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Identification of Residues in the Cysteine-rich Domain of Raf-1 That Control Ras Binding and Raf-1 Activity*

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David G. Winkler‡§, Richard E. Cutler, Jr.¶‖, Jonelle K. Drugan‡‡§§, Sharon Campbell‡‡§§, Deborah K. Morrison¶**, and Jonathan A. Cooper‡¶¶**

From the ‡Fred Hutchinson Cancer Research Center, Seattle, Washington 98109-1024, the ¶Molecular Basis of Carcinogenesis Laboratory, ABL-Basic Research, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702, and the ‡‡Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599

We have identified mutations in Raf-1 that increase binding to Ras. The mutations were identified making use of three mutant forms of Ras that have reduced Raf-1 binding (Winkler, D. G., Johnson, J. C., Cooper, J. A., and Vojtek, A. B. (1997) *J. Biol. Chem.* 272, 24402–24409). One mutation in Raf-1, N64L, suppresses the Ras mutant R41Q but not other Ras mutants, suggesting that this mutation structurally complements the Ras R41Q mutation. Missense substitutions of residues 143 and 144 in the Raf-1 cysteine-rich domain were isolated multiple times. These Raf-1 mutants, R143Q, R143W, and K144E, were general suppressors of three different Ras mutants and had increased interaction with non-mutant Ras. Each was slightly activated relative to wild-type Raf-1 in a transformation assay. In addition, two mutants, R143W and K144E, were active when tested for induction of germinal vesicle breakdown in *Xenopus* oocytes. Interestingly, all three cysteine-rich domain mutations reduced the ability of the Raf-1 N-terminal regulatory region to inhibit *Xenopus* oocyte germinal vesicle breakdown induced by the C-terminal catalytic region of Raf-1. We propose that a direct or indirect regulatory interaction between the N- and C-terminal regions of Raf-1 is reduced by the R143W, R143Q, and K144E mutations, thereby increasing access to the Ras-binding regions of Raf-1 and increasing Raf-1 activity.

The importance of the Raf protein kinases for cell growth and differentiation has been demonstrated in animals, tissue culture cells, *Drosophila*, and *Caenorhabditis elegans*. A highly conserved signal transduction pathway links growth factor receptors through the Ras small GTP-binding protein to the activation of the Raf-1 protein kinase. Raf-1 in turn can bind to, phosphorylate, and activate mitogen-activated protein kinase kinase 1 (MKK1)[1] and consequently the mitogen-activated protein kinase cascade (2–5).

Raf-1 regulation is a complex process that integrates information from multiple signaling pathways. The Raf-1 kinase domain is in the C-terminal half of the protein. The N-terminal half of the protein contains two conserved regions termed conserved region 1 (CR1) and conserved region 2 (CR2) (6) (Fig. 1). Deletion analysis and point mutations show that the CR1 and CR2 regions are inhibitory (4, 7, 8). Loading of Ras with GTP induces the binding of Raf-1 to Ras and the subsequent translocation of Raf-1 to the plasma membrane (9–12). This translocation is accompanied by an activation of the basal kinase activity of Raf-1 (13, 14). The Raf-1 kinase is further activated at the membrane by changes in inter- and intramolecular interactions and phosphorylation events (9, 15–19) (reviewed in Ref. 4).

Raf-1 binds to Ras in a GTP-dependent manner through a Ras-binding domain (RBD) in the N-terminal region of the CR1 domain of Raf. Mutations in the RBD block Ras binding and prevent activation by a number of stimuli (10, 11, 20–22). The RBD binds to Ras through an extensive interface (21, 23). Residues of the second β sheet and the first α helix of the RBD contact the Ras effector domain (residues 31–41), and Raf-1 or Ras mutations that map to this interface block binding.

Ras also interacts with the C-terminal part of the CR1 region of Raf, the cysteine-rich domain (CRD) (24, 25). The CRD binds to zinc and to phosphatidylserine as well as Ras (26, 27). Mutations that disrupt the structure of the CRD reduce binding to Ras *in vitro*, suggesting that the RBD and the CRD may both contribute to Raf-1 binding (25). However, CRD binding to prenylated Ras is not dependent on GTP, suggesting that the RBD, not the CRD, is the primary sensor of Ras activity (24, 25, 28, 29). The CRD has another important role; certain mutations in the CRD activate Raf-1 (17, 18, 29, 30). These data suggest that the CRD is important both for the low activity of unstimulated Raf-1 and for activation by Ras.

Raf-1 binds to 14-3-3 proteins, HSP90, and p50 (4, 31). 14-3-3 and HSP90 may be involved in the stabilization or activation of Raf-1 (32–37). 14-3-3 binds to Raf-1 at three characterized sites. The phosphorylated serines at Ser-259 and Ser-621 represent regulated 14-3-3- binding sites (38, 39). 14-3-3 also binds to the cysteine-rich domain in Raf-1 by a different mechanism, and availability of this 14-3-3- binding site may be activation state-dependent (29). The regulated nature of these three 14-

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‖ Current address: Sugen, Inc., 351 Galveston Dr., Redwood City, CA 94063-4720.

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¶¶ To whom correspondence should be addressed: Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N. (A2025), P. O. Box 9024, Seattle, WA 98109-1024. Tel.: 206-667-4454; Fax: 206-667-6522; E-mail: jcooper@fhcrc.org.

[1] The abbreviations used are: MKK1, mitogen-activated protein kinase kinase 1; GVBD, germinal vesicle breakdown; CRD, cysteine-rich domain; CR, conserved region; RBD, Ras binding domain; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; GMP-PNP, β:γ-imido-guanosine 5′-triphosphate; Cat, catalytic; Reg, regulatory.

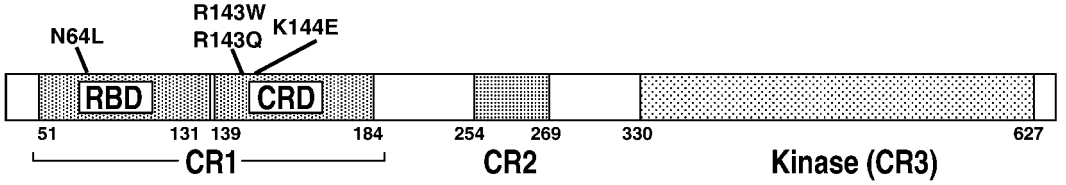

FIG. 1. **Schematic Raf-1 primary structure showing regions where sequence is conserved in the Raf family**. The CR1 contains the RBD and the CRD; the CR2 is rich in threonine and serine residues, and the CR3 contains the kinase domain. Also shown are the N64L, R143W, R143Q, and K144E mutations.

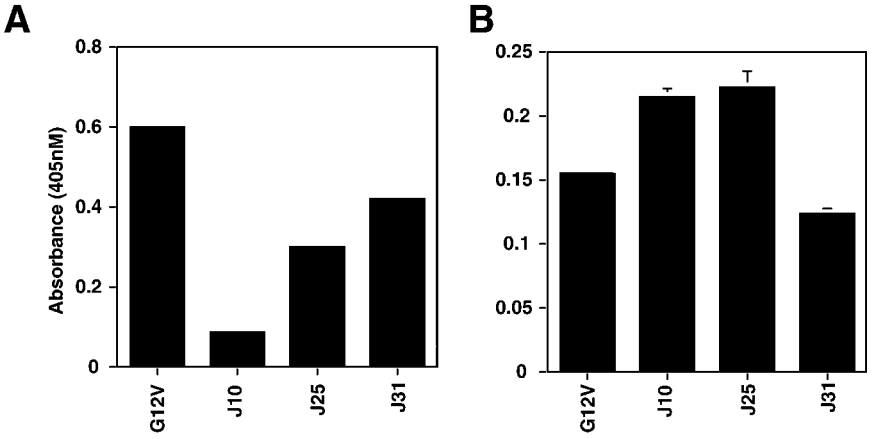

FIG. 2. **Ras specificity mutants were assessed for binding to the Raf RBD (*A*) and CRD (*B*) *in vitro*.** Fusion proteins were expressed in *E. coli* and purified, and binding was determined by ELISA (see "Experimental Procedures"). Results are summarized from an assay done in triplicate. *Error bars* are not shown when they were less than 5% of the mean.

3-3- binding sites presents the possibility that 14-3-3 can bind to alternative sites during Raf-1 activation (40).

We have recently utilized the yeast two-hybrid system to identify specificity mutants of Ras that bind other effector proteins but not Raf-1 (1) (see Table I for the mutations in these mutants). In the present study, we produced a collection of random mutations in Raf-1 and used the two-hybrid system to select mutations that restored Raf-1 binding to these Ras mutants. In one case we found a single mutation in the RBD (N64L) that directly interacts with the residue that was mutated in the Ras mutant it complements, suggesting structural complementarity. In all other cases the mutated residue was either Arg-143 or Lys-144 in the CRD. These mutants have generally increased interaction with mutant and non-mutant Ras and are somewhat activated *in vivo*. These mutations also decrease the ability of the isolated N-terminal regulatory region to inhibit the isolated C-terminal kinase domain. We propose that these mutations decrease an inhibitory interaction between the N-terminal regulatory region and the C-terminal kinase domain and expose the Raf-1 N terminus for Ras binding.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screens—The two-hybrid screens in this study used the L40 reporter strain with the dual reporters *HIS3* and *lacZ*. The strength of interactions between LexA fusions of Ras mutants expressed from pBTM116 and VP16 fusions of Raf-1 mutants in pVP16 was measured by examining the extent of color change in filter assays for β-galactosidase, using 0.75 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$). Filters were incubated at 30 °C for 1 h to overnight (41).

Activation of the *HIS3* reporter was indicated by plating cells on minimal media lacking histidine and containing increasing concentrations of 3-aminotriazole, an inhibitor of the *HIS3* gene product (41). Comparisons between transformants were made by plating serial dilutions of cells in drops onto plates in the absence and presence of increasing concentrations of 3-aminotriazole. The plating efficiency was calculated, and the *HIS3* reporter level was determined to closely relate to the relative activity of the *lacZ* reporter assayed in parallel.

Generation and Selection of Human Raf-1 Mutants—The Raf M1 library was generated by regionalized codon mutagenesis of residues 58–70 in Raf-1 (42). The Raf M2 library was generated by random PCR mutagenesis in the presence of $MnCl_2$ as described elsewhere (1). The region from residues 1 to 149 (prior to the *Hin*dIII site at codon 150) was then subcloned into a wild-type backbone in pVP16. Both of these libraries were transfected into the L40 yeast reporter strain along with V12G Ras and the J10, J25, and J31 Ras mutants in pBTM116 (see Table I for mutations present in the Ras mutants used). The transformants were plated onto selective media in the absence of histidine to select for Raf-1 mutants that had increased binding to the J10, J25, and J31 Ras mutants. Increased binding was confirmed by β-galactosidase assays. Plasmids were recovered from the positive clones, and the clones were retested for binding to the Ras mutants and for binding to MKK1a. False positives that did not retest and mutants that had lost the ability to bind to MKK1a were discarded. Mutations were sequenced on both strands using dye terminator sequencing.

Each VP16 Raf-1 mutant was then transfected with each LexA Ras mutant into L40, and the level of binding in the two-hybrid system was assessed as described above (41).

Protein Expression and Binding Assays—The Raf-CRD (Raf-1 residues 136–187) and the Ras variants used in the study were expressed as glutathione *S*-transferase (GST) fusion proteins and purified. The Ras mutants were eluted from the glutathione (GSH) beads and bound to GMP-PNP. The Raf-CRD was cleaved from the GSH beads using thrombin (26, 27).

The RBD (residues 51–131) was produced as a maltose-binding protein fusion in *Escherichia coli* and affinity purified on amylose agarose and eluted in 20 mM Tris, pH 8, 200 mM NaCl, 0.5 mM EDTA, and 10 mM maltose.

Enzyme-linked immunosorbent assays were employed to assess the interactions between the Ras mutants and the isolated Raf-1 CRD and RBD. The assays were similar to those described elsewhere (28). The Raf-1 fragments were immobilized on high affinity microtiter plates (Costar). Nonspecific interactions measured by using blank or MBP-coated plates (28). All assays were performed twice in triplicate.

14-3-3ζ Binding Assays—The DNA encoding the Raf-1 mutants were moved into pBTM116 and transfected into L40 along with pVP16 14-3-3ζ. The two-hybrid interaction between 14-3-3ζ and the Raf-1 mutants was assessed as described above (41).

The DNA encoding 14-3-3ζ was subcloned into pGEX3x for bacterial expression. GST and a GST 14-3-3ζ fusion protein were expressed in *E. coli* and affinity purified using GSH-Sepharose. Raf-1 mutants were subcloned into pCS3MT (43) and expressed in rabbit reticulocyte lysates according to the supplier's instructions (Promega), in the presence of [^{35}S]methionine. The reticulocyte lysates were diluted 1:10 in binding buffer (20 mM Tris 7.5, 1% Triton X-100, 10% glycerol, 50 mM NaF, 100 mM NaCl, 14 mM β-mercaptoethanol), and 5% of the reaction was run on SDS-polyacrylamide gel electrophoresis. The expression levels of the Raf-1 proteins were estimated by PhosphorImager analysis. Raf-1 proteins were incubated with 1 μg of GST or GST 14-3-3ζ on GSH beads in

1 ml of binding buffer for 2 h at 4 °C with end over end shaking. The beads were precipitated by centrifugation and washed 4 times with 1 ml of binding buffer. After the final wash, the beads were incubated with SDS-PAGE Loading buffer and analyzed by electrophoresis and PhosphorImager analysis. Similar results were obtained four times.

Enzyme-linked immunosorbent assays were employed to assess the interactions between the Ras mutants and the isolated Raf-1 CRD and14-3-3ζ. The assays were similar to those described elsewhere (29). The Raf-1 fragments were immobilized on high affinity microtiter plates (Costar). Nonspecific interactions were measured by using blank plates (29). All assays were performed twice in triplicate.

Transformation Assays—Focus formation assays were performed as described (29, 44). All of the plates received 1 μg of RhoA V14G in pLSXN vector, 1 μg of the Raf-1 mutant in pCS3MT vector, and 18 μg of sheared calf thymus DNA. To control for the effect of wild-type Raf, pCS3MT alone was used. The cells were transfected using the calcium phosphate precipitation method as described (44) and scored for G418 resistance and focus formation as described (44). Each data point presented is the summary of foci from 12 separate platings of transfected cells from 3 independent experiments.

Oocyte Injections—Wild-type full-length Raf-1 and Cat Raf constructs have been described (15, 45). The Reg Raf construct is described (46). Mutant Reg Raf constructs were generated by PCR amplification of Raf-1 residues 1–330 using mutant Raf-1 DNA as template. PCR products were cloned into the pA vector immediately downstream of the FLAG epitope tag. Mutations were also made in full-length Raf-1 in the pA vector by site-directed mutagenesis (Quickchange, Stratagene).

Oocytes were isolated and defolliculated as described previously. 8–12 h after isolation, oocytes were injected with approximately 30 ng of *in vitro* transcribed RNA encoding the full-length Raf-1 proteins or Reg Raf proteins. Oocytes expressing the Reg Raf proteins were then injected 4–8 h later with RNA encoding Cat Raf (15, 37, 45). The oocytes were scored for GVBD, as evidenced by the appearance of a white spot on the animal pole. This observation was verified by manual dissection of oocytes after fixation in 8% trichloroacetic acid.

Xenopus oocytes were lysed by trituration with a pipette tip in RIPA buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 units/ml), 20 mM leupeptin, 5 mM sodium vanadate). Insoluble material was pelleted by centrifugation and normalized for protein concentration. Raf-1, Reg Raf, and Cat Raf protein expression was assessed by Western blot (15, 37, 45).

TABLE I
Effects of mutagenesis on Ras/Raf-1 interaction

Strain L40 yeast cells were transformed with either wild-type (G12V) Ras or Ras specificity mutants, together with either wild-type Raf-1, the M1 library, or the M2 library. The frequency of colonies showing interaction between Ras and Raf-1 (His-positive phenotype) was calculated relative to the total number of transformants in each case. Individual substitutions present in each specificity mutant, J10, J25, and J31, are shown. Each of these mutants also contains the G12V-activating mutation.

	Ras mutant designation			
	G12V[a]	J10[b]	J25[c]	J31[d]
	no. interacting clones/10^4 transformants			
Raf-1 WT	10^4	0.48	0.08	0.60
Raf M1 library	10^4	0.65	2.6	2.3
Raf M2 library	10^4	14	3	18.0

[a] Mutation present is G12V.
[b] Mutations present are G12V and E31K.
[c] Mutations present are G12V, Q43E, D54N, and E63K.
[d] Mutations present are G12V, R41Q, and Y157N.

RESULTS

Characterization of Ras Mutants—We have recently used the yeast two-hybrid system to identify mutants of activated (G12V) Ras bind reduced levels of full-length Raf-1 (see Fig. 1 for a schematic of Raf-1) but bind normal levels of two other Ras effector domain-binding proteins (1). Curiously, these specificity mutants still bind to the Raf-1 RBD in yeast (Ref. 1, Fig. 3*A*). To test whether the Ras specificity mutants bind the RBD *in vitro*, an ELISA-based assay was used (Fig. 2). The RBD bound to the J10, J25, and J31 specificity mutants, although binding was reduced compared with wild-type (G12V) Ras. The CRD bound to wild type and mutants equally. These results suggest that the Ras specificity mutations reduce, but do not prevent, binding to the isolated RBD and have no effect on binding to the isolated CRD. Binding to full-length Raf-1 is strongly inhibited (1), however, suggesting that the Raf-1 kinase domain inhibits binding mediated by the RBD and CRD.

Mutations in Raf-1—We used the two-hybrid system to search for mutations in full-length Raf-1 that increased binding to the Ras specificity mutants. We expected that mutations in Raf-1 could increase binding in three ways as follows: by deleting the inhibitory Raf-1 kinase domain, by specifically complementing the Ras mutations, or by relieving the inhibitory effect of the C-terminal catalytic region. We made two libraries of random Raf-1 mutants, libraries M1 and M2. In library M1, the first loop of the RBD (codons 58–70) was mutagenized by regionalized codon mutagenesis (42). In library M2, codons 1–149 were mutagenized by low stringency PCR. These Raf-1 mutant libraries were transformed into the L40 two-hybrid reporter strain and screened for interaction with the Ras specificity mutants. Table I shows that the mutagenesis of Raf-1 increased the frequency of clones in which Ras and Raf-1 interact. Thus, for most combinations, the majority of identified clones contained mutations that caused increased Raf-1 interaction with mutant Ras.

Because truncation of the C terminus of Raf-1 increases interaction with the Ras mutants (Fig. 3*A*, compare RIP 7 and RIP 51 truncation mutants with full-length Raf-1) (1, 10), we

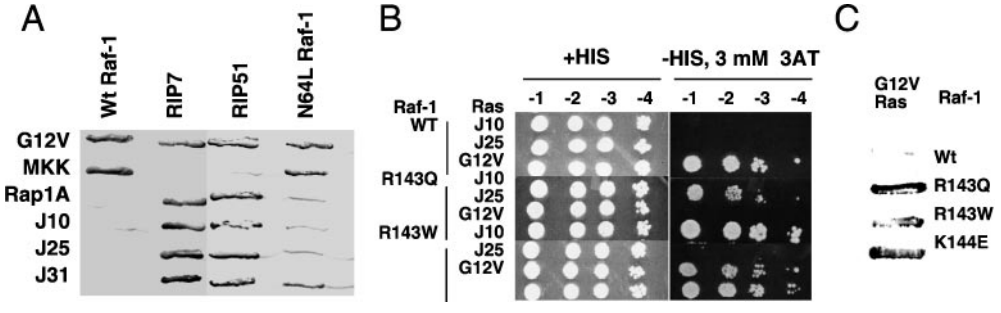

FIG. 3. **Interactions between Ras and Raf-1 mutants in yeast.** *A,* the N64L Raf-1 mutant suppresses the J31 Ras mutant but not the J10 or J25 Ras specificity mutants. A β-galactosidase filter assay (5-h development) showing the interaction of wild-type Raf-1, two characterized truncations of Raf-1 (RIP7 51–178, and RIP51 48–178 (10)), and N64L Raf-1 with G12V Ras, MKK1a, the Rap1A small G protein, and Ras specificity mutants. *Darker color* indicates binding. *B,* R143W and R143Q Raf-1 mutants suppress the J10 and J25 Ras mutants differentially and have increased binding to G12V Ras. Ten-fold dilutions of saturated cultures of yeast strains containing Ras and Raf-1 mutants were plated either on selective media containing His or on media lacking His and including 3 mM 3-aminotriazole. Representative of three assays. Increased growth and colony forming success indicates increased binding. *−1*, *−2*, *−3*, and *−4*, $\log_{10}$ of dilution. *C,* R143W, R143Q, and R144E have increased binding to G12V Ras. A β-galactosidase filter assay (1 h) showing the interaction of wild-type Raf-1, R143Q, R143W, and K144E with G12V Ras. *Darker color* indicates stronger interaction.

TABLE II
Specificity of Raf-1 suppressors

Mutants of Raf-1 were isolated as suppressors of Ras mutants with decreased binding to Raf-1. The Ras mutant that was used in the selection is listed. The Raf-1 mutants were re-transformed with activated Ras (G12V) and the Ras mutants into the L40 reporter strain, and the level of two-hybrid interaction was assessed using both the *HIS3* and *lacZ* reporters. The data are summarized with +++ indicating a very strong interaction, ++ indicating a strong interaction, + indicating a weak interaction, and − indicating the lack of an interaction. Assays not done are signified by ND.

Raf-1 mutant	Ras mutant used in selection	G12V	J10	J25	J31
Wild type		++	−	−	−
N64L	J31	++	−	−	++
R143Q[a]	J10 and J31	+++	+++	+	+++
R143W	J25 and J31	+++	+	+++	+++
K144E	J10	+++	+++	++	+++

[a] R143Q was isolated twice using Ras mutant J10, and a double mutant of Raf-1 T49S/R143Q was identified once with Ras mutant J10.

screened for truncation mutants using the C-terminal binding protein and Raf-1 substrate, MKK1. Raf-1 mutants that did not interact with MKK1 were likely to lack the C terminus and were discarded. The remaining eight clones were retested to ensure that all false positives and truncation mutants had been eliminated. The DNA was then sequenced, and codons 1–149 were recloned into a wild-type Raf-1 background to confirm that there were no hidden mutations outside of the mutagenized region.

The N64L Raf-1 Mutation—The specificity of the Raf-1 mutants was examined. The only missense mutant identified from the Raf M1 library was an N64L mutation in Raf-1, isolated because it increased binding to Ras mutant J31. Raf-1 N64L interacted with J31 but not J10 or J25 (Fig. 3*A*, Table II). Raf-1 N64L was the only allele-specific mutant identified. The basis for this specificity will be discussed below (see "Discussion").

Raf-1 CRD Mutants—We isolated seven other Raf-1 mutants from the Raf M2 library that were general suppressors. All of these mutants had changes in either codons 143 or 144 of Raf-1. The R143Q mutation was isolated four times, three times using Ras mutant J10 (once with a secondary T49S mutation), and once using J31. R143W was isolated once each using J25 and J31. K144E was isolated once using J10.

The mutations at codons 143 and 144 suppressed J10, J25, and J31 to different extents, suggesting some level of allelic specificity (Fig. 3*B*, Table II). Multiple transformations of bait and transactivator plasmids gave consistent results. The R143Q mutant was a good suppressor of J10 and J31 but only a weak suppressor of J25. On the other hand R143W was a good suppressor of J25 and J31 but a weak suppressor of J10. The R144E mutation was a good suppressor of J10 and J31 but not of J25. Importantly, the three mutations at codons 143 and 144 also increased the interaction of Raf-1 with wild-type Ras (G12V) suggesting a general effect on conformation (Fig. 3*C,* Table II). Although it is clear that the R143W, R143Q, and K144E mutations are general suppressors of the J10, J25, and J31 Ras mutations, the allelic specificity suggests that there are differences in the ways that these Raf-1 and Ras mutants interact.

14-3-3 Protein Binding to Raf-1 Mutants—A recent analysis showed that the isolated Raf-1 CRD binds to 14-3-3 and that a double mutation in the CRD, R143E/K144E, inhibited binding (29). To examine the binding of the Raf-1 mutants to 14-3-3ζ in the context of full-length Raf-1 protein, 14-3-3ζ was subcloned into pVP16, and the Raf-1 mutants were subcloned into pBTM116. A two-hybrid test showed interaction of Raf-1 with MKK1 and 14-3-3ζ but no interaction with VP16 alone. Binding of 14-3-3ζ to R143Q, R143W, and K144E Raf-1 mutants was equal to 14-3-3ζ binding to wild-type Raf-1 (data not shown). A

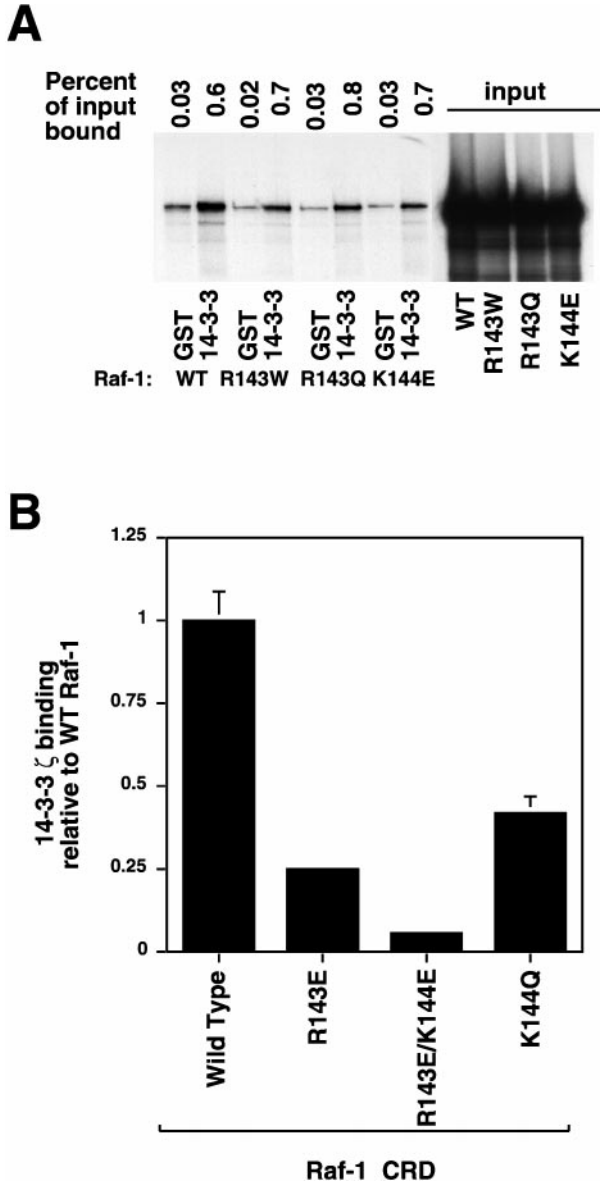

FIG. 4. **Binding of Raf-1 and CRD mutants to 14-3-3ζ.** *A,* the R143W, R143Q and K144E mutations do not affect the *in vitro* binding of full-length ^{35}S-labeled Raf-1 to 14-3-3ζ. Full-length wild-type or mutant Raf-1 was transcribed and translated *in vitro*. *In vitro* translations were incubated by GST alone or GST-14-3-3ζ, and bound proteins were detected by SDS-polyacrylamide gel electrophoresis and autoradiography. The percentage of binding compared with input into the binding assay was calculated using a PhosphorImager and is indicated *above each column*. *B,* mutations at codons 143 and 144 reduce Raf-1 CRD binding to 14-3-3ζ. Fusion proteins were expressed in *E. coli* and purified, and binding was determined by ELISA (see "Experimental Procedures"). Results are summarized from an assay done in triplicate. *Error bars* are not shown when they were less than 5% of the mean.

GST-14-3-3ζ fusion protein was then made in *E. coli* and purified using glutathione-Sepharose beads. The ability of *in vitro* translated Raf-1 protein to bind to the immobilized 14-3-3ζ was assessed, and no difference in the ability of 14-3-3ζ to bind to wild-type or mutant Raf-1 was seen (Fig. 4*A*). There are three characterized binding sites for 14-3-3 in the Raf-1 protein (29, 38). It is possible that 14-3-3 binding to the CRD site of the R143Q, R143W, and K144E mutant is reduced, as it is for the R143E/K144E mutant (29), but this reduction may be masked by continued binding to the two sites outside the CRD. Indeed, when mutations at codons Arg-143 and Lys-144 (R143E, K144Q, and R143E/K144E) were examined for *in vitro* 14-3-3ζ

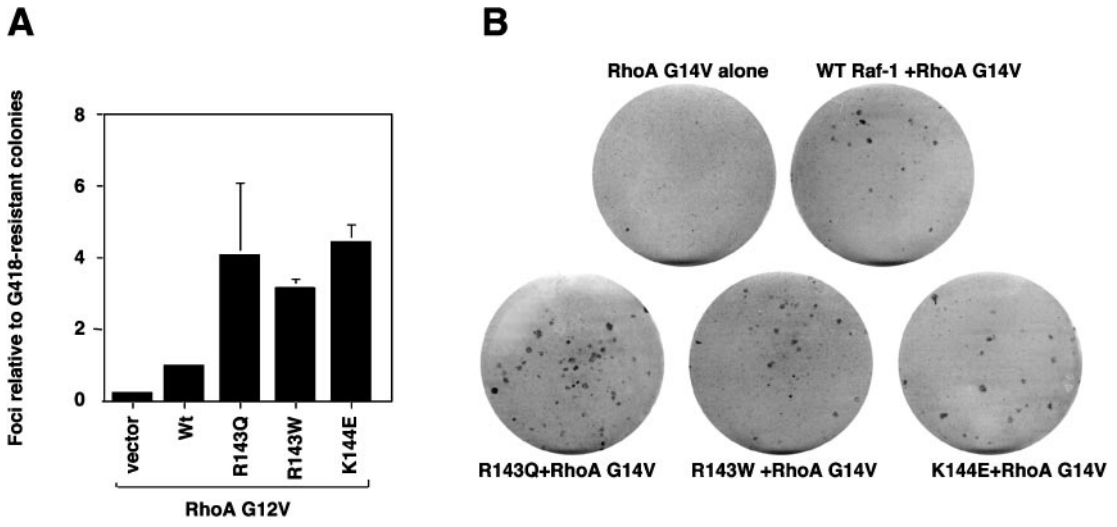

FIG. 5. **Focus formation by Raf-1 mutants in cooperation with Rho A G14V.** *A,* the number of Raf-1/RhoA induced foci relative to transfection efficiency are indicated. Each data point is a summary of 12 different platings in three independent experiments. *B,* representative foci on plates stained with crystal violet induced by G14V RhoA alone, G14V RhoA plus wild-type Raf-1, G14V RhoA plus Raf-1 R143W, G14V RhoA plus Raf-1 R143Q, and G14V RhoA plus Raf-1 K144E.

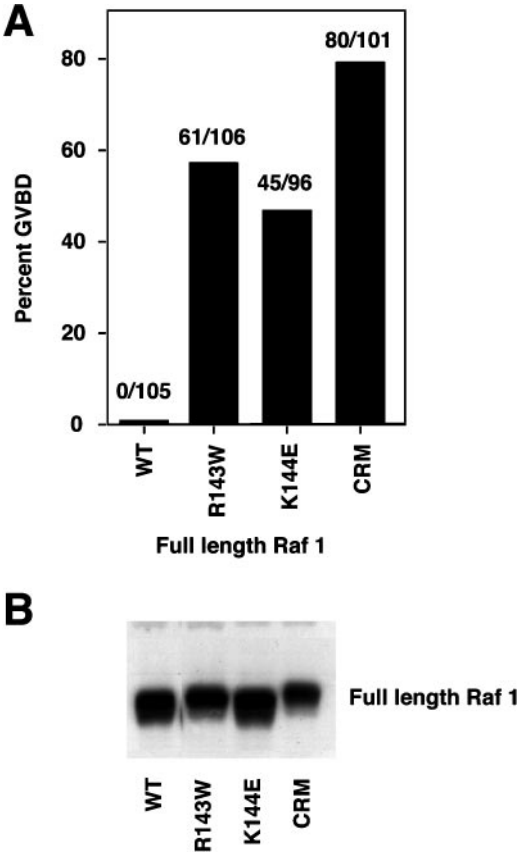

FIG. 6. **Raf-1 R143W and K144E mutants induce *Xenopus* oocyte meiotic maturation.** Stage 6 oocytes were injected with 30 ng of mRNA encoding wild-type, R143W, K144E, or CRM (an activated control) Raf-1. GVBD was scored 8–20 h after injection by the appearance of a white spot on the animal pole of the oocyte and confirmed by dissection of the oocytes. *A,* the percentage of oocytes undergoing GVBD is indicated by the *black bars*. The ratio of oocytes undergoing GVBD over the total number of oocytes injected is displayed *above each bar*. *B,* Western analysis for expression of FLAG tagged Raf-1 in injected oocytes.

binding in the context of the CRD, reduced 14-3-3ζ binding relative to the wild-type CRD was seen (Fig. 4*B*) (29). Thus general suppressor mutants R143W, R143Q, and K144E are likely to interfere with 14-3-3 binding to the CRD, but not 14-3-3 binding to other parts of Raf-1.

Increased Activity of Raf-1 Mutants—We tested whether the 143 and 144 Raf-1 CRD mutants were enzymatically activated. Neither direct *in vitro* kinase assays nor direct focus assays revealed any activation (data not shown), but two assays suggested that the mutants are more active than wild-type Raf-1.

The Raf-1 CRD mutants transform NIH 3T3 cells in cooperation with an activated allele of the RhoA small G protein (29). RhoA G14V or wild-type Raf-1 (data not shown) produce low numbers of foci if transfected into NIH 3T3 cells on their own. The numbers of transformed foci increase when activated RhoA G14V and Raf-1 are transfected together. The K144E, R143Q, and R143W mutants produce between 3 and 4 times more foci than wild-type Raf-1, showing that these mutants are activated (Fig. 5). This level of increase is similar to that reported for the R143E/K144E double mutant (29).

Activated Raf-1 stimulates cell cycle entry in *Xenopus* oocytes and leads to germinal vesicle breakdown (GVBD) (45). Approximately 100 oocytes were injected with RNA encoding wild-type, R143W, or K144E mutant Raf-1. The wild-type Raf-1 induced GVBD in very few of the oocytes. In contrast, the R143W and the K144E mutants both induced GVBD in ~60% of the oocytes (Fig. 6*A*). R143Q was not tested. For comparison, a known activated mutant of Raf-1, the CRM mutant, induced GVBD in 80% of the oocytes. The expression of the wild-type and mutant Raf-1 proteins in the oocytes was examined by Western blot and found to be equal (Fig. 6*B*).

Reduced Inhibition of the Raf-1 Catalytic Domain by the Mutant Raf-1 Regulatory Region—To test the basis for the activation of Raf-1, we measured the effect of the mutations on the interaction between the N-terminal regulatory region and the kinase domain. The catalytic domain of Raf-1 (Cat Raf) is deregulated, and injection of RNA encoding this domain into oocytes induces GVBD (46). The wild-type Raf-1 regulatory region (Reg Raf) can inhibit Cat Raf when supplied in *trans* (46). This inhibition requires the CRD but not the RBD (46). Reg Raf proteins containing R143Q, R143W, and R144E mutations did not inhibit Cat Raf-induced GVBD to the same extent as the wild-type Reg Raf (Fig. 7*A*), although they are expressed to an equal or higher level as assessed by Western blot (Fig. 7*B*). This reduction in GVBD inhibition by the mutant Reg Raf leads us to postulate that an inhibitory interaction between the N-terminal regulatory region of Raf-1 and the C-terminal catalytic domain of Raf-1 is reduced by mutations in the CRD. The destabilization of this interaction by these mu-

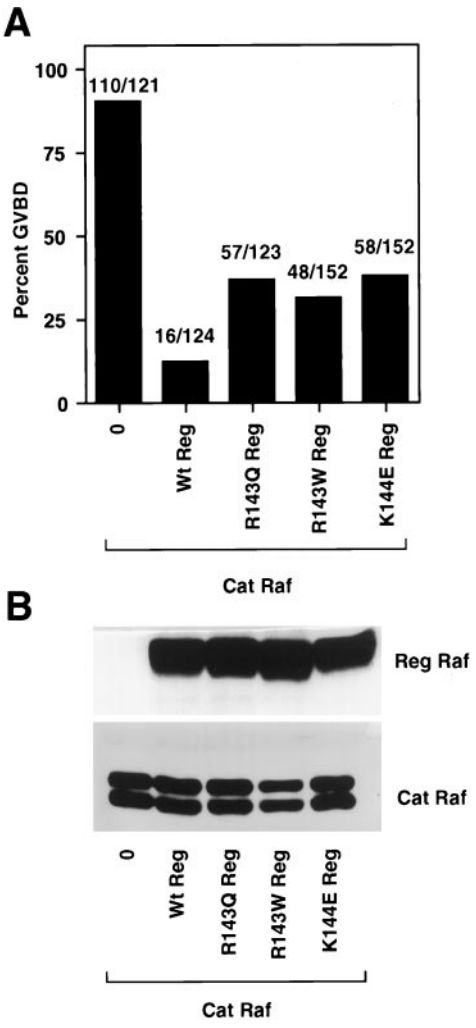

FIG. 7. **Inhibition of Cat Raf by Reg Raf is decreased by mutation of residues 143 and 144.** Wild-type and mutant Reg Raf mRNAs (30 ng) were injected into oocytes. After 4–8 h Cat Raf was injected, and GVBD was scored 8–20 h later by the appearance of a white spot on the animal pole and confirmed by dissection. *A,* the percentage of oocytes undergoing GVBD is represented by the *black bars*, and the ratio of oocytes undergoing GVBD over the total number of oocytes injected is displayed *above each bar*. *B,* Western analysis of the expression of FLAG-tagged Reg Raf and untagged Cat Raf.

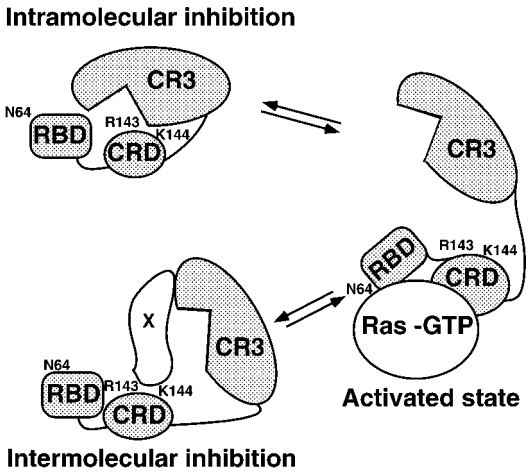

FIG. 8. **A model for Raf-1 activation.** Raf-1 is in an equilibrium between a closed (inactive) and open (active) conformation. The N64L mutation lies right at the interface between the RBD-binding site on Raf-1 for Ras and affects this interaction. The R143Q, R143W, and K144E mutations are within the CRD and affect the negative regulatory interaction between the N-terminal regulatory region and the C-terminal kinase domain. Consequently the open conformation is stabilized, allowing better access for Ras binding and higher Raf-1 activity. The negative regulatory interaction between the N-terminal regulatory region and the C-terminal kinase domain could be either intramolecular or intermolecular, perhaps involving 14-3-3 proteins.

tations would have two effects. It would increase the accessibility of the entire CR1 region for binding to Ras and would also allow for increased activity of the catalytic domain.

DISCUSSION

We used the two-hybrid system to isolate mutations in Raf-1 that increase binding to Ras specificity mutants (1). We identified one allele-specific suppressor that compensates for a specific structural change present in one mutant Ras. The N64L mutant specifically increases binding to a Ras mutant with an R41Q mutation. Arg-41 lies at the end of the effector domain. In the structural model for Ras binding to the RBD (23), the side chain of Ras Arg-41 interacts with that of Raf-1 Asn-64. It is likely that replacement of Asn-64 of the RBD with leucine compensates partly for the substitution of Arg-41 of Ras with glutamine.

We expected that mutations in Raf-1 that allowed for a relaxation of interdomain interactions would also increase binding to Ras. This expectation is based on the observation that Ras binds more weakly to full-length Raf-1 than to the RBD (10), suggesting that the Raf-1 kinase domain interferes with Ras binding to the RBD. Furthermore, the Ras specificity mutants have reduced binding to Raf-1 but significant binding to the RBD (Fig. 3*A,* Ref. 1), unlike some Ras effector domain mutants. Because the Ras specificity mutants still interact with the RBD and CRD, we predicted that mutations in full-length Raf-1 that expose the RBD and CRD would be general suppressors of the Ras mutants.

Mutations at codons 143 and 144 were repeatedly isolated in this screen as general suppressors of the Ras specificity mutants. The R143W, R143Q, and K144E mutants increase binding to wild-type activated Ras and to the J10, J25, and J31 specificity mutants (Fig. 3, *B* and *C*). The increased interactions of these mutants with wild-type Ras suggest an increased exposure of Ras-binding surfaces of the RBD, or the CRD, or both domains. However, there is some allelic specificity, implying that the CRD also contacts Ras directly (Fig. 3*B*, Table II). In particular the R143W mutant interacts better with J10 and J31 than J25, and the R143Q and K144E mutants interact better with J25 and J31 than J10.

Mutations in the CRD may increase Ras access to the RBD and CRD and simultaneously interfere with Ras-CRD binding. The balance between these effects could differ according to the specific mutant combination. The R143W mutation is non-conservative and may affect CRD folding, whereas R143Q and K144E are more conservative. Since the J10 Ras mutant has a lower affinity than J25 or J31 for RBD binding (Fig. 2*A*), it may rely more on the CRD for binding and is more adversely affected by the non-conservative R143W than the conservative R143Q or K144E Raf-1 mutations. The J25 and J31 Ras mutants rely less on the CRD for binding, and the increased exposure of the RBD and CRD in the R143W Raf-1 mutant may outweigh negative effects on Ras-CRD binding.

Residues 143 and 144 are prominently displayed on an exposed β strand within the CRD (27) and are conserved across the Raf family of protein kinases. Due to the surface localization and the charged nature of these residues, they may be involved in intermolecular or intramolecular interactions. A R143E/K144E double mutant, reversing the charge of this loop, was characterized previously (29). Like the R143Q, R143W, and K144E mutants isolated here, the R143E/K144E mutant can transform cells in cooperation with RhoA G14V. In isolation, the R143E/K144E CRD lost binding to 14-3-3ζ *in vitro* in

the absence of any loss of Ras binding (29), leading to the proposal that 14-3-3 binding to the CRD negatively regulates Raf-1. We failed to detect any reduction of 14-3-3ζ binding to full-length R143Q, R143W, and R144E mutants of Raf-1 either in the two-hybrid system or *in vitro*. However, *in vitro* binding studies with other mutations of codons 143 and 144 did reduce 14-3-3 binding to the isolated CRD (Fig. 4). Thus the effects of Arg-143 and Lys-144 substitutions on Ras binding and Raf-1 activity could be mediated in part by a shift of 14-3-3-binding sites from the CRD to other parts of the Raf-1 molecule.

Mutation of Arg-143 or Lys-144 partly activates Raf-1. Activation was too small to be detected by most assays used. However, all three mutants are activated in a sensitive focus formation assay in cooperation with RhoA G14V, and the R143W and K144E mutants are activated in the GVBD induction assay when compared with wild-type Raf-1. As the N-terminal regulatory region (Reg Raf) can inhibit the function of the catalytic region (Cat Raf) of Raf-1 assayed in *Xenopus* oocytes (46), we examined the effects of the R143W, R143Q, and R144E mutant Reg Raf on Cat Raf GVBD. All three of these mutations prevent Reg Raf from inhibiting Cat Raf.

These results fit a model that proposes a mutually inhibitory interaction between the N-terminal regulatory region and the catalytic region (Fig. 8) (46). This interaction both inhibits catalysis by the Raf-1 kinase domain and limits access by Ras to the RBD and CRD regions. Mutations that interfere with the inhibitory interaction are thus predicted to both activate the Raf-1 kinase domain and increase the binding of Ras to Raf-1 RBD and CRD. Site-directed mutations in the CRD can interfere with the inhibitory interaction (46), implicating the CRD in maintaining the inactive state. The present isolation of CRD mutations at codons 143 and 144 in a random screen further implicates the CRD, and residues 143 and 144 in particular, in negative regulation of Raf-1. However, our data do not address whether the interaction between the CRD and the kinase domain is direct or indirect. The CRD, through residues 143 and 144, could contact the kinase domain. Alternatively, an indirect interaction could be mediated by another protein *X* (Fig. 8). One candidate for *X* is 14-3-3, since mutations of residues 143 and 144 reduce Raf-1 CRD binding to 14-3-3 *in vitro* (Fig. 4*B*) (29).

White *et al.* (47) used a Ras effector domain mutant (E37G), which did not bind to Raf-1, to isolate Raf-1 mutants that restored binding. Like the suppressor mutants isolated in the present study, the suppressing mutation was not in the RBD. However, the Raf-1 mutation suppressing Ras E37G was in the CR2 region, not the CRD (47). As Ras E37G binds to the isolated Raf-1 RBD (data not shown), these data support the mutual inhibition model (Fig. 8), in which the CR2 domain of Raf-1 also contributes to the inhibited conformation. Interestingly, the mutated residue, Ser-257, is within one of the known 14-3-3-binding sites on Raf-1, again implicating 14-3-3 in inhibitory interactions.

The model for Raf-1 regulation resembles that proposed for Byr2, a Ras-regulated kinase from *Schizosaccharomyces pombe* (48). Like Raf-1, Byr2 has a C-terminal catalytic domain and an N-terminal regulatory region. Byr2 activation involves the binding of the Ras homolog, Ras1, the scaffolding protein Ste4, and phosphorylation by the protein kinase Shk. The N-terminal regulatory region (Reg Byr2) binds to the catalytic domain (Cat Byr2), and binding is disrupted by specific mutations (48). These mutations increase binding to Ras1 and Ste4 and activate the Byr2 kinase domain. Phosphorylation of the N terminus by Shk1 antagonized the interaction of Reg Byr2 with Cat Byr2 and also contributes to Byr2 activation. Inhibitory interactions between regulatory and catalytic regions of protein kinases is a common theme, and it will be important to uncover the structural basis in each case.

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