Communication

14-3-3 ζ Negatively Regulates Raf-1 Activity by Interactions with the Raf-1 Cysteine-rich Domain*

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Geoffrey J. Clark‡§, Jonelle K. Drugan§§, Kent L. Rossman¶, John W. Carpenter¶, Kelley Rogers-Graham†, Haian Fu†, Channing J. Der‡, and Sharon L. Campbell††

From the ‡Department of Pharmacology and the ¶Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599 and the †Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Although Raf-1 is a critical effector of Ras signaling and transformation, the mechanism by which Ras promotes Raf-1 activation is complex and remains poorly understood. We recently reported that Ras interaction with the Raf-1 cysteine-rich domain (Raf-CRD, residues 139–184) may be required for Raf-1 activation. The Raf-CRD is located in the NH2-terminal negative regulatory domain of Raf-1 and is highly homologous to cysteine-rich domains found in protein kinase C family members. Recent studies indicate that the structural integrity of the Raf-CRD is also critical for Raf-1 interaction with 14-3-3 proteins. However, whether 14-3-3 proteins interact directly with the Raf-CRD and how this interaction may mediate Raf-1 function has not been determined. In the present study, we demonstrate that 14-3-3 ζ binds directly to the isolated Raf-CRD. Moreover, mutation of Raf-1 residues 143–145 impairs binding of 14-3-3, but not Ras, to the Raf-CRD. Introduction of mutations that impair 14-3-3 binding resulted in full-length Raf-1 mutants with enhanced transforming activity. Thus, 14-3-3 interaction with the Raf-CRD may serve in negative regulation of Raf-1 function by facilitating dissociation of 14-3-3 from the NH2 terminus of Raf-1 to promote subsequent events necessary for full activation of Raf-1.

Substantial genetic, biochemical, and biological evidence supports the critical role of the Raf-1 serine/threonine kinase as a key downstream effector of Ras signaling and transformation (1, 2). Ras interaction with Raf-1 promotes the activation of Raf-1 in vitro, in part by facilitating its translocation from the cytoplasm to the plasma membrane. Activated Raf-1 phosphorylates and activates the mitogen-activated protein kinase kinases (MAPK1 kinases; also referred to as MEKs), which in turn phosphorylate and activate the p42 and p44 MAPKs. Activated MAPKs translocate to the nucleus where they regulate the activity of transcription factors such as Elk-1 (3).

Ras interaction with Raf-1 alone is not sufficient to cause full activation of Raf-1, but rather binding of Ras to Raf-1 initiates other events that lead to full activation. These additional events include tyrosine (4, 5) and serine/threonine (6–9) phosphorylation, phospholipid binding (10, 11), and interactions with other proteins that include members of the 14-3-3 protein family and 14-3-3-associated proteins (12–17). Hence, full kinase activation involves a complex multistep process that remains to be elucidated fully.

An additional complexity of Ras-mediated activation of Raf-1 is that the Ras/Raf-1 interaction is more convoluted than originally believed. We and others have shown recently that Ras interacts with two distinct Ras-binding domains in the NH2-terminal regulatory region of Raf-1 (18, 19). The first Ras-binding domain encompasses Raf-1 residues 55–131 (20, 21) and appears to interact with Ras prior to exposure of the second binding site (19). This second binding region is contained within the Raf-1 cysteine-rich domain (residues 139–184, designated the Raf-CRD; also called Raf-Cys or Raf-C1) that resembles the C1 domains of protein kinase C family members (22). Although the precise role of this second Ras/Raf-1 interaction is unclear, we recently determined that Ras interaction with the Raf-CRD is required for Ras transforming activity (19). We hypothesized that Ras interaction with Raf-1 residues 55–131 promotes Raf-1 association with the plasma membrane while Ras interaction with the Raf-CRD stabilizes this membrane association and/or relieves the regulatory effects of the NH2 terminus. Hence, additional interactions between Ras and Raf-CRD may promote further activation events that could involve interactions with 14-3-3 proteins or phospholipids.

14-3-3 proteins bind directly with Raf-1 in vitro and in vivo (13–17). However, the role of this interaction in Raf-1 function is unclear. Whereas some studies suggest that 14-3-3 may serve a role in activation (14, 16, 17, 23, 24), others support a negative regulatory role (25), and yet another indicates that 14-3-3 proteins are not essential for Raf-1 function (26). Moreover, there are conflicting reports regarding the relationship between the activated state of Raf-1 and its ability to bind 14-3-3 proteins (14, 15, 17).

Contradictory reports regarding the role of 14-3-3 proteins in Raf-1 activation may be due, in part, to the existence of multiple 14-3-3 binding sites in distinct NH2- and COOH-terminal sequences of Raf-1 (13–15). A 14-3-3 recognition consensus motif has been identified (27), and Raf-1 contains two phosphorylation sites that fit the consensus sequence: serine 259 in the NH2-terminal regulatory sequence downstream of the Raf-CRD and serine 621 in the COOH-terminal kinase domain. However, disruption of the structural integrity of the Raf-CRD can abolish 14-3-3 interactions (28), indicating that 14-3-3 binding sites distinct from this consensus motif may be present in the NH2 terminus of Raf-1. In fact, NH2-terminal fragments of Raf-1 that contain the Raf-CRD showed binding to 14-3-3 ζ
by yeast two-hybrid studies (25). However, whether 14-3-3 proteins can interact directly with sequences in the Raf-CRD, and what role, if any, such an interaction may play in the regulation of Raf-1 function has not been established.

In this report, we demonstrate that the isolated Raf-CRD binds directly to the ζ isoform of 14-3-3 and that mutations of residues 143–145 cause a loss of 14-3-3, but not Ras, binding in vitro. We also show that mutations that impair the 14-3-3/Raf-CRD association result in activation of Raf-1 transforming potential. Thus, 14-3-3 interaction with Raf-CRD may serve as a negative regulator of Raf-1 function. When taken together with our observation that Ras interaction with the Raf-CRD is essential for Ras transforming activity, we propose that Ras interaction with the Raf-CRD may disrupt the 14-3-3/Raf-CRD interaction to promote subsequent events that lead to full activation of Raf-1.

EXPERIMENTAL PROCEDURES

Generation, Expression, and Purification of Raf-1 Mutants—Oligonucleotide-directed mutagenesis of a cDNA sequence encoding Raf-1 residues 136–187 (encompassing the Raf-CRD) was utilized to generate sequences encoding specific mutants of Raf-CRD. Briefly, oligonucleotide primers containing the appropriate mutations were elongated by polymerase chain reaction, and the resulting products were ligated into the pCR<sup>TM</sup>1 vector (Invitrogen) using 5' BamHI or HindIII and 3' EcoRI restriction sites. Dideoxy sequence analysis was done to confirm the introduction of the correct mutations. The mutated cDNA sequences encoding Raf-1 residues 136–187 were isolated by digesting with HindIII/EcoRI or BamHI/EcoRI and ligated in-frame into the pGEX2T vector for expression of recombinant glutathione S-transferase (GST) Raf-CRD fusion proteins in the Escherichia coli strain BL21 (10).

To transfer the mutations into the full-length protein, a cassette was generated containing the 5' half of theraf-1 cDNA in which the sequence encoding the Raf-CRD is encompassed by unique BclI and SacI sites. Theraf-CRD variant sequences were amplified to carry the BclI and SacI sites at their termini, sequenced to confirm their fidelity, and cloned into theraf-1 cassette. The 5' fragments ofraf-1 containing the desired mutations were spliced to the 3' half of the cDNA using the unique BstXI site in theraf-1 cDNA sequence. Sequences encoding mutant Raf-CRDs were employed to generate sequences in wild type Raf-1 (28) and introduced into thepcDNA-hyg or pZip-NEO SV/1 (26) mammalian expression vector.

Enzyme-linked Immunoassorbent Assay (ELISA)—An ELISA protocol was employed to monitor Raf-CRD interactions with 14-3-3 ζ and Ras. All assays were performed at least twice in triplicate. Recombinant 14-3-3 ζ was expressed as an NH<sub>2</sub>-terminal polyhistidine-tagged fusion protein in Escherichia coli as previously described (30). Following immobilization of 14-3-3 ζ to a medium-binding polystyrene plate (Costar), a 1:1500 dilution of an anti-GST monoclonal antibody (Santa Cruz Biotechnology, Inc.) was employed to assess binding of each GST-Raf-CRD variant. Corresponding amounts of GST were used to control for background interactions.

Recombinant Ha-Ras was expressed and purified as described previously (31). Formation of the Ras and GMP-PCP (a nonhydrolyzable GTP analog, Boehringer Mannheim) complex for use in the ELISA experiments is described elsewhere (32). Ras/Raf-CRD interactions were assessed as described previously (18).

14-3-3 Binding Assay—Recombinant GST-Raf-CRD proteins were immobilized to glutathione-coated agarose beads and incubated with equimolar amounts of 14-3-3 ζ dimer. Raf-CRD proteins were separated from bound 14-3-3 proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after washing with 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 100 mM NaCl, 0.05% Tween 20, and 20 mM zinc. To identify 14-3-3 ζ, proteins were transferred to a polyvinylidene difluoride membrane, probed with a 1:2500 dilution of monoclonal antibody (Santa Cruz Biotechnology), and detected by an antibody bound to alkaline phosphatase. The amount of 14-3-3 ζ captured was assessed by scanning an equivalent gel band for the intensities of known protein concentrations. Nonspecific interactions between 14-3-3 proteins and GST were determined by the amount of captured 14-3-3 ζ with equivalent amounts of GST protein. All variants were analyzed in duplicate in at least two independent assays.

Transformation Assays—Primary focus-formation assays were conducted to quantitate the ability of each Raf-CRD mutant of Raf-1 in cooperation with activated RhoA(G63L) (33) to transform NIH 3T3 cells using procedures that have been described previously (34, 35). Also, secondary focus-formation assays were performed to quantitate the focus-forming potential of full-length Raf-CRD variants. Briefly, NIH 3T3 cells were transfected with 1 μg of plasmid DNA encoding each Raf-1 mutant protein using the calcium phosphate precipitation method as described (34) and were selected in 500 μg/ml G418 (Geneticin, Life Technologies, Inc.). Following selection, multiple drug-resistant colonies were pooled together (>100 colonies) and replated at 5 × 10<sup>4</sup> cells per 60-mm dish. The appearance of foci of transformed cells was quantitated after 14 days for both primary and secondary assays. Raf(Y340D) is a weakly transforming mutant of Raf-1 that contains a Y340D mutation (4, 35).

RESULTS AND DISCUSSION

The Raf-CRD Interacts Directly with 14-3-3 ζ in Vitro—Although the Raf-CRD has been implicated in interactions with 14-3-3 (25), direct binding has not been reported. To evaluate this possibility, we determined whether a bacterially expressed GST fusion protein that encompasses Raf-CRD (residues 136–187) could interact with bacterially expressed 14-3-3 ζ in vitro. By ELISA, we detected 14-3-3 ζ binding to GST-Raf-CRD, but not to GST alone (Fig. 1A). Thus, in addition to the two 14-3-3 consensus recognition motifs (27), 14-3-3 can interact with sequences contained within the Raf-CRD. Consequently, the Raf-CRD defines at least a second distinct 14-3-3 binding site in the NH<sub>2</sub>-terminal regulatory domain of Raf-1.

Mutation of Raf-1 Residues 143–145 Impairs 14-3-3 Interactions with the Raf-CRD—To identify specific residues within the Raf-CRD that are involved in interaction with 14-3-3 ζ, we introduced amino acid substitutions of conserved, charged, exterior residues in the Raf-CRD. This mutagenesis approach was employed because conservation of charged amino acids at the protein surface within protein families often indicates functional importance. Also, structural evidence suggests that charged residues in 14-3-3 proteins may be involved in binding interactions between 14-3-3 proteins and GST were determined by the amount of captured 14-3-3 ζ with equivalent amounts of GST protein. All variants were analyzed in duplicate in at least two independent assays.

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other proteins (36, 37).

The following mutations were introduced into GST-Raf-CRD and then analyzed for interaction with 14-3-3 by ELISA: R143E, R143E/K144E, K144Q, T145E, and D153N/K179Q. Although the D153N/K179Q double mutation did not affect binding, the substitution of R143E/K144E or T145E severely impaired Raf-CRD/14-3-3 interactions (Fig. 1A and Table 1). The impaired binding of the R143E/K144E mutant was the combined consequence of each substitution since the R143E mutation alone showed a partial reduction in binding.

Mutations That Impair 14-3-3 Binding Do Not Disrupt the Structure or Ras Binding Properties of the Raf-CRD—To address the possibility that the inability of the Raf-CRD mutants to interact with 14-3-3 is due to a collapse of the Raf-CRD structure, we assessed the structural integrity of the R143E/K144E mutant by nuclear magnetic resonance (data not shown). These nuclear magnetic resonance measurements indicated that the structure of the isolated Raf-CRD variant was unaltered. Moreover, none of the mutations analyzed in this study impaired expression of these variants as GST-fusion proteins (data not shown), providing further indication that these mutations do not diminish the structural integrity of the Raf-CRD. Thus, we conclude that the failure of the Raf-CRD mutants to bind 14-3-3 is not due to a collapse of the cysteinerich domain and instead is mediated by the isolated mutations.

We showed previously that the Raf-CRD can interact with Ras (18). To address the specificity of these point mutations on 14-3-3/Raf-CRD interactions, we assessed the effects of these substitutions on binding interactions between the Raf-CRD and Ras. ELISA analysis showed that all of the variants retained the ability to bind GTP-complexed, bacterially expressed Ha-Ras protein (Fig. 1B). Thus, mutation of Raf-1 residues 143–145 caused selective impairment of binding to 14-3-3 but not Ras. These results were unexpected since we speculated previously that these residues may represent a consensus Ras-GTP binding sequence shared between Raf-1 and other candidate Ras effectors (38). While these observations do not exclude their role in Ras interaction, it is clear that the integrity of each residue alone is dispensable for Ras binding.

Mutations That Impair 14-3-3 Binding Cause Activation of Raf-1 Transforming Potential—To determine the contribution of the 14-3-3/Raf-CRD interaction to Raf-1 function, we assessed the consequences of disrupting Raf-CRD/14-3-3 interactions on Raf-1 transforming potential. For these analyses, we introduced the R143E/K144E, T145E, and D153N/K179Q mutations into wild type, full-length Raf-1 (28).

We determined if the loss of 14-3-3/Raf-CRD interaction may potentiate the transforming activity of wild type Raf-1. For these analyses we used the weakly activated Raf(Y340D) mutant as a positive control. Raf(Y340D) shows strong focus-forming activity when assayed in a secondary focus-formation assay or when co-transfected with an expression construct encoding the constitutively activated RhoA(Q63L) mutant proteins in primary focus-formation assays (33). Raf-1 containing the R143E/K144E and T145E mutations showed a 5-fold enhanced focus-forming activity when compared with wild type Raf-1 (Fig. 2A). However, this activity was less than that seen with Raf(Y340D). In contrast, the D153N/K179Q mutant showed activity comparable with wild type Raf-1. Similarly, Raf-1 mutants deficient in 14-3-3 binding (R143E/K144E, T145E) showed 3-fold enhanced focus-forming activity when co-expressed with RhoA(Q63L). The focus-forming activity was comparable with that observed with Raf(Y340D) (Fig. 2B). Thus, loss of 14-3-3 binding to the Raf-CRD enhanced Raf-1 transforming potential, suggesting that 14-3-3 acts as a negative regulator of Raf-1 function via this interaction. These results are consistent with another report of an activating mutation in the Raf-CRD (26).

### Table 1

| Mutation      | Relative moles of 14-3-3 bound/mole of Raf-CRD | Transformation of NIH 3T3 cells |
|---------------|-----------------------------------------------|---------------------------------|
| None          | 1.00 ± 0.09                                   | ND                              |
| R143E         | 0.25 ± 0.01                                   | ND                              |
| R143E/K144E   | 0.06 ± 0.01                                   | ND                              |
| K144Q         | 0.42 ± 0.05                                   | ND                              |
| T145E         | 0.16 ± 0.07                                   | ND                              |
| D153N/K179Q   | 0.59 ± 0.04                                   | ND                              |

* Fraction 14-3-3 bound to Raf-CRD variant relative to amount bound to wild type Raf-CRD.

** Focus-forming activity in NIH 3T3 cell assay: −, no foci; +, 5–20 foci per dish.

* ND, not determined.

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**Fig. 2.** R143E/K144E and T145E mutants of Raf-1 are activated in their ability to generate foci in NIH 3T3 cells. Panel A, cells were transfected with 1 μg of pZip-NEO SV/X1 encoding each Raf-1 mutant protein, selected in G418, replated, and scored for the formation of foci after 16 days; panel B, cells were co-transfected with 1 μg of plasmid DNA encoding activated RhoA and 1 μg of pZip-NEO SV/X1-raf encoding wild type or mutant Raf-1. Foci were scored after 12–14 days. Data shown are derived from three independent assays.
Binding can cooperate with a transformation-defective Ras mutation that Raf-1 mutant proteins impaired in Raf-CRD/14-3-3 interactions cause a partial activation of Raf-1. This may be a preactivation step, and additional events are required to promote full activation. The ability of Ras and 14-3-3 to compete with each other for binding to the Raf-CRD and the role of other potential Raf-CRD-interacting components (e.g. phospholipids) in regulation of Raf-1 function are currently under investigation.

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