Elucidation of Binding Determinants and Functional Consequences of Ras/Raf-Cysteine-rich Domain Interactions*

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Raf-1 is a critical downstream target of Ras and contains two distinct domains that bind Ras. The first Ras-binding site (RBS1) in Raf-1 has been shown to be essential for Ras-mediated translocation of Raf-1 to the plasma membrane, whereas the second site, in the Raf-1 cysteine-rich domain (Raf-CRD), has been implicated in regulating Raf kinase activity. While recognition elements that promote Ras/RBS1 complex formation have been characterized, relatively little is known about Ras/Raf-CRD interactions. In this study, we have characterized interactions important for Ras binding to the Raf-CRD. Reconciling conflicting reports, we found that these interactions are essentially independent of the guanine nucleotide bound state, but instead, are enhanced by post-translational modification of Ras. Specifically, our findings indicate that Ras farnesylation is sufficient for stable association of Ras with the Raf-CRD. Furthermore, we have also identified a Raf-CRD variant that is impaired specifically in its interactions with Ras. NMR data also suggests that residues proximal to this mutation site on the Raf-CRD form contacts with Ras. This Raf-CRD mutant impairs the ability of Ras to activate Raf kinase, thereby providing additional support that Ras interactions with the Raf-CRD are important for Ras-mediated activation of Raf-1.

The best characterized Ras effector is the serine/threonine kinase Raf-1. Raf-1 propagates its signal via activation of the serine/threonine kinase MEK, which in turn, phosphorylates its substrate MAPK. MAPK then translocates to the nucleus, where it activates a variety of proteins including transcription factors (1, 2).

The amino terminus of Raf-1 contains two conserved regions (CR1 and CR2) that have been postulated to negatively regulate the carboxyl-terminal kinase domain (CR3) (reviewed in Refs. 1 and 3). While CR2 (residues 255–268) is rich in serine and threonine residues that become phosphorylated during Raf-1 activation (4, 5), CR1 of Raf-1 contains two Ras-binding domains. The first Ras-binding site encompasses Raf-1 residues 55–131 (Ras-binding site 1; RBS1) (6–8), with the second binding site located in the cysteine-rich domain (Raf-1 residues 139–184, Raf-CRD) (9–11).

Ras was initially believed to function by translocating Raf to the plasma membrane via interactions with RBS1 where other Ras-independent events, then led to Raf kinase activation. These Ras-independent activation events are thought to require modulation of both intra- and intermolecular interactions as well as Raf-1 phosphorylation (2, 12, 13). Although details regarding Raf activation upon Ras-mediated membrane recruitment of Raf still remain elusive, it has become apparent that even the role of Ras in mediating Raf-1 activation is more complex than originally envisioned. In fact, recent evidence supports a dual role for Ras in both membrane localization and allosteric activation of Raf-1. While Ras interaction with the RBS1 has been shown to facilitate membrane recruitment, we and others have found that interactions between Ras and the Raf-CRD play an important role in allosteric activation of Raf-1 by Ras (9–11, 14, 15). However, the recognition elements involved in binding of Ras to the Raf-CRD, and how this binding event is coupled to Raf kinase regulation is not clear. In this study, we have employed site-directed mutagenesis, binding analyses, in vivo Raf kinase assays, and NMR structural analyses to elucidate interactions important for Ras/Raf-CRD complex formation and the functional role these binding interactions play in regulation of Raf kinase activity.

Multiple regions of Raf appear to be involved in promoting binding interactions with the Raf-1 amino terminus. The binding of GTP to Ras causes a conformational change in two regions, commonly referred to as switch I and switch II (16–18). The GTP-dependent conformation of switch I is critical for high affinity interaction between Ras and RBS1 (19). However, conflicting findings exist on whether the CRD can bind Ras in the absence of post-translational modification, and whether the interaction displays guanine nucleotide dependence (9, 10). Regions flanking switch I appear important for mediating, at least in part, Ras/Raf-CRD interactions (10, 11), and may explain the weak GTP dependence we previously observed for the

The abbreviations used are: MEK, mitogen-activated protein kinase; CR3, nuclear single quantum coherence.

5'-y imidodiphosphate; ERK, extracellular signal-regulated kinase; MBP, myelin basic protein; PS, phosphatidylserine; HSQC, heteronuclear single quantum coherence.
mediated Raf kinase activity in binding both farnesylated and non-farnesylated Ras. This patch on the surface of the Raf-CRD that is selectively impaired to characterize a Raf-CRD variant, disrupted at a hydrophobic residue of the Raf-CRD important for Ras binding, we made residues at conserved surface-exposed residues, but relatively little is known about Raf-CRD mutations at conserved surface-exposed residues, which the ability to detect an interaction depends on the binding kinetics and assays employed. To resolve this apparent discrepancy, we have employed a fluorescence-based quantitative assay to determine the apparent dissociation constant between lipid modified and non-modified GTP- and GDP-bound Ras with the Raf-CRD.

Processed forms of both Ha-Ras and K-Ras appear necessary for in vitro and in vivo Ras-mediated activation of Raf-1 (14, 15, 20–23). Carboxyl-terminal processing differs in Ha-, K-, and N-Ras, and involves multiple modifications, including farnesylation, palmitoylation, carboxyl-terminal cleavage, and carboxy-methylylation (24). One common lipid modification, however, is farnesylation of the CAAX cysteine (20, 21). To assess whether farnesylation of Ras alone is required for stable association with the Raf-CRD, we prepared farnesylated Ha-Ras in vitro using purified bacterially expressed Ha-Ras (1–189) and recombinant farnesyl transferase. We then compared the binding of farnesylated to non-farnesylated bacterial expressed full-length Ha-Ras. While unmodified Ras showed a weak but detectable binding interaction with the Raf-CRD, higher affinity association was observed with farnesylated Ras. We had previously observed a weak, preferential binding of GTP-bound Ras relative to GDP-bound Ras to the Raf-CRD, when Ras is not processed by ELISA (11). Consistent with our previous observations, binding interactions between unprocessed Ras and the Raf-CRD do show a GTP dependence in our fluorescence-based assay. However, we observe little, if any, preference for GTP-bound Ras when Ras is farnesylated. This is in contrast to the strong GTP dependence observed for the Ras/Raf-RBS1 interaction (25).

While regions of Ras involved in Ras/RBS1 interactions have been characterized, relatively little is known about Raf-CRD residues important for associating with Ras. To elucidate residues of the Raf-CRD important for Ras binding, we made Raf-CRD mutations at conserved surface-exposed residues, based on our previously determined NMR solution structure of the Raf-CRD (26). Although we and others have identified Ras mutants that impair interactions between Ras and Raf-CRD (10, 11), Raf-CRD mutations that selectively interfere with Raf-CRD binding to Ras without disrupting interactions with the Raf-CRD’s other known ligands, phosphatidylinerse (27) and 14-3-3 proteins (28), have yet to be described. Here we characterize a Raf-CRD variant, disrupted at a hydrophobic patch on the surface of the Raf-CRD that is selectively impaired in binding both farnesylated and non-farnesylated Ras. This mutant, in the context of full-length Raf-1, is defective in Ras-mediated Raf kinase activity in vivo, supporting earlier findings that Ras interactions with the Raf-CRD are essential for Ras mediated Raf-1 activation (11).

To characterize the regions of the Raf-CRD that interact with the unprocessed form of Ras, we employed NMR spectroscopy to identify residues perturbed upon binding to unprocessed Ras. Results from these structural studies are consistent with our mutagenesis results and indicate that residues within the hydrophobic patch are important for Ras/Raf-CRD interactions.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Ras.** 14-3-3, and Raf-1 Fragments—Full-length Ha-Ras-(C118S/C181S/C184S) and Ha-Ras-(1–166) were expressed and purified as described (29). The Raf-CRD mutants were constructed using site-directed mutagenesis procedures described elsewhere (28). The Raf fragments (Raf-CRD and Raf-CRD-(149-151) variants) were expressed as glutathione S-transferase (GST) fusion proteins and purified by affinity chromatography as detailed in Ref. 27. 14-3-3 was a gift from Dr. Haian Fu, Emory) was expressed in bacteria as a His-tagged protein and purified using nickel affinity chromatography as described previously (31). After incubation, PThz was removed by the addition of nickel-agarose and the extent of Ha-Ras farnesylation was assayed by SDS-PAGE. Farnesylated Ha-Ras was separated from non-farnesylated Ha-Ras based on differential binding to bind to a phenyl-Sepharose matrix. FTase-treated Ras was added to a 5-mL phenyl-Sepharose CL-4B (Sigma) column that was previously equilibrated in 50 mM HEPES, 5 mM MgCl₂, 5 mM nucleotide, 1 mM DTT, and 0.1% N-octyl glycosorane. The column was then washed with 2 column volumes of the same buffer and farnesylated protein eluted with 50 mM HEPES, 5 mM MgCl₂, 5 mM nucleotide, 1 mM DTT, and 2.0% N-octyl glycosorane (Sigma).

**Synthesis of Mant-dGDP and Mant-GMP-P(NH)P—Mant-dGDP and mant-GMP-P(NH)P were synthesized as described previously (mant from Molecular Probes; dGDP and GMP-P(NH)P from Sigma) (32). The reaction was usually >80% complete and mant-mant was employed without further purification for incorporation into Ras.**

**Incorporation of Nucleotide into Ras**—250 μM Ha-Ras (farnesylated or non-farnesylated) was incubated for 2 h with gentle shaking in 32 mM Tris, pH 8.0, 200 mM (NH₄)₂SO₄, 0.1 mM EDTA, 5 mM DTT, 0.1% N-octyl glycosorane, and 2.5 mM nucleotide at 25 °C. When mant-GMP-P(NH)P labeling, 25 units of alkaline phosphatase linked to agarose (Sigma) were added per milligram of Ha-Ras. The labeling reaction was quenched by the addition of MgCl₂ to a final concentration of 20 mM. Ha-Ras was then exchanged into 20 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1% N-octyl glycosorane.

**Fluorescence Spectroscopy**—Fluorescence measurements were made using an SLM-Aminco 8100 fluorescence spectrophotometer with an excitation wavelength of 365 nm and an emission wavelength of 440 nm. Samples were under continuous stirring and thermostatted at 20 °C. Titrations were performed by adding known amounts of purified Raf-CRD to 1 μM mant-dGDP or mant-GMP-P(NH)P-labeled Ha-Ras (farnesylated or non-farnesylated) in 20 mM Tris, pH 7.5, 5 mM MgCl₂, 20 mM NaCl, 1 mM DTT and monitoring the subsequent increase in mant fluorescence intensity at 440 nm.

**Ras/Raf-CRD Direct Binding Assay**—200 pmol of the Raf-CRD fragment (Raf-1 residues 136–187) fused to GST was immobilized on GSH-agarose beads. The beads were then incubated with either 400 pmol of full-length farnesylated Ha-Ras-GDP or 400 pmol of processed K-Ras farnesyl-GDP that had been purified from baculovirus-infected S9 cells (a gift from Gideon Bollag and David Stokoe, Onyx, Richmond, CA) with gentle shaking to keep the beads suspended for 30 min. 100 μL of buffer (200 mM Hepes, pH 7.4, 5 mM MgCl₂, 20 μM NaCl, 30 μM GDP, 1 mM DTT, and 0.2% o-nitro β-o-glycosorane), the beads were washed once in 1 mL of the same buffer. The protein was quantitated by laser densitometry scanning of a Coomasie-stained SDS-PAGE, and the ratios of Gst-Raf were compared with the ratios of Gst-Raf alone. Assays were performed at least twice in duplicate.

**ELISA-based Ras/Raf-CRD, 14-33/Raf-CRD Binding Assays** and 14-3-3/Phosphatidyserine Competition Studies—Binding interactions between bacterially expressed Ha-Ras or 14-3-3 and the Raf-CRD were assessed by ELISA as described (9). For 14-3-3/ phosphatidylinerse competition studies, 200 pmol of GST-Raf-CRD and corresponding amounts of GST were incubated for 20 min at room temperature with 400 pmol of 14-3-3-3 in the presence and absence of 5 μM of disienyl phosphatidylinerse (Avanti Polar-Lipids, Inc.) and 1-palmitoyl-2-oleoyl phosphatidylinerse (Avanti Polar Lipids, Inc.) in 100 μl of 20 μM Hepes, pH 7.4, 5 mM MgCl₂, 50 mM NaCl, 20 μM NaCl, 1 mM DTT, and 10 μL of GSH-coated agarose beads. The presence of 14-3-3 was detected by Western blotting using a 1:2500 dilution of a polyclonal 14-3-3 antibody (Santa Cruz Biotechnology, Inc.). The amount of 14-3-3 was quantitated by laser densitometry. Assays were performed at least twice in duplicate.

In Vivo Raf Kinase Assays—COS cells were transiently transfected with 1 μg of mutant Ras and 1 μg of activated Ha-Ras, both in the vector pCGN using LipofectAMINE (33). After 48 h, the cells were shifted to 1% serum and incubated overnight. The cells were then lysed (150 μL NaCl, 50 mM Hepes, 1% Nonidet P-40, 0.5% deoxycholate, 5 mM EDTA, 5 mM ZnSO₄, 1 mM DTT, and 0.1% N-octyl glycosorane) and subjected to Western blotting using a 1:2500 dilution of a polyclonal 14-3-3 antibody (Santa Cruz Biotechnology, Inc.). The amount of 14-3-3 was quantitated by laser densitometry. Assays were performed at least twice in duplicate.
The purified protein was concentrated to 2 ml in 30 mM Tris acetate at the isolation of Ras and Raf mutants was examined by immunoblotting with myelin basic protein (MBP) (1 mg) in kinase buffer (10 mM Tris, pH 7.5, 10 mM MgCl₂, and 1 mM DTT) with [γ-32P]ATP at 30 °C for 20 min. The reaction was stopped by adding SDS loading buffer and resolved by SDS-PAGE. Incorporation of 32P into the MBP substrate was quantitated on a PhosphorImager. Expression of Ras and Raf mutants was examined by immunoblotting with anti-HA antibody (BabCo).

NMR Sample Preparation—Uniformly 15N-enriched wild type Raf-CRD or Raf-CRD-(149/151) protein was obtained by growing Escherichia coli with 99.8% 15NH₄Cl (Isotec) as the sole nitrogen source (26). The purified protein was concentrated to 2 ml in 30 mM Na₂SO₄, 10 μM ZnCl₂, 1 mM DTT and exchanged into NMR buffer (30 mM d₅-Tris-d₅-acetate (Isotec) at pH 6.5, 75 mM Na₂SO₄, 10 μM ZnCl₂, 1 mM d₅-DTT, 10% D₂O, 0.01% NaN₃) using an Amersham Pharmacia Biotech PD-10 gel filtration column. The final Raf-CRD (149/151) NMR sample contained 0.20 mM of uniformly 15N-enriched Raf-CRD. The wild type Raf-CRD NMR sample contained 0.28 mM buffer (10 mM Tris, pH 7.5, 10 mM MgCl₂, and 1 mM DTT) with 99.8% 15NH₄Cl (Isotec) as the sole nitrogen source (26). Phosphorylation of the NMR sample contained 0.28 mM of uniformly 15N-enriched Raf-CRD and unlabeled Ras ranged from 0.77 to 0.30 mM.

NMR Spectroscopy—NMR experiments were recorded on a Bruker AMX 500 (500 MHz) spectrometer at 12 °C or a Varian Inova 600 (600 MHz) spectrometer at 25 °C. Two-dimensional 1H-15N heteronuclear single quantum coherence spectroscopy (HSQC) experiments were performed with pulsed field gradient and water flip-back methods described previously (34). Data were acquired with 1024 × 256 complex data points and a spectral width of 7042.25 Hz for the 1H dimension and 1000 Hz for the 15N dimension on the Bruker AMX 500. HSQC experiments conducted on the Varian Inova 600 spectrometer were acquired with 1024 × 128 complex data points and a spectral width of 5000 Hz for the 1H dimension and 1700 Hz for the 15N dimension. NMR data were processed and analyzed using the program FELIX, version 97.2 ( Biosym Technology, San Diego).

NMR Chemical Shift Titration—Two-dimensional 1H-15N HSQC data were acquired on the Raf-CRD-Ras complexes at 4 different molar ratios: 1:2.75, 1:1.65, 1:1.1, and 1:0. The final concentration of 15N-Raf-CRD was 0.28 mM and unlabeled Ras ranged from 0.77 to 0.30 mM. Samples containing the highest molar ratio (1:2.75) of the Raf-CRD-Ras complex (0.28:0.77 mM) and Raf-CRD alone (0.28 mM) were prepared first. To prevent changes in sample volume and buffer composition, the other two complexes were made by exchanging equal volumes (200 μl) of these two samples. Proton and 15N resonance assignments of the Raf-CRD have been obtained previously in this laboratory (26).

RESULTS

The Farnesyl Moiety of Ras Mediates Interactions with the Raf-CRD—Processed forms of either Ha-Ras or K-Ras have been reported to be important for stable binding interactions with the Raf-CRD and for Ras-mediated activation of Raf-1. Although Ha-Ras and K-Ras possess distinct carboxyl-terminal lipid modifications, they are both farnesylated at the CAA cysteine (20–22). To assess whether farnesylation of Ras alone is required for stabilization of the Ras/Raf-CRD interaction, we prepared farnesylated Ha-Ras in vitro using purified bacterially expressed Ha-Ras-(1–189) and recombinant farnesyl transferase. Complex formation with the Raf-CRD can be detected with both farnesylated Ha-Ras and non-farnesylated Ras by ELISA (Fig. 1A), while only interactions with farnesylated Ras are observed by a direct binding assay (Fig. 1B). A likely explanation for the inability to detect non-farnesylated Ras association to the Raf-CRD in a direct binding assay is the assays relative insensitivity to weak binding interactions, particularly those with rapid rates of complex dissociation. Quantitation of the Ras/Raf-CRD interaction should aid in reconciling the apparent differences observed between the ELISA and direct binding assay. Hence, we have employed a binding assay based upon the fluorescence technique originally described by Wittinghofer and co-workers (25). Our method relies on an increase in apparent fluorescence intensity of mant-dGDP or mant-GMP-P(NH)P-labeled Ha-Ras upon Raf-CRD binding.

Assuming a simple one-site binding model, we have calculated an apparent K_d for the farnesylated Ha-Ras-GDP/Raf-CRD interaction of 33 ± 8.4 μM (Fig. 2). In addition, we have observed an apparent K_d for the farnesylated Ha-Ras-GMP-P(NH)P-Raf-CRD complex of 22 ± 6.2 μM (Fig. 2), but we cannot assert that these modest differences in binding affinity are statistically significant. This finding is consistent with previous reports from Hu et al. (10) that the Raf-CRD binds processed Ha-Ras in a nucleotide independent fashion (23).

FIG. 1. Binding of farnesylated and non-farnesylated Ras to the Raf-CRD. A, interactions between wild type Raf-CRD fused to GST and farnesylated or non-farnesylated Ha-Ras were assayed by ELISA. Assays were performed at least twice in triplicate, as described under “Experimental Procedures.” A representative data set is shown along with the resulting standard deviations from the mean. B, Ras binding to both wild type and mutant Raf-CRD were assessed by a direct binding assay using in vitro farnesylated and non-farnesylated Ha-Ras, as described under “Experimental Procedures.” Following separation by SDS-PAGE and detection by Coomassie R-250, the ratio of Ras bound to Raf-CRD fused to GST (dark bars) was quantitated by laser densitometry scanning and compared with the ratio of Ras bound to GST alone (light bars). All assays are performed at least twice in triplicate. Data from one representative assay and the resulting standard deviations from the mean are shown.
More importantly, however, we have determined that fully processed K-Ras4B binds the Raf-CRD with similar affinity to that of Ha-Ras that has farnesylation as its only post-translational modification (data not shown). Due to technical difficulties stemming from solubility limitations of the Raf-CRD, we were unable to reach binding saturation and therefore not able to calculate reliable \( K_d \) values for unprocessed Ras/Raf-CRD interactions.

**Mutation of a Hydrophobic Surface on the Raf-CRD Impairs Interactions with Both Farnesylated and Non-farnesylated Ha-Ras**—We recently solved the NMR solution structure of the Raf-CRD (26) and found it to be structurally similar to CRDs found in protein kinase C- \( \delta \) and -\( \gamma \) isoforms (36–38). We were intrigued by the existence of a solvent-exposed hydrophobic patch encompassing residues Leu\( ^{149} \), Phe\( ^{151} \), and Phe\( ^{158} \) on the surface of the Raf-CRD. We suspected that this hydrophobic region might be involved in contacting the farnesyl moiety of Ras, since the Raf-CRD preferentially interacts *in vitro* with farnesylated Ras. To test this hypothesis, we disrupted the hydrophobic patch by creating a L149T/F151Q Raf-CRD variant.

To assess possible structural perturbations resulting from mutation of residues Leu\( ^{149} \) and Phe\( ^{151} \), we evaluated the HSQC \( ^{1}H^{15}N \) NMR spectral changes of the mutant protein relative to the wild type Raf-CRD. Results from these analyses are illustrated in Fig. 3. We observed multiple peaks for some amide proton resonances in the HSQC \( ^{1}H^{15}N \) spectrum of Raf-CRD-(L149T/F151Q) (data not shown), suggesting that this mutant form of Raf-CRD can adopt multiple conformations in the region of the L149T/F151Q mutation. The Raf-CRD-(L149T/F151Q) variant, however, shows little if any difference in \( ^{1}H \) and \( ^{15}N \) chemical shift values relative to wild type Raf-CRD throughout the majority of the protein, indicating very similar global fold and local protein environments. Notable differences in chemical shift values are confined to the residues immediately proximal to and including those mutated, as illustrated in Fig. 3, indicating that only the region of the protein near the mutation is perturbed by the introduction of the L149T/F151Q mutation.

As shown in Fig. 4, the L149T/F151Q variant showed reduced binding to farnesylated Ras relative to wild type Raf-CRD in both a direct binding assay and a fluorescence based assay (Fig. 4, A and C). We also observed a reduction in binding of the Raf-CRD-(L149T/F151Q) variant to non-farnesylated Ha-Ras, as detected by both ELISA and fluorescence-based assays (Fig. 4, B and C), indicating that interactions with both farnesylated and non-farnesylated Ras are, at least in part, mediated by hydrophobic contacts.

**Mutation of a Hydrophobic Surface on the Raf-CRD Retains Interactions with 14-3-3\( ^{\delta} \) and Phosphatidylserine**—In addition to binding Ras, the Raf-CRD also interacts with 14-3-3 and PS (27, 28). Therefore, to assess whether this mutation specifically impairs interactions with Ras or also affects binding to 14-3-3 and PS, we employed ELISAs and competition binding studies, respectively. The Raf-CRD-(L149T/F151Q) displays impaired interactions with farnesylated Ha-Ras, processed K-Ras4B (data not shown), and unprocessed Ha-Ras. As shown in Fig. 5, this impairment is specific for Ras, as the Raf-CRD-(L149T/F151Q) variant does not effect binding to 14-3-3\( ^{\delta} \) as detected by ELISA (Fig. 5A) or the ability of PS to compete with 14-3-3 for contacting the Raf-CRD in a competition binding assay where we assessed the amount of 14-3-3 bound to Raf-CRD in the presence or absence of PS (Fig. 5B). The previously described Raf-CRD-(R143E/K144E) mutant was included in these assays as a negative control (28).

**Raf kinase Activation**—Our identification of a Raf-CRD variant that is selectively impaired in Ras, but not phosphatidylserine or 14-3-3\( ^{\delta} \), binding provides a novel reagent to assess the effect of Ras-Raf-CRD interactions in mediating Raf-1 activation. For these analyses, we introduced L149T/F151Q into full-length Raf-1 and compared the ability of Ras to stimulate the kinase activity of equal amounts of wild type and mutant protein Raf-1 protein in COS cells. The data are shown in Fig. 6. We have previously shown that the Raf R143E/K144E mutant is defective in both 14a3-3 and PS binding and possesses enhanced transforming potential (28), and proposed that 14-3-3 acts as a negative regulator of Raf-1 function by this interaction. In contrast, the Raf(L149T/F151Q) mutant that is defective in Ras/Raf-CRD interactions, exhibits no transforming potential and a severely impaired kinase activity in the pres-
ence of activated Ras. While the basal kinase activities of wild type and L149T/F151Q mutant Raf-1 showed no significant differences in this assay, the very low unstimulated activities make it difficult to draw a firm conclusion regarding the effect that the L149T/F151Q mutation has on the basal activity of the kinase.

NMR Chemical Shift Mapping—Although substitution of residues Leu149 and Phe151 results in impaired interaction with Ras, it was not clear whether the hydrophobic patch containing these residues is a direct point of contact with Ras or if the mutations perturb other residues/regions of the Raf-CRD involved in binding Ras. To elucidate the residues involved in...

Fig. 4. Interactions of Ha-Ras-GDP with wild type or mutant Raf-CRD. A, Ras-binding properties of mutant Raf-CRD were assessed by a direct binding assay using in vitro farnesylated Ha-Ras, as described under “Experimental Procedures.” Following separation by SDS-PAGE and detection by Coomassie R-250, the ratio of Ras bound to Raf-CRD fused to GST (dark bars) was quantitated by laser densitometry scanning and compared with the ratio of Ras bound to GST alone (light bars). All assays are performed at least twice in triplicate. Data from one representative assay and the resulting standard deviations from the mean are shown. B, interactions between mutant Raf-CRD fused to GST and non-farnesylated Ha-Ras were also assayed by ELISA. Ras bound to Raf-CRD fused to GST is denoted by dark bars whereas Ras bound to GST alone is represented by light bars. Assays were performed at least twice in triplicate, as described under “Experimental Procedures.” A representative data set is shown along with the resulting standard deviations from the mean. C, fluorescence assay based on an increase in apparent fluorescence intensity of mant-dGDP labeled Ha-Ras upon the addition of Raf-CRD. denotes farnesylated Ras and wild type Raf-CRD; denotes farnesylated Ras and wild type Raf-CRD. M, denote farnesylated Ras and Raf-CRD-(149/151); [4], non-farnesylated Ras-(L149T/F151Q) mutant Raf-CRD.

Fig. 5. The mutant Raf-CRD-(L149T,F151Q) retains interactions with 14-3-3ζ and phosphatidylserine. A, the ability of the Raf-CRD-(L149T/F151Q) variant to interact with 14-3-3ζ was assessed by ELISA as described under “Experimental Procedures.” Dark bars (■) symbolize Raf-CRD and the light bars (□) represent interactions between GST and 14-3-3. B, since phosphatidylserine can compete with the Raf-CRD-(L149T/F151Q) variant to bind 14-3-3 in the presence of phospholipids. The amount of 14-3-3 captured in the presence (■) and absence (□) of PS is plotted here. Assays were performed three times in duplicate. The previously described R143E/K144E mutant was included in these assays as a negative control.
The Raf-CRD 149/151 Mutant Severely Impairs Ras-Mediated Raf-1 Activation

![Diagram of Raf-CRD and Ras interactions](image)

**Fig. 6. Effect of the 149/151 mutation on Ras-mediated Raf-1 activation.** 1 μg of pCGN-hyg vector encoding HA-tagged wild type or mutant Raf proteins were co-transfected with pCGN-hyg Ha-Ras 61L into COS-7 cells using LipofectAMINE (Life Technologies, Inc.). After 48 h the cells were shifted to 1% serum and incubated overnight. The cells were then lysed and examined for the expression of Raf protein by Western blot using an anti-HA antibody (BabCo). Raf-1 kinase activity was examined by immunoprecipitating the Raf protein with C-12 polyclonal antisera (Santa Cruz) and adding recombinant MEK, ERK, and MBP substrate to generate a coupled assay for 32P incorporation into MBP. The data presented are representative of two separate assays.

**DISCUSSION**

In this work, we have characterized binding contacts important for interactions between Ras and the Raf-CRD and Ras-mediated Raf-1 activation. In particular, post-translational...
lipid modification of Ras imparts higher affinity interactions with the Raf-CRD. The binding of post-translationally modified Ras to Raf-1 has been shown to be critical for membrane recruitment of Raf-1. However, it also appears important for directly regulating Raf-1 activity, as Stokoe and McCormick (12) have shown that purified processed K-Ras-GTP, but not unmodified K-Ras-GTP or farnesylated K-Ras-GDP, is capable of stimulating Raf-1 to the same extent as activated processed Ha-Ras using an in vitro reconstitution system (20). Moreover, in a cell-free assay, fully processed K-Ras has been shown to be more active than unmodified K-Ras in Ras-dependent ERK kinase stimulator-mediated activation of MAP kinase (21, 22).

Carboxyl-terminal processing differs in Ha-, K-, and N-Ras, and involves multiple modifications, including farnesylation, palmitoylation, carboxyl-terminal cleavage, and carboxyl-methylation (24). However, processed forms of both Ha-Ras and K-Ras can promote Ras-mediated activation of Raf-1, with the only common lipid modification being farnesylation. These results suggest that the farnesyl moiety alone may be critical for promoting Ras-dependent activation of Raf-1. To characterize post-translationally modified sRas important for binding to the Raf-CRD, we prepared full-length farnesylated Ha-Ras, and found that farnesylation of Ras alone was sufficient to confer higher affinity association with the Raf-CRD, relative to bacterially expressed unprocessed Ras. Consistent with this observation, McGeady et al. (35) have shown that farnesylation of Ha-Ras is sufficient to cause activation of MAP kinase in a cell- and membrane-free Ras-dependent MAP kinase activation system (35). These observations, in combination with earlier findings that post-translationally modified Ha-Ras confers stable association with the Raf-CRD (10), provide additional support that it is the farnesyl moiety that conveys this additional binding affinity to the Raf-CRD, rather than any other form of post-translational modification and may play a critical role in Ras-dependent activation of Raf-1.

We have also examined non-farnesylated Ha-Ras’ interactions with the Raf-CRD and determined that both GDP- and GMP-P(NH)P-bound Ha-Ras associate with the Raf-CRD. Our results do indicate, however, that non-processed forms of Ras bind the Raf-CRD with at least 10-fold lower affinity relative to farnesylated Ras. These observations agree with our previous results that demonstrated binding of non-processed Ras to the Raf-CRD. The binding of post-translationally modified sRas has been shown to be more active than unmodified K-Ras in Ras-dependent ERK kinase stimulator-mediated activation of MAP kinase (21, 22).

GTP-dependent binding was not observed in our studies of Raf-CRD interactions with farnesylated Ras as well as in earlier studies conducted with processed Ha-Ras (10). A possible explanation for this apparent discrepancy is that farnesylation of Ras confers additional binding affinity for the isolated Raf-CRD independent of Ras’ activated state. Hence, the modest GTP-preference observed in the unprocessed form of Ras may not be detectable in the presence of the additional binding energy supplied, in a nucleotide independent manner, upon farnesylation or processing.

While some information currently exists on regions of Ras important for association with the Raf-CRD, very little is known about Ras-CRD residues that facilitate binding to Ras. To elucidate residues of the Raf-CRD important for Ras binding, we prepared Raf-CRD mutants and assessed the binding of these mutants to both farnesylated and non-farnesylated Ha-Ras. We also employed NMR approaches to map the binding interface between Ha-Ras and the Raf-CRD. A Raf cystein-rich domain variant that disrupts a hydrophobic patch on the surface of the Raf-CRD was generated. This Raf-CRD-(149/151) variant is impaired selectively in its ability to bind Ras, but not phosphatidylinerse or 14-3-3. Moreover, the Raf-CRD-(149/151) is defective for activation by Ras. This result indicates that Ras interactions with the Raf-CRD act to positively regulate Ras-1 function, perhaps by facilitating release of negative regulatory constraints by the dissociation of 14-3-3 and/or intramolecular interactions (3, 28, 39). This, in turn, may facilitate Raf-1 kinase activation. We cannot exclude the possibility, however, that the Leu<sup>145</sup>-Phe<sup>151</sup> mutation disrupts positive regulatory interactions with an, as of yet, uncharacterized ligand.

The Raf-CRD-(149/151) variant binds with reduced affinity to both the farnesylated and non-farnesylated forms of Ras. These findings indicate that the hydrophobic surface patch does not specifically recognize the farnesyl group but is also important for binding interactions with an epitope present on non-farnesylated Ras. Moreover, our observations that residues 148–150 and 158–164 were perturbed in NMR chemical shift mapping experiments provide further support of the importance of this hydrophobic region in mediating binding contacts with unprocessed Ras. Although spectral changes are also observed for Raf-CRD residues 144 and 145, our recent characterization of the K144E Raf-CRD variant indicates that this mutation does not reduce Ras binding to the Raf-CRD (28).

Hence, the observed spectral changes associated with residues 144 and 145 may reflect changes in their chemical environment due to Ras binding at nearby residues. The NMR data suggests that one additional region may be involved in contacting unprocessed Ras, as residues 174–177 are also sensitive to the binding of Ha-Ras-GDP to the Raf-CRD. However, it is possible that the chemical shift differences in this region are due to indirect effects of Ras binding since we have mutated Thr<sup>178</sup> of the Raf-CRD and seen no effects on Ras, 14-3-3, or phosphatidylycerse binding (data not shown).

While we and others have identified Ras regions important for binding the Raf-CRD (10, 11), this report now characterizes residues within the Raf-CRD that are involved in contacting Ras. Additionally, we have shown that disruption of this region of the Raf-CRD that contacts Ras results in a form of the Raf-1 kinase that is biologically inactive, strongly suggesting a necessity for direct contact between Ras and the Raf-CRD for Ras-mediated activation of Raf-1. Furthermore, although the non-farnesylated form of Ras clearly contains a subset of binding determinants for the Raf-CRD, our data indicate that the farnesyl moiety confers higher affinity interactions with the Raf-CRD.

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It is unclear at this time if the farnesyl group directly interacts with the Ras-CRD and/or structures an epitope on Ras (e.g., the COOH terminus) such that it confers additional binding affinity for the Ras-CRD. However, it is clear that the higher affinity binding of processed Ras to the Ras-CRD is mediated by the addition of the farnesyl moiety, rather than further processing (carboxyl-terminal proteolysis, methylation, and palmitoylation), and that the Ras-CRD recognizes a subset of Ras-GTP binding determinants distinct from those that interact with RBS-1 (10, 11, 42).

The data presented here help to elucidate the regions of the Ras-CRD that are involved in Ras binding and subsequent Raf-1 activation events following membrane localization. Moreover, our observation that higher affinity interaction between the Ras-CRD and Ras requires Ras farnesylation elucidates over, our observation that higher affinity interaction between Ras-CRD and/or structures an epitope on Ras (e.g., the COOH terminus) such that it confers additional binding affinity for the Ras-CRD. However, it is clear that the higher affinity binding of processed Ras to the Ras-CRD is mediated by the addition of the farnesyl moiety, rather than further processing (carboxyl-terminal proteolysis, methylation, and palmitoylation), and that the Ras-CRD recognizes a subset of Ras-GTP binding determinants distinct from those that interact with RBS-1 (10, 11, 42).

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