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Biochemistry

A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function

(signal transduction/GTP-binding protein/protein-serine/threonine kinases)

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ABSTRACT **Ras and Raf-1 are key proteins involved in the transmission of developmental and proliferative signals generated by receptor and nonreceptor tyrosine kinases. Genetic and biochemical studies demonstrate that Raf-1 functions downstream of Ras in many signaling pathways. Although Raf-1 directly associates with GTP-bound Ras, an effect of this interaction on Raf-1 activity *in vivo* has not been established. To examine the biological consequence of the Ras/Raf-1 interaction *in vivo*, we set out to identify key residues of Raf-1 required for Ras binding. In this report, we show that a single amino acid mutation in Raf-1 (Arg^{89} to Leu) disrupted the interaction with Ras *in vitro* and in the yeast two-hybrid system. This mutation prevented Ras-mediated but not tyrosine kinase-mediated enzymatic activation of Raf-1 in the baculovirus/Sf9 expression system. Furthermore, kinase-defective Raf-1 proteins containing the Arg^{89} → Leu mutation were no longer dominant-inhibitory or capable of blocking Ras-mediated signal transduction in *Xenopus laevis* oocytes. These results demonstrate that the association of Raf-1 and Ras modulates both the kinase activity and the biological function of Raf-1 and identify Arg^{89} as a critical residue involved in this interaction. In addition, the finding that tyrosine kinases can stimulate the enzymatic activity of Raf-1 proteins containing a mutation at the Ras-interaction site suggests that Raf-1 can be activated by Ras-independent pathways.**

The Raf-1 and Ras protooncogene products serve as central intermediates in many signaling pathways by connecting upstream tyrosine kinases with downstream serine/threonine kinases, such as mitogen-activated protein kinase (MAPK) and MAPK kinase (MKK, also known as MEK) (1, 2). Ras is a membrane-localized guanine nucleotide-binding protein that is biologically active in the GTP-bound state (3, 4). Raf-1 is a protein-serine/threonine kinase located primarily in the cytosol (5, 6). Growth factors that stimulate cellular protein-tyrosine kinase activity enhance both the kinase activity of Raf-1 and the proportion of Ras bound to GTP (reviewed in ref. 7). The activation of Raf-1 in many cases is dependent on the activity of Ras, suggesting that Raf-1 functions downstream of Ras (8–10). Further evidence positioning Raf-1 downstream of Ras comes from studies using deregulated and dominant-inhibitory mutants of Ras and Raf-1 in mammalian cells (9–15), as well as from studies examining developmental pathways in *Drosophila melanogaster*, *Xenopus laevis*, and *Caenorhabditis elegans* (16–19). Recently, Raf-1 has been shown to interact directly with GTP-bound forms of Ras *in vitro* and in yeast two-hybrid expression systems (20–25). Ras has also been reported to coimmunoprecipitate with Raf-1 from stimulated, but not unstimulated, mammalian cells (26, 27). On the basis of these experiments and genetic and biochemical studies positioning Raf-1 downstream of Ras, Raf-1 has been proposed to be a direct effector of Ras. However, whether Raf-1 activity is modulated by the association with Ras has not been reported.

In this study, we identify Arg^{89} of Raf-1 to be a critical residue required for Ras/Raf-1 interaction. In addition, we find that mutation of this conserved site alters both the enzymatic and the biological activities of Raf-1.

MATERIALS AND METHODS

Construction and Expression of Raf-1 Mutants. The Raf-1^{R89L} plasmid, encoding Raf-1 with an Arg^{89} → Leu mutation, was generated by site-directed mutagenesis using the full-length wild-type (WT) Raf-1 construct, pKS:WT Raf-1, and a custom oligonucleotide primer to introduce the desired base change (18). To obtain kinase-defective (KD) Raf-1^{R89L}, pKS:KD Raf-1 (18) was digested with *Bgl* II and *Eco*RI, and a 387-bp fragment containing the S621A mutation present in KD Raf-1 was subcloned into the corresponding sites of pKS:Raf-1^{R89L}. The specific base changes in all mutant constructs were confirmed by sequence analysis. Sequences encoding the entire WT Raf-1, Raf-1^{R89L}, KD Raf-1, and KD Raf-1^{R89L} proteins were then inserted into the appropriate expression vectors.

***In Vitro* Binding Studies.** WT Raf-1 Ras-interaction domain (RID) and Raf-1^{R89L} RID sequences (aa 51–131 of Raf-1) were amplified by PCR and cloned into a maltose-binding protein (MBP) fusion vector for expression in *Escherichia coli* (24). Sequences of the RID inserts were confirmed by sequence analysis. *In vitro* binding assays using the MBP–RID fusion proteins were performed as described (24).

Transactivation Assays in the Yeast Two-Hybrid System. Activation of the *HIS3* and *lacZ* reporter constructs was detected by growth and color assays (24).

Immunoprecipitation and *in Vitro* Raf-1 Kinase Assays. Infected Sf9 insect cells were lysed 48 hr postinfection in ice-cold lysis buffer [20 mM Tris, pH 8.0/137 mM NaCl/10% (vol/vol) glycerol/1% (vol/vol) Nonidet P-40/0.1% (wt/vol) SDS/0.5% (wt/vol) sodium deoxycholate/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride/1 mM aprotinin/20 μM leupeptin/5 mM sodium vanadate]. Insoluble material was removed by centrifugation and cell lysates were equalized for Raf-1 protein expression. Raf-1 proteins were immunoprecipitated with antibodies to Raf-1 (28) and the antigen–antibody complexes were collected with protein A-Sepharose. The Raf-1 immunoprecipitates were washed three times with lysis buffer and once with kinase buffer (30 mM Hepes, pH 7.4/1 mM dithiothreitol/7 mM $MnCl_2$/5 mM $MgCl_2$/15

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Abbreviations: D-Raf, *Drosophila* Raf; GST, glutathione *S*-transferase; FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; GTP[γS], guanosine 5′-[γ-thio]triphosphate; GVBD, germinal-vesicle breakdown; KD, kinase-defective; MBP, maltose-binding protein; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; RID, Ras-interaction domain; WT, wild type.

μM ATP). The precipitated complexes were then incubated at 25°C for 15 min in 40 μl of kinase buffer containing 20 μCi of [γ-^{32}P]ATP (1 μCi = 37 kBq) and 0.1 μg of purified 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA)-treated MKK1. The MKK1 (kindly provided by P. Dent and T. Sturgill, University of Virginia) used in these assays was purified from Sf9 cells infected with a recombinant baculovirus encoding MKK1 and was treated with FSBA to inactivate the autokinase activity of MKK1. Kinase assays were terminated by the addition of 4% SDS/80 mM dithiothreitol/10% glycerol, the samples were resolved by SDS/PAGE, and phosphoproteins were visualized by autoradiography.

RNA Transcription and Injection of *Xenopus* Oocytes. For the production of RNA *in vitro*, a *Bam*HI fragment encoding the various Raf-1 proteins was inserted into the *Bgl* II site of the vector pT764T, which contains the 5′ and 3′ flanking regions of the *Xenopus* β-globin mRNA from pSP64T (29) proximal to the T7 promoter of pT7/T319U (Ambion, Austin, TX). The Tpr–Met and Ha-Ras^{V12} (encoding Val^{12}) constructs used for *in vitro* transcription were described previously (18). All plasmids were linearized with the appropriate restriction enzyme, and capped RNA transcripts were synthesized as specified by the vendor (Ambion) by using either T7 or SP6 RNA polymerase.

Xenopus oocytes were collected and prepared for injection as described (18). At 18 hr after isolation, oocytes were injected with 30 ng of RNA encoding the various Raf-1 proteins. Eight to 24 hr later, oocytes were injected with 30 ng of Ha-Ras^{V12} or Tpr–Met RNA. Oocytes were scored for germinal-vesicle breakdown (GVBD), as evidenced by the appearance of a white spot at the animal pole. This observation was verified by manual dissection of oocytes after fixation in 8% trichloroacetic acid. Histone H1 kinase assays were performed on extracts prepared from four oocytes (30).

RESULTS

Identification of Arg^{89} as a Residue of Raf-1 Required for Ras/Raf-1 Interaction. A two-hybrid screen for proteins that interact with human c-Ha-Ras previously showed that residues 51–131 within the CR1 domain of mammalian Raf-1 might be sufficient to bind Ras·GTP in this system (24). Sequences within this putative RID are highly conserved among the Raf family of proteins (5, 17, 31), and analysis of *Drosophila* Raf (D-Raf) mutations has revealed that an arginine-to-leucine substitution at position 217 (analogous to Arg^{89} of Raf-1) within this domain results in a partial loss of D-Raf function (31). Therefore, we performed experiments to ascertain whether mutation of this conserved arginine residue could alter Raf-1 function and whether this residue was required for the association with Ras.

We first examined the effect of the arginine-to-leucine mutation on the Ras/Raf-1 interaction by using wild-type and mutant RID fusion proteins. The RID sequences were cloned from Raf-1 by PCR, inserted into the appropriate vectors, and expressed as a fusion protein with MBP in *E. coli* and as a fusion protein with the transactivation domain of herpes simplex virus protein VP16 in yeast. An Arg^{89} → Leu mutant (R89L) of RID was also generated and cloned into the same vectors. In association experiments performed *in vitro*, MBP–RID^{WT} bound to a glutathione *S*-transferase (GST)–Ras fusion protein complexed with guanosine 5′-[γ-thio]triphosphate (GTP[γS]), but not to GST–Ras·GDP or GST (Fig. 1). In contrast, MBP–RID^{R89L} did not bind to GST–Ras·GTP[γS] (Fig. 1). In the yeast two-hybrid system, coexpression of VP16–RID^{WT}, but not VP16–RID^{R89L}, with LexA–Ras^{V12} transactivated *HIS3* and *lacZ* reporter gene transcription (data not shown). Transactivation of the reporter constructs was not detected with combinations of VP16–RID^{WT} with effector domain mutants of LexA–Ras^{V12}

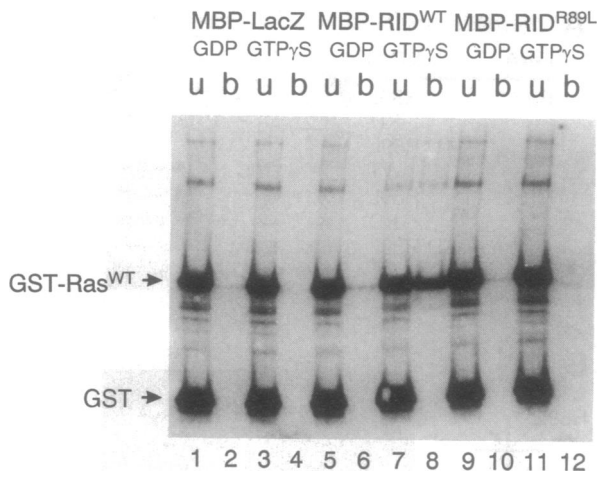

FIG. 1. Interaction of Raf-1 RID fusion proteins and Ras *in vitro*. Control (MBP–LacZ), WT (MBP-RID^{WT}), and R89L mutant (MBP–RID^{R89L}) fusion proteins were bound to amylose resin, and the immobilized proteins were incubated with a mixture of free GST and GST–Ras^{WT} that had been preincubated with either GDP or GTP[γS] (24). After incubation at 4°C for 1 hr, the resin was sedimented and the supernatant consisting of the unbound fraction was assayed. The resin was then washed, the bound proteins were eluted with maltose, and an aliquot of the bound fraction was sampled. To detect the added GST and GST-Ras^{WT}, equal samples of the bound (b, even-numbered lanes) and unbound (u, odd-numbered lanes) fractions were analyzed by SDS/PAGE and immunoblotting with antibodies to GST.

(data not shown). These results indicate that the RID region of Raf-1 is sufficient for binding to the activated form of Ras and that the R89L mutation inhibits this interaction.

Full-length WT and KD Raf-1 cDNAs encoding Leu^{89} were generated by site-directed mutagenesis. These alleles were then inserted into the VP16 vector and tested for interaction with LexA–Ras^{V12} in the two-hybrid system (Fig. 2). LexA–MKK1a (32) was used as a positive control for expression of the mutant VP16–Raf hybrid proteins, since MKK has been shown to interact with Raf-1 *in vitro* as well as in a two-hybrid assay (25, 33). All VP16–Raf mutants interacted strongly with LexA–MKK1a, and WT and KD Raf-1 VP16 hybrids interacted with LexA–Ras^{V12}. In contrast, the Raf-1^{R89L} and KD Raf-1^{R89L} mutants of VP16–Raf did not interact with LexA–Ras^{V12}, as judged by the failure to transactivate *lacZ* (Fig. 2*A*) or *HIS3* (Fig. 2*B*) expression. These experiments indicate that the R89L mutation inhibits the interaction of full-length Raf-1 with Ras.

Enzymatic Activity of Raf-1 Proteins Containing the R89L Mutation Can Be Induced by Src but not by Ras. To determine the effect that the R89L mutation has on the enzymatic activity of Raf-1, we performed *in vitro* kinase assays. WT Raf-1 and Raf-1^{R89L} proteins were expressed in the baculovirus/Sf9 expression system in the absence or presence of activated Src and Ras proteins, and their ability to autophosphorylate or to phosphorylate purified recombinant MKK1 was examined (Fig. 3). In addition, KD Raf-1 was included as a control to ensure that the kinase activity was due to Raf-1. The baculovirus/Sf9 expression system was chosen for this analysis because mutant proteins could be examined in the absence of endogenous mammalian Raf-1 protein and because this system has previously been used to characterize the catalytic activities of both wild-type and mutant Raf-1 proteins (34–36). Coexpression with either Ras or Src increased the basal level of WT Raf-1 kinase activity (as measured by autophosphorylation or phosphorylation of MKK1), and this activity was synergistically enhanced upon coexpression with both Ras and Src (Fig. 3*A*), as previously reported (34, 35). In contrast, the kinase activity of Raf-1^{R89L}

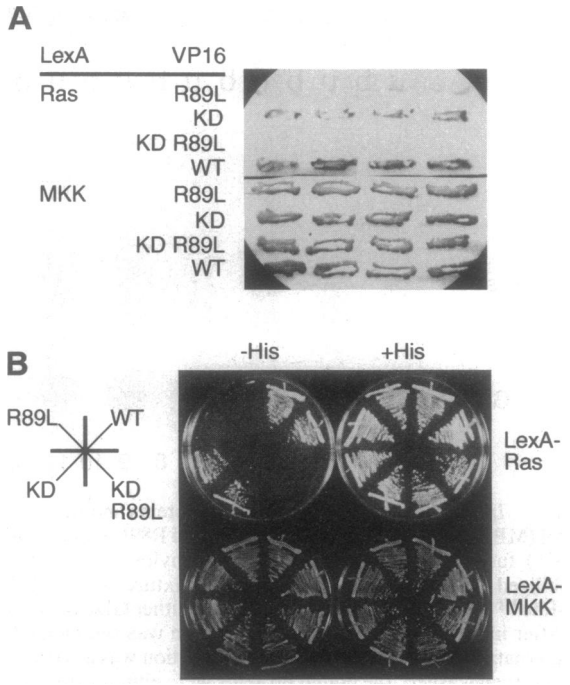

FIG. 2. Interaction of WT or mutant Raf-1 proteins with Ras and MKK in the yeast two-hybrid expression system. Yeast strain L40 (24) was transformed with plasmids encoding LexA–Ras^{V12} (LexA–Ras) or LexA–MKK1a (LexA–MKK) together with plasmids encoding WT Raf-1 (WT), Raf-1^{R89L} (R89L), KD Raf-1 (KD), or KD Raf-1^{R89L} (KD R89L) VP16–Raf hybrid proteins. Transformants were selected on plates lacking leucine and tryptophan. (*A*) Four independent transformants of each combination of plasmids were grown on plates lacking leucine and tryptophan and then analyzed for β-galactosidase activity. A nitrocellulose filter replica of the plate was incubated with 0.75 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside for 4 hr (lower half) or 20 hr (upper half). Dark color indicates transactivation of the *lacZ* reporter gene. (*B*) Two independent transformants of each combination of plasmids were streaked onto plates lacking leucine and tryptophan (+His, right) or lacking leucine, tryptophan, and histidine (−His, left). All VP16–Raf mutants transactivated the *HIS3* reporter gene when coexpressed with LexA–MKK1a, but only WT Raf-1 and KD Raf-1 transactivated *HIS3* when coexpressed with LexA–Ras^{V12}.

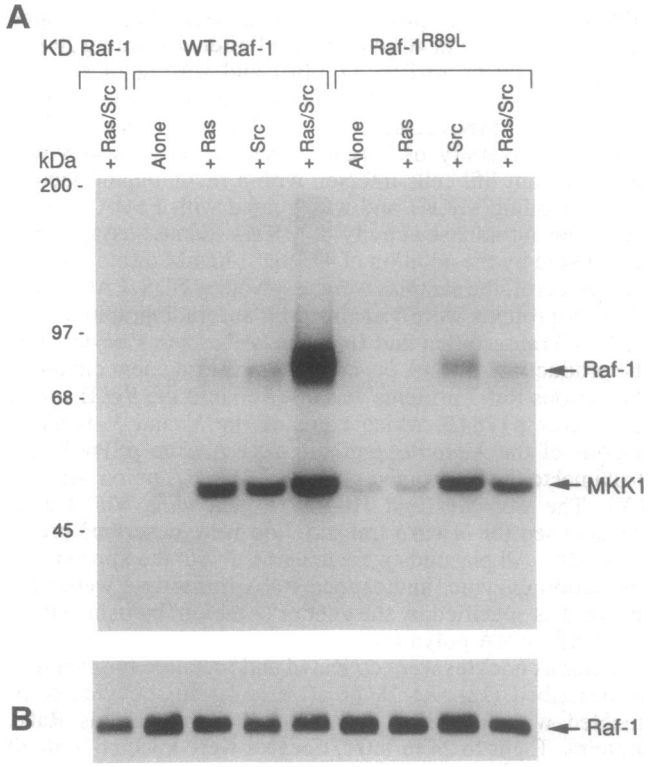

FIG. 3. Analysis of the *in vitro* kinase activity of Raf-1^{R89L}. (*A*) WT Raf-1, KD Raf-1, and Raf-1^{R89L} were expressed by recombinant baculoviruses in Sf9 cells in the absence (Alone) or presence of activated Ras (+Ras), activated Src (+Src), or Ras and Src (+Ras/Src). Raf-1 proteins were immunoprecipitated from Sf9 cell lysates and *in vitro* kinase assays were performed. Purified recombinant MKK1 that had been treated with FBSA (to inactivate MKK1 autokinase activity) was added as an exogenous substrate. Assays were terminated by the addition of 4% SDS/80 mM DTT/10% glycerol, the samples were resolved by SDS/7.5% PAGE, and the phosphoproteins were visualized by autoradiography. (*B*) Immunoprecipitated Raf-1 was detected by immunoblotting with antibodies to Raf-1. Molecular size markers are shown at left.

was activated when coexpressed with Src, but not when coexpressed with Ras. Moreover, coexpression with both Src and Ras did not synergistically enhance the activity of Raf-1^{R89L}. These results demonstrate that the R89L mutation does not inactivate or constitutively activate the catalytic activity of Raf-1 but does alter the Ras-mediated enzymatic activation of Raf-1.

KD Raf-1 Proteins Containing the R89L Mutation Fail to Block Ras-Mediated Signaling Events in *Xenopus* Oocytes. We then evaluated the effect of the R89L mutation on Raf-1 function *in vivo*. Since Raf-1 is ubiquitously expressed in all cell types (37), the impact of the Ras-binding mutation on Raf-1 function would be obscured by the presence of endogenous biologically active Raf-1. Therefore, we assessed the effect of this mutation by using KD Raf-1 proteins that have been shown to block Ras-mediated signaling pathways in NIH 3T3 mouse cells and in *Xenopus* oocytes (12, 13, 18, 19, 38). Experiments were performed in stage VI-arrested oocytes to determine whether the R89L mutation altered the dominant-inhibitory activity of KD Raf-1. *Xenopus* oocytes were chosen for this assay because they represent a synchronized population of cells and because the expression level and biological phenotype of the mutant proteins could be monitored easily. Expression of Ha-Ras^{V12} (an oncogenic form of Ras) or Tpr–Met (an oncogenic activated form of hepatocyte growth factor receptor tyrosine kinase) in oocytes has been shown to induce GVBD, histone H1 kinase activity (an indicator of p34^{cdc2} kinase activity), and MAPK activity (39, 40). As we previously reported (18), GVBD and H1 kinase activity mediated by Tpr–Met or Ha-Ras^{V12} was blocked in oocytes expressing KD Raf-1, but not in oocytes expressing WT Raf-1 (Fig. 4). MAPK activation, as measured by the tyrosine phosphorylation and electrophoretic mobility shift of MAPK, was also blocked in oocytes expressing KD Raf-1 (Fig. 5; shown are lysates prepared from Ha-Ras^{V12} injected oocytes; oocytes injected with Tpr–Met gave similar results). These results demonstrate that KD Raf-1 proteins can act in a dominant-inhibitory manner to block oocyte maturation induced by components of the receptor tyrosine kinase pathway. In contrast, GVBD, H1 kinase activity, and MAPK were still induced by Ha-Ras^{V12} and Tpr–Met in oocytes expressing KD Raf-1^{R89L} (Figs. 4 and 5). In addition, an electrophoretic shift that correlates with increased Raf-1 phosphorylation (15) was observed for WT Raf and KD Raf-1^{R89L} but not for KD Raf-1 (Figs. 4 and 5), indicating that the shift in mobility is maturation dependent and does not require the catalytic activity of the injected Raf-1. These results suggest that KD Raf-1 proteins block oocyte maturation by interacting with Ras and inhibiting its ability to transmit signals. These findings further indicate that the R89L mutation disrupts this interaction so that KD Raf-1 no longer can block Ras-mediated signaling in a dominant-inhibitory manner.

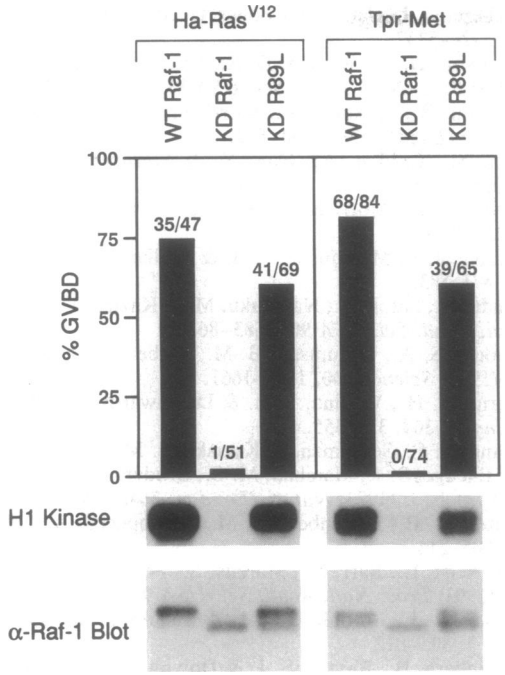

FIG. 4. Analysis of the dominant-inhibitory effects of wild-type and mutant Raf-1 proteins in *Xenopus* oocytes. Oocytes preinjected with capped transcripts encoding WT Raf-1, KD Raf-1, or KD Raf-1^{R89L} (KD R89L) (30 ng per oocyte) were microinjected with capped transcripts encoding Ha-Ras^{V12} or Tpr–Met (15 ng per oocyte). At 18–24 hr after injection, oocytes were either scored for GVBD or were homogenized in 1% Nonidet P-40 lysis buffer as described (18). The percentage of oocytes undergoing GVBD is represented by the bars, and the ratio of the number of oocytes with GVBD to the total number injected is displayed above each bar (the numbers listed represent the results of three experiments). Histone H1 kinase assays were performed on extracts from four injected oocytes and the autoradiograph is displayed below each bar. Expression of Raf-1 proteins in injected oocytes was analyzed by SDS/PAGE and immunoblotting with Raf-1 antibodies (α-Raf-1).

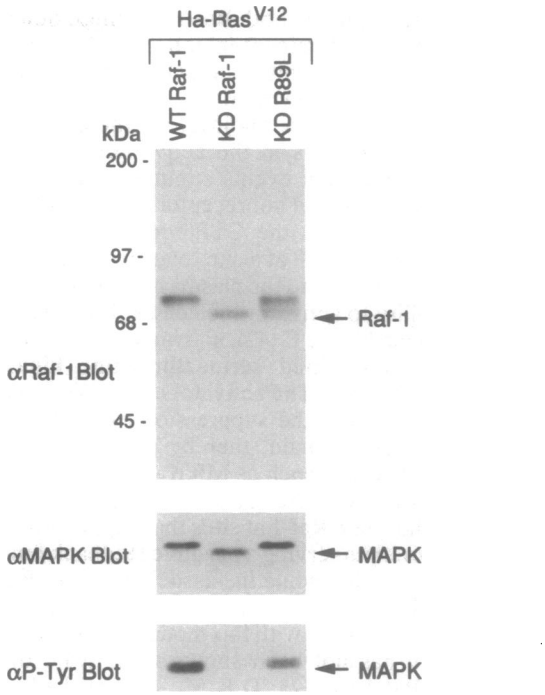

FIG. 5. Effect of wild-type and mutant Raf-1 proteins on the activation of MAPK. Lysates were prepared from Ha-Ras^{V12}-injected oocytes that had been preinjected with WT Raf-1, KD Raf-1 or KD Raf-1^{R89L}. The lysates (1.5 oocyte equivalents per lane) were resolved by SDS/8% PAGE and examined by immunoblotting with antibodies to Raf-1 (αRaf-1), MAPK (αMAPK), or phosphotyrosine (αP-Tyr). Molecular size markers are shown at left.

DISCUSSION

In this report, we identify a residue of Raf-1 required for Ras binding and show the importance of this interaction for the Ras-mediated enzymatic and biological activities of Raf-1. Arg^{89}, located in the CR1 regulatory region, was chosen as a potential site for Ras interaction because of the identification of a homologous mutation that affects late zygotic functions of the D-Raf protein (31). The R89L mutation in mammalian Raf-1 disrupted the GTP-dependent association of Ras with both the full-length Raf-1 protein in the two-hybrid system and the isolated Raf-1 RID fusion proteins *in vitro*. These results suggest that Arg^{89} of Raf-1 either is part of a recognition site for the association of Raf-1 with Ras or is a residue involved in maintaining the stability of the Ras/Raf-1 interaction. However, the R89L mutation did not affect the interaction of Raf-1 with MKK, indicating that Arg^{89} is a residue required for the association with Ras but not with MKK. These findings are consistent with previous studies indicating that the C-terminal, catalytic domain of Raf-1 is sufficient for interactions with MKK, whereas the N-terminal region, containing the RID, is sufficient for association with Ras (25).

To evaluate the effect that mutation of the Ras-interaction site has on the enzymatic activity of Raf-1, *in vitro* kinase assays were performed with baculovirus-expressed proteins. The R89L mutation prevented the kinase activation of Raf-1 induced by coexpression with Ras but had no effect on the activation induced by Src. These findings demonstrate that the Ras-mediated activation of Raf-1 requires the binding of Ras to the regulatory region of Raf-1. However, tyrosine kinases can activate Raf-1 through mechanisms that do not involve Ras. This finding is consistent with previous studies showing that even in the presence of dominant-inhibitory Ras (Ras^{N17}) protein-tyrosine kinases can enhance Raf-1 activity in this system (34). In addition, mutations have been made in Raf-1 that render the protein unable to be phosphorylated or activated by tyrosine kinases yet still capable of being activated by Ras (35). These results and those described in this report suggest that in the baculovirus/Sf9 expression system, Raf-1 can be activated by at least two separate mechanisms, one mediated by activated tyrosine kinases and one propagated by activated Ras proteins. The existence of Ras-independent pathways for Raf-1 activation may explain why the homologous mutation in D-Raf (R217L) causes only partial loss of function (31). Alternatively, D-Raf^{R217L} may interact to a limited degree with Ras.

To test the mechanism by which kinase-inactive Raf-1 proteins interfere with the propagation of growth signals, we introduced the R89L mutation into a dominant-inhibitory Raf-1 protein (18). When expressed in *Xenopus* oocytes, mutation of the Ras-interaction site resulted in KD Raf-1 protein (KD Raf-1^{R89L}) that could no longer inhibit meiotic maturation and MAPK activation induced by oncogenic Ras (Ha-Ras^{V12}) or by an activated receptor tyrosine kinase (Tpr–Met). Since the MAPK pathway is activated in oocytes expressing KD Raf-1^{R89L}, these results suggest that while KD Raf-1^{R89L} can interact with MKK in the two-hybrid system, KD Raf-1^{R89L} does not prevent MKK activation by sequestering it *in vivo*. Furthermore, these experiments indicate that dominant-inhibitory Raf-1 mutants function in part by binding to activated Ras molecules and thus prevent their interaction with the endogenous Raf protein. If Ras has other effector molecules that cooperate with Raf-1 to transduce the biological effects of Ras, then overexpression of KD Raf-1

may block signaling by both Raf-1 and these other effectors. In this case, the "dominant-inhibitory" effects of KD Raf-1 may more closely resemble a blockade of Ras function than a blockade of Raf-1 function.

As a summary of our findings, we therefore propose the following role for Ras in the activation of Raf-1. Growth factors and mitogenic events stimulate the tyrosine kinase activity of receptor and nonreceptor tyrosine kinases and the conversion of Ras to the GTP-bound form (7, 41). Raf-1 interacts with Ras·GTP at a site requiring Arg^{89}, inducing the translocation of Raf-1 to the plasma membrane and increasing its accessibility to activator molecules. Raf-1 activators may include protein kinase C (42), activated tyrosine kinases (35), or as yet unidentified serine/threonine kinases, phosphatases, or ligands. The activators help to relieve the Raf-1 kinase domain from the suppression exerted by the N-terminal regulatory domain, thereby allowing Raf-1 to phosphorylate substrates such as MKK. Following phosphorylation of substrates by Raf-1, other serine/threonine kinases then phosphorylate Raf-1 at sites that suppress activity, such as Ser^{259} (36), thus serving a negative feedback function. This model suggests that while the association with Ras is necessary, the binding itself may not be sufficient for full Raf-1 activation. Consistent with this model, the binding of Raf-1 to Ras *in vitro* has not been shown to activate the catalytic activity of Raf-1 (ref. 23; D.K.M., unpublished results).

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