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## Oncogenic Ras Activation of Raf/Mitogen-Activated Protein Kinase-Independent Pathways Is Sufficient To Cause Tumorigenic Transformation

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**Substantial evidence supports a critical role for the activation of the Raf-1/MEK/mitogen-activated protein kinase pathway in oncogenic Ras-mediated transformation. For example, dominant negative mutants of Raf-1, MEK, and mitogen-activated protein kinase all inhibit Ras transformation. Furthermore, the observation that plasma membrane-localized Raf-1 exhibits the same transforming potency as oncogenic Ras suggests that Raf-1 activation alone is sufficient to mediate full Ras transforming activity. However, the recent identification of other candidate Ras effectors (e.g., RalGDS and phosphatidylinositol-3 kinase) suggests that activation of other downstream effector-mediated signaling pathways may also mediate Ras transforming activity. In support of this, two H-Ras effector domain mutants, H-Ras(12V, 37G) and H-Ras(12V, 40C), which are defective for Raf binding and activation, induced potent tumorigenic transformation of some strains of NIH 3T3 fibroblasts. These Raf-binding defective mutants of H-Ras induced a transformed morphology that was indistinguishable from that induced by activated members of Rho family proteins. Furthermore, the transforming activities of both of these mutants were synergistically enhanced by activated Raf-1 and inhibited by the dominant negative RhoA(19N) mutant, indicating that Ras may cause transformation that occurs via coordinate activation of Raf-dependent and -independent pathways that involves Rho family proteins. Finally, cotransfection of H-Ras(12V, 37G) and H-Ras(12V, 40C) resulted in synergistic cooperation of their focus-forming activities, indicating that Ras activates at least two Raf-independent, Ras effector-mediated signaling events.**

Ras proteins act as molecular switches that cycle between active GTP- and inactive GDP-bound forms (reviewed in references 6 and 9) and function as essential components of signal transduction pathways that regulate cell growth and differentiation (reviewed in references 21, 36, and 51). Upon activation by ligand-stimulated receptors, activated Ras proteins transmit their signals to a cascade of serine/threonine kinases (reviewed in references 59, 64, 68, and 86). Activated Ras complexes with and promotes activation of the Raf-1 serine/threonine kinase (50, 76, 78, 79, 87). Raf-1 in turn activates mitogen-activated protein kinase (MAPK) kinases (MEK1 and MEK2), which in turn activate p42 and p44 MAPKs/extracellular signal-regulated kinases (ERKs) (16, 34, 41, 42, 61, 88). Activated MAPKs then translocate into the nucleus, where they phosphorylate and activate nuclear transcription factors such as Elk-1 (30), resulting in immediate-early gene induction (reviewed in references 31 and 47).

The critical involvement of the Raf/MEK/MAPK cascade in mediating Ras transformation is supported by a number of experimental observations. First, kinase-deficient mutants of Raf-1, MEKs, and MAPKs have been shown to block Ras-mediated signaling events and transformation (14, 37, 39, 58,

67, 81). Second, constitutively activated mutants of Raf-1 exhibit strong transforming activities in rodent fibroblast transformation assays (7, 73). For example, the potent transforming activity of the plasma membrane-targeted Raf-1 has prompted suggestions that Ras may function primarily to promote the translocation of Raf-1 from the cytosol to the plasma membrane, where subsequent Ras-independent events trigger Raf-1 kinase activation (43, 74). Consistent with this possibility, constitutively activated Raf-1 is sufficient to overcome the loss of Ras function caused by the Ras(17N) dominant negative or the Y13-259 anti-Ras neutralizing antibody (24, 71). Third, constitutively activated MEKs can cause tumorigenic transformation of NIH 3T3 cells (3, 45). Finally, genetic studies with *Drosophila melanogaster* and *Caenorhabditis elegans* have shown that gain-of-function mutations in Raf, MEK, or MAPK homologs can overcome defects in Ras (18, 28, 44, 75). Taken together, these observations support the possibility that Ras-mediated activation of the Raf/MEK/MAPK pathway is both necessary and sufficient for oncogenic Ras transformation.

Despite the evidence that Raf-1 is a critical downstream effector of Ras function, there is increasing evidence that Ras may mediate its actions through the activation of multiple downstream effector-mediated pathways (23). For example, the existence of Raf-independent Ras signaling pathways is suggested by the expanding roster of candidate Ras effectors that have been identified (27, 33, 38, 58, 65, 66, 72, 76–78). Like Raf-1, these functionally diverse proteins show preferential binding to the active GTP-bound form of Ras, and this interaction requires an intact Ras effector domain (amino acids 32 to 40). For example, although the two Ras GTPase-activating

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proteins (GAPs) clearly function as negative regulators of Ras, there is also evidence that they serve a dual role and may also be important downstream targets of Ras (reviewed in references 6 and 25). Yeast two-hybrid studies have identified two guanine nucleotide exchange factors (GEFs) for Ras-related proteins RalA and RalB (RalGDS and RGL/Rsb3), as well as additional novel proteins, as candidate Ras effectors (33, 38, 72, 77, 78). Phosphatidylinositol-3-OH kinase has also been shown to complex with Ras and to exhibit the properties of a Ras effector (65). Finally, additional candidate Ras effectors include mammalian Rin-1, MEK kinase 1, and AF6/Rsb1, *Saccharomyces cerevisiae* adenyl cyclase (CYR1), and the *Schizosaccharomyces pombe* byr2 and scd1 proteins (10, 27, 66, 76–78). AF6 has been previously defined as part of a fusion protein coded for by a hybrid gene caused by a translocation in acute lymphocytic leukemia and may be a critical Ras effector in hematopoietic systems (40). To date, the precise role of these candidate effectors in mediating Ras downstream signal transduction and transformation remains to be established.

Additional evidence for the involvement of multiple downstream events is provided by observations from genetic, biochemical, and biological studies that suggest that Ras acts through members of the Rho family of Ras-related proteins (RhoA, RhoB, RhoC, RhoG, Rac1, Rac2, CDC42, and TC10). First, genetic studies of fission yeast *S. pombe* Ras (*ras1*) function have identified two distinct *ras1* effector-mediated activities (10). One involves *ras1* interaction with byr2 (a MEK kinase homolog), and the other involves *ras1* interaction with scd1 (a putative Rho GEF). scd1 in turn may regulate the function of the *cdc42sp* Rho family protein. Second, Swiss 3T3 mouse fibroblast microinjection studies showed that the oncogenic Ras-induced alterations of actin cytoskeletal organization (5) may be mediated via activation of two Rho family proteins, Rac1 and RhoA (62, 63). Third, the inhibition of oncogenic Ras transformation by dominant negative mutants of three Rho family proteins (RhoA, RhoB, and Rac1) demonstrated that full Ras transformation was dependent on Rho protein function (37, 56, 58). In contrast, interfering Rac1 or RhoA mutants weakly blocked Raf transforming activity (37, 58). Finally, the synergistic transforming activity seen after activated Raf-1 is coexpressed with constitutively activated RhoA or Rac1 suggests that coordinate activation of the Raf/MEK/MAPK pathway and Rho proteins may be required for full Ras transformation (37, 58). How Rho protein function may contribute to Ras transformation is presently not known. Rho family proteins have been shown to be regulators of actin cytoskeletal organization (reviewed in references 11, 19, and 26) and may also trigger changes in gene expression by modulating the activities of various transcription factors (13, 32, 49, 53).

Clear genetic evidence that Ras mediates transformation by activating Raf-dependent and Raf-independent pathways was demonstrated by our discovery of H-Ras effector domain mutants that separate the abilities of H-Ras to interact with different downstream targets (83). Mutations in the Ras effector domain (residues 32 to 40) can impair Ras transforming activity and interaction with effector proteins without causing alterations in intrinsic GDP and GTP regulation (reviewed in reference 48). Although one of these mutants [H-Ras(12V, 37G)] failed to bind Raf-1, it retained the ability to synergistically enhance the transforming activity of another H-Ras effector domain mutant [H-Ras(12V, 35S)] which retained full-length Raf-1 binding activity (1, 20, 70, 83). This observation suggested that H-Ras(12V, 37G) retained the ability to trigger a Raf-independent signaling pathway that was required for full

Ras transformation. The identity of this Raf-independent signaling pathway(s) has not been established.

In the present study, we have analyzed the role of Raf-independent signaling pathways in growth transformation by using two H-Ras effector domain mutants, H-Ras(12V, 37G) and H-Ras(12V, 40C), that failed to bind full-length Raf-1 but that could bind to other candidate effectors in yeast two-hybrid analyses (83). We observed that both mutants were impaired in their abilities to stimulate the Raf/MAPK/Elk pathway, and yet they retained the ability to cause a potent tumorigenic transformation of some cell strains. However, the transformed phenotype induced by the Raf-binding defective mutants in these strains was distinct from that caused by oncogenic Ras and instead was indistinguishable from the transformed focus and morphology seen with Rho- or Rho GEF-transformed NIH 3T3 cells (35, 37, 56). Finally, H-Ras(12V, 37G) and H-Ras(12V, 40C) cooperated and showed synergistic induction of transformed foci, suggesting their abilities to trigger at least two distinct, Raf-independent pathways that promote Ras transformation. Taken together, our observations indicate that coordinate activation of Raf-dependent and Raf-independent pathways by oncogenic Ras may be required to mediate full Ras-induced transforming activity. Furthermore, the activation of the Raf-independent pathway(s) is sufficient to cause cellular transformation and requires the participation of Rho family proteins.

#### MATERIALS AND METHODS

**Molecular constructs.** All *ras* expression constructs encode mutant versions of the transforming human H-Ras(12V) protein. pHP5-H-*ras*(12V), pHP5-H-*ras*(12V,35S), pHP5-H-*ras*(12V,37G), and pHP5-H-*ras*(12V,40C) are expressed as LexA-binding domain fusions, and pGAD-*raf1* is expressed as a GAL4 activation domain fusion and has been described previously. The pDCR-*ras*(12V), pDCR-*ras*(12V,35S), pDCR-*ras*(12V,37G), and pDCR-*ras*(12V,40C) mammalian constructs encode effector domain mutants of H-Ras(12V) in which expression is under the control of the cytomegalovirus promoter (83). pZIP-*raf*(340D) and pCEP4-*raf*BXB encode mutants of Raf-1 which contain either a single amino acid substitution (Y-340→D) (22) or an N-terminal truncation of the Ras-binding domain (80), respectively, and have been described previously (37, 83). pZIP-*rhoA*(63L) encodes a transforming mutant human RhoA protein which contains a substitution analogous to the Q-61→L mutation which activates Ras transforming activity (17, 37). pGEX-*mek1*(WT), pGEX-*mek1*(KR), pGEX-*erk1*(KR), and pGEX-*c-jun*(WT) were used as described previously to generate substrates for the kinase assays (2, 82).

**Yeast two-hybrid assay.** The yeast strains and media for performance of the yeast two-hybrid binding analyses were described previously (48, 77). Like H-*ras*(37G), H-*ras*(40C) was isolated from a PCR-derived randomly mutagenized H-*ras* library which was fused to the GAL4 DNA-binding domain and found to show no interaction with Raf-1 fused to the GAL4 activation domain. Additional Ras binding partners were expressed as GAL4 activation domain fusions containing the Ras binding sequences from RalGDS, AF6 (residues 1 to 180), *cyr1*, and byr2 (77). AF6 was previously described in a single example as a fusion partner for ALL-1 in acute lymphoblastic leukemias (55). Interactions between Ras-GAL4 DNA-binding domain fusions and Ras binding partner-GAL4 activation domain fusions were performed in *S. cerevisiae* reporter strain YPB2 and assayed for  $\beta$ -galactosidase activity on filters.

**Cell culture and transformation assays.** NIH 3T3(CSHL) and NIH 3T3(UNC) are two independent strains of NIH 3T3 fibroblasts that were isolated for their flat shapes and low rates of spontaneous transformation and have been used previously for Ras transformation studies (12, 83). DNA transfections were done by the calcium phosphate precipitation technique as previously described (12, 85). Ten nanograms of pDCR-*ras*H(12V), 100 ng of the effector domain mutants, 100 ng of pCEP4-*raf*BXB, 1  $\mu$ g of pZIP-*raf*(340D), and 1  $\mu$ g of pZIP-*rhoA*(63L) were used in each dish for transformation studies with NIH 3T3(UNC) cells. Transfected cells were maintained in growth medium, and the appearance of transformed foci was quantitated after 14 to 16 days. To establish NIH 3T3(UNC) cell lines expressing each protein, the transfected cells were selected in growth medium containing 400  $\mu$ g of Geneticin (G418; GIBCO/BRL) per ml. NIH 3T3(UNC) cells expressing oncogenic and various effector domain mutants were examined, and their growth rates and saturation densities on plastic, their abilities to proliferate in low serum (1%) or soft agar (0.3%), and their abilities to form tumors after being inoculated subcutaneously into athymic nude mice ( $10^5$  cells per site) were compared by procedures that we have described previously (12).

The ability of Raf-1 and RhoA mutant proteins to modulate the focus-forming

activity of the effector domain mutants was determined by cotransfection focus-formation assays. Cultures were transfected with oncogenic Ras or effector domain mutants alone or together with plasmid DNA expression vectors encoding activated Raf-1 (340D or BXB) or RhoA(63L) proteins. The appearance of transformed foci was quantitated after 14 to 16 days. Data gathered were representative of two or more independent determinations, with each determination representing the number of foci seen in four dishes.

**Protein expression.** Protein expression from the exogenously introduced wild-type and mutant *H-ras* cDNA sequences in stably transfected NIH 3T3(UNC) cells was determined by Western blot (immunoblot) analyses of G418-selected cell lysates. Ras expression was determined with the mouse 146-3E4 anti-H-Ras-specific monoclonal antibody (Quality Biotech) (15). Detection of antibody was done by enhanced chemiluminescence (Amersham).

**Transient transfection luciferase assays.** To compare the abilities of the different effector domain mutants to induce transcriptional activation of the Ras-responsive Elk-1 transcription factor, NIH 3T3(UNC) cells were transiently cotransfected with 250 ng of plasmid DNA encoding mutant Ras together with 2.5  $\mu$ g of the Gal-*luc* and 250 ng of the Gal-*elk* fusion constructs (provided by M. Karin and R. Treisman, respectively) by procedures described previously (29). Forty-eight hours after transfection, cell lysates were prepared in 200  $\mu$ l of luciferase cell lysis buffer. Fifty microliters of the lysate was then assayed in a luminometer with 100  $\mu$ l of ATP and luciferin reagent. The fold luciferase activity was then calculated by dividing the luciferase activity of the Ras mutants by the activity of the sample containing the vector alone as described previously. The data represent two experiments performed in duplicate.

**In vitro Raf kinase and MAPK assays.** NIH 3T3(UNC) cells expressing oncogenic Ras and various effector domain mutants were lysed in a modified RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 5 mM EDTA, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.5], 1 mM sodium vanadate, 50 mM NaF, and phosphatase inhibitors). Raf-1 or MAPK/ERK was then immunoprecipitated with anti-Raf-1 (C-12) or anti-ERK1 (C-16) rabbit polyclonal antibody (Santa Cruz Biotech), respectively. The Raf-1 immunocomplex kinase assay was then carried out in a coupled assay. Raf was incubated with 2 to 5  $\mu$ g of wild-type MEK1 for 15 min and then with 2 to 5  $\mu$ g of the kinase-deficient mutant of MAPK for 15 min in a kinase assay in the presence of a solution containing 25 mM HEPES (pH 7.5), 25 mM Mg<sub>2</sub>Cl<sub>2</sub>, 2 mM dithiothreitol, 0.1 M Na<sub>3</sub>VO<sub>4</sub>, phosphatase inhibitors, and [ $\gamma$ -<sup>32</sup>P] ATP at room temperature. The MAPK immunocomplex assay was carried out by incubating the immunoprecipitated MAPK with 12  $\mu$ g of myelin basic protein in a kinase assay for 30 min at room temperature. The reactions were then stopped with 2 $\times$  sodium dodecyl sulfate (SDS) sample buffer. The proteins were then separated on an SDS-15% polyacrylamide gel and visualized by autoradiography (2).

**In vitro JNK activation assay.** NIH 3T3(UNC) cells stably expressing each effector domain mutant were lysed in modified RIPA buffer. Lysate containing approximately 30  $\mu$ g proteins was incubated with 1 to 5  $\mu$ g of GST-c-Jun(1-79) substrate (provided by M. Karin) in a kinase assay as described above. Proteins were then separated by SDS-polyacrylamide gel electrophoresis, and phosphorylation of the substrate was observed following autoradiography (82). Transient Jun kinase (JNK) assays were carried out by transfection of COS-7 cells with 1  $\mu$ g of each effector domain mutant along with 1  $\mu$ g of pCMV-(M2)JNK with Lipofectamine, according to the manufacturer's procedures (GIBCO/BRL). The cells were then lysed on day 3 with modified RIPA buffer. JNK was immunoprecipitated with the M2 Flag antibody (IBI) and used in a kinase assay as described above in the presence of 2 of GST-c-Jun(1-79).

**Immunofluorescence analyses.** For visualization of stress fibers and focal adhesion components, cells were plated on coverslips in growth medium (35). After 16 to 24 h, the cells were fixed in 3.7% formaldehyde in phosphate-buffered saline for 7 min, washed in Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl [pH 7.6], 0.1% sodium azide), and permeabilized with 0.5% Triton X-100 in Tris-buffered saline for 6 min. Double labeling was performed as described previously (35). Briefly, polymerized actin was stained with either 600 mU of tetramethyl rhodamine isothiocyanate-phalloidin per ml or 5 U of fluorescein isothiocyanate-phalloidin (Molecular Probes, Inc., Eugene, Ore.) per ml. The focal adhesion protein, vinculin, was treated with 7f9 antivinculin monoclonal antibody and then with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) or tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Chemicon International, Inc., Temecula, Calif.).

## RESULTS

**The Raf-binding defective mutants of H-Ras are defective in activation of the Raf/MAPK/Elk pathway.** We recently showed that the H-Ras(12V, 37G) mutant could complement the transforming activity of the H-Ras(12V, 35S) mutant, indicating that Raf-independent pathways activated by Ras can contribute to transformation (83). We sought to determine if activation of Raf-independent pathways could in themselves lead to tumorigenic transformation. However, the H-Ras(12V, 37G)

mutant had no focus-forming activity in the NIH 3T3(CSHL) strain (83). We have observed that different isolates of NIH 3T3 cells differ with respect to their abilities to form foci in response to activated Rho family members. Whereas the NIH 3T3(UNC) strain is responsive to transformation by constitutively activated mutants of RhoA and Rac1 (37), the NIH 3T3(CSHL) strain is not (83a). Therefore, we sought to characterize the transforming activity of the H-Ras(12V, 37G) mutant in NIH 3T3(UNC) and other cell lines. For these purposes, we decided to test another effector loop mutant of H-Ras, H-Ras(12V, 40C), which also failed to bind to full-length Raf-1 in the two-hybrid system (Fig. 1A) but which retained the ability to interact with another candidate target, AF6, which has been described previously (Table 1 shows the matrix of mutant H-Ras interactions with candidate effectors) (77).

First, we determined if H-Ras(12V, 37G) and H-Ras(12V, 40C) were impaired in their abilities to upregulate the Raf/MAPK pathway after being expressed in cells of the NIH 3T3(UNC) strain. These cells were stably transfected with H-Ras(12V) or each of the effector domain mutants. Multiple G418-resistant colonies (>100) were pooled, and Western blot analysis showed that they expressed comparable levels of mutant H-Ras protein (Fig. 1B). Whereas cells expressing H-Ras(12V) and H-Ras(12V, 35S) exhibited elevated Raf-1 kinase activities (20- and 6-fold, respectively) after being assessed in a Raf-1 immunocomplex-coupled in vitro kinase assay, cells expressing H-Ras(12V, 37G) or H-Ras(12V, 40C) showed the same low Raf-1 kinase activity seen in untransfected NIH 3T3(UNC) cells (Fig. 1C). These observations are consistent with the yeast two-hybrid binding results and demonstrated that both H-Ras(12V, 37G) and H-Ras(12V, 40C) were impaired in their abilities to activate Raf-1 kinase in mammalian cells.

Consistent with their impaired abilities to upregulate Raf-1 kinase activity, cells expressing H-Ras(12V, 37G) and H-Ras(12V, 40C) showed no significant increase in p42 and p44 MAPK activities, as measured by their abilities to phosphorylate myelin basic protein in vitro (Fig. 1D). In contrast, both H-Ras(12V)- and H-Ras(12V, 35S)-expressing cells showed elevated MAPKs (7- and 3-fold elevations, respectively). Finally, we analyzed the ability of these effector domain mutants to activate the Elk-1 transcription factor in transiently transfected NIH 3T3(UNC) cells. Elk-1 has been shown to be a substrate for MAPK phosphorylation, and Elk-1 transcriptional activity can be stimulated by oncogenic Ras activation of the Raf/MEK/MAPK pathway (46, 60). We found that H-Ras(12V, 35S) but not H-Ras(12V, 37G) or H-Ras(12V, 40C), retained the ability to stimulate transcriptional activity of Elk-1 (Fig. 1E). Thus, both Raf-binding defective mutants were impaired in their abilities to activate components downstream of Ras and Raf-1.

**Ras mutants which are defective in activation of the Raf/MAPK pathway cause tumorigenic transformation.** We next determined whether H-Ras(12V, 37G) or H-Ras(12V, 40C) retained the ability to cause transformation of NIH 3T3(UNC). In contrast to the NIH/3T3(CSHL) strain, the NIH 3T3(UNC) strain was able to have foci induced by these mutants, although the frequency was greatly attenuated (60- and 110-fold reductions, respectively) compared with the focus-forming activity of H-Ras(12V) ( $4 \times 10^3$  to  $8 \times 10^3$  transformed foci per  $\mu$ g of transfected DNA) (Fig. 2). Similarly, we observed that these Ras effector domain mutants also caused focus formation in Rat-1 fibroblasts and RIE-1 rat intestinal epithelial cells (52a).

We next characterized the growth properties of NIH 3T3(UNC) cells stably expressing each mutant Ras protein. For these analyses, we used both mass populations of newly transfected, G418-resistant cells (pooled from more than 100 drug-

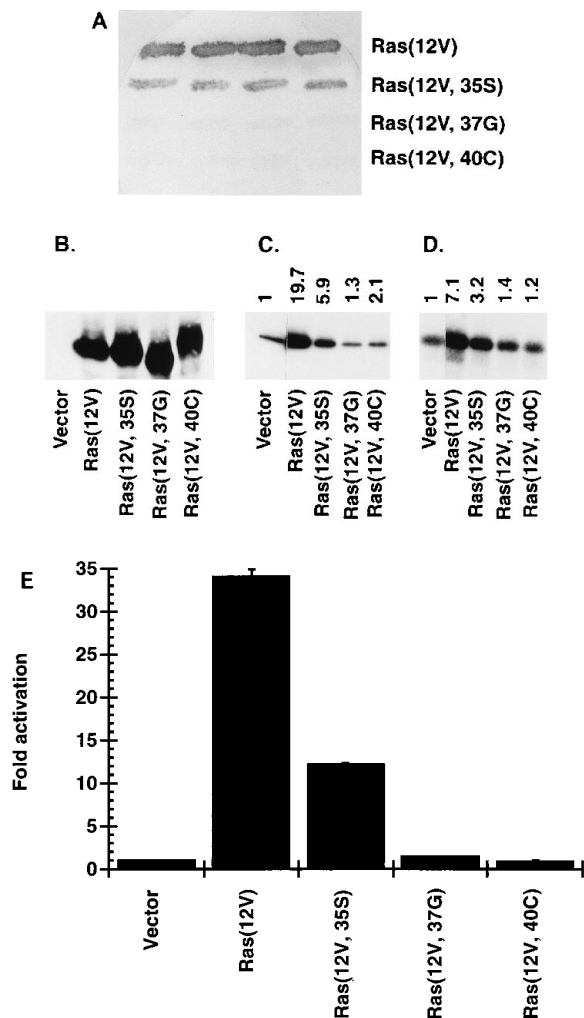


FIG. 1. H-Ras(12V, 35S), but not H-Ras(12V, 37G) or H-Ras(12V, 40C), interacts with and causes activation of Raf-1 and MAPKs and Elk-1 transcription. (A) Yeast two-hybrid analysis was done to determine if the different Ras effector domain mutants are impaired in their abilities to bind full-length Raf-1.  $\beta$ -Galactosidase activity was determined by filter assay. Yeast patches containing interacting protein pairs are dark, owing to  $\beta$ -galactosidase activity and X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) cleavage product. Yeast patches containing noninteracting protein pairs remain white, owing to the absence of detectable  $\beta$ -galactosidase activity. (B) Western blot analysis with the 146 anti-H-Ras-specific monoclonal antibody was done to quantitate the level of mutant Ras expression in each NIH 3T3(UNC) cell line transfected with the indicated expression construct. The data shown are representative of three independent Western blot analyses with lysates generated from equivalent numbers of cells expressing each mutant protein. (C) In vitro Raf kinase assays were done on immunoprecipitated Raf-1 from lysates of equivalent cell numbers of NIH 3T3(UNC) cells stably expressing each mutant H-Ras protein with a Raf-immunocomplex-coupled kinase assay. Raf-1 was immunoprecipitated with the C-12 Raf-1 polyclonal antibody (Santa Cruz Biotech) and then incubated with wild-type MEK1, and this incubation was followed by incubation with kinase-deficient p42 MAPK/ERK2(KR) in the presence of [ $\gamma$ - $^{32}$ P]ATP (2). Numbers indicate fold elevation relative to vector-transfected cells. The data shown are representative of four independent assays. (D) The level of activated MAPKs in lysates from NIH 3T3(UNC) cells stably expressing each mutant H-Ras protein was determined by an in vitro immunocomplex kinase assay with the C-16 anti-ERK1 (Santa Cruz Biotech) rabbit polyclonal antibody and by a subsequent incubation with myelin basic protein as a substrate in presence of  $\gamma$ - $^{32}$ P (2). Numbers indicate fold elevation relative to vector-transfected cells. The data shown are representative of four independent assays. (E) NIH 3T3(UNC) cells were transiently transfected with expression constructs encoding each H-Ras mutant (250 ng) along with 2.5  $\mu$ g of Gal-*luc* and 250 ng of Gal-*elk* plasmid DNAs to determine the activation of Elk-1 transcriptional activity in a luciferase assay with 100  $\mu$ l of ATP and luciferin reagent in a luminometer (29). The data shown are the fold activation for duplicate transfections and are representative of three independent assays.

TABLE 1. Interaction between Ras effector domain mutants and Ras binding partners<sup>a</sup>

Mutant protein	Raf-1	byr2	AF6/Rsb1 <sup>b</sup>	RalGDS	RGL/Rsb3 <sup>b</sup>	cyr1
H-Ras(12V)	+	+	+	+	+	+
H-Ras(12V, 35S)	+	-	±	+	+	-
H-Ras(12V, 37G)	-	+	+	+	+	+
H-Ras(12V, 40C)	-	-	+	-	-	-

<sup>a</sup> +, strong interaction; -, no interaction; ±, weak interaction.

<sup>b</sup> Isolated by yeast two-hybrid library screening as described previously (77).

resistant colonies) as well as clonal populations which were established from individual G418-resistant transformed colonies. Mass populations of cells expressing H-Ras(12V, 37G) or H-Ras(12V, 40C) showed enhanced growth properties and higher saturation densities compared with untransformed NIH 3T3(UNC) cells, comparable in magnitude to those observed for cells expressing H-Ras(12V) or H-Ras(12V, 35S) (Fig. 3). Additionally, mass populations of NIH 3T3(UNC) cells expressing H-Ras(12V, 37G) and H-Ras(12V, 40C) also proliferated in growth medium supplemented with low serum (1%) and formed colonies in soft agar (Table 2). Most importantly, cells expressing either Raf-binding defective mutant caused rapid tumor formation after being inoculated into nude mice (Table 2). Clonal populations derived from individual G418-resistant, transformed colonies that possessed comparable levels of protein expression also exhibited essentially the same enhanced growth properties in all of these growth assays (data not shown). These results clearly demonstrate that oncogenic Ras can cause potent tumorigenic transformation by activation of a Raf/MAPK-independent pathway(s). Furthermore, the potent growth transformation induced by H-Ras(12V, 37G) or H-Ras(12V, 40C) contrasts dramatically with their weak focus-forming activities and suggests that focus-forming potential is not an accurate measure of their growth transforming activities.

**H-Ras(12V, 37G) and H-Ras(12V, 40C) cause morphological transformation indistinguishable from that induced by constitutively activated Rho proteins.** H-Ras(12V, 37G) and H-Ras(12V, 40C) caused the appearance of transformed foci which were clearly distinct from the transformed foci induced by oncogenic H-Ras(12V) mutants (Fig. 4A and B). Whereas H-Ras(12V) or H-Ras(61L), as well as transforming mutants of Raf-1 (data not shown), caused the appearance of large, swirled foci that contained highly refractile and spindle-shaped cells, H-Ras(12V, 37G) and H-Ras(12V, 40C) caused the appearance of circle-shaped foci that contained densely packed nonrefractile cells (Fig. 4A). The appearance of these foci was indistinguishable from that of the transformed foci caused by constitutively activated mutants of Rho family proteins (RhoA, RhoB, and Rac1) (Fig. 4A) (37, 56) or Rho GEFs (e.g., Dbl and Vav) (35). Consistent with its retention of a Raf/MAPK signaling activity, H-Ras(12V, 35S) also induced Ras- or Raf-like transformed foci (Fig. 4A). Finally, the cellular morphology of isolated NIH 3T3(UNC) cells stably expressing H-Ras(12V, 37G) or H-Ras(12V, 40C) was also very distinct from the highly refractile, spindle-shaped morphology of H-Ras(12V)- or H-Ras(12V, 35S)-transformed cells. Instead, their cellular morphologies were essentially the same as those of cells transformed by activated RhoA(63L) (Fig. 4B) (35). Cells expressing either Raf-binding defective H-Ras mutant or RhoA(63L) retained the nonrefractile appearance and well adherent nature of untransformed NIH 3T3 cells.

Because the two Raf-binding defective mutants caused

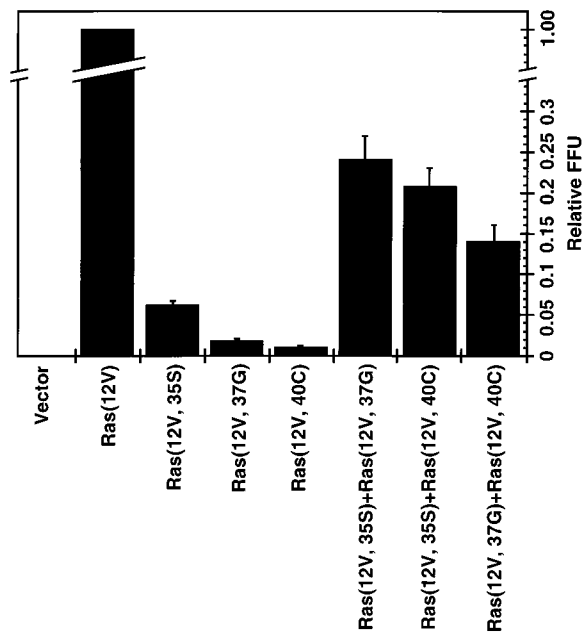


FIG. 2. H-Ras(12V, 37G) and H-Ras(12V, 40C) exhibit focus-forming activities which show synergistic cooperation. NIH 3T3(UNC) cells were transfected with plasmid constructs encoding the indicated Ras mutant proteins, either alone [10 ng per dish for H-Ras(12V) and 100 ng per dish for each effector domain mutant] or at a concentration of 50 ng for each effector domain mutant. The number of transformed foci was quantitated after 14 to 16 days. Focus-forming activities were normalized to the activities seen with H-Ras(12V) ( $6.16 \times 10^3$  foci per  $\mu\text{g}$  of transfected DNA). The data shown represent the average of three dishes and are representative of three independent experiments. FFU, focus-forming units.

transformed foci and cellular morphologies that were similar to those caused by transforming mutants of Rho proteins or Rho GEFs, we next analyzed the cytoskeletal organization of cells expressing these mutant proteins. We previously reported that whereas oncogenic Ras-transformed cells exhibit a loss of actin stress fibers and focal adhesions, RhoA- and Dbl-transformed cells retain well organized actin stress fibers and abundant focal adhesions (35). Indirect immunofluorescence analysis showed that like H-Ras(12V)-transformed cells, H-Ras(12V, 35S)-transformed cells exhibit disrupted actin stress fibers and reduced focal adhesions (Fig. 4C). In contrast, both H-Ras(12V, 37G)- and H-Ras(12V, 40C)-transformed cells retain organized actin stress fibers and abundant focal adhesions characteristic of untransformed NIH 3T3(UNC) cells (35). Thus, like constitutively activated mutants of RhoA, Rac1, Dbl, and Vav, H-Ras(12V, 37G) and H-Ras(12V, 40C) cause tumorigenic transformation without causing a concomitant loss of actin cytoskeletal organization.

**H-Ras(12V, 37G) and H-Ras(12V, 40C) activate JNK, as do members of the Rho family of proteins.** Recent studies demonstrated that two members of the Rho family (Rac1 and CDC42Hs), as well as Rho GEFs (e.g., Dbl), caused activation of the stress-activated protein kinase (SAPK/JNK), but not the p42 and p44 MAPKs, in transient transfection assays (13, 37, 49, 53). In contrast, oncogenic Ras caused strong activation of MAPKs and also stimulated SAPK/JNK. Since H-Ras(12V, 37G) and H-Ras(12V, 40C) induced transforming properties similar to those caused by mutant Rho proteins, we determined if these two mutants retained the ability to activate SAPK/JNK. Consistent with our previous observation with HeLa cells (83), H-Ras(12V) caused a 10-fold activation of

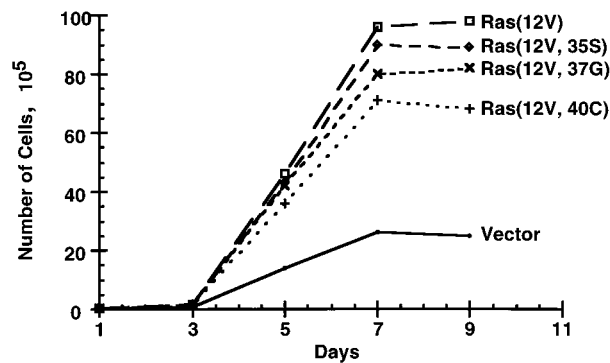


FIG. 3. NIH 3T3(UNC) cells expressing H-Ras(12V, 37G) and H-Ras(12V, 40C) show enhanced growth rates and saturation densities. Sixty-millimeter-diameter dishes were plated with  $10^5$  cells, and the number of cells was determined on days 1, 3, 5, and 7. The values represent the average of duplicate dishes.

JNK, whereas all three effector domain mutants retained partial ability to activate JNK (2.3- to 3.6-fold activation) in a transient transfection JNK assay in COS cells (Fig. 5A). We also observed that NIH 3T3(UNC) cells stably expressing H-Ras(12V, 37G) and H-Ras(12V, 40C) had approximately 10-fold higher levels of JNK activities than untransformed cells and that these activities were equivalent to those seen in H-Ras(12V)- as well as RhoA(63L)- and Rac1(115I)-transformed cells (Fig. 5B). These observations, when taken together with that of the Rho-like transformed phenotype caused by these two mutants, further suggest the convergence of Rho family protein-stimulated pathways in mediating, in part, the transforming actions of H-Ras(12V, 37G) and H-Ras(12V, 40C).

**Coexpression of the Raf-binding defective mutants of Ras with activated Raf(340D) caused synergistic enhancement of their focus-forming activities.** Consistent with the proposal that oncogenic Ras induces transformation by coordinated activation of Raf/MEK/MAPK and Rho protein pathways, we and others have observed that coexpression of activated Raf-1 with activated Rac1 or RhoA caused a dramatic synergistic enhancement in focus formation (37, 57, 58). Therefore, if the transforming pathways of H-Ras(12V, 37G) and H-Ras(12V, 40C) converge on the activation of Rho family proteins, we anticipated that coexpression of activated Raf(340D), which is a weakly transforming Raf mutant, would also synergistically enhance the focus-forming activities of Ras effector domain mutants.

Transfection of weakly activated Raf(340D) alone does not

TABLE 2. Transforming properties of Ras effector domain mutants in NIH 3T3(UNC) cells

Mutant protein	Growth in low serum <sup>a</sup>	Growth (%) in soft agar <sup>b</sup>	No. of sites forming tumors/no. of sites inoculated <sup>c</sup>
Vector	—	0.0	0/4
H-Ras(12V)	+	7.2	4/4
H-Ras(12V, 35S)	+	5.7	4/4
H-Ras(12V, 37G)	+	2.0	4/4
H-Ras(12V, 40C)	+	2.3	4/4

<sup>a</sup> Growth in Dulbecco modified Eagle medium supplemented with 1% calf serum. —, no colonies formed; +, colonies formed.

<sup>b</sup> Percentage of cells plated that formed colonies in 0.35% agar after 14 days.

<sup>c</sup> Progressive tumor formation (>1 cm in diameter) was detected for animals inoculated with  $10^5$  cells expressing H-Ras(12V), H-Ras(12V, 35S), H-Ras(12, 37G), and H-Ras(12V, 40C) after 10, 12, 20, and 20 days, respectively.

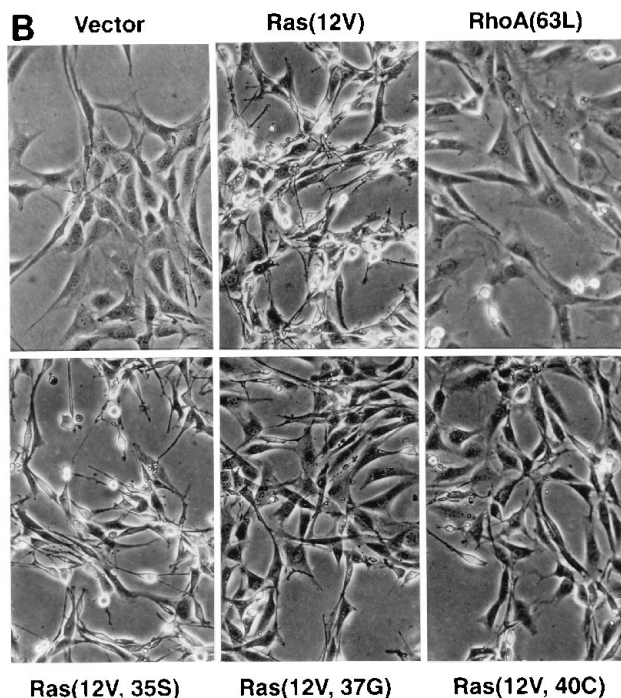
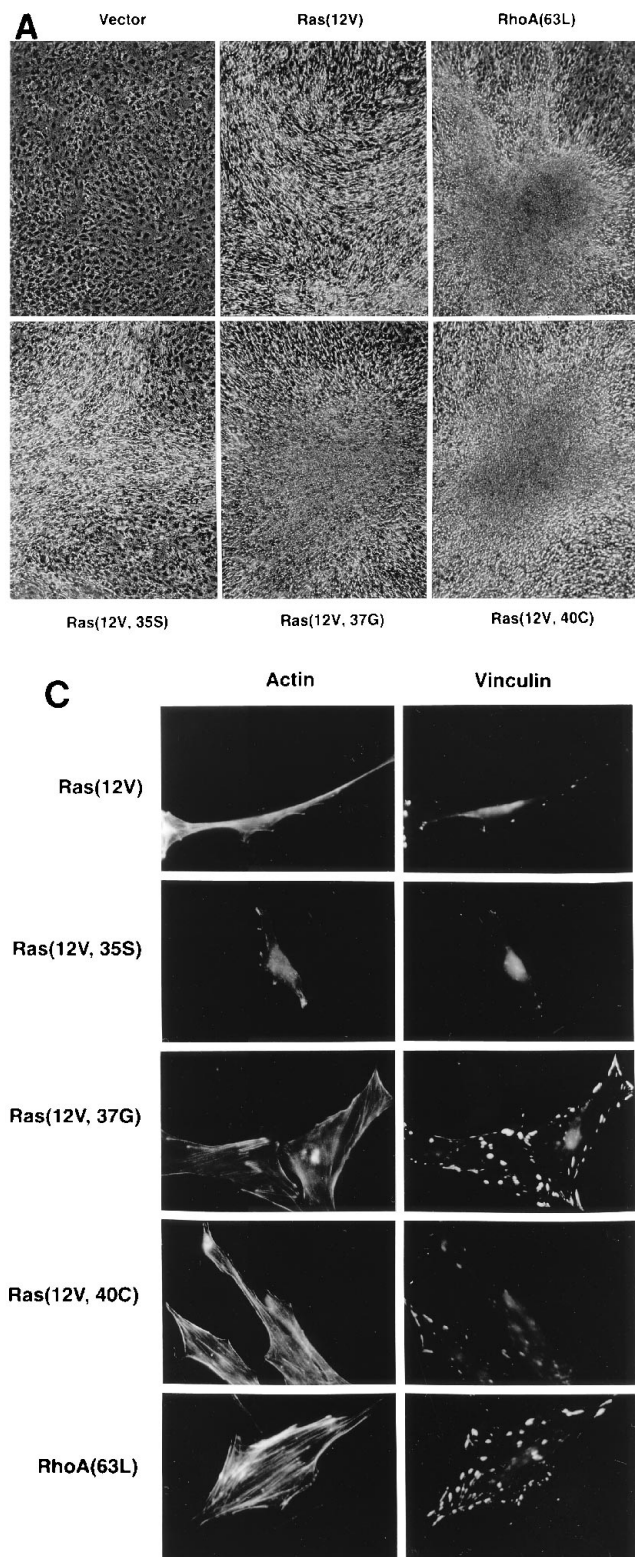


FIG. 4. H-Ras(12V, 37G) and H-Ras(12V, 40C) cause transformed focus and cell morphologies, as well as cytoskeletal organization changes, similar to those caused by transforming RhoA(63L). (A) Representative transformed foci from cultures transfected with expression plasmids encoding the indicated proteins. (B) The morphologies of NIH 3T3(UNC) cells stably transfected with plasmid constructs encoding the indicated proteins. Multiple G418-resistant colonies (>500) were pooled together to establish each cell line. (C) Actin stress fibers and focal adhesions were visualized by indirect immunofluorescence with phalloidin and antivinulin staining, respectively.

cause the appearance of transformed foci (37). However, when we cotransfected expression constructs of Raf(340D) together with constructs encoding H-Ras(12V, 37G) or H-Ras(12V, 40C), we observed a 7- to 10-fold synergistic enhancement of their focus-forming activities in NIH 3T3(UNC) cells (Fig. 6A

and B). A similar cooperation was also seen in both NIH 3T3 (CSHL) and NIH 3T3(UNC) transfection assays after the Raf-binding defective mutants were cotransfected with the Raf-BXB mutant, which is activated by an N-terminal truncation and removal of the Ras-binding domain (data not shown). In addition to enhanced focus-forming activities, we also observed that coexpression of Raf(340D) also restored a Ras-like appearance to the transformed foci induced by H-Ras(12V, 37G) (Fig. 6C) or H-Ras(12V, 40C) (data not shown). Thus, coexpression of activated Raf(340D) with either Raf-binding mutant restored both potent focus-forming activity and the appearance of transformed foci which are more characteristic of those seen with H-Ras(12V).

**Coexpression of the RhoA(19N) dominant inhibitory mutant blocked the focus-forming activities of H-Ras(12V, 37G) and H-Ras(12V, 40C).** We and others recently showed that coexpression of dominant negative mutants of RhoA, RhoB, and Rac1 impaired oncogenic Ras-transforming activity (37, 56, 58). Since the transformed phenotype caused by H-Ras(12V, 37G) and H-Ras(12V, 40C) was indistinguishable from those caused by activated Rho proteins, we suspected that coexpression of the RhoA(19N) dominant inhibitory mutant would inhibit the focus-forming activities retained by the two Raf-binding deficient Ras mutants. Because transfection of H-Ras(12V, 37G) or H-Ras(12V, 40C) alone exhibited poor focus-forming activity, we determined if coexpression of RhoA(19N) could inhibit the potent focus-forming activities of these two mutants after they were coexpressed with Raf(340D). As can be seen from Fig. 6D, RhoA(19N) greatly impaired the

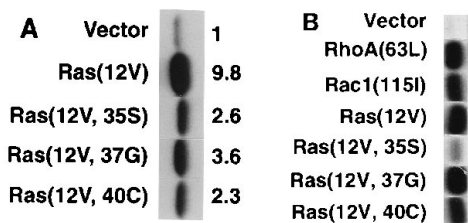


FIG. 5. H-Ras(12V, 37G) and H-Ras(12V, 40C) retained the ability to cause transient or constitutive stimulation of JNK activation. JNK activity was measured by immunocomplex kinase assays with transiently transfected COS (A) or stably transfected NIH 3T3(UNC) (B) cells with each pDCR-*ras* construct. Numbers represent fold activation relative to vector-transfected cells. The data shown are representative of three independent assays.

focus-forming activities seen in cultures cotransfected with Raf(340D) and either H-Ras(12V, 37G) or H-Ras(12V, 40C) but caused at most a partial inhibition (~20%) of H-Ras(12V, 35S) focus-forming activity. This result is consistent with the relative inability of RhoA(19N) to block the transforming Raf22W mutant (37). Thus, these results suggest that the transforming activities of the two Ras mutants defective in Raf binding require a RhoA or RhoA-related protein function.

**H-Ras(12V, 37G) and H-Ras(12V, 40C) cooperate and cause synergistic enhancement of focus-forming activity.** The binding activities of the H-Ras(12V, 35S), H-Ras(12V, 37G), and H-Ras(12V, 40C) mutants are all distinct (Table 1), suggesting that the mutants may each possess distinct effector pathways. Our previous observation that H-Ras(12V, 35S) and H-Ras(12V, 37G) can cooperate and show a synergistic enhancement (8- to 10-fold) of focus-forming activity in NIH 3T3(CSHL) cells (83) suggested that H-Ras(12V, 37G) retained a Raf-independent function that contributed to Ras transforming activity. To determine if H-Ras(12V, 40C) retained a distinct activity which could cooperate with H-Ras(12V, 35S) or H-Ras(12V, 37G), we performed cotransfection analyses with these mutants in NIH 3T3(UNC) cells. Cotransfection of H-Ras(12V, 40C) with either H-Ras(12V, 35S) or H-Ras(12V, 37G) caused greatly enhanced focus-forming activities that were three and fivefold higher, respectively, than the additive activities of each mutant analyzed alone (Fig. 2). Thus, H-Ras(12V, 40C) provided a complementary activity which cooperated with the activities of the other two effector domain mutants. It is unlikely that upregulation of the Raf/MEK/MAPK pathway is responsible for the synergistic transforming activity seen with these two Raf-binding defective effector domain mutants, since their coexpression failed to activate Elk-1 transcription activity (data not shown). Furthermore, since H-Ras(12V, 37G) and H-Ras(12V, 40C) are both defective in Raf-1 activation, their complementary activities suggest the existence of at least two distinct Raf-independent signaling pathways that mediate Ras transformation. This enhancement was quantitative, since the resulting foci retained a Rho-like appearance. Finally, cotransfection of all three effector domain mutants did not result in a transforming activity that was comparable to oncogenic Ras-induced transformation. So, collectively, the three mutants together are probably still deficient in other pathways that contribute to full Ras transformation.

## DISCUSSION

We have used two effector domain mutants of oncogenic Ras [H-Ras(12V, 37G) and H-Ras(12V, 40C)] which are impaired in their abilities to bind to Raf-1 in yeast two-hybrid protein-protein binding analyses and do not activate the Raf/

MAPK pathway in mammalian cells to investigate the contribution of Raf-independent pathways to the oncogenic potential of Ras. Our results clearly demonstrate that Ras can promote potent tumorigenic transformation and growth in soft agar and low serum by stimulation of Raf-independent signaling pathways. Furthermore, we observed that the transformed phenotype caused by these two Raf-binding defective Ras mutants was distinct from that caused by oncogenic H-Ras(12V) and, instead, was indistinguishable from the transformed phenotype caused by constitutively activated RhoA, RhoB, and Rac1 proteins. Moreover, the transforming activity of these two Ras mutants is blocked by dominant negative RhoA. Our results suggest that the transforming actions of these two Raf-binding mutants are convergent with and dependent upon Rho family proteins. We also observed that H-Ras(12V, 37G) and H-Ras(12V, 40C) cooperate in the induction of foci, indicating that each mutant retains a distinct Raf-independent activity, thus raising the possibility that activation of either of two Raf-independent Ras signaling pathways may lead to tumorigenic transformation. The identification of multiple numbers of candidate Ras effectors by yeast two-hybrid library screening (33, 38, 72, 77, 78), by association with Ras in mammalian cells (65), or by yeast genetic analyses (10, 27, 77) further supports the notion that Ras proteins interact with multiple effectors. The multiplicity of Ras pathways in mammalian cells is echoed by studies of simple eukaryotes (10, 84).

Since mutated *ras* sequences are frequently associated with human carcinomas, Ras-mediated activation of Raf-independent pathways may be critical for the tumorigenicity of these cells. In fact, focus assays with a single fibroblast cell line do not necessarily reflect the essential roles of Ras in tumors. We have observed variation in response to Ras mutants even among strains of NIH 3T3 cells. Although both H-Ras(12V, 37G) and H-Ras(12V, 40C) can cooperate with Raf-1 in NIH 3T3(CSHL), both mutants showed a complete loss of focus-forming activity in transfected NIH 3T3(CSHL) cells (83). Furthermore, preliminary results suggest that these mutants cannot induce tumorigenic transformation of NIH 3T3(CSHL) cells. These results contrast dramatically with our results with NIH 3T3(UNC) cells, which can be transformed by both Raf-dependent and Raf-independent Ras signaling pathways. Yet other cell lines exhibit different sensitivities. Both H-Ras(12V, 37G) and H-Ras(12V, 40C) caused focus formation of Rat-1 fibroblast and RIE-1 rat intestinal epithelial cells, whereas Ras-mediated activation of the Raf/MAPK pathway alone is not sufficient for transformation of this and other epithelial cell lines (52).

While we cannot rule out the possibility that very low levels of Raf kinase activity might be responsible for the transforming activities of H-Ras(12V, 37G) and H-Ras(12V, 40C) mutants, the transforming properties of these mutants were distinct from those of H-Ras(12V) and, instead, were indistinguishable from those of Rho family proteins and their activators. First, these proteins induced similar types of foci with little change in cell morphology and actin cytoskeletal organization. Second, while cells expressing Rho or either H-Ras(12V, 37G) or H-Ras(12V, 40C) exhibited potent growth-transformed properties, such as growth in low serum, soft agar, and nude mice, which were comparable to those seen with H-Ras(12V)-transformed cells, activated Rho and these mutant Ras proteins only weakly induced foci (37, 56, 58). Third, we recently observed that activated Raf and Rho proteins cooperated and cause synergistic transforming activity (37, 58), and both Raf-binding defective mutants of Ras also showed the ability to cooperate with activated Raf. Furthermore, the recent observation by Bottorff and colleagues has demonstrated that chi-



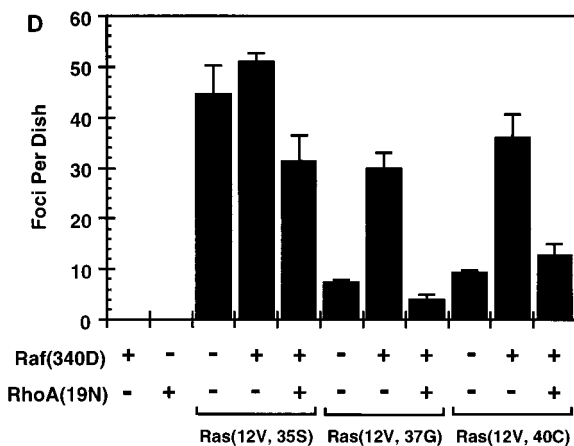
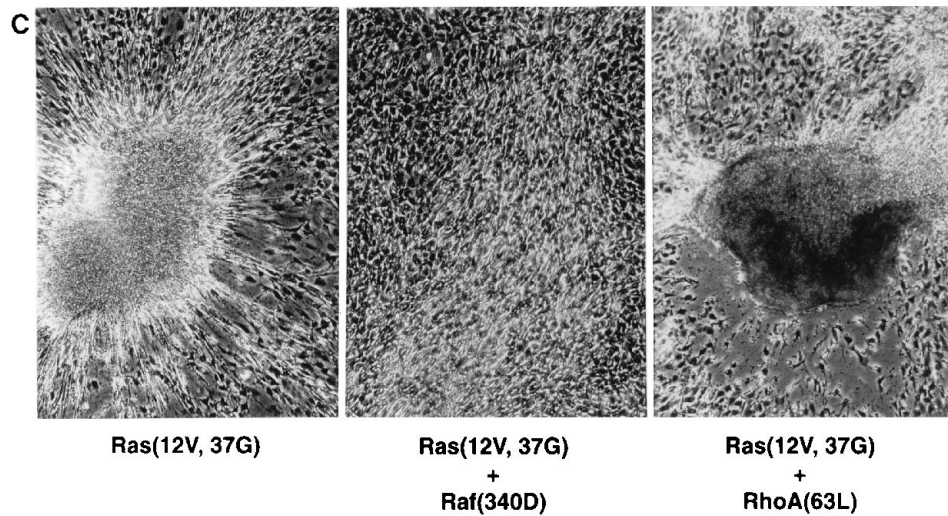
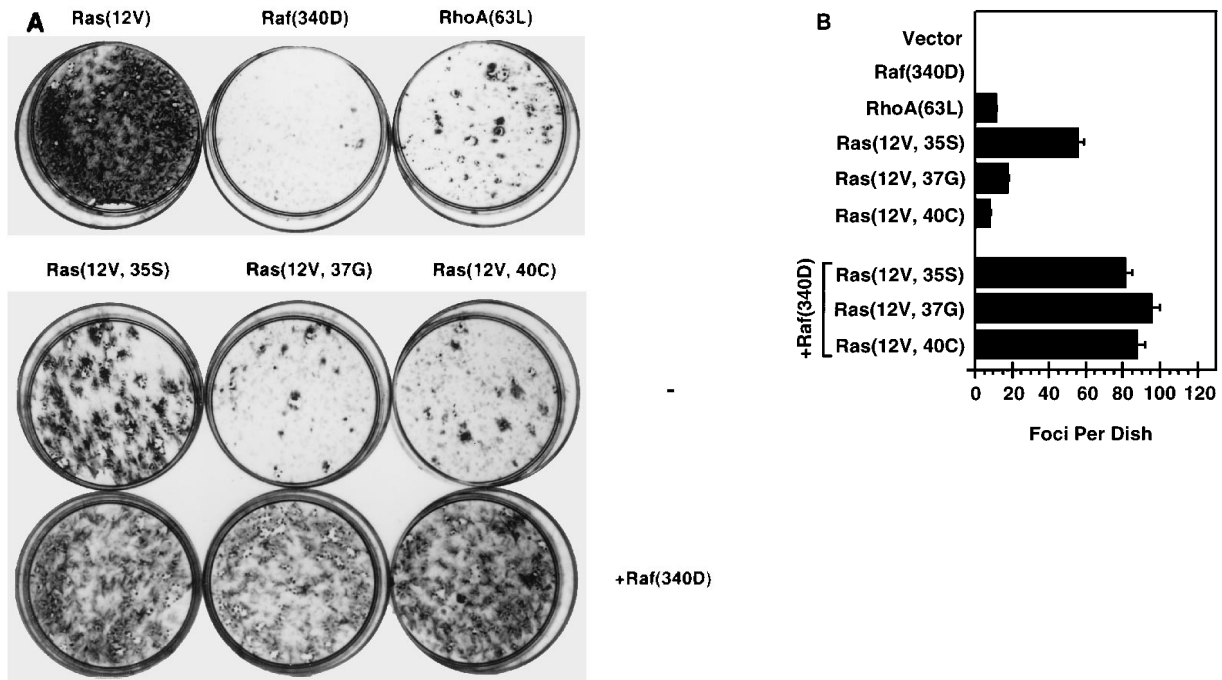


FIG. 6. Activated Raf(340D) and dominant negative RhoA(19N) caused differential abilities to modulate the transforming activities of each effector domain mutant. (A) NIH 3T3(UNC) cells were transfected with pDCR-*ras* encoding the indicated Ras protein, either alone or together with pZIP-*rhoA*(63L) or pZIP-*raf*(340D) plasmids. After 14 days, the dishes were stained with 0.1% crystal violet to better visualize the transformed foci. (B) Quantitation of the dishes shown in panel A. The data represent the average of three dishes and are representative of three independent assays. (C) The appearance of H-Ras(12V, 37G)-induced transformed foci, either alone or with cotransfection of pZIP-*raf*(340D) or RhoA(63L). (D) The ability of the RhoA(19N) dominant negative mutant to inhibit the synergistic focus-forming activity observed with the coexpression of Raf(340D) and each Ras mutant was determined. Transformed foci were quantitated after 14 days. The data represent the average of three dishes and are representative of three independent assays.

meras of Ras in which the effector domain is replaced by the RhoA, Rac1, or CDC42 effector regions can cause foci in a Rat-2 cell line containing a hyperactivated mutant of MEK1 (8). Since these chimeras are defective in Raf binding and activation, this observation further supports the model in which coordinate activation of Raf- and Rho-dependent pathways are required for cellular transformation. Fifth, coexpression of activated Raf converted the transformed morphology caused by RhoA(63L), as well as those of H-Ras(12V, 37G) and H-Ras(12V, 40C), to resemble that caused by activated H-Ras(12V). Sixth, the Raf-binding deficient mutants and activated Rho mutants showed the same ranges of host strain sensitivity: all induced foci and tumorigenicity in NIH 3T3 (UNC) but not in NIH 3T3(CSHL). Finally, both Rho and Ras mutants caused constitutive upregulation of JNK activity. Therefore, Ras and Rho most likely have convergent mechanisms of action.

Further experiments indicate that the Raf-independent mechanisms are dependent upon Rho or Rho-like proteins. For example, an interfering form of RhoA blocked focus development by Raf(340D) and H-Ras(12V, 37G) or Raf(340D) and H-Ras(12V, 40C) but did not block focus induction by H-Ras(12V, 35S) or activated Raf (37). Moreover, Rho protein function is required for full Ras transforming activity (37, 56, 58), strongly suggesting that Ras-induced Raf-independent pathways may themselves regulate Rho or Rho-mediated activities. Consistent with this idea, we have observed that yeast *S. pombe* *ras1* activates a pathway that involves a Rho GEF and Rho protein (10). Finally, microinjection analyses with Swiss 3T3 cells showed that oncogenic Ras caused membrane ruffling which was dependent on Rac1 function (62, 63). Further biochemical studies will be needed to rigorously test this particular hypothesis. Although it is possible that the G-37 or C-40 mutation has converted Ras into mutant proteins that directly bind Rho effectors, the significant sequence divergence of the Ras and Rho family protein effector domain sequences argue against this possibility.

Observations by others that Rho family proteins are regulators of actin cytoskeletal organization (11) have prompted suggestions that Ras-induced morphologic transformation may be mediated by the activities of Rho proteins. However, cells transformed by constitutively activated Rho proteins alone do not exhibit the highly refractile, elongated, and spindle-shaped morphology which is characteristic of Ras- or Raf-transformed cells (4, 37, 54, 58, 69). Furthermore, the loss of Raf binding results in Ras mutants which cause the same limited morphologic transformation seen with activated Rho proteins. Therefore, the activities which mediate Ras morphologic transformation more likely represent the action of Ras through the Raf/MAPK pathway. This possibility is supported by observations that constitutively activated mutants of MEK1 cause the same morphologic transformation caused by Ras (3, 45).

In summary, Ras appears to act through multiple pathways. These pathways can act independently, or synergistically, to cause cellular transformation. Cell type differences can influence the sensitivity to transformation by Raf and Raf-independent pathways. Whereas the Raf/MAPK pathway alone is sufficient to cause transformation of NIH 3T3 fibroblasts, Raf-independent pathways are essential for tumorigenic transformation of RIE-1 and other epithelial cells (52). Thus, it is important to resolve what these pathways are and to establish their contributions to tumorigenicity. The use of Ras effector domain mutant proteins which show differential impairments of their abilities to bind to distinct Ras effectors will be important in deciphering the complex nature of Ras-mediated signal transduction and transformation. As demonstrated by this and

our recent study (83), such mutants can be used to define the importance of a candidate effector target for Ras function. Furthermore, the tumorigenic profile of transgenic animals harboring mutants of Ras deficient in specific effector interactions will determine the contribution of each pathway to tumorigenesis. Establishing a role for Raf-independent signaling pathways in human carcinogenesis may identify new directions for antagonizing Ras for cancer treatment.

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