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Activation of Rac1, RhoA, and Mitogen-Activated Protein Kinases Is Required for Ras Transformation

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Although substantial evidence supports a critical role for the activation of Raf-1 and mitogen-activated protein kinases (MAPKs) in oncogenic Ras-mediated transformation, recent evidence suggests that Ras may activate a second signaling pathway which involves the Ras-related proteins Rac1 and RhoA. Consequently, we used three complementary approaches to determine the contribution of Rac1 and RhoA function to oncogenic Ras-mediated transformation. First, whereas constitutively activated mutants of Rac1 and RhoA showed very weak transforming activity when transfected alone, their coexpression with a weakly transforming Raf-1 mutant caused a greater than 35-fold enhancement of transforming activity. Second, we observed that coexpression of dominant negative mutants of Rac1 and RhoA reduced oncogenic Ras transforming activity. Third, activated Rac1 and RhoA further enhanced oncogenic Ras-triggered morphologic transformation, as well as growth in soft agar and cell motility. Finally, we also observed that kinase-deficient MAPKs inhibited Ras transformation. Taken together, these data support the possibility that oncogenic Ras activation of Rac1 and RhoA, coupled with activation of the Raf/MAPK pathway, is required to trigger the full morphogenic and mitogenic consequences of oncogenic Ras transformation.

The three Ras proteins (H-, K-, and N-Ras) are members of a large superfamily of regulatory proteins whose activities are controlled by regulated GDP/GTP cycling (3, 4). Ras activity is controlled by guanine nucleotide exchange factors (GEFs; SOS and RasGRF/CDC25) which promote formation of active Ras-GTP, as well as GTPase-activating proteins (GAPs; p120-GAP and NF1-GAP), which act as negative regulators and promote formation of inactive Ras-GDP. Whereas members of the Ras branch of this superfamily (e.g., R-Ras and TC21/R-Ras2) are regulators of signaling pathways that control cell growth and differentiation (11, 16, 24, 35), Rho family proteins control signaling pathways that regulate actin cytoskeletal organization (8, 15, 17). Rho proteins share approximately 30% amino acid identity with Ras proteins, and their GDP/GTP cycles are regulated by distinct Rho GAPs and GEFs (Dbl family proteins) (3, 36). To date, at least eight distinct Rho family proteins (RhoA, RhoB, RhoC, RhoG, Rac1, Rac 2, TC10, and CDC42Hs) have been identified in mammalian cells (4).

Considerable biochemical and genetic evidence suggests that a Raf-1-triggered cascade of serine/threonine kinases is important in mediating the mitogenic and transforming action of Ras proteins (29, 40). Ras proteins directly bind to and promote the activation of Raf-1, which in turn activates mitogen-activated protein kinase (MAPK) kinases (also designated MEKs), which then trigger the activation of p42 and p44 MAPKs. Activated MAPKs in turn translocate to the nucleus, where they phosphorylate and regulate the activities of nuclear transcription factors which cause changes in gene expression that control cell proliferation. The importance of this cascade to oncogenic Ras transformation is supported by observations that kinase-deficient mutants of Raf-1 and MEK can block Ras-mediated transformation (5, 10, 27) and that constitutively activated mutants of Raf-1 and MEK can cause malignant transformation (10, 27, 30). Furthermore, kinase-deficient mutants of MAPKs have also been shown to be inhibitors of Ras-mediated signaling events as well as Raf-mediated transforming activity (33, 45, 46). Thus, the activities of Raf and MEKs are essential for oncogenic Ras transformation. However, whether MAPKs are also critical for Ras transformation has not been demonstrated.

A number of experimental observations suggest that Ras may trigger its actions via pathways which are distinct from the Raf/MAPK pathway. For example, in addition to Raf, other candidates for Ras effector targets have been identified. These include the two Ras GAPs, which may function as both negative regulators and downstream targets of Ras (3, 24). Two-hybrid yeast studies have repeatedly identified RalGDS and RalGDS-related proteins, which function as GEFs for the Ras-related protein Ral (1), as putative Ras effectors (21, 26, 44). Finally, phosphatidylinositol-3-OH kinase has also been shown to complex to Ras and to exhibit properties of a Ras effector (41). Like Raf, these proteins show preferential binding to the active, GTP-bound form of Ras, and this association requires an intact Ras effector domain (Ras residues 32 to 40). However, the role of these putative Ras effectors in mediating oncogenic Ras signal transduction and transformation is presently not known.

Recent biochemical, biological, and genetic evidence has implicated a second Ras-mediated signaling pathway which is distinct from the Raf/MAPK pathway and involves Rho family proteins. First, Ridley et al. performed microinjection studies which showed that oncogenic Ras triggered the activation of Rac1 and RhoA in signaling pathways that regulated the actin cytoskeleton (37, 38). Oncogenic Ras was observed to trigger actin filament accumulation at the plasma membrane, to form membrane ruffles via a Rac-dependent process, and to be involved in actin stress fiber formation and focal adhesion de-

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velopment via a Rho-dependent process (37, 38). Activated Rac1 was shown to trigger membrane ruffling and Rho-dependent actin stress fiber and focal adhesion formation, while activated RhoA stimulated only stress fiber and focal adhesion formation. These observations suggested the existence of a cascade in which oncogenic Ras induces the activation of Rac1, which in turn activates RhoA (37, 38). However, whether these transiently activated changes in actin organization are important for stable Ras transformation has not been addressed. Second, genetic studies addressing the function of the *Schizosaccharomyces pombe* Ras homolog (ras1) showed that ras1 triggered two distinct signaling pathways (7). One involves the byr2 serine/threonine kinase, which is homologous to Raf, and the other involves a Rho family protein (CDC42sp) and a Rho GEF (Scd1). Furthermore, these studies showed that scd1 directly complexed with ras1 and displayed the properties of a downstream effector of ras1 function. Finally, White et al. recently described results which support the existence of a Ras effector-mediated signaling pathway which is distinct from Raf-mediated events yet is required for full Ras transformation (47). Taken together, these observations support a model in which Ras function is mediated by the activities of at least two distinct signaling pathways.

We describe results from three complementary approaches to address the possibility that Rac1 and RhoA function downstream of oncogenic Ras and that their activities are required for Ras transformation. We observed that coexpression of activated Raf with constitutively activated mutants of Rac1 and RhoA caused a dramatic synergistic enhancement of transforming activity, that dominant negative mutants of Rac1 and RhoA inhibited oncogenic Ras transforming activity, and that activated Rac1 and RhoA greatly potentiated oncogenic Ras-mediated morphologic transformation. Finally, we demonstrated that MAPK function is also required for Ras transformation. Taken together, these observations support the proposal that oncogenic Ras requires the coordinate activation of a Raf/MEK/MAPK pathway and a Rac/Rho pathway for full malignant transformation.

MATERIALS AND METHODS

Molecular constructs. Oligonucleotide-directed mutagenesis was used to generate mutant versions of human *rac1* and *rhoA* (provided by A. Hall) by *Taq* PCR-mediated DNA amplification approaches which we have described previously (23). The resulting mutated sequences were first verified by dideoxy sequencing and then introduced into the unique *Bam*HI site in the pZIP-NeoSV(x)1 retrovirus vector (Neo^r). pZIP-*ras*(61L) and pMUT-*ras*(61L) contain cDNA and genomic sequences, respectively, that encode a highly transforming mutant of the human H-Ras protein. The cDNA sequence encoding Raf(340D) (provided by D. Morrison) was removed from the pBS vector (13) and inserted into the *Bam*HI site of pZIP-NeoSV(x)1. pfos encodes the transforming viral Fos protein (provided by Charles Van Beveren). pCMV-based vector constructs encoding wild-type and kinase-deficient mutant (which contain a K-to-R mutation in their ATP-binding sites) human p42 and p44 MAPKs have been described previously and were provided by M. Cobb (39).

Cell culture and transformation assays. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. DNA transfections were done by the calcium phosphate precipitation technique as previously described (9). Cells were transfected with plasmid constructs encoding the indicated proteins at 10 ng per dish [Ras(61L)] or 2 μg per dish [Rac1(115I), RhoA(63L), or Raf(340D)]. Transformed foci were quantitated after 14 to 16 days. Transfected cultures were stained with 0.4% crystal violet to better visualize transformed foci. Transfected cultures were also maintained in growth medium containing G418 at 400 μg/ml (geneticin; GIBCO/BRL) to establish cell lines that stably expressed normal or mutant proteins. The growth properties of NIH 3T3 cells expressing wild-type or mutant Ras, Rac1, or RhoA proteins were compared in terms of their growth rates and saturation densities on plastic, their abilities to proliferate in low serum concentrations (1%) or soft agar (0.3%), and their ability to form tumors when inoculated into athymic nude mice (10^5 cells per site) by procedures that we have described previously (9).

The ability of Rac1, RhoA, and MAPK mutant proteins to modulate Ras(61L) or Fos focus-forming activity was determined by cotransfection focus inhibition assays by procedures similar to those that we have previously described for Raf dominant negative proteins (5). Cultures were transfected with either pMUT-1 plasmid DNA (10 ng per dish), pfos plasmid DNA (500 ng per dish) alone, or pfos plasmid DNA (500 ng per dish) plus plasmid DNA expression vectors encoding either wild-type or mutant Rac1, RhoA, or MAPK proteins. The appearance of transformed foci was quantitated after 14 to 16 days. Relative focus-forming units were calculated on the basis of the number of transformed foci present in control dishes, which were transfected with empty pZIP-NeoSV(x)1 retrovirus vector [4×10^3 to 6×10^3 foci per μg of transfected *ras*(61L) plasmid DNA]. Data shown are representative of two or more independent determinations, with each determination representing the average number of foci from four dishes.

TABLE 1. Focus-forming activities of Rac1 and RhoA mutant proteins

Protein	Substitution	Expression[a]	NIH 3T3 focus-forming activity[b]	Tumor growth in nude mice[c]
Ras(WT)	Wild type	+	0.00	0/4
Ras(61L)	61 (Gln→Leu)	+	1.00	4/4
Ras(116I)	116 (Asn→Ile)	+	0.22	3/3
Rac1(WT)	Wild type	+	0.00	0/6
Rac1(61L)	61 (Gln→Leu)	ND[d]	0.00	ND
Rac1(115I)	115 (Asn→Ile)	+	0.00[e]	6/6
RhoA(WT)	Wild type	+	0.00	0/6
RhoA(63L)	63 (Gln→Leu)	+	0.01	6/6
RhoA(117I)	117 (Asn→Ile)	ND	0.00	ND

[a] Expression of exogenously introduced *ras*, *rac1*, and *rhoA* cDNAs was determined by Western blot analysis as described in Materials and Methods. Whereas we found that untransfected NIH 3T3 cells showed very low (±; H-Ras and Rac1) or no (−; RhoA) detectable levels of endogenous protein, we readily detected severalfold-higher levels (+) of protein expression in the stably transfected mass populations.

[b] Focus-forming activities in NIH 3T3 transfection assays were normalized to the activities observed with Ras(61L) (4×10^3 to 6×10^3 foci per μg of plasmid DNA = focus-forming units).

[c] Number of animals positive for tumor growth/number of animals inoculated. Data are from two independent assays. Ras(61L)-transformed cells formed large tumors (~1 cm in diameter) after 1 to 2 weeks, whereas Rac1(115I)- and RhoA(63L)-transformed cells caused large tumors (~1 cm in diameter) after 10 to 12 weeks.

[d] ND, not determined.

[e] Rac1(115I) showed focus-forming activity only after G418 selection of transfected cells.

Protein expression. Protein expression from the exogenously introduced *ras*, *rac1*, or *rhoA* cDNA sequences were determined by Western immunoblot analyses of G418-selected cell lysates. H-Ras expression was determined with the 146-3E4 mouse anti-H-Ras monoclonal antibody (Quality Biotech). RhoA expression was determined with the RhoA (119) rabbit anti-RhoA polyclonal antiserum, which recognizes RhoA residues 119 to 132 but does not recognize the closely related RhoB or RhoC proteins (Santa Cruz Biotechnology). Rac1 expression was determined with the Rac1 (C-11) rabbit polyclonal antiserum, which recognizes the Rac1 C terminus but does not recognize Rac2 or other Ras-related proteins (Santa Cruz Biotechnology). Antibody was detected by enhanced chemiluminescence (Amersham).

Transient-transfection CAT assays. To compare the ability of Ras, Rac1, or RhoA proteins to induce transcriptional activation of Ras-responsive promoter elements, NIH 3T3 cells were transiently cotransfected with 0.5 μg of plasmid DNA encoding mutant Ras or 5 μg of plasmid DNA encoding normal or mutant Rac1 or RhoA proteins together with 1 μg of the pB4X-CAT reporter plasmid (provided by B. Wasylyk) (6). This reporter plasmid contains the chloramphenicol acetyltransferase (CAT) gene driven by a minimal promoter that contains four tandem copies of the Ras-responsive promoter element from the polyomavirus enhancer (*ets*/AP-1 sequences). At 48 h after transfection, cell lysates were prepared, and the CAT activity induced by each *ras*, *rac1*, or *rhoA* construct was assayed as described previously (20).

MAPK activation. Activation of the MAPK pathway in NIH 3T3 cells expressing Ras(61L), Rac1(115I), or RhoA(63L) was determined essentially as described previously (46). Briefly, total-cell extracts were prepared in Laemmli sample buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to Immobilon membranes (Millipore) for analysis by Western blotting with the 691 anti-MAPK antiserum (Santa Cruz Biotechnology) to detect the phosphorylated active and nonphosphorylated inactive forms of $p42^{MAPK}$/ERK2 and $p44^{MAPK}$/ERK1.

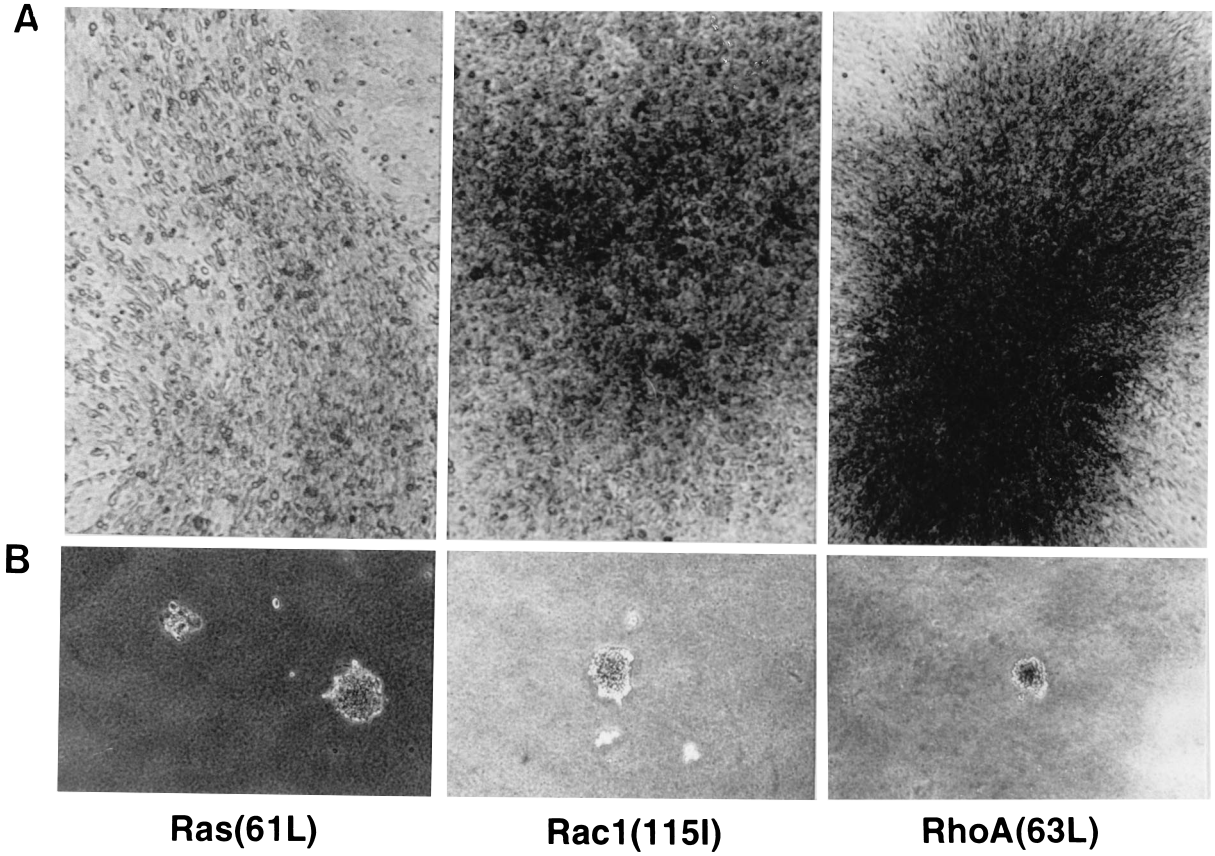


FIG. 1. Aberrant Rac1 and RhoA function causes transformation of NIH 3T3 cells. (A) Rac1(115I)- and RhoA(63L)-induced foci are distinct from Ras(61L)-transformed foci. (B) Rac1(115I)- and RhoA(63L)-transformed NIH 3T3 cells form colonies in soft agar. Colonies from Ras(61L)-transformed cells are shown after 10 days, whereas colonies from Rac1(115I)- or RhoA(63L)-transformed cells are shown after 30 days. Ras(61L)-transfected cells showed 5 to 10% colony-forming efficiency, whereas Rac1(115I)- and RhoA(63L)-transfected cells showed 0.1 to 0.5% and 0.5 to 1% frequencies, respectively.

Cell motility analysis. NIH 3T3 cells stably expressing the indicated proteins were plated at low density onto 60-mm tissue culture dishes and incubated overnight in growth medium. The growth medium was then removed and replaced with serum-free medium. Cell movement was then recorded with a time-lapse video recorder with a 60-fold time compression. The rates of cell motility were calculated by measuring the displacement of individual cells at 37°C at 10-min intervals over a 1-h period. Rates are reported as an average of values for at least 20 samples.

RESULTS

Constitutively activated mutants of Rac1 and RhoA exhibit weak focus-forming activity in NIH 3T3 cells. Our first approach to defining the contribution of Rac1 and RhoA function to Ras transforming activity was to determine the biological consequences of constitutive Rac1 and RhoA activities in NIH 3T3 cells. For these studies, we generated mutant versions of these two Rho family proteins which contained single amino acid substitutions analogous to those that activate Ras transforming potential (Table 1). Ras(61L) and Ras(116I) are constitutively activated mutants as a consequence of impaired intrinsic and GAP-stimulated GTPase activities or greatly enhanced GDP-GTP exchange rates, respectively. The transforming potentials of the Rac1 and RhoA mutants were then compared with those of constitutively activated forms of Ras in NIH 3T3 focus formation assays.

As described previously (11a), Ras(61L) showed potent focus-forming activity when transfected at 10 ng of plasmid DNA per dish ($>4 \times 10^3$ foci per μg). In contrast, we observed no focus-forming activity when plasmid constructs encoding wild-type or mutant Rac1 proteins were transfected at concentrations of up to 2 μg of DNA per dish (Table 1). Similarly, no focus-forming activity was observed in cultures transfected with vectors encoding RhoA(WT) or RhoA(117I). However, we did observe a very low frequency of transformed foci in cultures transfected with pZIP-*rhoA*(63L). This activity was approximately 60-fold lower than that observed with Ras(61L), and the appearance of RhoA(63L)-induced foci was very distinct from that of Ras(61L)-induced foci (Fig. 1A). Whereas Ras(61L) caused the appearance of foci which had a swirled appearance and contained very refractile, elongated, spindle-shaped cells, RhoA(63L)-induced foci were more compact and consisted of densely packed cells that were not very refractile in appearance.

Although initially we saw no focus-forming activity for any of the Rac1 mutant proteins, we did observe the appearance of transformed foci from NIH 3T3 cells stably expressing the Rac1(115I) mutant. These transformed foci appeared in cultures of cells which were plated from pooled populations of G418-resistant colonies after transfection with pZIP-*rac1*(115I). Rac1(115I)-induced foci showed a similar appearance to RhoA(63L)-induced foci (Fig. 1A). Because these transformed foci occurred after only one passage of the G418-resistant colonies, we suspect that these morphologically transformed subpopulations may represent cells that express higher levels of the mutant Rac1 protein rather than cells that had acquired secondary changes to complement Rac1(115I) activity. Rac1(115I) focus-forming activity was comparable to the

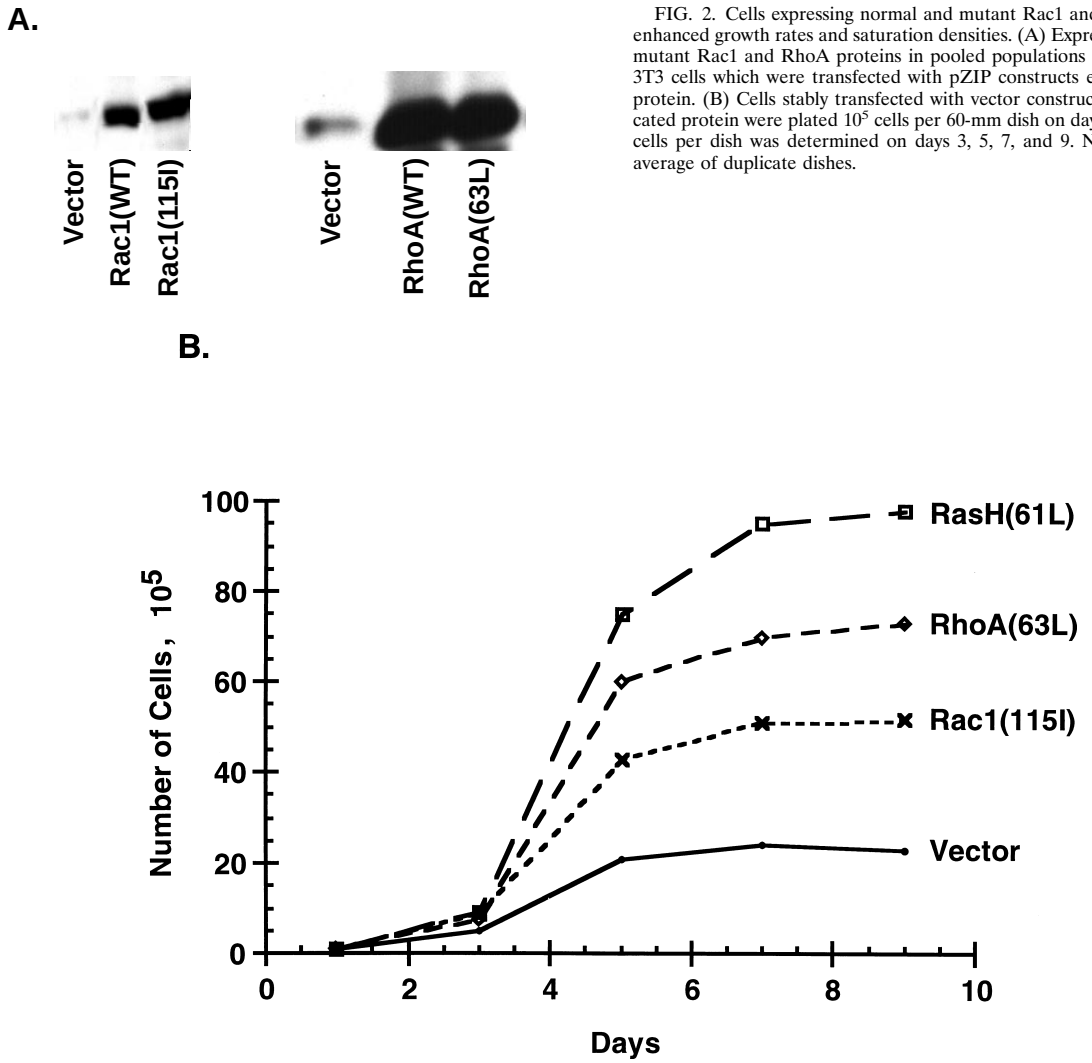


FIG. 2. Cells expressing normal and mutant Rac1 and RhoA proteins show enhanced growth rates and saturation densities. (A) Expression of wild-type and mutant Rac1 and RhoA proteins in pooled populations of G418-selected NIH 3T3 cells which were transfected with pZIP constructs encoding the indicated protein. (B) Cells stably transfected with vector constructs expressing the indicated protein were plated 10^5 cells per 60-mm dish on day 1, and the number of cells per dish was determined on days 3, 5, 7, and 9. Numbers represent the average of duplicate dishes.

potency that we have observed for previously described transforming mutants of MEK (10, 30).

Expression of activated Rac1(115I) and RhoA(63L) enhances the growth of NIH 3T3 cells. The weak focus-forming activity observed with Rac1(115I)- and RhoA(63L)-transfected cultures suggested that these two mutant proteins altered the growth properties of NIH 3T3 cells. To evaluate this possibility, we compared the in vitro and in vivo growth properties of cells expressing either wild-type or mutant versions of these two proteins with Ras(61L)-transformed cells. To avoid clonal variations in our analyses, we used pooled populations of multiple G418-resistant colonies (>100 colonies), rather than isolated transformed foci, for the different biological assays. Parallel cultures of NIH 3T3 cells stably transfected with the empty pZIP-NeoSV(x)1 vector or pZIP-*ras*(61L) were also established and used as control cell lines for these analyses. Protein expression from the exogenously introduced *ras*, *rac1*, and *rhoA* cDNAs was confirmed by Western blot analysis and showed levels which were severalfold above the endogenous levels (Fig. 2A; Table 1).

Cells stably expressing Rac1(115I) or RhoA(63L) did not exhibit the same morphologic transformation observed with Ras(61L)-transformed cells. Instead, these cells retained the poorly refractile and highly adherent properties which are characteristic of untransformed NIH 3T3 cells. In contrast, we observed that like Ras-transformed cells, cells stably expressing Rac1(115I) or RhoA(63L) formed colonies in soft agar (Fig. 1B), displayed increased growth rates and saturation densities (Fig. 2B), and proliferated in low serum concentrations (1%) (data not shown). However, Ras(61L)-transformed cells typically showed more enhanced growth potential than did either Rac1(115I)- or RhoA(63L)-transformed cells in each of these growth assays. For example, whereas Ras(61L)-transformed cells formed large soft agar colonies within 2 weeks, Rac1(115I)- and RhoA(63L)-transformed cells formed smaller colonies after 4 weeks. Our observations with RhoA(63L)-transformed cells are consistent with previous observations that RhoA exhibits weak transforming potential (2, 34, 42).

We also evaluated the ability of the different cell populations to form tumors when inoculated into athymic nude mice (Table 1). As described previously, Ras(61L)-expressing cells showed progressive tumor formation (~1 cm in diameter) within 1 to 2 weeks whereas Ras(WT)-expressing cells failed to show any tumor formation for up to 8 weeks (11, 16). RhoA (63L)- and Rac1(115I)-expressing cells formed progressive tumors (~1 cm in diameter) after 10 to 12 weeks, whereas cells

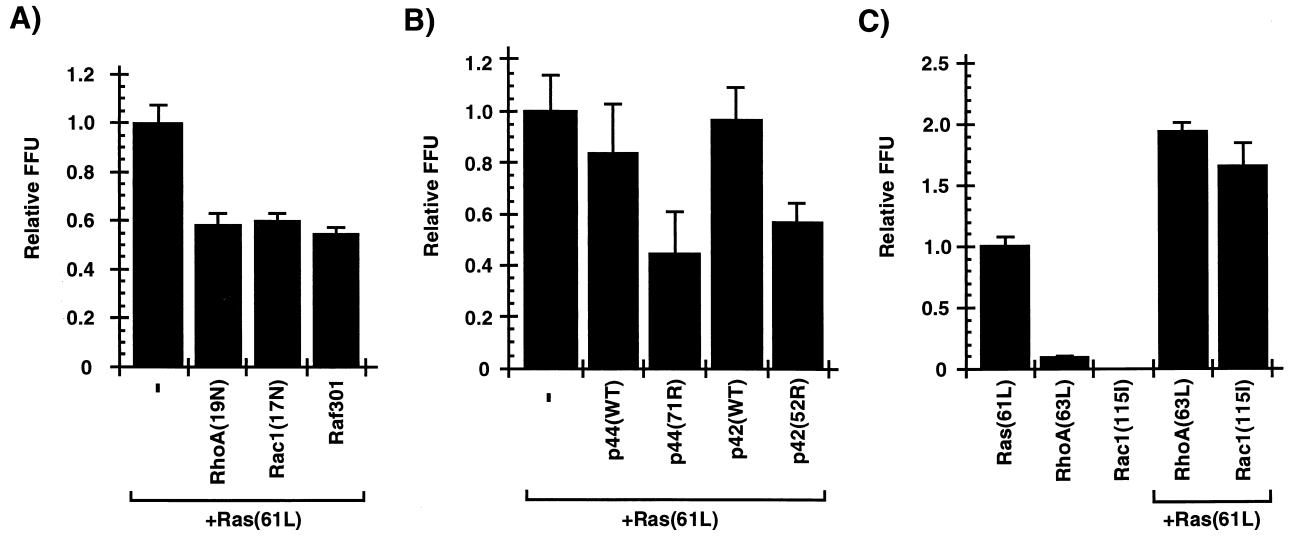


FIG. 3. Requirement for Rac1, RhoA, and MAPKs for Ras or Raf transformation. (A) Dominant negative mutants of Rac1 and RhoA reduce Ras(61L) focus-forming activity. NIH 3T3 mouse fibroblasts were transfected with 50 ng of pMUT-1 DNA [encoding RasH(61L)] per dish, either alone or together with 1 μg of pZIP-NeoSV(x)1 expression constructs of dominant negative Rac1(17N), RhoA(19N), or Raf301. (B) Cotransfection of kinase-deficient but not wild-type MAPKs causes inhibition of Ras(61L) focus-forming activity. (C) RhoA(63L) and Rac1(115I) potentiate oncogenic Ras(61L) focus formation.

transfected with the empty-vector control remained negative for tumor growth for up to 12 weeks. Thus, like Ras(61L), both Rac1(115I) and RhoA(63L) enhanced growth potential and caused malignant transformation of NIH 3T3 cells. Finally, our observation that tumor-derived cell lines showed higher expression of Rac1(115I) and RhoA(63L) is consistent with a transforming action of these two mutated proteins (data not shown).

Constitutively activated mutants of Rac and Rho are not activators of the Raf/MAPK pathway. Since it has been shown that Rac1 and RhoA function downstream of Ras (37, 38), we determined whether constitutively activated forms of these proteins could trigger downstream signaling events associated with oncogenic Ras-mediated activation of the Raf/MAPK pathway. Oncogenic but not normal Ras can activate transcription from reporter plasmids that contain Ras-responsive promoter elements upstream of the CAT gene (20), and dominant negative mutants of Raf and MAPKs can block this activity (5, 46). For these analyses, we used a reporter plasmid in which CAT gene expression is regulated by a minimal promoter which contains the *ets*/AP-1 Ras-responsive promoter in transient-transfection assays. Whereas oncogenic Ras(61L) caused a 4- to 13-fold activation of transcription, no activation was observed with wild-type or mutant versions of *rac1* or *rhoA* genes (data not shown). Finally, we also determined whether Rac1(115I)- or RhoA(63L)-transformed cells possessed the constitutively activated levels of p42 and p44 MAPKs that we and others have observed in Ras-transformed NIH 3T3 cells (11, 16, 22). In contrast to Ras(61L)-transformed cells, neither RhoA(63L)- nor Rac1(115I)-transformed cells showed significant levels of activated MAPKs (data not shown). Thus, both Rac1 and RhoA may cause transformation via pathways which are distinct from the Ras-activated Raf/MAPK pathway.

Dominant negative mutants of Rac1 and RhoA inhibit Ras transformation. If Rac1 and RhoA activation is required for Ras transforming activity, we anticipated that blocking the functions of Rac1 and RhoA should impair Ras-mediated transformation. Consequently, we generated dominant inhibitory mutants of these two proteins, analogous to the Ras(17N) dominant inhibitory protein (14), designated Rac1(17N) and RhoA(19N). Rac1(17N) has previously been shown to function as a dominant negative inhibitor of Rac function (38). NIH 3T3 cells were then transfected with expression constructs of Ras(61L), either alone or together with Rac1(17N) or RhoA (19N). Whereas cultures transfected with Ras(61L) alone caused the appearance of more than 60 transformed foci per dish, cotransfection with the Rac1 and RhoA mutants caused an approximately 50% reduction in transformed foci (Fig. 3A). This degree of inhibition is comparable to the inhibition seen with the well-characterized Raf301 dominant negative mutant (Fig. 3A) (5). The incomplete inhibition seen with the Raf, Rac1, and RhoA dominant negative mutants reflects in part their incomplete cotransfection into all cells that acquire Ras and in part the likelihood that each dominant negative mutant alone will impair only a subset of the multiple signaling events required for full Ras transforming activity.

Finally, we observed that NIH 3T3 cells stably expressing Ras(61L) and either Rac1(17N) or RhoA(19N) (Fig. 4A) showed a partial reversion of the morphologic transformation caused by the expression of Ras(61L) alone (Fig. 4B). Similar to untransformed NIH 3T3 cells, these coselected cells lacked the spindle-shaped, highly refractile morphology of Ras(61L)-transformed cells. The partial inhibition of Ras transformation may reflect the likelihood that Rac1 and RhoA dominant negative mutants will impair only a subset of signaling events required for full Ras transformation. Nevertheless, these results are consistent with the focus inhibition data and suggest that Rac1 and RhoA activities are required for full Ras transforming activity.

MAPKs are essential for oncogenic Ras transforming activity. Our observations with Rac1 and RhoA suggested that a Ras-mediated signaling pathway involving these Ras-related proteins, together with the Raf/MAPK pathway, is required for full Ras transformation. However, although constitutively activated MAPKs are observed in Ras-transformed NIH 3T3 cells (11, 16, 22), it has not been established whether their activities are required for full oncogenic Ras transformation. We and others have previously shown that kinase-deficient mutants of p42 and p44 MAPKs can function as dominant inhibitory proteins and block Ras-mediated signal transduction

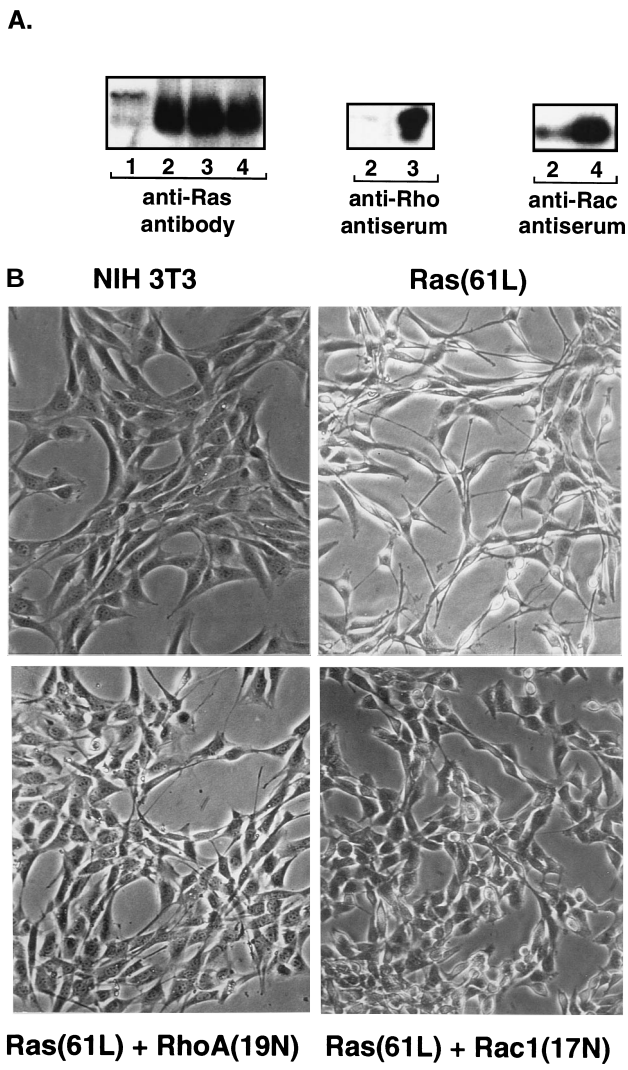


FIG. 4. Coexpression of Rac1(17N) and RhoA(19N) decreases oncogenic RasH(61L)-induced morphologic transformation. NIH 3T3 cells were transfected with 2 μg of pMUT-1 (Neo^s) and 50 ng of pZIP-Rac1(17N) or pZIP-RhoA(19N) (both Neo^r). Similar to the potent growth-inhibitory activity of the Ras(17N) dominant negative mutant, only rare G418-resistant colonies were detected in drug-selected NIH 3T3 cells transfected with the Rac1(17N) or RhoA(19N) expression vectors, indicating that both mutant proteins were also growth inhibitory (results not shown). Therefore, isolation of G418-resistant colonies was observed only when these expression constructs were cotransfected with the Neo^r Ras(61L) expression plasmid. (A) Transfected cells were maintained in growth medium supplemented with 400 μg of geneticin (G418) per ml to select for the cells that are expressing Ras(61L) and the dominant negative mutant proteins. Western blot analysis showed expression of dominant negative Rac1(17N) and RhoA(19N) proteins in cells which coexpress oncogenic RasH(61L). Lanes: 1, vector only; 2, Ras(61L); 3, Ras(61L) plus RhoA(19N); 4, Ras(61L) plus Rac1(17N). (B) Approximately 30% of the cotransfected cells showed a flatter, less refractile morphology. Shown is a representative clonal population of cells which coexpress Ras(61L) and the indicated dominant negative protein.

and Raf transforming activity (33, 45, 46). To extend these observations, we performed cotransfection studies to determine if coexpression of kinase-deficient MAPKs can inhibit Ras transformation. Whereas cotransfection with expression vectors encoding wild-type p42 or p44 MAPKs did not reduce Ras(61L) focus-forming activity, cotransfection with either kinase-deficient p42(52R) or p44(71R) caused a 50% reduction in Ras(61L)-induced focus-forming activity (Fig. 3B). This degree of inhibition is comparable to what we and others have seen with kinase-deficient Raf dominant negative proteins. Finally, no reduction in the number of G418-resistant colonies or reduction in v-*fos* focus-forming activity was observed in NIH 3T3 cultures transfected with expression vectors encoding these mutant MAPKs. Thus, the reduction in Ras focus formation is not likely to be a consequence of a nonspecific inhibitory action. These results suggest that oncogenic Ras activation of the Raf/MAPK pathway, as well as Rac1 and RhoA, is required for full Ras transformation.

Coordinate expression of activated Rac1 or RhoA with Raf causes synergistic enhancement of transforming activity. Our observation that dominant negative mutants of Rac1 and RhoA, as well as MAPKs, blocked Ras focus-forming activity suggested that the coordinate activation of a Raf/MAPK and a Rac/Rho pathway may be required for full oncogenic Ras transforming activity. If this is so, we anticipated that the coordinate expression of activated Raf, together with activated Rac1 or RhoA, may cause a synergistic enhancement of transformation. For these experiments, we used a weakly transforming mutant of human c-Raf-1, designated Raf(340D) (13). The Raf-1 tyrosine residue at position 340 is phosphorylated in response to the activation of various activated tyrosine kinases, and the substitution of a charged residue (Y340D) at this position has been shown to activate Raf transforming potential (13).

For these assays, expression plasmids for Rac1(115I), RhoA (63L), or Raf(340D) were transfected at DNA concentrations that resulted in very low or no focus-forming activity (Fig. 5). However, we observed that cotransfection of either Rac1(115I) or RhoA(63L) with Raf(340D) resulted in a dramatic enhancement of focus-forming activity. Coexpression of Rac1(115I) with Raf(340D) resulted in the appearance of more than 20 foci per dish, whereas parallel dishes transfected with either alone did not show any transformed foci (Fig. 3C and 5). Similarly, the very weak focus-forming activity observed with RhoA(63L) alone was enhanced more than 35-fold when it was cotransfected with nontransforming levels of Raf(340D) (Fig. 5). These results are consistent with a model in which oncogenic Ras requires the coordinate activation of a Raf- and a Rho-mediated pathway for full transforming potential.

Activated Rac1(115I) and RhoA(63L) enhances oncogenic Ras(61L) morphologic transformation. To evaluate the contribution of activated Rac1 and RhoA to the transformed phenotype of oncogenic Ras-expressing cells, we determined if the coordinate expression of activated Rac1(115I) or RhoA(63L) would further enhance any properties of oncogenic Ras-transformed cells. First, although Ras(61L) already showed potent focus-forming activity, we observed that cotransfection of activated Rac1(115I) or RhoA(63L) caused a 70 to 100% enhancement of Ras(61L) focus-forming activity (Fig. 3C). We also found that the appearance of the transformed foci was dramatically altered in these cotransfection assays. Whereas Ras (61L)-induced foci contained very refractile, spindle-shaped cells, cotransfection with either Rac1(115I) or RhoA(63L) caused the appearance of the transformed foci was dramatically altered in these cotransfection assays. Whereas Ras(61L)-induced foci contained very refractile, spindle-shaped cells, cotransfection with either Rac1(115I) or RhoA(63L) caused the appearance of transformed foci that contained very rounded, refractile, and poorly adherent cells (Fig. 6A and B). These striking alterations in cell morphology were unexpected in light of the fact that we saw only limited alterations in cell morphology in cells transformed by either Rac1(115I) or RhoA(63L) alone.

We also determined the consequences of Rac1(115I) or

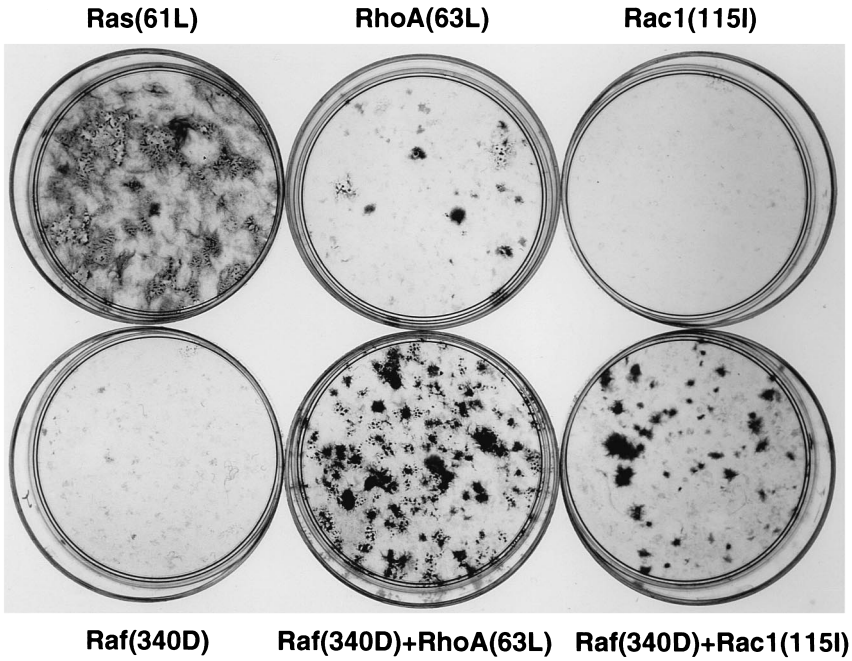


FIG. 5. Cotransfection of activated Rac1(115I) or RhoA(63L) with activated Raf(340D) causes synergistic enhancement of focus-forming activity. Whereas transfection of 1 μg of pZIP-*raf*(340D), which encodes a weakly transforming Raf mutant protein (13), alone showed no focus-forming activity, cotransfection with constitutively activated Rac1(115I) or RhoA(63L) showed greatly enhanced focus-forming activities. Cultures transfected with either Rac1(115I) or RhoA(63L) showed no and low focus-forming activity, respectively. Representative dishes were stained with crystal violet to visualize transformed foci.

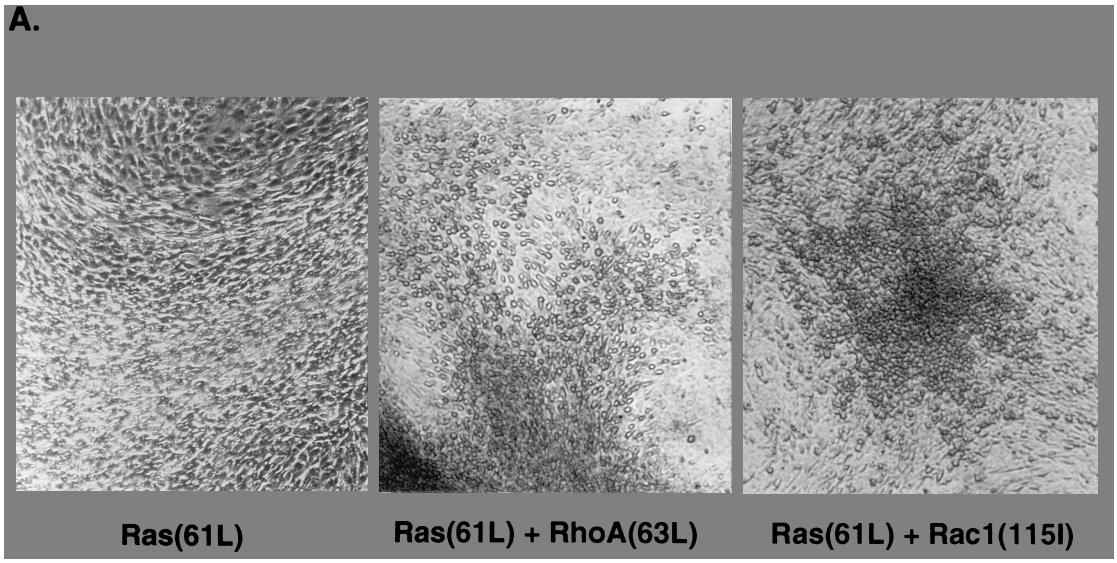
RhoA(63L) coexpression on the properties of Ras-transformed cells. First, we observed that their coexpression enhanced the frequency, size, and appearance of Ras(61L)-transformed colonies in soft agar (Fig. 6C). In particular, whereas colonies formed by Ras-transformed cells were round clusters that possessed a smooth and uniform surface, cells coexpressing either Rho family protein showed uneven surfaces that contained extensions which suggested that they exhibited an enhanced ability to migrate. Consistent with this, we observed that cells coexpressing Rac1(115I) and Ras(61L) showed greater motility than did cells expressing either protein alone (Fig. 6D). Finally, time-lapse video analysis showed that cells coexpressing Rac1(115I) and Ras(61L) displayed significantly more membrane-ruffling activity than did cells transformed by either protein alone (data not shown). Thus, constitutive activation of Rac1 and RhoA greatly potentiated several aspects of the transformed phenotype of Ras-transformed cells.

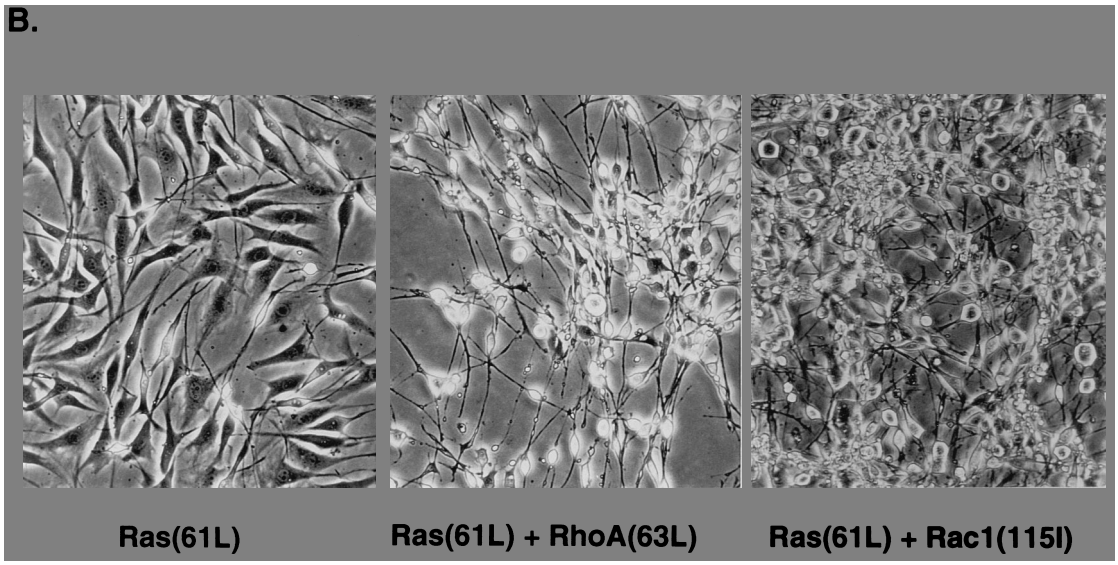
DISCUSSION

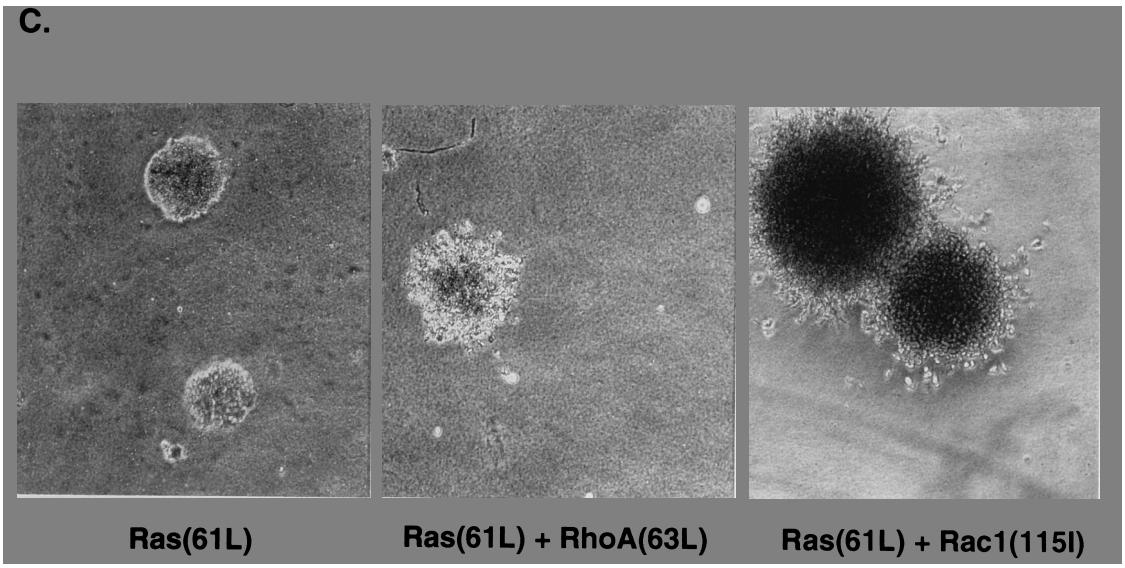
Observations from several independent lines of study suggest that oncogenic Ras may mediate its actions through a Raf-independent pathway that involves members of the Rho family of Ras-related proteins. First, microinjection studies showed that both Rac and Rho functions are required for oncogenic Ras-mediated changes in the actin cytoskeleton (37, 38). Second, genetic studies have implicated a second Ras-mediated pathway, involving Rho family proteins, which controls cell morphology in *S. pombe* (7). Third, random-mutagenesis studies identified a mutant of Ras that no longer bound Raf but still retained an activity required for Ras transforming potential (47). In the present study, we observed that dominant inhibitory mutants of Rac1 and RhoA blocked oncogenic Ras transforming activity whereas weakly transforming mutants of Rac1 and RhoA cooperated with a weakly transforming mutant of Raf-1 and caused potent transforming activity; we also observed that activated Rac1 and RhoA further enhanced oncogenic Ras-mediated morphologic transformation and cell motility. These results provide further support for a Ras-triggered activation of a Rho-mediated signaling pathway and demonstrate an important contribution of Rac- and Rho-induced changes in the actin cytoskeleton to oncogenic Ras transformation of NIH 3T3 cells. Since we also observed that kinase-deficient MAPKs mutants inhibited Ras transformation, we propose that oncogenic Ras activation of the Raf/MAPK pathway and of a Rac/Rho pathway may together be required for the mitogenic and morphogenic events associated with Ras transformation (Fig. 7).

Although our observations that Rac1 and RhoA activities can modulate Ras and Raf transforming activities may simply reflect the complementary actions of two independent signaling pathways, these results are consistent with the observations from microinjection and yeast genetic studies and support a model that Rac and Rho are downstream mediators of oncogenic Ras transformation. Furthermore, we have recently observed that two effector domain mutants of oncogenic Ras that are defective for interaction with Raf (47) and MAPK activation retain transforming activities similar to those of activated Rac1 and RhoA proteins (25). We have also observed that coexpression of activated Raf(340D) restored Ras-like transforming activities to these transformation-impaired Ras mutants (25). These observations support the possibility that oncogenic Ras can activate Rho family proteins via a downstream, effector-mediated process.

Presently, it is not clear how oncogenic Ras might regulate Rac1 and RhoA function. However, the identification of Ras effector targets which are distinct from Raf suggests the possible presence of linkages between Ras and these Ras-related proteins. Like Raf, p120-GAP, NF1-GAP, RalGDS and related proteins, and PI3K all show preferential binding to the


A.
Ras(61L)
Ras(61L) + RhoA(63L)
Ras(61L) + Rac1(115I)


B.
Ras(61L)
Ras(61L) + RhoA(63L)
Ras(61L) + Rac1(115I)


C.
Ras(61L)
Ras(61L) + RhoA(63L)
Ras(61L) + Rac1(115I)

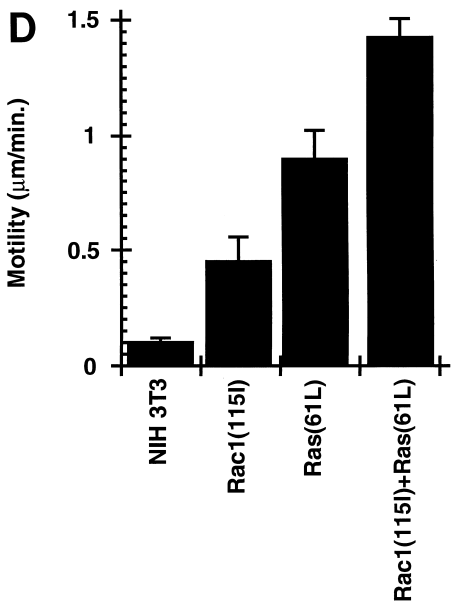


FIG. 6. RhoA(63L) and Rac1(115I) enhance the transformed properties of oncogenic Ras(61L)-transformed cells. Coexpression of RhoA(63L) or Rac1 (115I) enhanced the morphologic transformation of Ras(61L)-induced foci (A) as well as the appearance of individual cells (B), the number and appearance of colonies in soft agar (day 20) (C), and cell motility (D). NIH 3T3 mouse fibroblasts were transfected with 50 ng of pMUT-1 DNA [encoding RasH(61L)] per dish, either alone, or together with 1 μg of pZIP-NeoSV(x)1 expression constructs of constitutively activated Rac1(115I) or RhoA(63L).

activated, GTP-complexed form of Ras, and this binding is impaired by mutations in the Ras effector domain (3, 21, 26, 41, 44). Thus, Rac1 and RhoA may be activated via Ras interaction with one of these putative downstream effectors. For example, studies by McGlade et al. indicated that p120-GAP may link Ras with pathways that control the actin cytoskeleton (31). They observed that overexpression of a truncated form of p120-GAP, which lacked the GTPase-activating domain, caused alterations in the actin cytoskeleton and cell morphology. This linkage may be a consequence of the p190 GAP-associated protein, which functions as a Rho GAP (43). Similarly, phosphatidylinositol-3-OH kinase has been shown to be an upstream activator of Rho family proteins and may link the Ras and Rho pathways (28, 48). We are presently evaluating the possible connection between different Ras effectors and the functional interaction between oncogenic Ras and Rho family proteins.

A second possible connection between Ras and Rho proteins may be fostered by proteins that coordinately regulate Ras and Rho activities. In addition to the GAP-associated p190 protein, which may coordinately regulate the GTPase activities of Ras and Rho proteins (32, 43), Ras GEFs may function as bifunctional proteins that coordinately activate Ras and Rho proteins. In addition to CDC25-homologous domains required for Ras GEF activity, both SOS and RasGRF/CDC25 contain sequences which show strong amino acid identity with the protein encoded by the *dbl* oncogene (36). Dbl has been shown to function as a GEF for several members of the Rho branch of the Ras superfamily (RhoA and CDC42Hs) (18, 19), and its transforming activity is consistent with constitutive activation of Rho proteins (22). Thus, while no Rho GEF activity has presently been described for the Dbl homology domains of SOS or RasGRF/CDC25 (36), it remains possible that these proteins can coordinately regulate the activities of Ras and Rho family proteins. Finally, a third possible connection may be a consequence of an oncogenic Ras-triggered autocrine loop, via upregulation of transforming growth factor α, to trigger cell surface receptor-mediated activation of signaling pathways that lead to the activation of Rho family proteins. This possibility is supported by the observation that various growth factors trigger Rac- and Rho-dependent membrane ruffling and stress fiber formation (38).

Our observation that coexpression of activated Rac1 and RhoA with oncogenic Ras resulted in highly refractile, poorly adherent cells supports a role for these two Rho family proteins in mediating oncogenic Ras-triggered morphologic transformation. However, these results contrast with observations that the transient consequences of Rac1 and RhoA activation are to promote the development of actin stress fibers and the formation of focal adhesion contacts (37, 38). Furthermore, we previously observed that RhoA-transformed cells do not show the same disruption in cell morphology associated with Ras-transformed cells but instead show an enhancement of actin stress fibers and focal adhesions (22). One possible explanation for these apparently contrasting actions of Rac1 and RhoA may be that the chronic activation of these two proteins, in the context of other Ras-mediated signaling events, may lead to a disruption, rather than a promotion, of the actin cytoskeleton and focal adhesions. Alternatively, the aberrant overexpression of Rac1 and RhoA activities may simply deregulate the interactions of actin structures with other cytoskeletal components, thereby leading to a loss of cytoskeletal organization.

While the combined observations from microinjection studies, yeast genetic analyses, and Ras transformation studies support the existence of a Ras-mediated signaling pathway involving Rho family proteins, more evidence is clearly required to confirm the model proposed in Fig. 7. For example, although genetic studies support the divergence of these two pathways at the level of Ras (7), the potent morphologic transformation

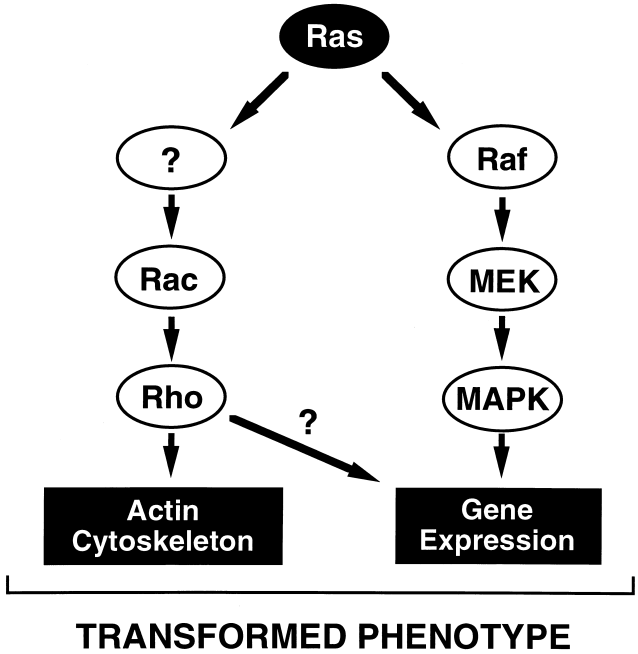


FIG. 7. Oncogenic Ras coordinately activates the Raf/MAPK and Rac/Rho pathways. From the combined observations in previous studies and the present study, it appears that oncogenic Ras transforming activity is mediated by at least two distinct signaling pathways.

associated with constitutively activated mutants of Raf-1 argues against a divergence of a Rac/Rho pathway at the level of Ras effector interaction. Like the remarkable convergence of observations from biochemical and biological studies of mammalian cells, together with genetic studies of *Caenorhabditis elegans*, *Drosophila melanogaster*, and yeasts, that established the now well-defined Ras→Raf→MEK→MAPK pathway (12, 24, 35), further studies are required to provide a clear delineation of the mechanism by which Rho family proteins modulate Ras signal transduction and transformation. Nevertheless, it is becoming increasingly clear that Ras proteins do not simply function in a single linear signaling pathway which connects the cell surface with the nucleus and that Ras function will be mediated by multiple signaling pathways.

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