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A role for Rho in Ras transformation

(Rac/stress fiber/cell proliferation)

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ABSTRACT **The small GTP-binding proteins Rac and Rho are key elements in the signal-transduction pathways respectively controlling the formation of lamellipodia and stress fibers induced by growth factors or oncogenic Ras. We recently reported that Rac function is necessary for Ras transformation and that expression of constitutively activated Rac1 is sufficient to cause malignant transformation. We now show that, although expression of constitutively activated V14-RhoA in Rat 1 fibroblasts does not cause transformation on its own, it strongly cooperates with constitutively active RafCAAX in focus-formation assays in NIH 3T3 cells. Furthermore, dominant-negative N19-RhoA inhibits focus formation by V12-H-Ras and RafCAAX in NIH 3T3 cells, and stable coexpression of N19-RhoA and V12-H-Ras in Rat1 fibroblasts reverts Ras transformation. Interestingly, stress fiber formation is inhibited in V12-H-Ras lines and restored by coexpression of N19-RhoA. We conclude that Rho drives at least two separate pathways, one that induces stress fiber formation and another one that is important for transformation by oncogenic Ras.**

Ras proteins occupy key control points in important biological functions ranging from cell differentiation to cell proliferation (1, 2). Ras proteins function as molecular switches, cycling between the inactive GDP-bound state and the active GTP-bound state (3). Ras genes have been found to be mutated in ≈30% of human tumors (4, 5). Stimulation of Ras leads to activation of the mitogen-activated protein (MAP) kinase cascade composed of Raf, MAP kinase/extracellular signal-regulated kinase kinase (MEK), and extracellular signal-regulated kinase (ERK), which in turn leads to transcriptional activation of a variety of targets (1, 2).

Transformation by oncogenic Ras is also accompanied by dramatic alterations in the actin cytoskeleton, the most striking of which is a decrease in stress fiber formation and focal adhesions (6, 7). Key players controlling the organization of the actin cytoskeleton are GTPases of the Rho family of small GTP-binding proteins: In mammalian cells, Cdc42 is involved in the extension of filipodia (8, 9), Rac controls lamellipodia and their ruffling behavior, and Rho regulates the formation of stress fibers and focal adhesions (10, 11).

It has recently become apparent that Rho family GTPases can be organized in cascades: In Swiss 3T3 fibroblasts, for example, microinjection studies have indicated that Cdc42 activates Rac, which in turn leads to Rho activation (8, 9, 12). Interesting, in both higher eukaryotes and in yeast, GTPases of the Ras subfamily can activate cascades of Rho family members: In Swiss 3T3 fibroblasts, Ras activates the Rac/Rho pathway (10), in *Saccharomyces cerevisiae*, RSR1/BUD1 acts upstream of CDC42 (13), and in *Saccharomyces pombe*, Ras likely modulates the activity of Cdc42 (14).

The observation that in Swiss 3T3 fibroblasts oncogenic Ras induces lamellipodia and stress fibers in a manner that depends on Rac and Rho function (10) raises the question as to the role of Rac and Rho proteins in Ras transformation. We recently showed that Rac indeed is essential in transformation by Ras and that expression of the constitutively active mutant V12Rac1 causes malignant transformation on its own (15). With respect to the oncogenic potential of Rho, two studies have indicated that either overexpression of wild-type or constitutively active Rho can transform NIH 3T3 fibroblasts (16, 17), although this conclusion could not be totally substantiated in a later report (18). An involvement of Rho in oncogenesis is also suggested by the existence of the dbl family of oncogenes, which all contain a domain that for some members, notably *dbl, ost, Ibc,* and *Tiam1*, has been shown to possess exchange activity for Rho-family members *in vitro* (9–22). Interestingly, in contrast with cells transformed by Ras or Ras guanine nucleotide releasing factor (RasGRF), *dbl*- and *vav*-transformed fibroblasts display well-formed stress fibers reminiscent of cells transfected with an activated Rho mutant (7).

To study the possible role of Rho in the control of cell proliferation, we established stable Rat1 fibroblast lines expressing V14-RhoA, a constitutively active mutant, or N19-RhoA, which is expected to function as a dominant-negative mutant, by analogy with dominant-negative N17-Ras and N17-Rac (10, 23), and studied the actin cytoskeleton and growth properties of these lines. We also investigated the role of Rho in Ras transformation, making use of focus-formation assays in NIH 3T3 cells and by establishing Rat1 lines coexpressing V12-H-Ras and N19-RhoA.

MATERIALS AND METHODS

Site-Directed Mutagenesis and Plasmid Construction. Full-length wild-type RhoA cDNA was released from pGEM11Z-G14 RhoA (from J. Hancock, University of Queensland Medical School, Australia) and cloned into pBluescript. This cDNA was provided with a Kozak sequence and a Myc tag encoding the epitope MEQKLIEEDL (24) at the 5′ end by PCR. Subsequently, mutagenesis of Ala → Val at codon 14 and Thr → Asn at codon 19 was achieved with a pAltered-1 mutagenesis kit (Promega). Tetracycline-repressable expression plasmids pU-MycRhoA-V14 and pU-MycRhoA-N19 were obtained by subcloning the mutated cDNAs into the pUHD10-3 vector (25). pEXV-MycRhoA-V14 and pEXV-MycRhoA-N19 were made by subcloning the respective cDNAs into pEXV3. The pBabe-MycRhoA-N19 used in the clonability assay to test for N19-RhoA toxicity was made by subcloning the MycRhoA-N19 cDNA into pBabepuro (from E. Porfiri, Onyx Pharmaceuticals).

Establishment of Cell Lines. Rat1 lines expressing N19-RhoA were established by the same procedure used for the V14-RhoA lines (15). The lines were maintained in high-glucose (4.5 g/liter) Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 μg/ml, G418 at

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Abbreviation: MAP kinase, mitogen-activated protein kinase.

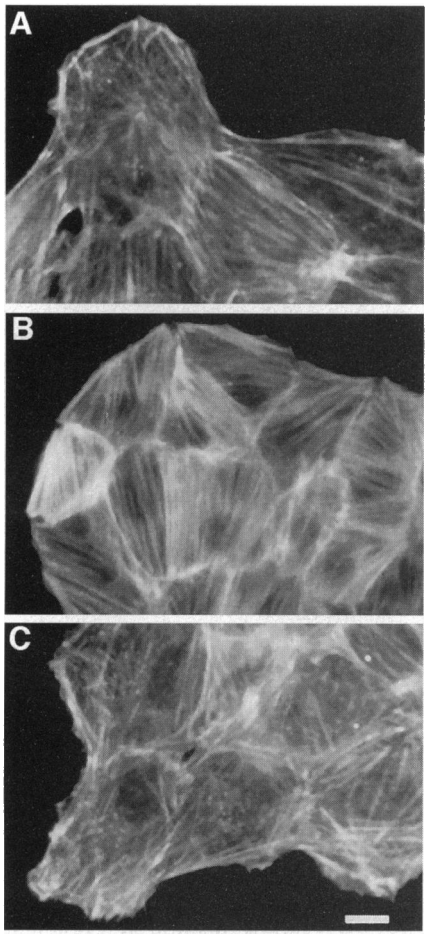

FIG. 1. Phalloidin staining of the actin cytoskeleton of Rat1 fibroblasts expressing vector (*A*), V14-RhoA (*B*), and N19-RhoA (*C*). (Bar = 10 μm.)

400 μg/ml, puromycin at 2 μg/ml, and tetracycline at 2 μg/ml and kept at 37° C and 5% CO_2/95% air. Tetracyline was withdrawn 2 days before the start of each experiment.

Rat1 fibroblast lines expressing V12-H-Ras were obtained by transfection of the tTA (tetracycline-controlled transactivator)-expressing Rat1-R12 (from D. Resnitsky, Weizmann Institute, Israel) with pEXV-H-Ras-V12. Rat 1 fibroblasts coexpressing V12-H-Ras and N19-RhoA were obtained by cotransfection of Rat1-R12 with pEXV-H-Ras-V12 and pU-MycRhoA-N19. These lines were maintained under the same conditions as the mutant RhoA lines, except for using hygromycin B at 200 μg/ml instead of puromycin.

Expression of mutant RhoA protein was determined by immunoblot analysis using the 9E10 anti-Myc antibody (24) with the procedure outlined in the enhanced chemiluminescence kit (Amercham). V12-H-Ras expression was detected by immunoprecipitation with anti-Ras antibody Y13-238 (Oncogene Science), followed by immunoblotting with anti-Ras antibody 6B7.

Characterization of Cell Proliferation. Soft-agar colony formation, tumor growth in athymic nude mice, and focus-formation assays were studied as described (15).

Microscopy and Immunofluorescence. For immunofluorescence, induced cells were grown on 12-mm diameter coverslips. Immunofluorescence procedures, observation, and photography were done as described (26), except that cells were stained in addition with fluorescein isothiocyanate–phalloidin at 100 nM. Micrographs of foci were taken on a TMS inverted microscope, equipped with a 10× 0.25 numerical aperture objective and a polaroid camera (Nikon).

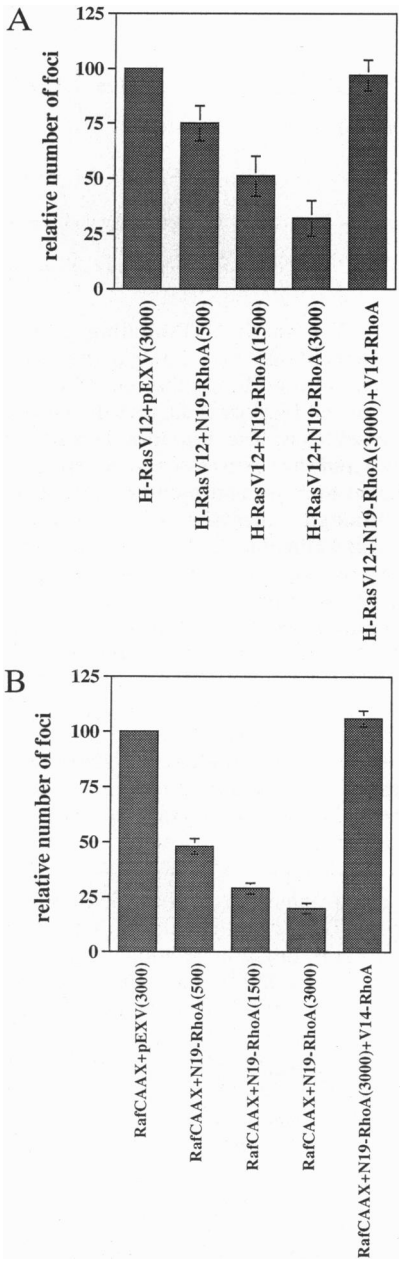

FIG. 2. N19-RhoA inhibits NIH 3T3 focus formation by V12-H-Ras and RafCAAX. (*A*) Plasmid concentrations per 10-cm dish were 2 ng of pEXV-H-Ras-V12, 100 ng of pEXV-MycRhoA-V14, and ng indicated in parentheses for pEXV-MycRhoA-N19. Number of foci per dish were normalized against those produced by V12-H-Ras; the average number of foci produced by V12-H-Ras was 49. (*B*) Plasmid concentratons per 10-cm dish were 500 ng of pEXV-EERafCAAX, 100 ng of pEXV-MycRhoAV14, and ng indicated in parentheses for pEXV-MycRhoA-N19. Number of foci per dish were normalized against those produced by RafCAAX; the average number of foci produced by RafCAAX was 75. Error bars indicate the SE of two to four independent experiments; each experiment was done in duplicate.

RESULTS

Characterization of Mutant RhoA Cell Lines. To study the role of RhoA in the control of cell proliferation, we constructed the constitutively active V14-RhoA and the putative dominant-negative N19-RhoA mutants and subsequently established stable Rat1 fibroblast lines expressing these mutants from a tetracycline-repressable promotor (25). Fluorescent phalloidin staining of V14-RhoA-expressing Rat1 fibroblasts revealed a strong increase in stress fiber formation over vector

controls (Fig. 1 *A* and *B*), consistent with earlier reports using microinjection of recombinant V14-RhoA protein or expression of L63-RhoA in 3T3 fibroblasts (7, 11). Interestingly, in spite of this increase in stress fibers, V14-RhoA-expressing cells showed significantly less spreading than controls (Fig. 1*B*). This result would suggest that activation of Rho increases actomyosin-based cell contractility, in agreement with previous studies (27–29). Rat1 fibroblasts expressing dominant-negative N19-RhoA, in contrast, tend to show a slight inhibition of stress fiber formation: the stress fibers appear to be thinner and fewer in number (Fig. 1*C*). These observations agree with earlier studies using microinjection of *Clostridium botulinum* C3 exoenzyme, which inactivates Rho by ADP-ribosylation (11), and also suggest that the level of inhibition achieved by the dominant-negative N19-RhoA is rather low.

To study the role of Rho in cell transformation, we investigated the ability of the mutant RhoA lines to grow in soft agar but could find no colony formation, even after 5 weeks of incubation time, whereas V12-Rac1-expressing lines generated colonies after 1 week (15). When tested for tumorigenicity *in vivo*, V14-RhoA-expressing cells also failed to induce tumors in nude mice (data not shown). Therefore, expression of constitutively active RhoA does not lead to a transformed phenotype.

Rho Is Necessary for Transformation by Ras and RafCAAX. It has been reported (10) that in Swiss 3T3 fibroblasts microinjection of oncogenic Ras induces stress fiber formation in a C3 exoenzyme-dependent fashion, indicating that Ras can activate Rho. To establish the role of Rho in Ras transformation, we performed focus-formation assays in NIH 3T3 cells using V12-H-Ras with and without cotransfected N19-RhoA. N19-RhoA caused a dose-dependent inhibition of Ras-induced focus formation (Fig. 2*A*). Interestingly, N19-RhoA inhibited focus formation by RafCAAX, which constitutively activates the MAP kinase pathway (26, 30), even more efficiently than it inhibited focus formation by V12-H-Ras (Fig. 2*B*). To test whether N19-RhoA would inhibit cell growth in a nonspecific manner, we transfected NIH 3T3 cells with N19-RhoA under selection, using a plasmid carrying both the N19-RhoA cDNA and the puromycin resistance gene (see *Materials and Methods*). We found that the efficiency of colony formation was 298 ± 16 (SEM, $n = 3$) colonies per μg of plasmid equivalent, which is not significantly different from the colony number obtained with the control plasmid, 266 ± 54 (SEM, $n = 3$). This result indicates that the inhibition of Ras and RafCAAX focus formation by N19-RhoA was not due to a toxic effect. This conclusion is further supported by the observation that coexpression of V14-RhoA effectively rescues the inhibitory effect of N19-RhoA on focus formation by Ras and RafCAAX (Fig. 2 *A* and *B*). Taken together, these experiments show that Rho is necessary for transformation by both Ras and RafCAAX.

To further explore the role of Rho in Ras transformation we established stable Rat1 fibroblast lines expressing V12-H-Ras with or without N19-RhoA. Cells expressing V12-H-Ras alone look fusiform and tend to grow in foci (Fig. 3*A*). Cells coexpressing V12-H-Ras and N19-RhoA, however, have a close to normal morphology (Fig. 3*C*). Moreover, stress fiber formation that is inhibited in Ras-transformed cells (compare Figs. 1*A* and 3*B*) is restored in V12-H-Ras/N19-RhoA-coexpressing cells (Fig. 3*D*). Coexpresson of N19-RhoA also completely inhibits growth in soft agar. Three out of four V12-H-Ras lines tested grew very efficiently (>20%) in soft agar, whereas the remaining line grew with a lower efficiency (<1%). The two V12-H-Ras/N19-RhoA-coexpressing lines we obtained completely lost the capability of anchorage-independent growth, although the V12-H-Ras expression levels were even higher than those of the lines expressing V12-H-Ras alone (Fig. 3*E*). We therefore conclude that Rho plays a crucial role in Ras transformation.

Constitutively Active RhoA Cooperates with RafCAAX in Cell Transformation. Previous experiments indicated that the Rac and MAP kinase pathways bifurcate at the level of Ras (15). Consistent with this result, activated V12-Rac1 strongly synergizes with RafCAAX in focus-formation assays in NIH 3T3 cells (15). As previous observations on Rho-controlled stress fiber formation in Swiss 3T3 cells indicated that Rho acts downstream of Rac (10), we also used focus-formation assays to test for synergy between V14-RhoA and RafCAAX. Cotransfection of V14-RhoA with RafCAAX in NIH 3T3 cells, at plasmid concentrations that produce few foci when transfected individually, caused a dramatic increase in focus formation (Fig. 4), suggesting a high degree of cooperativity between the Rho and MAP kinase pathways. The morphology

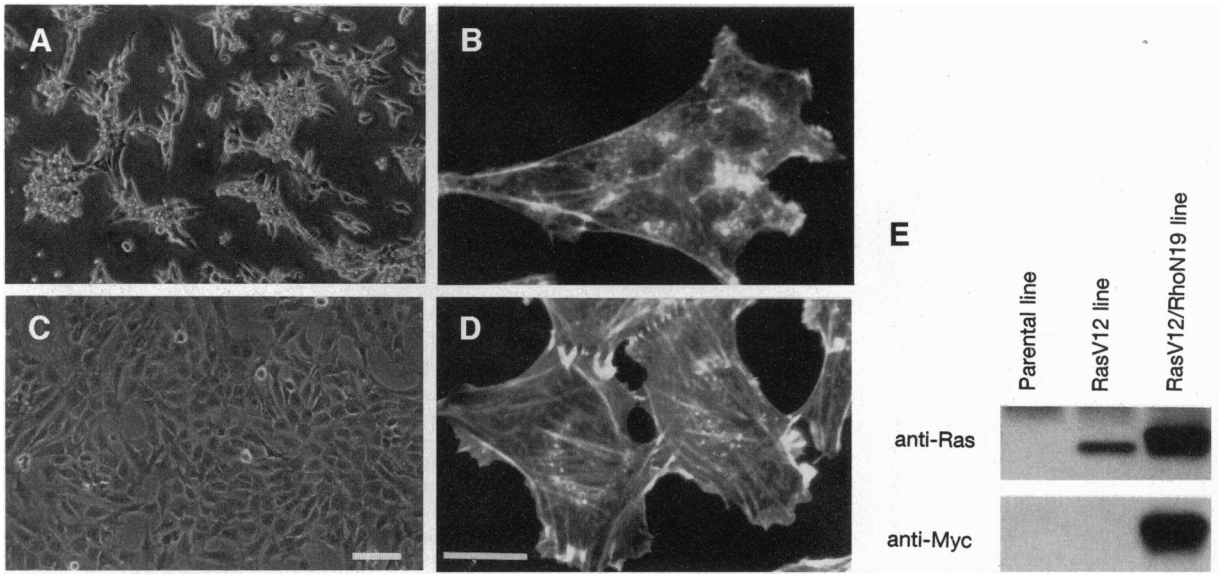

FIG. 3. Morphology and phalloidin staining of Rat1 fibroblasts expressing V12-H-Ras alone or coexpressing V12-H-Ras and N19-RhoA. Phase-contrast pictures of V12-H-Ras cells (*A*) and V12-H-Ras/N19-RhoA cells (*C*). (Bar = 100 μm.) Phalloidin staining of V12-H-Ras cells (*B*) and V12-H-Ras/N19-RhoA cells (*D*). (Bar = 25 μm). (*E*) Immunoblot showing expression levels of V12-H-Ras using 6B7 anti-Ras antibody (*Upper*) and N19-RhoA using anti-Myc antibody (*Lower*) in the parental, V12-H-Ras, and V12-H-Ras/N19-RhoA lines.

of the various foci is also quite revealing. NIH 3T3 cells transformed by Ras are spindle-shaped, creating the highly characteristic swirling pattern of Ras foci (Fig. 5*A*). RafCAAX foci are very similar to those of Ras, except that they are smaller in size and tend to be less dense (Fig. 5*B*). V14-RhoA foci are extremely dense, and the cells are rounded and tend to pile up (Fig. 5*C*) (18). The morphology of V14-RhoA/RafCAAX foci is more variable. Most foci have a morphology that is intermediate between those of V14-RhoA and RafCAAX on their own: the foci are denser than those of RafCAAX, whereas the cells toward the periphery of the foci are more fusiform than in V14-RhoA foci (Fig. 5*D*). Interestingly, an analogous situation occurs in the cooperativity between V12-Rac1 and RafCAAX. V12-Rac1 foci are very small, with the cells piling up, although the foci are less compact than those of V14-RhoA. V12-Rac1 foci are also marked by a very high frequency of multinucleated giant cells (Fig. 5*E*). As with Rho, V12-Rac1/RafCAAX foci have an appearance intermediate between V12-Rac1 foci and RafCAAX foci (Fig. 5*F*).

DISCUSSION

Our results indicate an important role for Rho in cell proliferation. Although expression of constitutively active RhoA, unlike constitutively active Rac1 (15), is not sufficient to transform Rat1 fibroblasts, it does cooperate with activation of the MAP kinase pathway in NIH 3T3 transformation. Furthermore, Rho is necessary for Ras transformation, as shown by focus-formation assays in NIH 3T3 fibroblasts and the reversal of cell morphology and restoration of anchorage dependence in Rat1 fibroblasts. The stronger inhibition of N19-RhoA on soft-agar growth of Ras-transformed cells in comparison with the inhibitory effect on Ras focus formation is likely due to the fact that soft-agar growth provides a more stringent criterion for transformation than does focus formation (for example see ref. 18).

The crucial role of Rho in Ras transformation indicated by our observations agrees with previous studies showing that microinjection of constitutively active Ras or Rac proteins induces stress fiber formation in a Rho-dependent fashion (10, 31). Our results are therefore consistent with a model in which Rho acts downstream of Ras and Rac, relaying a proliferative signal driven by oncogenic Ras (Fig. 6). This model also would suggest that at least part of the proliferation-stimulating signal of Rac (15) might be mediated by Rho.

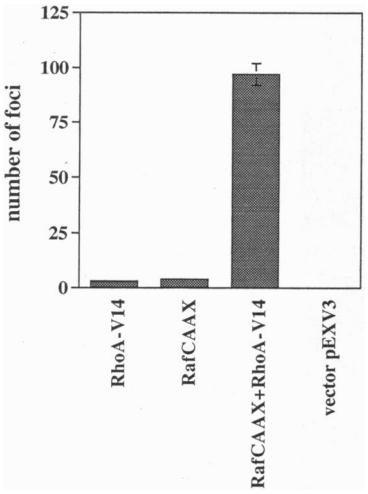

FIG. 4. V14-RhoA syngergizes with RafCAAX in NIH 3T3 focus formation. Plasmid concentrations per 10-cm dish were 500 ng of pEXV3 vector and 50 ng of pEXV-MycRhoA-V14 and pEVX-EERafCAAX, respectively. Error bars indicate the SE of five independent experiments; each experiment was done in duplicate.

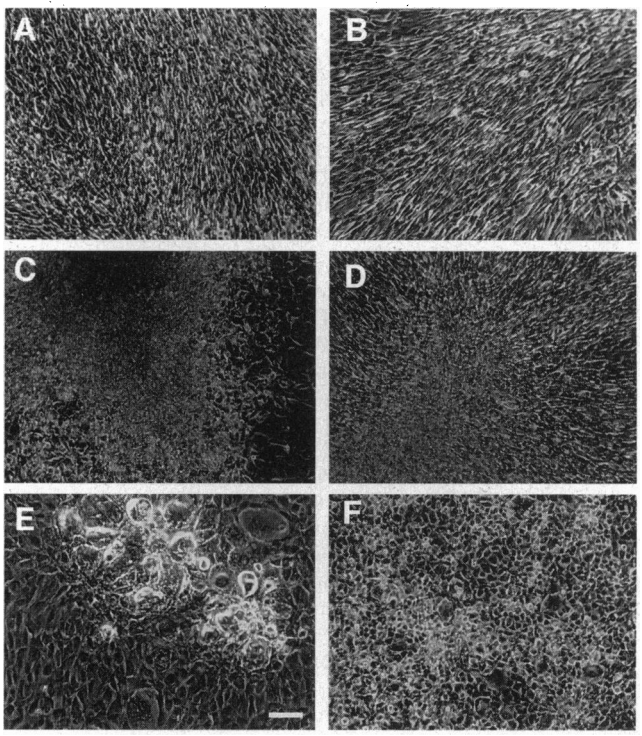

FIG. 5. Morphology of NIH 3T3 foci. Phase-contrast pictures of foci transformed by V12-H-Ras (*A*), RafCAAX (*B*), V14-RhoA (*C*), cotransfection of V14-RhoA and RafCAAX (*D*), V12-Rac1 (*E*), and cotransfection of V12-Rac1 and RafCAAX (*F*). (Bar = 100 μm.)

As activation of Rho thus far has been strongly associated with stress fiber formation (11), this model appears at variance with the observations that Ras transformation leads to the dissolution of stress fibers (6, 7) (see also Fig. 3), which implies that stress fibers are not essential for Ras transformation. Taken together, these data are consistent with a model in which Rho activates multiple pathways and in which the signaling cascade involved in cell proliferation is distinct from the one controlling stress fiber formation. On the basis of our findings, we also propose that the proliferation pathway driven by Rho cooperates with the Raf/MAP kinase pathway and provides a signal that downregulates stress fiber formation (Fig. 6). This result is consistent with the observation that the induction of stress fibers by microinjection of oncogenic Ras proteins is transient (10).

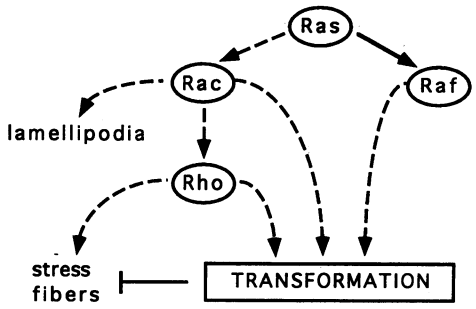

FIG. 6. Bifurcation model of Ras signaling. Ras drives both the Raf/MAP kinase and Rac/Rho pathways to transform cells. Rac stimulates at least two signaling cascades, one inducing lamellipodia and the other leading to activation of Rho (31). Rho also activates at least two pathways, one involved in stress fiber formation, whereas the other synergizes with Raf and possibly with other downstream elements of the MAP kinase pathway to stimulate cell proliferation. The growth control arm of Rho in cooperation with RafCAAX is involved in the inhibibition of stress fiber formation observed in Ras-transformed cells.

A possible mechanism for an inhibitory signal involved in the dissolution of stress fibers might be provided by the downregulation of the tropomyosin isoforms TM1 and TM2 seen in Ras-transformed fibroblasts (32). In addition, expression of TM1 in these Ras-transformed cells to levels that correspond to those found in normal cells caused partial reversion of the transformed phenotype, notably an increase in spreading and stress fiber formation and a decrease in anchorage-independent growth (32), suggesting that the downregulation of TM1 and possibly other isoforms of tropomyosin contributes to establishment of the transformed phenotype. Similar observations have been made with respect to the actin-binding proteins vinculin and α-actinin (33, 34).

Our observations that V14-RhoA foci have a morphology that is totally different from that of V12-H-Ras foci, whereas the morphology of RafCAAX foci is identical to that of V12-H-Ras foci, are also in line with the idea that the contribution of the Rho pathway to transformation by Ras is independent of the previously recognized role of Rho in control of cell shape and organization of the actin cytoskeleton. Moreover, the distinct morphology of V12-Rac1 foci suggests that this conclusion may hold for Rac as well.

Our observation that focus formation by RafCAAX is inhibited by coexpression with N19-RhoA suggests a potential role for Rho in the MAP kinase pathway, in agreement with the inhibitory effect of C3 exoenzyme on extracellular signal-regulated kinase 2 (ERK2) activation by growth factors (35, 36). In a linear pathway, this result would argue for the involvement of Rho somewhere between Raf and ERK. However, V14-RhoA fails to activate ERK2 in several different cell systems (37, 38). Furthermore, the high degree of cooperativity between the Rac/Rho and MAP kinase pathways suggested by the focus-formation assays, as well as the clearly distinct morphologies of the Rac, Rho, and RafCAAX foci, also indicate that the Rac/Rho and MAP kinase pathways are distinct from each other. These pathways are likely to bifurcate at the level of Ras (15). The inhibitory effect of N19-RhoA on transformation by RafCAAX could also be explained by a model in which Rho mediates an autocrine loop driven by RafCAAX. Such autocrine factors could include lipid growth factors such as lysophosphatidic acid (39), which can activate Rho independently of Rac (10).

The Rho-driven signal-transduction pathway involved in the control of cell proliferation still needs to be charted out. Rho has been shown to regulate a variety of signal-transduction elements—namely, phosphatidylinositol 4-phosphate 5-kinase, phosphatidylinositol (4,5)bisphosphate 3-kinase, and phospholipase D (40–42). Rho function is also necessary for the activation of focal adhesion kinase and MAP kinases by growth factors (35). Moreover, Rho also regulates serum response factor-dependent transcription (36). Which of these activities are mediating cell proliferation is unclear at this moment. It is also possible that yet-to-be-identified pathways are involved.

In summary, we have established a crucial function for Rho in transformation by oncogenic Ras. This, together with our previous finding that Rac is also necessary for Ras transformation, indicates that inhibitors of the Rac/Rho pathway will provide another approach to cancer therapy.

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