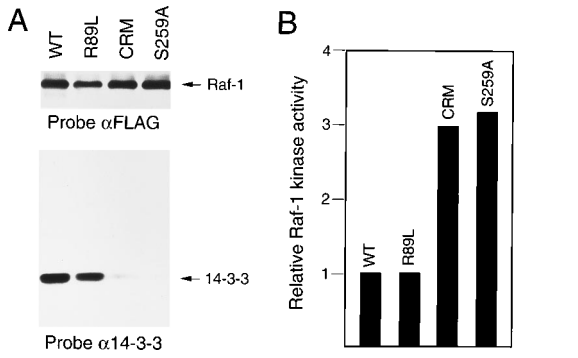

FIG. 6. Raf-1 mutants unable to stably interact with 14-3-3 have enhanced kinase activity in human 293 cells. Constructs encoding WT, R89L–, CRM–, and S259A–FLAG–Raf-1 proteins were transiently transfected into 293 cells. Twenty-four hours after transfection, cells were placed in serum-free medium and incubated 18 h before lysis. (A) FLAG–Raf-1 proteins were immunoprecipitated from cell lysates and examined for the presence of Raf-1 and 14-3-3 by immunoblotting with antibodies to Raf-1 (αRaf-1) and 14-3-3 (α14-3-3). (B) In vitro protein kinase assays with FSBA-treated MKK1 as an exogenous substrate were performed on the FLAG–Raf-1 immunoprecipitates. The amount of ^{32}P incorporated into MKK1 was determined, and the results are expressed relative to the Raf-1 kinase activity present in immunoprecipitates of WT FLAG–Raf-1.

in vivo sites of Raf-1 phosphorylation, and Raf-1 proteins containing mutations in these sites have been generated (6, 31). Therefore, we tested whether any of our phosphorylation site mutants might be defective in 14-3-3 binding. In addition, because previous studies indicated that 14-3-3 associates with Raf-1 at multiple sites, with the primary interaction site located in the amino-terminal regulatory domain, we also tested other Raf-1 proteins containing mutations in this domain. The Raf-1 mutant proteins were expressed in Sf9 cells, and the association with 14-3-3 was examined in coimmunoprecipitation experiments. By immunoblot analysis, 14-3-3 was not detected in immunoprecipitates of two mutant proteins, S259A–Raf-1 and CRM–Raf-1 (Fig. 5). An interaction with 14-3-3 was observed for all other mutants but was somewhat reduced in immunoprecipitates of S621A–Raf-1 (Fig. 5). However, all of the Raf-1 mutant proteins associated to similar extents with hsp90, another protein which has been shown to coimmunoprecipitate with Raf-1 (Fig. 5). In addition, we found that S259A–Raf-1 and CRM–Raf-1 were able to interact with Ras (see Fig. 8); however, they were unable to associate in vitro with GST–14-3-3 (data not shown), suggesting that these proteins contain mutations which specifically inhibit the Raf-1–14-3-3 interaction. S259A–Raf-1 contains a serine-to-alanine substitution at amino acid position 259, a site of in vivo phosphorylation located in the conserved serine- and threonine-rich CR2 domain (31). CRM–Raf-1 contains cysteine-to-serine changes at amino acids 165 and 168, and mutation of these residues disrupts a conserved cysteine repeat located in the CR1 domain (19, 36).

To further evaluate the S259A–Raf-1 and CRM–Raf-1 mutants, we transiently transfected FLAG epitope-tagged versions of these proteins into human 293 cells. Epitope-tagged proteins were used such that the mutant transfected proteins could be distinguished from the endogenous Raf-1 present within the cells. By immunoblot analysis, anti-FLAG immunoprecipitates of WT Raf-1 and R89L–Raf-1 did contain 14-3-3 (Fig. 6A). In contrast, CRM–Raf-1 and S259A–Raf-1 again failed to efficiently coimmunoprecipitate 14-3-3 (Fig. 6A). When the enzymatic activities of these proteins were measured, both S259A–Raf-1 and CRM–Raf-1 had approximately threefold increased kinase activity compared with WT Raf-1 (Fig. 6B).

Raf-1 mutant proteins defective in the ability to interact with 14-3-3 are biochemically and biologically activated in *Xenopus* oocytes. To assess the biological activities of S259A–Raf-1 and CRM–Raf-1, we examined the ability of these proteins to induce the meiotic maturation of *Xenopus* oocytes. As we have previously reported, WT Raf-1 was not enzymatically activated or capable of inducing maturation when expressed alone in stage VI oocytes (6–8) (Fig. 7A). However, expression of Ha-Ras^{V12} in oocytes did induce meiotic maturation resulting in GVBD (7) (Fig. 7A). Furthermore, coexpression of Ha-Ras^{V12} with WT Raf-1 activated the kinase activity of the WT Raf-1 protein (Fig. 7B). When the Raf-1 mutant proteins were expressed in oocytes, we found that S259A–Raf-1 induced GVBD in 96% of the oocytes and CRM–Raf-1 induced GVBD in 98% of the oocytes (Fig. 7A). In addition, CRM–Raf-1 and S259A–Raf-1 proteins isolated from oocyte lysates were enzymatically activated and coexpression with Ha-Ras^{V12} did not further stimulate the kinase activity of these proteins (Fig. 7B).

Activation of CRM–Raf-1 and S259A–Raf-1 is independent of Ras. Because CRM–Raf-1 and S259A–Raf-1 were biologically and biochemically activated in the oocyte system and because their activity was not influenced by coexpression with Ha-Ras^{V12}, we examined whether these proteins function in a Ras-independent manner. For these studies, we generated mutant Raf-1 proteins which were not only defective in their ability to bind 14-3-3 but which were also defective in their ability to interact with Ras. Previously, we have shown that mutation of a single amino acid in Raf-1 (R89L) disrupts the interaction with Ras in vitro and in vivo (8). Therefore, we constructed Raf-1 proteins that contained the R89L mutation together with either the CRM or S259A mutation. To confirm that the Raf-1 proteins containing the R89L mutation were defective in their ability to interact with Ras, these mutants were expressed in Sf9 cells and the association with Ras was examined in coimmunoprecipitation experiments. By immunoblot analysis, we found that while CRM–Raf-1 and S259A–

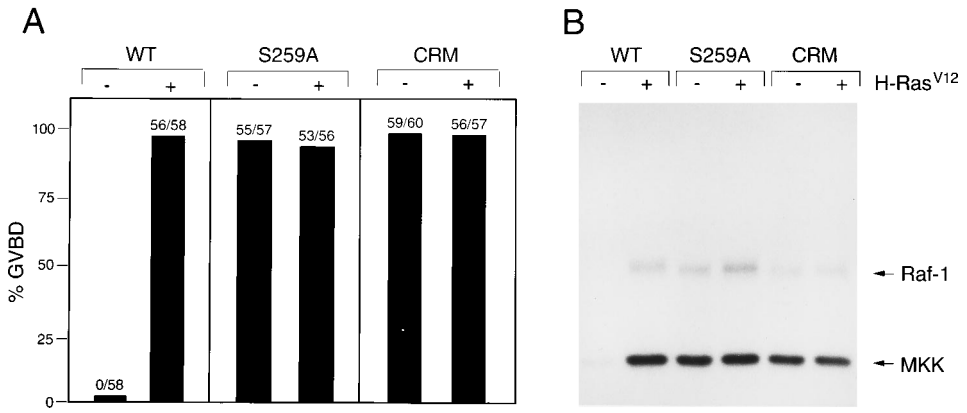

FIG. 7. Raf-1 mutants defective in 14-3-3 association are enzymatically and biologically activated in *Xenopus* oocytes. (A) Induction of meiotic maturation by the expression of WT and mutant Raf-1 proteins in stage VI *Xenopus* oocytes. RNA (~30 ng) encoding WT, S259A–, or CRM–FLAG–Raf-1 was injected into defolliculated stage VI oocytes. Four hours later, oocytes were injected (+) or not injected (−) with H-Ras^{V12} RNA. Oocytes were scored for GVBD within 24 h of the first injection. (B) In vitro kinase activity of WT and mutant Raf-1 proteins expressed in *Xenopus* oocytes. Lysates were prepared at GVBD, and FLAG–Raf-1 proteins were immunoprecipitated from the lysates of three infected oocytes. In vitro protein kinase assays were performed with purified FSBA-treated MKK1 as an exogenous substrate. Assays were terminated by the addition of gel loading buffer, the samples were resolved by electrophoresis on SDS–10% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography.

Raf-1 did associate with Ras, R89L–CRM–Raf-1 and R89L–S259A–Raf-1 did not (Fig. 8). To next examine the biological activity of these Raf-1 mutants, the ability of these proteins to induce the meiotic maturation of *Xenopus* oocytes was assessed. We found that both R89L–CRM–Raf-1 and R89L–S259A–Raf-1 efficiently induced GVBD in oocytes (Fig. 9A) and were catalytically activated (Fig. 9B). These results suggest that the biologically activated CRM–Raf-1 and S259A–Raf-1 proteins have bypassed the requirement for Ras association.

DISCUSSION

In this study, we examined the role of 14-3-3 in modulating the enzymatic activity and biological function of Raf-1. Our results identify residues of Raf-1 required for the association with 14-3-3 and suggest a mechanism whereby 14-3-3 interacts with Bcr and Raf-1. In addition, our findings indicate that association with 14-3-3 alone is not sufficient to activate Raf-1 and that Raf-1 mutants unable to stably interact with 14-3-3 are biologically activated in a Ras-independent manner. These data argue that 14-3-3 alone is not a direct activator of Raf-1 and suggest that 14-3-3 proteins influence Raf-1 function by other mechanisms.

The 14-3-3 family of proteins recently received much attention when they were found to associate with several proto-oncogene and oncogene products. The initial reports demonstrating the interaction between 14-3-3 isoforms and Raf-1 were definitive, with the interaction detected with numerous techniques and approaches (9, 12, 14, 21). However, what was not evident from these studies was the functional significance of the interaction. Several lines of evidence suggested that 14-3-3 modulated Raf-1 function and raised the question of whether 14-3-3 directly activated Raf-1. Overexpression of

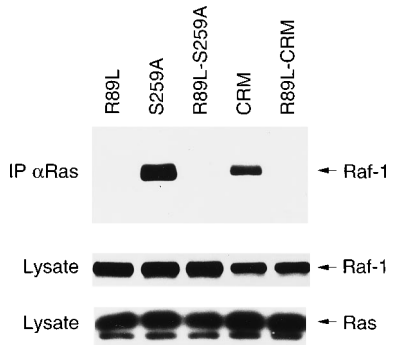

FIG. 8. Association of Raf-1 mutant proteins with Ras in Sf9 cells. Sf9 cells coexpressing Ras and R89L–, CRM–, S259A–, R89L–S259A–, or R89L–CRM–Raf-1 proteins were lysed in NP-40 lysis buffer, and the Ras proteins were immunoprecipitated. The immunoprecipitates (IP) were resolved by electrophoresis on an SDS–10% polyacrylamide gel and examined for the presence of Raf-1 by immunoblotting with antibodies to Raf-1. Total lysates were examined for the expression of the mutant Raf-1 proteins and Ras by immunoblotting with antibodies to Raf-1 and Ras.

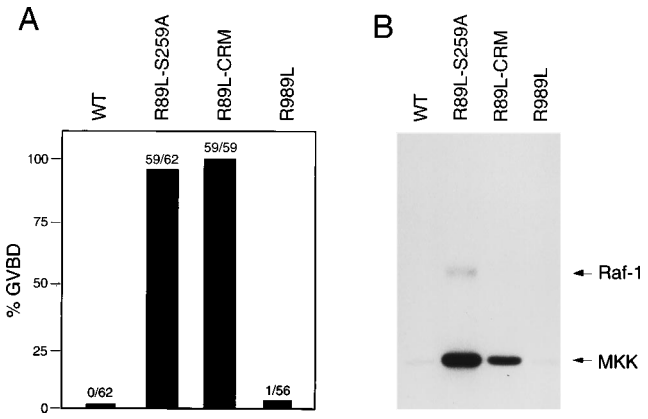

FIG. 9. Raf-1 mutants defective in 14-3-3 and Ras association are enzymatically and biologically activated in *Xenopus* oocytes. (A) Induction of meiotic maturation by the expression of WT and mutant Raf-1 proteins in stage VI *Xenopus* oocytes. RNA (~30 ng) encoding WT, R89L-S259A–, or R89L–CRM–Raf-1 proteins was injected into defolliculated stage VI oocytes. Oocytes were scored for GVBD within 24 h of injection. (B) In vitro kinase activity of WT and mutant Raf-1 proteins expressed in *Xenopus* oocytes. Lysates were prepared at GVBD, and Raf-1 proteins were immunoprecipitated from the lysates of three injected oocytes. In vitro protein kinase assays were performed with purified FSBA-treated MKK1 as an exogenous substrate. Assays were terminated by the addition of gel loading buffer, the samples were resolved by electrophoresis on SDS–10% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography.

BMH1, the yeast homolog of 14-3-3, enhanced the function of mammalian Raf-1 in *Saccharomyces cerevisiae* and was required for Raf-1 to be activated by Ras in this system (21). Overexpression of human 14-3-3β or ζ in yeast cells rescued a defect in a signaling pathway required for Raf-dependent growth, and Raf-1 immunoprecipitated from rescued yeast cells overexpressing 14-3-3 had three- to fourfold more activity than cells lacking 14-3-3 (12). Finally, injection of mRNA encoding mammalian 14-3-3 into *Xenopus* oocytes induced meiotic maturation and increased Raf-1 kinase activity in immunoprecipitates (9). However, several observations argued that 14-3-3 enhanced Raf-1 function in ways other than through direct activation. In vivo, 14-3-3 proteins are constitutively associated with Raf-1 regardless of the cellular location or activation state of Raf-1 (12, 14). In addition, while Irie et al. (21) found that the addition of purified 14-3-3 enhanced the activity of mammalian Raf-1 immunoprecipitated from yeast, Fu et al. (14) found that most preparations of purified 14-3-3 (four out of five) had no effect on Raf-1 activity. Therefore, the published reports were unclear as to the biological role of the Raf-1–14-3-3 interaction.

To begin our analysis of the functional significance of the Raf-1–14-3-3 association, we examined the effect of 14-3-3 binding on the enzymatic activity of Raf-1 in vitro. Because Raf-1 is constitutively associated with 14-3-3 in vivo, we first identified conditions that would disrupt the Raf-1–14-3-3 interaction (cell lysis in buffers containing SDS and sodium deoxycholate). Raf-1 isolated in the absence of 14-3-3 was then evaluated for enzymatic activity. In our experiments, the in vitro kinase activity of Raf-1 associated with 14-3-3 was equivalent to that of Raf-1 not associated with 14-3-3. We also found that the addition of purified 14-3-3 to purified Raf-1 in vitro did not alter the catalytic activity of Raf-1, regardless of whether the purified Raf-1 was inactive or partially activated by Ras. Therefore, with purified components and MKK as a Raf-1 substrate, our biochemical studies failed to demonstrate that the Raf-1–14-3-3 interaction was an activating event in vitro. In contrast, previous studies showing an effect of 14-3-3 on Raf-1 activity in vitro were performed with Raf-1 immunoprecipitated under conditions that preserved the endogenous association of 14-3-3 with Raf-1 (14, 21). Thus, the activational effects observed might reflect the modulation of some other factor present within the immunoprecipitates. Furthermore, in one report, the Raf-1 activity was assayed indirectly in a coupled reaction measuring the phosphorylation of kinase-inactive MAPK (21). When Raf-1 activity is measured in an indirect assay, small variations in activity may appear larger because of signal amplification, leading to an overestimation of Raf-1 activity. Our results, however, do not rule out the possibility that 14-3-3 might be a cofactor required for Raf-1 activation in some circumstances and cell contexts.

To address the biological role of the Raf-1–14-3-3 interaction in vivo, we performed experiments to identify Raf-1 mutant proteins that fail to stably interact with 14-3-3. From these studies, we found that Cys-165 and Cys-168 as well as Ser-259 are residues of Raf-1 required for 14-3-3 association and constitute two separate sites of interaction. Both Cys-165 and Cys-168 and Ser-259 were required for this interaction, but mutation of either prevented stable association with 14-3-3. This finding is consistent with previous yeast two-hybrid experiments and in vitro binding studies indicating that 14-3-3 interacts with Raf-1 at multiple sites (12, 14, 21). Cys-165 and Cys-168 are located in the CR1 domain downstream of sequences required for Ras interaction and are part of a conserved cysteine repeat that forms a zinc binding motif (18, 45). Ser-259 is a conserved site of in vivo phosphorylation located in the CR2 domain (18, 31). Consistent with the observation that a serine phosphorylation site is required for the interaction with 14-3-3, we found that dephosphorylated Raf-1 was unable to bind GST–14-3-3 in vitro. Phosphatase treatment also prevented the association of GST–14-3-3 and the Bcr protein. In addition to Raf-1, Bcr, Bcr/Abl, and polyomavirus middle T antigen have recently been shown to be complexed with 14-3-3 in vivo (33, 37). Both cysteine- and serine-rich regions are common elements present within these proteins and have been proposed to be determinants for 14-3-3 binding (30, 37). Previous studies have also shown that 14-3-3 binds to phosphorylated but not dephosphorylated tryptophan hydrolase (15). These findings, together with our results, suggest that binding to phosphorylated residues may be a common mechanism whereby 14-3-3 interacts with other proteins.

If 14-3-3 does play a role in modulating Raf-1 function, then mutational analysis should show a correlation between 14-3-3 association and the biological and enzymatic activity of Raf-1. After the identification of residues required for the Raf-1–14-3-3 interaction, we then examined whether mutation of these sites altered Raf-1 function. When expressed in human 293 cells, epitope-tagged versions of S259A–Raf-1 and CRM–Raf-1 had increased catalytic activity compared with WT Raf-1. S259A–Raf-1 and CRM–Raf-1 proteins were also capable of inducing the meiotic maturation of *Xenopus* oocytes. In addition, the biological activity of the S259A–Raf-1 and CRM–Raf-1 proteins did not require an interaction with Ras, as shown by the observation that mutation of a residue required for Ras binding (R89L) had no effect on the ability of these mutants to induce oocyte maturation. Furthermore, coexpression with Ha-Ras^{V12} did not enhance the kinase activity of S259A–Raf-1 and CRM–Raf-1 in oocytes. Therefore, our analysis indicates that Raf-1 proteins defective in their ability to interact with 14-3-3 were biologically and biochemically activated in a Ras-independent manner.

In conclusion, our studies of the Raf-1–14-3-3 interaction indicate that the association with 14-3-3 is not an activating event or essential for Raf-1 activity. Previous data suggesting that 14-3-3 modulates Raf-1 function in vivo were from studies performed with systems overexpressing 14-3-3 or with a yeast system, in which Raf-1 is not normally expressed. Several explanations could account for the observed effects of 14-3-3. 14-3-3 may play a structural role in stabilizing the conformation of Raf-1 or in facilitating the interaction of Raf-1 with other signaling proteins or activators. Alternatively, 14-3-3 may mediate intracellular trafficking involved in the translocation of Raf-1 from the cytosol to the membrane, where it becomes activated. In these cases, the overexpression of 14-3-3 may increase the amount of Raf-1 that becomes functionally competent. A hypothesis consistent with our data is that 14-3-3 functions to keep Raf-1 in an inactive state in quiescent cells and that in response to signaling events may participate in the activation of Raf-1. Further experimentation will be necessary to fully elucidate the precise biological role of the Raf-1–14-3-3 interaction.

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REFERENCES

1. **Aitken, A., D. B. Collinge, B. P. H. van Heusden, T. Isobe, P. H. Roseboom, G. Rosenfeld, and J. Soll.** 1992. 14-3-3 proteins: a highly conserved, widespread family of eukaryotic proteins. Trends Biochem. Sci. **17:**498–501.
2. **Cleghon, V., and D. K. Morrison.** 1994. Raf-1 interacts with Fyn and Src in a non-phosphotyrosine-dependent manner. J. Biol. Chem. **269:**17749–17755.
3. **Dent, P., Y.-H. Chow, J. Wu, D. K. Morrison, R. Jove, and T. W. Sturgill.** 1994. Expression, purification, and characterization of recombinant MAP kinase kinases, MKK1 and MKK2. Biochem. J. **303:**105–112.
4. **Dent, P., and T. W. Sturgill.** 1994. Activation of (His)6-Raf-1 in vitro by partially purified plasma membranes from v-Ras-transformed and serum-stimulated fibroblasts. Proc. Natl. Acad. Sci. USA **91:**9544–9548.
5. **Dickson, B., F. Sprenger, D. Morrison, and E. Hafen.** 1992. Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. Nature (London) **360:**600–603.
6. **Fabian, J. R., I. O. Daar, and D. K. Morrison.** 1993. Critical tyrosine residues regulate the enzymatic and biological activity of the Raf-1 kinase. Mol. Cell. Biol. **13:**7170–7179.
7. **Fabian, J. R., D. K. Morrison, and I. Daar.** 1993. Requirement for Raf and MAP kinase function during the maturation of *Xenopus* oocytes. J. Cell Biol. **122:**645–652.
8. **Fabian, J. R., A. B. Vojtek, J. A. Cooper, and D. K. Morrison.** 1994. A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. Proc. Natl. Acad. Sci. USA **91:**5982–5986.
9. **Fantl, W. J., A. J. Muslin, A. Kikuchi, J. A. Martin, A. M. MacNicol, R. W. Gross, and L. T. Williams.** 1994. Activation of Raf-1 by 14-3-3 proteins. Nature (London) **371:**612–614.
10. **Finney, R., S. Robbins, and M. J. Bishop.** 1993. Association of Ras and Raf-1 in a complex correlates with activation of a signal transduction pathway. Curr. Biol. **3:**805–812.
11. **Ford, J. C., F. AlKhodairy, E. Fotou, K. S. Sheldrick, D. J. F. Griffiths, and A. M. Carr.** 1994. 14-3-3 protein homologs required for the DNA damage checkpoint in fission yeast. Science **265:**533–535.
12. **Freed, E., M. Symons, S. G. Macdonald, F. McCormick, and R. Ruggieri.** 1994. Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. Science **265:**1713–1716.
13. **Fu, H., J. Coburn, and J. Collier.** 1993. The eukaryotic host factor that activates exoenzyme S of *Pseudomonas aeruginosa* is a member of the 14-3-3 protein family. Proc. Natl. Acad. Sci. USA **90:**2320–2324.
14. **Fu, H., K. Xia, D. C. Pallas, C. Cui, K. Conroy, R. P. Narsimhan, H. Mamon, R. J. Collier, and T. M. Roberts.** 1994. Interaction of the protein kinase Raf-1 with 14-3-3 proteins. Science **266:**126–129.
15. **Furukawa, Y., N. Ikuta, S. Omata, T. Yamauchi, T. Isobe, and T. Ichimura.** 1993. Demonstration of the phosphorylation-dependent interaction of tryptophan hydroxylase with the 14-3-3 protein. Biochem. Biophys. Res. Commun. **194:**144–149.
16. **Hallberg, B., S. I. Rayter, and J. Downward.** 1994. Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. J. Biol. Chem. **269:**3913–3916.
17. **Han, M., A. Golden, Y. Han, and P. W. Sternberg.** 1993. *C. elegans lin-45 raf* gene participates in *let-60 ras*-stimulated vulval differentiation. Nature (London) **363:**137–140.
18. **Heidecker, G., J. L. Cleveland, T. Beck, M. Huieihel, W. Kolch, S. M. Storm, and U. R. Reddy.** 1989. Role of *raf* and *myc* oncogenes in signal transduction, p. 339–374. *In* N. H. Colburn (ed.), Genes and signal transduction in multistage carcinogenesis. Marcel Dekker, Inc., New York.
19. **Heidecker, G., M. Huleihel, J. L. Cleveland, W. Kolch, T. W. Beck, P. Lloyd, T. Pawson, and U. R. Rapp.** 1990. Mutational activation of c-*raf*-1 and definition of the minimal transforming sequence. Mol. Cell. Biol. **10:**2503–2512.
20. **Heidecker, G., W. Kolch, D. K. Morrison, and U. R. Rapp.** 1991. The role of Raf-1 phosphorylation in signal transduction. Adv. Cancer Res. **58:**53–73.
21. **Irie, K., Y. Gotoh, B. M. Yashar, B. Errede, E. Nishida, and K. Matsumoto.** 1994. Stimulatory effects of yeast and mammalian 14-3-3 proteins on the Raf protein kinase. Science **265:**1716–1719.
22. **Koide, H., T. Satoh, M. Nakafuku, and Y. Kaziro.** 1993. GTP-dependent association of Raf-1 with Ha-Ras: identification of Raf as a target downstream of Ras in mammalian cells. Proc. Natl. Acad. Sci. USA **90:**8683–8686.
23. **Kolch, W., G. Heidecker, G. Kochs, R. Hummel, H. Vahidi, H. Mischak, G. Finkenzeller, D. Marme, and U. R. Rapp.** 1993. Protein kinase Cα activates Raf-1 by direct phosphorylation. Nature (London) **364:**249–252.
24. **Krieg, P. A., and D. A. Melton.** 1984. Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAS. Nucleic Acids Res. **12:**7057–7070.
25. **Kyriakis, J. M., T. L. Force, U. R. Rapp, J. V. Bonventre, and J. Avruch.** 1993. Mitogen regulation of c-Raf-1 protein kinase activity toward mitogen-activated protein kinase-kinase. J. Biol. Chem. **268:**16009–16019.
26. **Leevers, S. J., H. F. Paterson, and C. J. Marshall.** 1994. Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature (London) **369:**411–414.
27. **Moodie, S. A., B. M. Willumsen, M. J. Weber, and A. Wolfman.** 1993. Complexes of Ras-GTP with Raf-1 and mitogen-activated protein kinase kinase. Science **260:**1658–1661.
28. **Moodie, S. A., and A. Wolfman.** 1994. The 3Rs of life: Ras, Raf and growth regulation. Trends Genet. **10:**44–48.
29. **Morrison, D. K.** 1990. The Raf-1 kinase as a transducer of mitogenic signals. Cancer Cells **2:**377–382.
30. **Morrison, D. K.** 1994. 14-3-3: modulators of signaling proteins? Science **266:**56–57.
31. **Morrison, D. K., G. Heidecker, U. R. Rapp, and T. D. Copeland.** 1993. Identification of the major phosphorylation sites of the Raf-1 kinase. J. Biol. Chem. **268:**17309–17316.
32. **Nishida, E., and Y. Gotoh.** 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Biochem. Sci. **18:**128–131.
33. **Pallas, D. C., H. Fu, L. C. Haehnel, W. Weller, R. J. Collier, and T. M. Roberts.** 1994. Association of polyomavirus middle tumor antigen with 14-3-3 proteins. Science **265:**535–537.
34. **Pumiglia, K., Y.-H. Chow, J. Fabian, D. Morrison, S. Decker, and R. Jove.** 1995. Raf-1 N-terminal sequences necessary for the Ras-Raf interaction and signal transduction. Mol. Cell. Biol. **15:**398–406.
35. **Rapp, U. R.** 1991. Role of Raf-1 serine/threonine protein kinase in growth factor signal transduction. Oncogene **6:**495–500.
36. **Rapp, U. R., G. Heidecker, M. Huleihel, J. L. Cleveland, W. C. Choi, T. Pawson, J. N. Ihle, and W. B. Anderson.** 1988. *raf* family serine/threonine protein kinase in mitogen signal transduction. Cold Spring Harbor Symp. Quant. Biol. **53:**173–184.
37. **Reuther, G. W., H. Fu, L. D. Cripe, R. J. Collier, and A. M. Pendergast.** 1994. Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. Science **266:**129–133.
38. **Roberts, T. M.** 1992. A signal chain of events. Nature (London) **360:**534–535.
39. **Smith, M. R., S. J. DeGudicibus, and D. W. Stacey.** 1986. Requirement for c-ras proteins during viral oncogene transformation. Nature (London) **320:**540–543.
40. **Stanton, V. P., Jr., D. W. Nichols, A. P. Laudano, and G. M. Cooper.** 1989. Definition of the human *raf* amino-terminal regulatory region by deletion mutagenesis. Mol. Cell. Biol. **9:**639–647.
41. **Stokoe, D., S. G. Macdonald, K. Cadwallader, M. Symons, and J. F. Hancock.** 1994. Activation of Raf as a result of recruitment to the plasma membrane. Science **264:**1463–1467.
42. **Troppmair, J., J. T. Bruder, H. App, H. Cai, L. Liptak, J. Szeberenyi, G. M. Cooper, and U. R. Rapp.** 1992. Ras controls coupling of growth factor receptors and protein kinase C in the membrane to Raf-1 and B-Raf protein serine kinases in the cytosol. Oncogene **7:**1867–1873.
43. **Tsuda, L., Y. H. Inoue, M.-A. Yoo, M. Mizuno, M. Hata, Y.-M. Lim, T. Adachi-Yamada, H. Ryo, Y. Masamune, and Y. Nishida.** 1993. A protein kinase similar to MAP kinase activator acts downstream of the Raf kinase in Drosophila. Cell **72:**407–414.
44. **Van Aelst, L., M. Barr, S. Marcus, A. Polverino, and M. Wigler.** 1993. Complex formation between Ras and Raf and other protein kinases. Proc. Natl. Acad. Sci. USA **90:**6213–6217.
45. **Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
46. **Warne, P. H., P. R. Viciana, and J. Downward.** 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature (London) **364:**352–355.
47. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell **14:**723–731.
48. **Williams, N. G., T. M. Roberts, and P. Li.** 1992. Both $p21^{ras}$ and $pp60^{v\text{-}src}$ are required, but neither alone is sufficient, to activate the Raf-1 kinase. Proc. Natl. Acad. Sci. USA **89:**2922–2926.
49. **Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis.** 1992. *ras* mediates nerve growth factor receptor modulation and three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell **68:**1041–1050.
50. **Zhang, X. F., J. Settleman, J. M. Kyriakis, E. Takeuchi-Suzuki, S. J. Elledge, M. S. Marshall, J. T. Bruder, U. R. Rapp, and J. Avruch.** 1993. Normal and oncogenic $p21^{ras}$ proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature (London) **364:**308–313.