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Ras Interaction with Two Distinct Binding Domains in Raf-1 May Be Required for Ras Transformation*

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Although Raf-1 is a critical Ras effector target, how Ras mediates Raf-1 activation remains unresolved. Raf-1 residues 55–131 define a Ras-binding domain essential for Raf-1 activation. Therefore, our identification of a second Ras-binding site in the Raf-1 cysteine-rich domain (residues 139–184) was unexpected and suggested a more complex role for Ras in Raf-1 activation. Both Ras recognition domains preferentially associate with Ras-GTP. Therefore, mutations that impair Ras activity by perturbing regions that distinguish Ras-GDP from Ras-GTP (switch I and II) may disrupt interactions with either Raf-1-binding domain. We observed that mutations of Ras that impaired Ras transformation by perturbing its switch I (T35A and E37G) or switch II (G60A and Y64W) domain preferentially diminished binding to Raf-1-(55–131) or the Raf-1 cysteine-rich domain, respectively. Thus, these Ras-binding domains recognize distinct Ras-GTP determinants, and both may be essential for Ras transforming activity. Finally, since Ha-Ras T35A and E37G mutations prevent Ras interaction with full-length Raf-1, we suggest that Raf-Cys is a cryptic binding site that is unmasked upon Ras interaction with Raf-1-(55–131).

Ras proteins are molecular switches controlled by GDP/GTP cycling (1). These proteins are transiently activated in response to ligand-stimulated receptor tyrosine kinases (2, 3). Upon activation, Ras complexes with and promotes activation of the Raf-1 serine/threonine kinase (4, 5). Raf-1 then activates mitogen-activated protein kinase (MAPK)[1] kinases (MEKs), which in turn phosphorylate and activate MAPKs. Activated MAPKs translocate to the nucleus where they regulate the activities of Elk-1 and other nuclear transcription factors (6).

Although substantial evidence supports the importance of Ras-Raf-1 interactions for Ras-mediated signaling and transformation, the precise role of Ras in activating Raf-1 remains unresolved (7). The potent transforming activity of membrane-targeted forms of Raf-1 suggests that Ras-mediated translocation of Raf-1 to the plasma membrane is an important step in Raf-1 activation (8, 9). However, it is clear that Ras-Raf-1 interaction alone is not sufficient, and subsequent Ras-independent events are required for full Raf-1 kinase activation. For example, there is evidence that Raf-1 interaction with 14-3-3 proteins (10–14), lipids (15), and protein kinases (16–19) contributes to full Raf-1 kinase activation. Whether Ras simply promotes Raf-1 membrane association or also modulates the subsequent activation events is presently unclear.

Yeast two-hybrid and *in vitro* binding studies demonstrated that Raf-1 residues 55–131 are sufficient for stable association with Ras (20–22). Additionally, recent structural studies conducted with both Ras and the Ras-related protein, Rap1A, indicate that Raf-1 residues 55–131 interact with residues 33–41 in the Ras effector region (23, 24). Finally, the critical role for Raf residues 55–131 in Ras-mediated activation of Raf-1 is demonstrated by the ability of a point mutation, Raf (R89L), to disrupt Ras-Raf-1 binding and Raf-1 kinase activation (25). However, observations that mutations outside the Ras effector domain impair Ras-Raf-1 binding and Ras-mediated cell signaling (26–28) suggest that other Ras recognition elements may contribute to Raf-1 kinase regulation.

We have recently characterized a second Ras-GTP-binding site, located in the cysteine-rich domain of Raf-1 (residues 139–184), which interacts with Ras both *in vitro* and *in vivo* (29). Additionally, peptides from this region blocked Ras-mediated activation of MAPKs (30). The importance of a second Ras-binding domain in Raf-1 is supported by a report that mutations in the Raf-1 cysteine-rich region reduced Raf-1-Ras binding by 55% and Raf-1 kinase activity by 60–90% (27). Moreover, specific mutations in the cysteine-rich or kinase domains of D-Raf reversed the loss of function of a D-Raf variant containing a mutation analogous to c-Raf-1(R89L), leading Perrimon and co-workers (31) to speculate that the cysteine-rich domain formed negative regulatory contacts with the kinase domain, which are relieved upon binding Ras. However, it is presently unclear if the Raf-1 cysteine-rich domain recognizes Ras-GTP binding determinants distinct from those that interact with Raf-1 residues 55–131 and if both Ras-Raf-1 interacting sites are required for Ras signaling and transformation.

Both Ras recognition sites of Raf-1 preferentially bind Ras-GTP. Therefore, mutations that impair Ras transforming activity by causing disruptions in regions of Ras whose conformation differs between Ras-GDP and Ras-GTP (switch I and II) may disrupt interactions with either Ras-binding site of Raf-1. To investigate the significance of the two Ras-binding sites in

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; GST, glutathione *S*-transferase; GMPPCP, guanosine 5′-(β,γ-methylenetriphosphate).

Raf-1 and to elucidate the regions of activated Ras-GTP important for interactions with the Raf-1 cysteine-rich region, we determined if mutations that abolished Ras transforming activity also perturbed Ras interaction with the two distinct Ras-interacting fragments of Raf-1. Our observations suggest that the Raf-1 cysteine-rich domain contains a cryptic binding site, which recognizes region(s) of Ras different from those that bind Raf-1 residues 55–131, and both of these Ras-interacting sites may be necessary for Ras-mediated transformation.

EXPERIMENTAL PROCEDURES

Generation of Ha-Ras Mutants and Expression Constructs—Generation of the mammalian expression constructs encoding Ha-Ras(G12V), Ha-Ras(G12V, E37G), Ha-Ras(T35A), Ha-Ras(Q61L), Ha-Ras(G60A), and Ha-Ras(Y64W) have been described elsewhere (26, 32–34). Mutant Ha-*ras* (wild type or Q61L) sequences encoding T35W or I36W substitutions were generated by oligonucleotide-directed mutagenesis using the Mutator Kit (Stratagene, La Jolla, CA) and confirmed by dideoxy sequencing method using Sequenase (U. S. Biochemical Corp.). The mutant Ha-*ras* sequences were then introduced into the pAT-*ras* bacterial expression vector and into the pZIP-NeoSV(X)1 retrovirus vector for expression in mammalian cells (36).

Expression and Purification of Ras and Raf-1 Proteins—Ha-*ras* sequences encoding Ha-Ras(G12V) and Ha-Ras(G12V, E37G) were introduced into the pGEX bacterial expression vector, expressed as glutathione *S*-transferase (GST) fusion proteins, and cleaved from GST using thrombin (Sigma). The remaining Ha-Ras mutant proteins were expressed and purified as described previously (37). Complex formation of Ras and GMPPCP, a non-hydrolyzable GTP analog (Boehringer Mannheim), is described elsewhere (38). Raf-N (residues 2–140) and Raf-Cys (residues 136–188) were provided by S. Ghosh (22, 29) and were also prepared as GST-fusion proteins (15).

Cell Culture and Transformation Assays—NIH 3T3 cells were transfected using previously described procedures (39), and the appearance of transformed foci was quantitated after 14–16 days. Cell lines with stable expression of each mutant Ha-Ras protein were established by maintaining transfected cultures in growth medium supplemented with G418 at 400 μg/ml (Geneticin, Life Technologies, Inc.). Tumorigenicity was determined by subcutaneous inoculation of each cell line (1×10^5 cells/site) into athymic nude mice as described previously (39).

Direct Ras Binding Assay—Ras-GMPPCP was labeled with stoichiometric amounts of [γ-^{32}P]GTP. Ras-GTP (0.2 nM) was incubated with 0.5 mM Raf-1-GST fusion fragments in 20 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 1 mg/ml bovine serum albumin, and 0.3% Triton X-100 as described elsewhere (40). The Ras-Raf-1 complex was isolated by binding the GST-fusion fragment to glutathione-coated agarose beads, and the amount of radiolabeled Ras bound was determined by scintillation counting. Assays were performed at least three times in duplicate with GST background values subtracted from the amount of Ras-GTP detected.

Enzyme-linked Immunosorbent Assay—An enzyme-linked immunosorbent capture assay was employed as a second, distinct assay to further assess the binding profiles of several non-transforming Ras mutants and has been summarized elsewhere (29). Essentially, 100 pmol of Ras-GMPPCP was plated onto 96-well microtiter plates for 2 h at room temperature. The ability of various Ha-Ras mutants to capture 100 pmol of Raf-GST fusion fragments and corresponding amounts of GST in a buffer containing 10 mM Na_2HPO_4, pH 7.4, 0.5% gelatin, 0.05% Tween 20, and 0.2% sheep serum was measured by an anti-GST antibody (Santa Cruz Biotechnology, Inc.) at a 1:2500 dilution.

RESULTS AND DISCUSSION

Oncogenic Ras Interaction with Raf-1 Residues 55–131 Alone Is Not Sufficient for Ras Transforming Activity—Although we recently demonstrated that the cysteine-rich domain of Raf-1 (residues 139–184) associates with Ras both *in vitro* and *in vivo* (29), the role of this interaction in Ras transforming activity is unclear. Given the preferential binding between active GTP-bound Ras and fragments encoding both the cysteine-rich domain (Raf-1 residues 136–188, Raf-Cys) and Raf-1 residues 55–131 (Raf-1 residues 1–140, designated Raf-N) (22, 29), we anticipated that Raf-N and Raf-Cys are likely to interact with regions of Ras whose conformation is sensitive to its guanine nucleotide-bound state. Two such regions of Ras identified to undergo conformational changes between GTP- and GDP-bound forms of Ras are commonly referred to as switch I (residues 30–38 in loop 2/β strand 3) and switch II (residues 59–76 of loop 4 and helix 2) (41, 42). Thus, we evaluated the consequences of mutations that impair oncogenic Ras transforming activities via perturbations in switch I or II on Raf-N and Raf-Cys binding. These analyses may elucidate the region(s) of Ras involved in binding Raf-Cys and establish a role for this interaction in Ras transformation.

TABLE I
Consequences of switch I or II disruptions on Ras transforming activity and Ras binding to Raf-N and Raf-Cys in vitro

Mutation	NIH 3T3 FFU[a]	Switch disruption	*In vitro* binding		
			Raf-1	Raf-N	Raf-Cys
None[b]	+	−	+	+	+
G60A	−[c]	II	+	+	−
Y64W	−[c]	II	ND[d]	+	−
T35A	−	I	−	−	+
E37G	−	I	−[e]	−	+
T35W	−	I and II	ND	−	−
I36W[f]	+	None	ND	+	+

[a] Consequences of each mutation on oncogenic Ras(G12 or Q61L) focus-forming activity in NIH 3T3 transformation assays; focus-forming activity lost (−) or retained (+) is indicated.
[b] Oncogenic Ras(12V) or Ras(61L).
[c] Abolishes v-Ha-Ras transforming activity (26, 43).
[d] ND, not determined.
[e] Determined by yeast two-hybrid binding analysis (32).
[f] Introduction of I36W caused activation of wild type Ha-Ras transforming potential, showed approximately 10% of the focus-forming activity seen with Ha-Ras(Q61L) ($3–4 \times 10^3$ transformed foci/μg of plasmid DNA), and caused tumor formation of mice inoculated with NIH 3T3 cells.[2]

The biochemical and biological consequences of each mutation to Ras are summarized in Table I. Like wild type Ras, two different transforming mutants of Ras (G12V and Q61L) retained high affinity binding to both Raf-N and Raf-Cys (Fig. 1*A*). Given the GTP dependence of Ras interactions with the two Ras-Raf-1 interacting domains of Raf-1, taken together with recent data showing that Raf-1 residues 55–131 bind directly to the switch I domain of Ras (23, 24), we postulated that Raf-Cys requires the switch II domain for its interaction with Ras. Hence, to better characterize the regions of Ras required for Raf-Cys binding, we determined whether a mutation that disrupted switch II, but not switch I, altered Ras binding to Raf-N or Raf-Cys.

For these studies, we used Ras G60A and Y64W switch II mutants that abolish oncogenic Ras transforming activity (26, 43). Ras residue Gly-60 interacts with the γ-phosphate of GTP, and this interaction is believed to be crucial for propagating conformational changes within the switch II domain of Ras. Trypsin cleavage profiles and fluorescence analysis of mutations analogous to the Ras G60A substitution in other GTP-binding proteins ($G_{s\alpha}$ and EF-Tu) indicate that this mutation disrupts switch II but not switch I (44–46). Moreover, fluorescence analysis using 8-anilino-1-naphthalenesulfonic acid dye complexed with wild type and G60A Ras-GTP provides additional evidence that substitution of alanine for glycine at position 60 alters the active conformation of Ha-Ras (26).

Although we detected an association between Raf-N and both the Ras(G60A) and Ras(Y64W) variants, we did not observe complex formation between these Ras mutants and Raf-Cys (Fig. 1*B*). Thus, these results show that whereas Raf-N interacts with switch I (23, 24), Raf-Cys requires an intact switch II region for binding to Ras. This is consistent with a previous observation that mutations in the switch II region of Ras (including residue 64) perturb interactions between Ras and various putative effector proteins (47). Furthermore, the observa-

tion that the G60A and Y64W mutations abolish Ras transforming activity without impairment of Raf-N binding suggests that Ras interaction with Raf-N alone is not sufficient for Ras biological activity.

The Ras Interaction Site in Raf-Cys Requires an Intact Switch II Domain for Ras Binding and Is Cryptic in Raf-1—Previous studies have shown that the T35A and E37G effector domain mutations abolished Ras binding to full-length Raf-1 and impaired oncogenic Ras transforming activity (32, 48). Consistent with the inability of these switch I mutants to bind full-length Raf-1, we were unable to detect a stable complex between T35A and E37G variants of Ras and Raf-N *in vitro* (Fig. 1*B*). However, the ability to bind the isolated Raf-Cys domain was retained (Fig. 1*B*). These results are also consistent with earlier observations that Ras-Raf-N (residues 55–131) binding contacts involve the Ras effector domain (23, 24) and that these interactions are required for Ras-mediated activation of Raf-1 (25). Finally, we had previously observed that mutations R256G, S257L, and S257P in the highly conserved central region of Raf-1 (commonly referred to as the CR2 domain) or the removal of Raf-1 COOH-terminal residues 247–648 are required to allow Ras(G12V, E37G) interaction with Raf-1 (32), indicating that the Ras binding determinants in Raf-Cys are masked in full-length Raf-1 (Fig. 2).

The inability of Raf-N to complex with the biologically inactive T35A mutant may result from diminished interactions between position 35, magnesium, GTP, and possibly the Asp-38 residue (48, 49). It is also not surprising that the E37G mutation disrupted interactions with Raf-N, as the x-ray structure of the binding interface between Raf-1 residues 55–131 and Rap1A showed that the Glu-37 residue of Rap1A was in close contact with this Raf-1 sequence and was involved in water-mediated protein-protein interactions. Rap1A shares complete identity with Ras residues 32–40 and can associate with most Ras effectors, including Raf-1, RalGDS, and Ras GTPase-activating proteins (50–52).

Tryptophan Substitutions at Ras Residues 35 or 36 Cause Opposing Biological Consequences—We also determined the consequences of two adjacent Ras effector domain mutations, T35W and I36W, on the properties of normal or oncogenic Ras (Table I). We found that the Ras T35W mutant, but not I36W, abolished the transforming activity of Ras(Q61L). Instead, the I36W mutation alone was sufficient to activate wild type Ras transforming potential. A similar gain of function mutation at this position has been described previously (53). In contrast to the minor perturbations observed in the switch I region of Ras(I36W), NMR structural studies of the Ras(T35W) mutant showed pronounced alterations in both switch I and switch II.[2] Consistent with our suggestion that Ras interaction with both Raf-N and Raf-Cys is important for Ras transformation, we found that Ras(I36W) retained binding to both Raf-N and Raf-Cys (Fig. 1*A*), whereas the nontransforming Ras(T35W) mutant failed to interact with either Raf-1 sequence (Fig. 1*B*).

Our analyses of mutations that impair Ras transformation as a consequence of perturbations to either the Ras switch I or switch II domain suggest that Ras transforming activity requires interaction with two distinct NH_2-terminal Raf-1 sequences. Furthermore, Raf-N and Raf-Cys demonstrated opposing binding profiles with Ras proteins containing mutations that disrupt switch I (T35A and E37G) *versus* switch II (G60A and Y64W), indicating that they recognize distinct Ras GTP-binding elements. These observations, together with our dem-

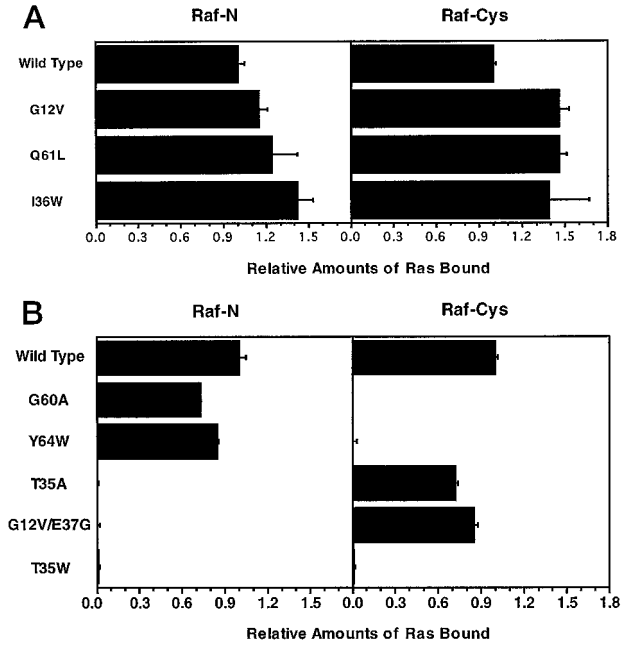

FIG. 1. **Both Ras-binding domains of Raf-1 appear necessary for Ras-mediated transformation.** Two distinct Raf-1 fragments were tested for their ability to bind various mutants of Ras as described under "Experimental Procedures." *A*, transforming Ras mutants bound both Raf-N and Raf-Cys. *B*, Ras variants with switch I or II defects lacked the ability to interact with at least one Ras recognition fragment.

[2] J. K. Drugan, R. Khosravi-Far, C. J. Der, and S. L. Campbell, manuscript in preparation.

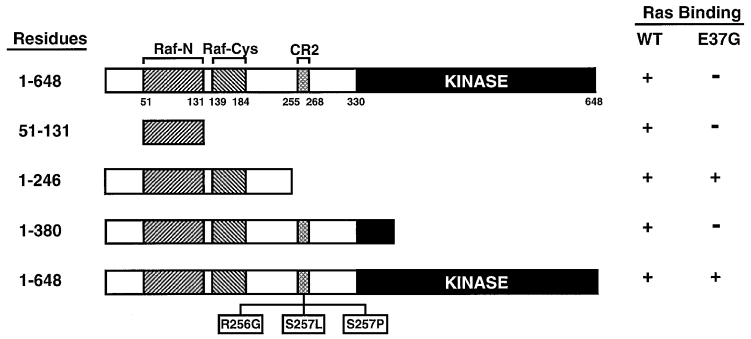

FIG. 2. **Ras(G12V, E37G) abolishes Ras interaction with Raf-N but not the isolated Raf-Cys domain.** Yeast two-hybrid analysis was done to determine the ability of Ras(G12V, E37G) to interact with different fragments of Raf-1 by procedures described previously (32). The indicated Raf-1 residues were fused to transcriptional activating domains in the yeast reporter strain L40 as described below. A positive interaction (+) was determined by growth on medium lacking histidine and by a positive indication of β-galactosidase activity using filter assays; (−) indicates no interaction. At least four independent yeast colonies expressing the indicated pairs were tested. Raf-1 residues 51–131 were fused to the VP-16-activating domain (a gift from A. Vojtek and J. Cooper) (20) while the remaining Raf-1 sequences were fused to the GAD-activating domain. The Raf-1 CR2 mutants (R256G, S257L, S257P) were isolated and characterized previously (32). *WT*, wild type.

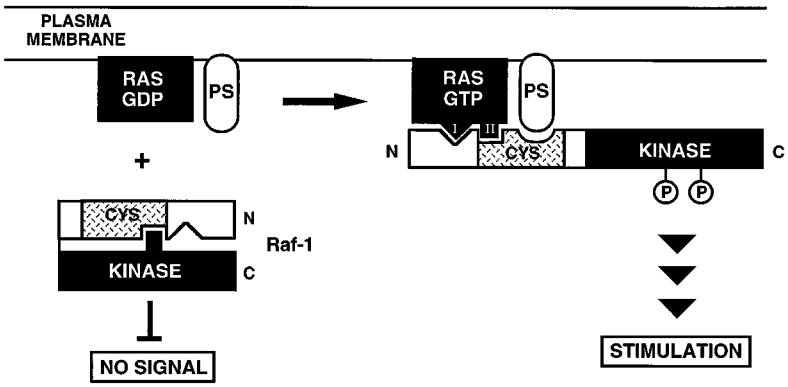

FIG. 3. **Model for the role of the Raf-1 cysteine-rich region in Raf-1 kinase activation.** Binding of Ras-GTP to Raf-N promotes membrane localization of Raf-1 and exposes Ras-binding elements in the adjacent Raf-Cys domain for interaction with Ras-GTP and possibly phosphatidylserine (*PS*). These interactions, in turn, facilitate the removal of any autoinhibitory contacts with the kinase domain. Following phosphorylation of Raf-1 residues, Raf-1 kinase is stabilized in its active configuration and stimulates the MEK/MAPK cascade.

onstration that peptides containing a consensus Ras binding sequence from Raf-Cys can block Ras activation of MAPKs (30), provide strong evidence that Ras interaction with Raf-Cys is a critical step in Ras-mediated activation of Raf-1.

Previous studies determined that Raf-N represents a minimal Ras binding sequence and that a mutation in this Raf-1 domain (R89L) prevented Ras interaction and activation of full-length Raf-1 (25). Therefore, we speculated that the Ras-binding elements in Raf-Cys are cryptic in the intact unstimulated Raf-1 protein (29). In support of this hypothesis, we have shown in the present study that two Ras effector domain mutations (T35A and E37G), which impair Ras binding to both full-length Raf-1 and Raf-N, did not abolish Ras binding to the isolated Raf-Cys domain.

Recent observations that addition of the Ras membrane-targeting sequence onto Raf-1 caused activation of Raf-1 transforming activity suggested that Ras binding to Raf-N was important for Ras-mediated translocation of Raf-1 from the cytosol to the plasma membrane (8, 9). Once at the membrane, Ras-independent events have been proposed to trigger Raf-1 kinase activation. However, our observation that the G60A and Y64W mutations retained interaction with Raf-N yet abolished oncogenic Ras transforming activity suggests that Ras interaction with Raf-Cys is also required for Raf-1 activation. Therefore, Ras interaction with both Raf-N and Raf-Cys may be necessary to promote Raf-1 association with the plasma membrane. However, several lines of evidence suggest that the NH_2-terminal half of Raf-1 serves to negatively regulate the activity of the COOH-terminal kinase domain because mutation, insertion, or deletion of these regions results in oncogenic activation of the Raf-1 kinase (54–56). Therefore, Ras binding to Raf-Cys may relieve the negative regulatory action of the Raf-1 NH_2 terminus to allow other events to activate Raf-1. This hypothesis is consistent with our observation that the Ras-binding site in Raf-Cys is cryptic in full-length Raf-1. Additionally, it provides an explanation for previous findings that specific mutations in the cysteine-rich and kinase domains of D-Raf reverse the loss of function associated with a D-Raf mutation in the Raf-N site that abolishes Ras binding.

In light of our observations, we propose a model for the role of Ras-mediated activation of Raf-1 via two distinct Ras binding sequences in Raf-1 (Fig. 3). Binding of the Ras-GTP switch I domain to Raf-N promotes both membrane localization of Raf-1 and exposes residues in Raf-Cys for binding Ras-GTP and possibly other membrane components. Interactions with Raf-Cys then trigger loss of the negative regulatory activity of the Raf-1 NH_2 terminus to allow subsequent Ras-independent events to promote Raf-1 kinase activation.

The Raf-Cys zinc finger motif also contains binding determinants for phosphatidylserine (15) and 14-3-3 proteins (7). Hence, Ras interaction with Raf-Cys may modulate their activities to mediate Raf-1 activation. By analogy with the cysteine-rich domain of protein kinase C (35, 57–59), the Ras-binding site in Raf-Cys may functionally substitute for diacylglycerol, and synergistic binding of Ras and phosphatidylserine may be involved in release of negative regulatory constraints between the NH_2 and COOH termini of Raf-1. It is also possible that exposure of the Ras-binding elements in Raf-Cys may require release of 14-3-3 proteins (14). In summary, the Ras-Raf-Cys interaction may induce the removal of negative regulatory action in the Raf-1 NH_2 terminus and consequently facilitate Raf-1 activation by additional events such as phosphorylation of select residues in Raf-1.

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