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Cdc42 Regulates Anchorage-Independent Growth and Is Necessary for Ras Transformation

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The Rho family members Cdc42, Rac, and Rho play a central role in the organization of the actin cytoskeleton and regulate transcription. Whereas Rac and Rho have been implicated in transformation by oncogenic Ras, the role of Cdc42 in this process remains unknown. In this study, we found that Rat1 fibroblasts expressing constitutively active V12-Cdc42 were anchorage independent and proliferated in nude mice but failed to show enhanced growth in low serum. Similar to V12-Rac1-expressing Rat1 fibroblasts, V12-Cdc42 lines displayed a high frequency of multinucleated cells. Interestingly, coexpression of dominant negative N17-Rac1 blocked the V12-Cdc42-induced multinucleated phenotype but not growth in soft agar, indicating that Cdc42 controls anchorage independence in a Rac-independent fashion. We also showed that dominant negative N17-Cdc42 inhibited Ras focus formation and anchorage-independent growth and caused reversion of the transformed morphology, indicating that Cdc42 is necessary for Ras transformation. N17-Cdc42 caused only partial inhibition of Ras-induced low-serum growth, however. In contrast, whereas N17-Rac1 also effectively inhibited Ras-induced anchorage independence, it did not revert the morphology of Ras-transformed cells. N17-Rac1 strongly inhibited low-serum growth of Ras-transformed cells, however. Together, these data provide a novel function for Cdc42 in cell proliferation and indicate that Cdc42 and Rac play distinct roles in growth control and Ras transformation.

Rho family GTPases, which include the various isoforms of Rac, Rho, and Cdc42, are members of the Ras superfamily and act as binary molecular switches, cycling between the inactive GDP-bound and active GTP-bound forms (3). The activity of Rho proteins is controlled by several accessory factors, guanine nucleotide exchange factors, GTPase-activating proteins (GAPs), and GDP dissociation inhibitory factors.

Rho family proteins play an important role in signal transduction pathways that link plasma membrane receptors to the organization of the actin cytoskeleton and to cell adhesion (27). In fibroblasts, Rho regulates the formation of focal adhesions and stress fibers, Rac mediates lamellipodium formation (40, 41), and Cdc42 controls the formation of filopodia (24, 30). Both Rac and Cdc42 also regulate the formation of focal complexes (30). Rho-related proteins also participate in a variety of other cellular processes which are accompanied by a reorganization of the actin cytoskeleton, such as cell aggregation, motility, and cytokinesis (11, 49).

Functional studies on Rac and Cdc42 have relied on the use of dominant negative versions of these proteins in which Thr-17 has been changed to Asn. Indeed, these dominant negative mutants have been shown to inhibit the activation of the respective endogenous GTPases with remarkable specificity. For instance, bradykinin-induced filopodium formation in Swiss 3T3 cells is abolished by microinjection of N17-Cdc42 protein but is not affected by N17-Rac1 (24); conversely, induction of lamellipodia by microinjected *n*-chimaerin is inhibited strongly by N17-Rac1 but not at all by N17-Cdc42 (23).

Microinjection studies of Swiss 3T3 fibroblasts have shown that Cdc42 can promote lamellipodium formation in a Rac-dependent manner, indicating that Cdc42 can activate Rac. Furthermore, it has been shown that Rac can induce stress fiber formation in a Rho-dependent manner (30, 34, 41). This observation indicates that Rac can activate Rho and suggests the existence of a Cdc42-Rac-Rho GTPase cascade (5). In another study, however, Cdc42 was shown to inhibit stress fiber formation (24). Therefore, the precise relationship between Cdc42 and Rho remains to be established.

Over the past several years it has become evident that Rho family proteins, in addition to regulating the organization of the actin cytoskeleton, play an important role in cell proliferation (48). It has been shown that expression of activated forms of Rac and Rho can transform fibroblasts (2, 35, 37, 44) and that dominant negative versions of both Rac and Rho inhibit transformation by oncogenic Ras (19, 36–38), indicating that Rac and Rho are essential for Ras transformation. These results suggest that Rac and Rho may act downstream of Ras in the control of cell proliferation. This is consistent with recent observations showing that Ras transformation is mediated by several independent pathways, including the ERK kinase cascade (17, 20, 28, 53, 54). It is possible however, that Rac and Rho also control autocrine loops which are activated by oncogenic Ras and are necessary for the maintenance of the transformed phenotype.

RhoA, Rac1, and Cdc42 have also been implicated in the regulation of transcriptional activation (14, 31) and of DNA synthesis (32). Furthermore, Rac1 and Cdc42 were shown to modulate the activity of the JNK (Jun N-terminal kinase)/stress-activated protein kinase signaling pathway (7, 29).

While roles for Rac and Rho in cell proliferation and Ras transformation have been established, the role of Cdc42 in cell growth control and transformation remains unclarified. We show here that Cdc42 controls anchorage requirement independently of Rac and plays an important role in Ras morphological transformation. Our findings also indicate that the roles of Cdc42 and Rac in Ras transformation are distinct.

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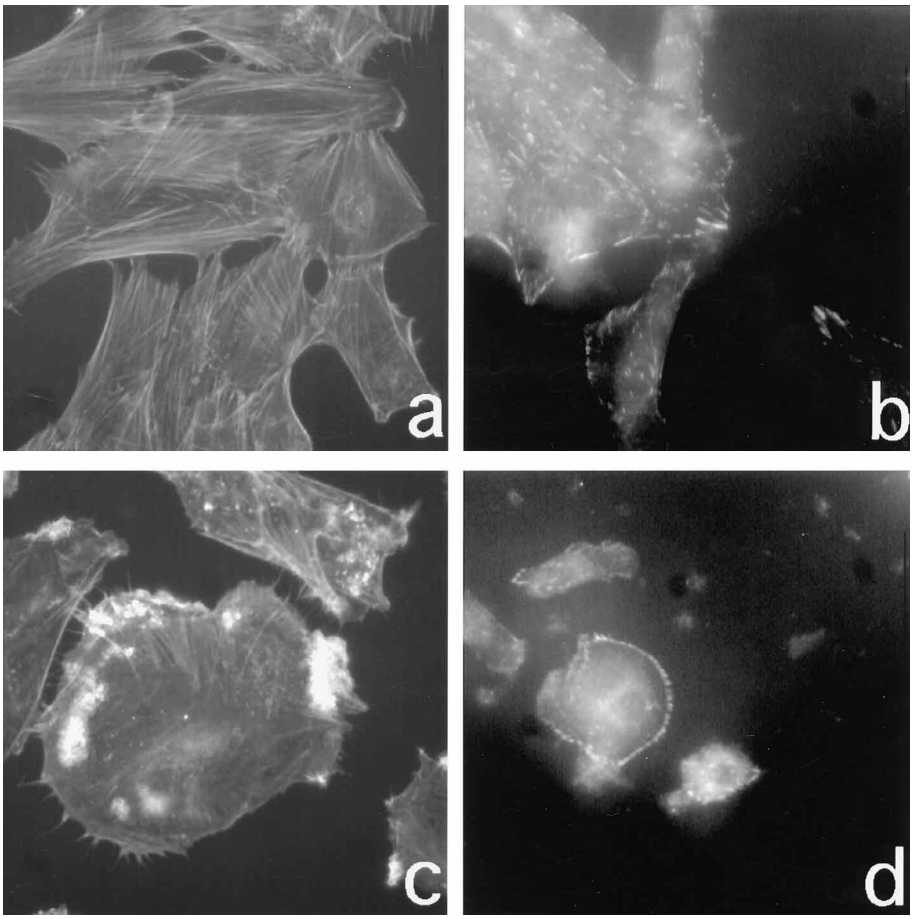

FIG. 1. V12-Cdc42 induces a reorganization of the actin cytoskeleton and focal complexes in Rat1 fibroblasts. Shown are FITC-phalloidin staining (a and c) and vinculin staining (b and d) of Rat1 cells expressing vector (a and b) and V12-Cdc42 (c and d).

MATERIALS AND METHODS

Plasmids. V12-Cdc42 and N17-Cdc42 (Hs isoform; gifts of M. Hart [12]) were expressed in a pCMVneoMyc vector, a modified pCMV plasmid with a G418 resistance gene and a Myc tag sequence at the N terminus. pEXV-MycN17-Cdc42 (gift of A. Hall [30]) is the G25K isoform. pEXV-V12-H-Ras, pEXV-MycN17-Rac1, and pEXV-EE-RafCAAX have been previously described (37). pSV2neo (45) was used for G418 selection in some of the cotransfections. The mutations in the Cdc42 cDNA were confirmed by sequencing. For construction of pyDF30-WASP-GBD, a human WASP gene PCR fragment spanning from GCTCTAGACTGGCGACAGTGGACATC to GGCCTTAAGTCACGCCTC ATCTCCTGCCGC, a region which contains the GTPase binding domain (GBD) (50), was cloned into the *Xba*I and *Eco*RI sites of the pyDF30 vector, which provides a Kozak sequence (22) and start ATG to a FLAG epitope at the 5′ end. The insert in the resulting plasmid was checked by DNA sequencing. Expression of the WASP-derived GBD (WASP-GBD) was tested in transient transfection and demonstrated by Western blotting.

Stable transfections. All transfections were performed by the calcium phosphate precipitation method (45). Transfectants were selected with 400 μg of G418 per ml, and colonies were picked about 2 weeks postselection. Cell lines expressing oncogenic H-Ras used in this study came from two sources; one was previously cloned (38), and the other resulted from V12-H-Ras/pSV2neo/N17-Rac1 cotransfection in which N17-Rac1 expression was undetectable by Western blotting with the anti-Myc monoclonal antibody 9E10 (9). Rat1 fibroblast lines expressing V12-Rac1 and its vector control line have been described previously (37).

Immunoprecipitation and Western blotting. Expression levels of V12-H-Ras were determined by immunoprecipitation (42) of 500 μg of total cell lysate with the Ras monoclonal antibody Y13-238 (Ras Ab-2; Oncogene Science, Inc.), followed by Western blotting with the Ras monoclonal antibody 6B7. Expression of Myc-tagged Cdc42 mutant proteins was detected by Western blotting with 9E10 or a polyclonal antibody raised against a C-terminal peptide of Cdc42, which was affinity purified. N17-Rac1 expression in the cotransfected cells was obtained by immunoblotting using 9E10 or an affinity-purified Rac1 polyclonal antibody. For Western blotting analysis, 25 μg of total lysate was denatured and resolved on a sodium dodecyl sulfate–4 to 20% polyacrylamide gel (Novex), transferred onto a polyvinylidene difluoride membrane (Immobilon), probed with the antibody indicated, and visualized by enhanced chemiluminescence reagents (Amersham) according to the protocol provided by the manufacturer.

Tissue culture and characterization of growth rates. Rat1 fibroblasts and all derived stable lines were maintained in Dulbecco's modified Eagle (DME) high-glucose (4.5 g/liter) medium (DME-Hg) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM glutamine, and G418 (400 μg/ml; for derived stable lines only). To study the proliferation properties of the various stable lines both in 0.5 and 10% serum conditions, cells were trypsinized and plated in duplicate wells in the appropriate medium. At each time point, cells were trypsinized and counted in a hemocytometer.

Soft agar growth assays. A total of 1,000 or 3,000 cells was sandwiched between 1 ml of 0.6% bottom agar and 0.3% top agar (Difco) in a six-well plate. Duplicate wells were tested for each cell line. The cells were fed with 1 ml of top agar once every week. Colonies were scored after 3 to 5 weeks.

In vivo tumor formation assay. Cells were trypsinized and washed twice with cold phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} and resuspended in serum-free DME-Hg containing 400 μg of G418 per ml at a concentration of 10^7 cells per 0.2 ml. In vivo tumor formation was initiated by injecting 0.2 ml of the processed cells subcutaneously into each flank of three 10-week-old athymic nude mice. Tumors formed were measured weekly.

Immunofluorescence analysis. Cells growing on coverslips were fixed with 4% formaldehyde in Ca^{2+}/Mg^{2+}-free PBS and washed several times with Ca^{2+}/Mg^{2+}-free PBS containing 0.1% Triton X-100. The fixed cells were then stained with fluorescein isothiocyanate (FITC)-phalloidin to visualize filamentous actin. For vinculin staining, cells were fixed with 4% formaldehyde–0.1% Triton X-100 in Ca^{2+}/Mg^{2+}-free PBS and stained with an antivinculin monoclonal antibody (Sigma).

Micrographs. Phase-contrast micrographs were taken on a TMS inverted microscope equipped with a 10× 0.25 numerical aperture objective and a Po-

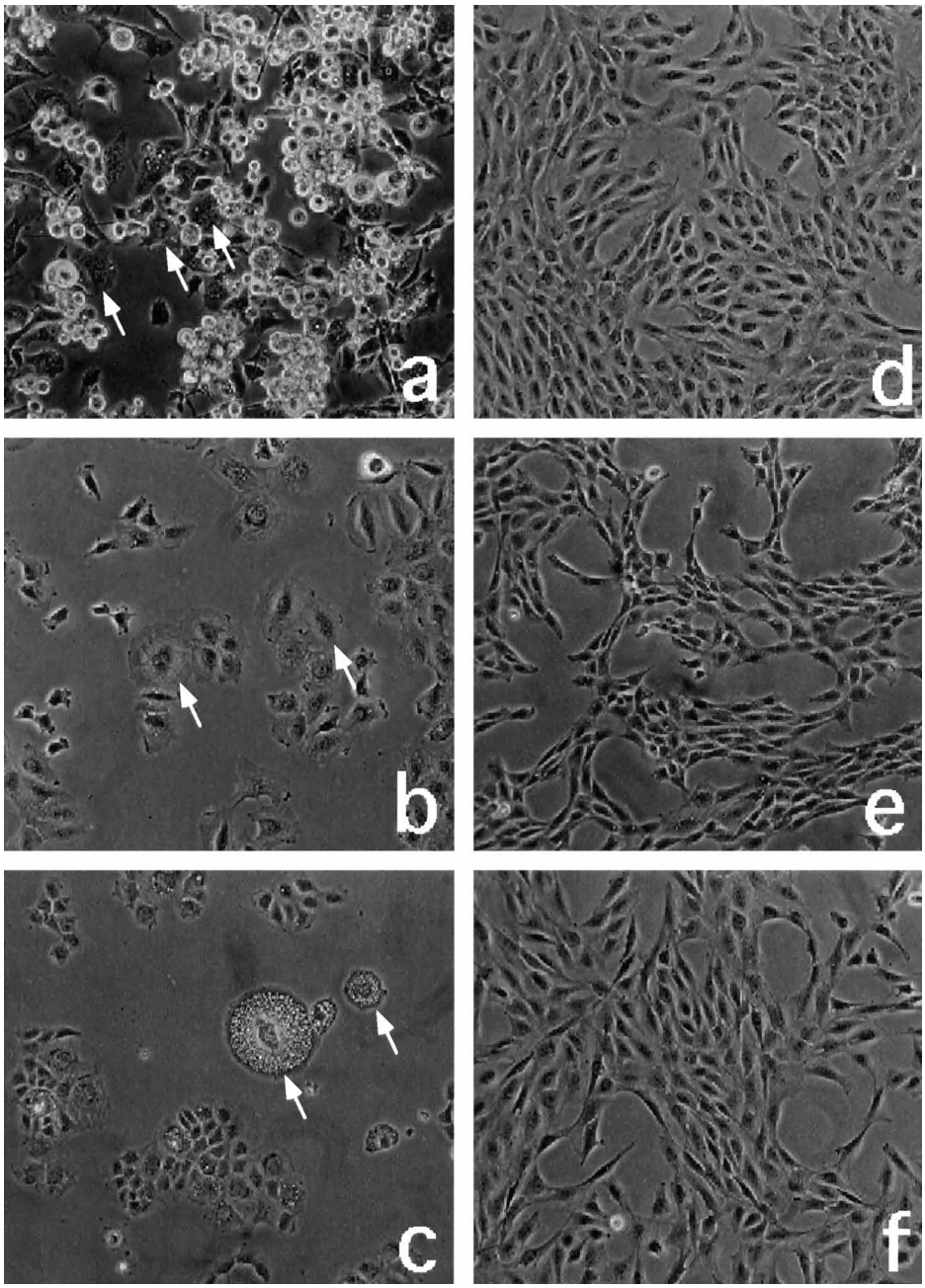

FIG. 2. Morphology of Rat1 fibroblast lines expressing mutant Cdc42 and Rac. Phase-contrast micrographs show the cell morphology of lines expressing mutant Cdc42 and Rac. Shown are the following (the percentages of multinucleated cells [determined for 300 to 600 cells] are indicated in parentheses): (a) V12-Cdc42, clone 1, early passage (18% ± 2%); (b) V12-Cdc42 clone 9, later passage (7% ± 1%); (c) V12-Rac1 (8% ± 1%); (d) N17-Cdc42 (2% ± 1%); (e) V12-Cdc42/N17-Rac1 (2% ± 1%); (f) vector control (1% ± 1%). Examples of multinucleated cells are indicated by arrows.

laroid camera (Nikon). Immunofluorescence micrographs were obtained with a charged-coupled device (CCD) camera as previously described (47).

Lamellipodium formation assay. Cells were plated on coverslips and serum starved overnight. Cells were incubated with platelet-derived growth factor BB (PDGF-BB; 10 ng/ml) for 4 min and fixed and stained with phalloidin as described above. The number of lamellipodia per cell was determined from CCD camera micrographs.

Focus formation assays. NIH 3T3 cells were transfected as described above for stable transfection. Cells were grown in DME low-glucose medium supplemented with 10% donor calf serum in 10% CO_2. Transfected NIH 3T3 cells were washed about 18 h posttransfection with Tris saline (pH 7.1) containing 20 mM HEPES and incubated in 5% donor calf serum. Medium was changed every other day. Assays were stopped after 14 to 21 days, and the number of foci was scored.

RESULTS

Characterization of V12-Cdc42-expressing Rat1 fibroblasts. To investigate the role of Cdc42 in the control of cell growth, we established stable lines of Rat1 fibroblasts which constitutively express Cdc42 mutant proteins. Phalloidin staining of cells expressing constitutively active V12-Cdc42 revealed a strong induction of filopodium formation (Fig. 1a and c). These cells also displayed an increase in lamellipodium formation, suggesting an increase in Rac activity (41). These phenotypes are similar to previous observations obtained by microinjection of V12-Cdc42 protein into Swiss 3T3 cells, indicating that Rac

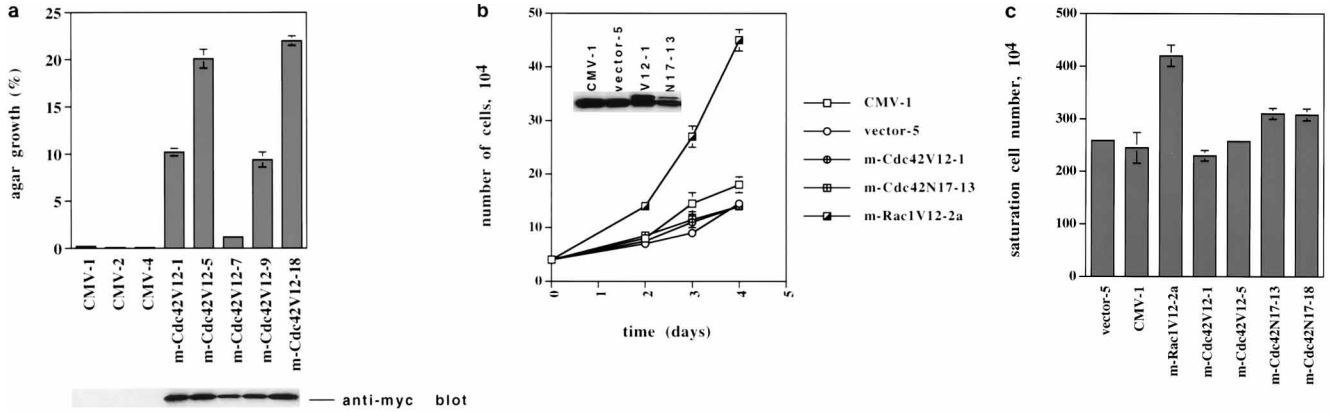

FIG. 3. V12-Cdc42 induces anchorage-independent growth. (a) Soft agar assays were carried out as described in Materials and Methods. The percentage of growth represents the ratio of the number of colonies obtained to the total number of cells seeded. Expression of V12-Cdc42 in each cell line was detected by anti-Myc antibody 9E10 (bottom panel). (b) Low-serum (0.5% FBS) growth curves of mutant Cdc42 lines, a line expressing V12-Rac1, and vector controls. CMV-1 is a control for the mutant Cdc42 lines, and vector-5 is a control for the line expressing V12-Rac1. The various lines were plated at 4×10^4 cells/well in 12-well plates. Data shown are representative of two independent clones. Expression of Cdc42 was detected by using an antibody raised against a C-terminal peptide of Cdc42 (inset). The upper and lower bands correspond to recombinant and endogenous Cdc42, respectively. (c) Saturation densities in 10% FBS. The various lines were plated at 10^5 cells/well in six-well plates. Data shown are the averages of duplicate wells, with error bars indicating the spread between duplicates. Data shown are representative of two to three independent experiments.

can act downstream of Cdc42 in the control of lamellipodia (24, 30). V12-Cdc42-expressing lines also displayed a high frequency of multinucleated cells (Fig. 2a and b), suggesting a defect in cleavage furrow formation. V12-Rac1-expressing lines are also characterized by a large number of multinucleated cells (Fig. 2c), suggesting that Rac may act downstream of Cdc42 in the control of cleavage furrow formation as well. In agreement with previous observations (41), V12-Rac1-expressing cells display strong pinocytotic activity (Fig. 2c, arrows). The pinocytotic nature of these vesicles was confirmed by time-lapse video microscopy. V12-Cdc42-expressing cells also show an increase in pinocytosis relative to vector controls, but it is less marked than that in the V12-Rac1 lines (compare Fig. 2a to c).

In addition to these phenotypes, stable expression of V12-Cdc42 also caused a marked reduction in stress fibers. Vinculin staining indicated that expressing of V12-Cdc42 disrupted the stress fiber-linked focal adhesions in the central area of the cell and promoted the formation of focal complexes at the periphery (Fig. 1b and d). These peripheral focal complexes are presumably regulated by Cdc42 and Rac (30).

N17-Cdc42-expressing cells showed a morphology which was similar to that of vector controls (Fig. 2d and f). In addition, the actin cytoskeleton was not affected and no inhibition of filopodium formation could be observed (data not shown), suggesting that the levels of N17-Cdc42 expression obtained might not be sufficient to alter the organization of the cytoskeleton in these cells. Expression levels of V12-Cdc42 were similar to or lower than those of endogenous Cdc42; levels of N17-Cdc42 in the lines which we obtained were significantly lower (Fig. 3b, inset; Fig. 4b, bottom panel). Nevertheless, similar levels of N17-Cdc42 expression (see Fig. 6b) caused dramatic effects in V12-H-Ras/N17-Cdc42-coexpressing lines (see below).

Expression of V12-Cdc42 causes anchorage-independent growth. All V12-Cdc42-expressing cell lines tested showed significant growth in soft agar, while none of the vector control clones did (Fig. 3a). The efficiency of soft agar growth approximately correlated with V12-Cdc42 expression levels (Fig. 3a, bottom panel). We also observed that cells expressing high levels of V12-Cdc42 tended to round up and detach from the substratum (Fig. 2a). This phenomenon was most marked at low passage numbers and gradually disappeared with higher passage numbers. When collected, these cells maintained a rounded morphology and efficiently proliferated in suspension (data not shown), consistent with a role for Cdc42 in the regulation of anchorage independence.

In contrast to V12-Rac1 lines (37), V12-Cdc42 lines did not show an increase in low (0.5%)-serum growth (Fig. 3b) and did not grow to higher saturation density in the presence of 10% serum (Fig. 3c). Moreover, whereas expression of N17-Rac1 strongly inhibits low-serum growth and saturation density in 10% serum (37), N17-Cdc42 did not have any discernible effect on these parameters (Fig. 3b and c). Together, these data indicate that Cdc42 controls anchorage dependence of growth but not mitogenicity in Rat1 fibroblasts.

We also examined V12-Cdc42-expressing cells for tumor formation in nude mice. All lines expressing V12-Cdc42 tested showed tumorigenicity, while vector control lines did not cause tumors (Table 1). Thus, expression of constitutively active Cdc42 is sufficient for malignant transformation.

Cdc42 controls cell proliferation independently of Rac. The induction of lamellipodia and the increase in the number of multinucleated cells caused by expression of V12-Cdc42 are consistent with previous studies, which indicated that Cdc42 can act upstream of Rac in a signaling cascade which controls the organization of the actin cytoskeleton (30). It was therefore of interest to examine whether Rac is also necessary for anchorage-independent growth caused by V12-Cdc42. To address this question, we generated stable lines coexpressing V12-Cdc42 and N17-Rac1. The expression levels of V12-Cdc42 obtained in three of the V12-Cdc42/N17-Rac1-coexpressing lines were comparable to those of the lines only expressing V12-Cdc42 (Fig. 4, bottom panel). N17-Rac1 expression levels in the coexpressing lines are shown in Fig. 6d.

The generation of multinucleated cells caused by V12-Cdc42 (up to 18% in some cell lines) was strongly inhibited by coexpression of N17-Rac1 (2% of total cells, compared to 1% in vector controls [see also Fig. 2]). In addition, PDGF-induced formation of lamellipodia was also inhibited in V12-Cdc42/N17-Rac1-coexpressing lines (Fig. 4a). In contrast, soft agar growth of the V12-Cdc42 lines was not significantly inhibited by coexpression of N17-Rac1 (Fig. 4b). These data indicate

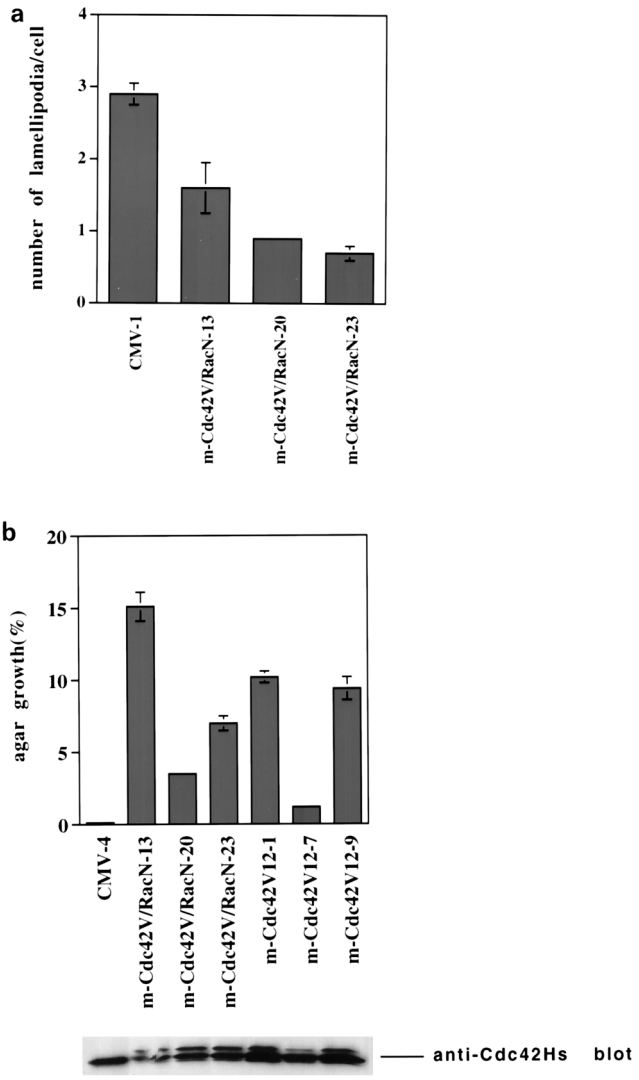

FIG. 4. N17-Rac1 coexpression does not inhibit V12-Cdc42-induced soft agar growth. (a) PDGF-BB-induced lamellipodium formation is inhibited in V12-Cdc42/N17-Rac1-coexpressing cells. The number of lamellipodia/cell represents the average value determined on three groups of 15 to 20 cells each. Quantitation of lamellipodia induced by PDGF-BB is described in Materials and Methods. (b) Soft agar growth was performed as indicated in Materials and Methods. Data shown are the averages obtained from two independent experiments performed in duplicate. Error bars indicate the spread between the two independent experiments. V12-Cdc42 expression was detected by immunoblotting with the antipeptide Cdc42 antibody (bottom panel). The upper and lower bands correspond to recombinant and endogenous Cdc42, respectively.

that Cdc42 controls multiple pathways: one or more pathways which control lamellipodia and cleavage furrow formation and are Rac dependent, and another pathway which regulates anchorage-independent growth and is Rac independent.

Cdc42 plays a role in transformation by oncogenic Ras. Having identified a novel function for Cdc42 in cell proliferation, we next studied the role of Cdc42 in Ras transformation. Coexpression of N17-Cdc42 caused a dose-dependent inhibition of focus formation by V12-H-Ras in NIH 3T3 cells (Fig. 5a). To test for possible toxicity of N17-Cdc42, we transfected NIH 3T3 cells with pCMVneoMycN17-Cdc42 under selection by G418. Colony formation efficiency was 3,080 ± 88 (standard error of the mean, $n = 4$) per μg of plasmid equivalent, comparable to that obtained with the vector pCMVneoMyc, 3,000 ± 49 (standard error of the mean, $n = 4$) per μg of plasmid equivalent indicating that the inhibition of Ras focus formation by N17-Cdc42 was not due to a toxic effect. These results therefore indicate that Cdc42, like Rac and Rho (19, 36–38), is essential for Ras transformation. We also examined the effect of inhibiting Cdc42 function on Ras transformation by using WASP-GBD (WASP was recently shown to be a Cdc42-specific effector) (1, 50). WASP-GBD also strongly inhibited focus formation by V12-H-Ras (Fig. 5a). In addition, both N17-Cdc42 and WASP-GBD inhibited focus formation induced by RafCAAX (Fig. 5b), which constitutively activates the ERK kinase pathway by virtue of localizing Raf to the plasma membrane (26, 47). We therefore conclude that Cdc42 is necessary for transformation by both Ras and RafCAAX.

To further study the role of Cdc42 in Ras transformation, we generated stable Rat1 lines coexpressing V12-H-Ras and N17-Cdc42. Ras expression levels in the V12-H-Ras/N17-Cdc42-coexpressing lines were similar to those in lines expressing V12-H-Ras alone (Fig. 6a). Coexpression of N17-Cdc42 strongly inhibited Ras-induced anchorage-independent growth (Fig. 6e) and potently reverted the transformed morphology of V12-H-Ras-expressing cells (Fig. 7), confirming that Cdc42 is essential for Ras transformation.

Cdc42 and Rac play distinct roles in Ras transformation. Although N17-Cdc42 and N17-Rac1 both inhibit Ras focus formation (Fig. 5a and references 19 and 37), stable Rat1 fibroblast lines expressing N17-Rac1 show strongly diminished proliferation potential in low serum (37), whereas lines expressing N17-Cdc42 are not inhibited in low serum (Fig. 3b). This finding suggested that Cdc42 and Rac may play distinct roles in Ras transformation. To address this question, we also generated stable Rat1 lines coexpressing V12-H-Ras and N17-Rac1. Expression levels of V12-H-Ras in V12-H-Ras/N17-Rac1-coexpressing lines were similar to those in lines expressing V12-H-Ras alone (Fig. 6c). Like N17-Cdc42, N17-Rac1 strongly inhibited the ability of these cells to grow in soft agar (Fig. 6e). However, whereas expression of N17-Cdc42 strongly reverted the transformed morphology of V12-H-Ras-expressing cells, expression of N17-Rac1 only marginally affected this aspect of Ras transformation (Fig. 7a to d). In addition, in line with the effects of dominant negative Rac and Cdc42 in nontransformed cells (Fig. 3b and reference 37), N17-Rac1 strongly inhibited low-serum growth of Ras-transformed cells, whereas N17-Cdc42 merely lowered the growth rate in low serum to that of vector control cells (Fig. 7e). Together, these results indicate that Cdc42 and Rac1 play distinct roles in Ras transformation.

To examine the potential role of filopodia in cell transformation, we stained with phalloidin the V12-H-Ras-transformed Rat1 fibroblasts and the cells coexpressing V12-H-Ras and N17-Cdc42 or N17-Rac1. We could not detect any significant change in filopodium formation in V12-H-Ras-expressing cells or V12-H-Ras/N17-Cdc42-coexpressing cells with respect to

TABLE 1. Tumor formation of V12-Cdc42Hs-expressing cells[a]

Cell line	Tumor latency (wk)	Tumors incidence
V12-Cdc42-1	2	6/6
V12-Cdc42-7	3	6/6
V12-Cdc42-9	2	6/6
CMV-2	NA	0/6
CMV-4	NA	0/6

[a] CMV-2 and -4 represent vector control lines. NA, not applicable.

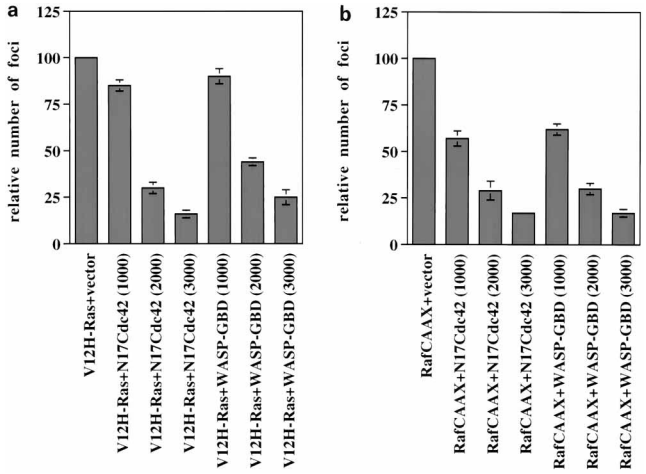

FIG. 5. N17-Cdc42 and WASP-GBD inhibit focus formation by V12-H-Ras and RafCAAX. Focus formation assays were carried out in NIH 3T3 cells as described in Materials and Methods. Plasmid amounts per 100-mm-diameter dish were 2 ng of pEXV-V12-H-Ras (a), 350 ng of pEXV-EE-RafCAAX (b), 2,000 ng of vector, and the amounts (in nanograms) of pEXV-Myc-N17-Cdc42 and pWASP-GBD indicated in parentheses. Error bars indicate the spread of data obtained in two independent experiments performed in duplicate.

vector control cells (Fig. 8a to c), suggesting that the expression level of N17-Cdc42 which is sufficient to inhibit Ras transformation is not sufficient to inhibit filopodium formation. Interestingly, however, filopodium formation was strongly stimulated in the V12-H-Ras/N17-Rac1-coexpressing cells (Fig. 8d). These observations therefore do not reveal any correlation between filopodium formation and anchorage-independent growth (Fig. 6e) and suggest that Cdc42 controls filopodium formation and anchorage independence via independent pathways.

DISCUSSION

In this report, we show that expression of constitutively active Cdc42 is sufficient to cause anchorage-independent growth and that Cdc42 is essential for Ras transformation, providing a novel role for Cdc42 in the control of cell proliferation and Ras transformation. Our data also indicate that the roles of Cdc42 and Rac in Ras transformation are distinct: Cdc42 is critical for Ras-induced transformed morphology, whereas Rac is essential for mitogenicity. In addition, both Cdc42 and Rac are necessary for anchorage-independent growth caused by Ras transformation.

We showed that dominant negative N17-Cdc42 inhibits Ras transformation both in focus formation assays in NIH 3T3 fibroblasts and in stable cotransfectants in Rat1 fibroblasts. This result strongly suggests that activation of a signaling pathway controlled by Cdc42 is necessary for Ras transformation. We further confirmed the involvement of Cdc42 in Ras transformation by making use of a dominant negative version of WASP, a Cdc42-specific effector (1, 50).

Transformation by most oncogenes is characterized by the acquisition of both anchorage independence and the ability to grow in low serum. Our observation that expression of V12-Cdc42 causes abrogation of anchorage, but not growth factor requirements, suggests that Cdc42 may relay an adhesion-induced signal which is necessary for normal cell proliferation. Interestingly, constitutive activation of focal adhesion kinase (FAK) also causes anchorage independence in the absence of low-serum growth (10). In addition, FAK binds to Graf, which has been shown to possess GAP activity toward Cdc42 (13), indicating the possibility of cross talk between the FAK and Cdc42 pathways. It will therefore be of interest to investigate the precise relationship between the FAK and Cdc42 signaling cascades.

Recent work using stable NIH 3T3 lines expressing the Rho guanine nucleotide exchange factors lbc and dbl has indicated that activation of Rho may also induce anchorage-independent growth without having a significant effect on growth rates in low serum (43). Rat1 fibroblast lines expressing constitutively active V14-RhoA do not show induction of anchorage-independent growth, however (38). Interestingly, dbl-transfected

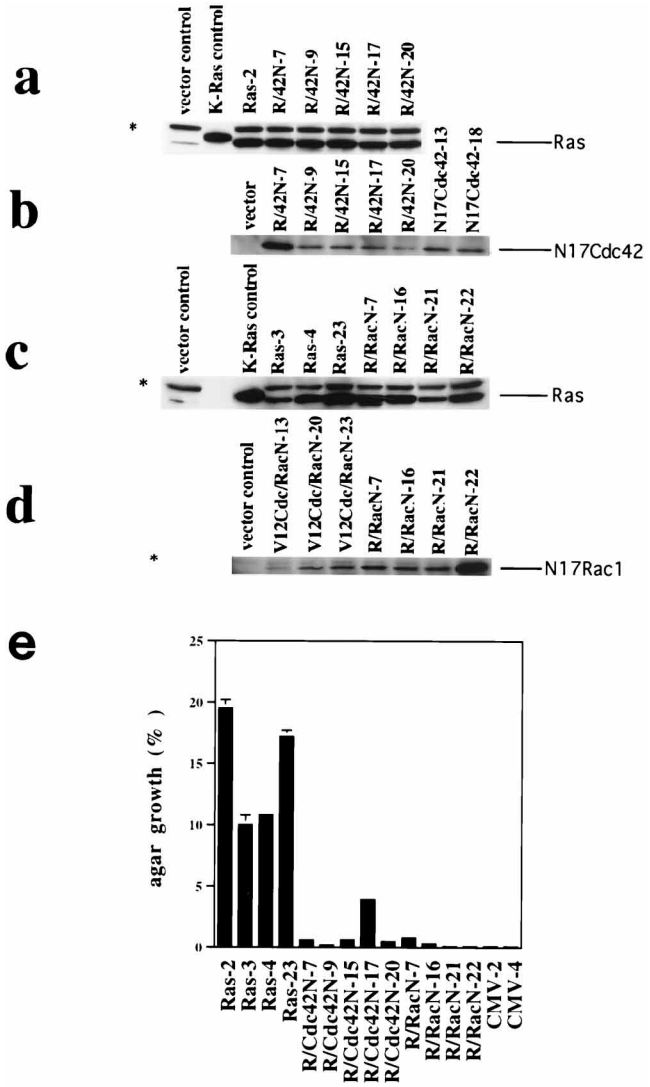

FIG. 6. Both N17-Cdc42 and N17-Rac1 inhibit soft agar growth induced by V12-H-Ras, as indicated by stable coexpression of V12-H-Ras and N17-Cdc42 or V12-Rac1. V12-H-Ras expression (a and c) was determined by immunoprecipitation with Y13-238 and Western blotting with 6B7; Cdc42 and Rac1 expression (b and d) was determined by Western blotting with anti-Myc antibody 9E10 and affinity-purified anti-Rac antibody, respectively. *, a nonspecific band. R/42N designates V12-H-Ras/N17-Cdc42-coexpressing clones; R/RacN designates V12-H-Ras/N17-Rac1-coexpressing clones; V12-Cdc/RacN designates V12-Cdc42/N17-Rac1-coexpressing clones. Coexpression of dominant negative Cdc42 or Rac1 blocks soft agar growth induced by V12-H-Ras (e). Soft agar assays were performed as described above. Error bars represent the spread of data from two independent experiments performed in duplicate.

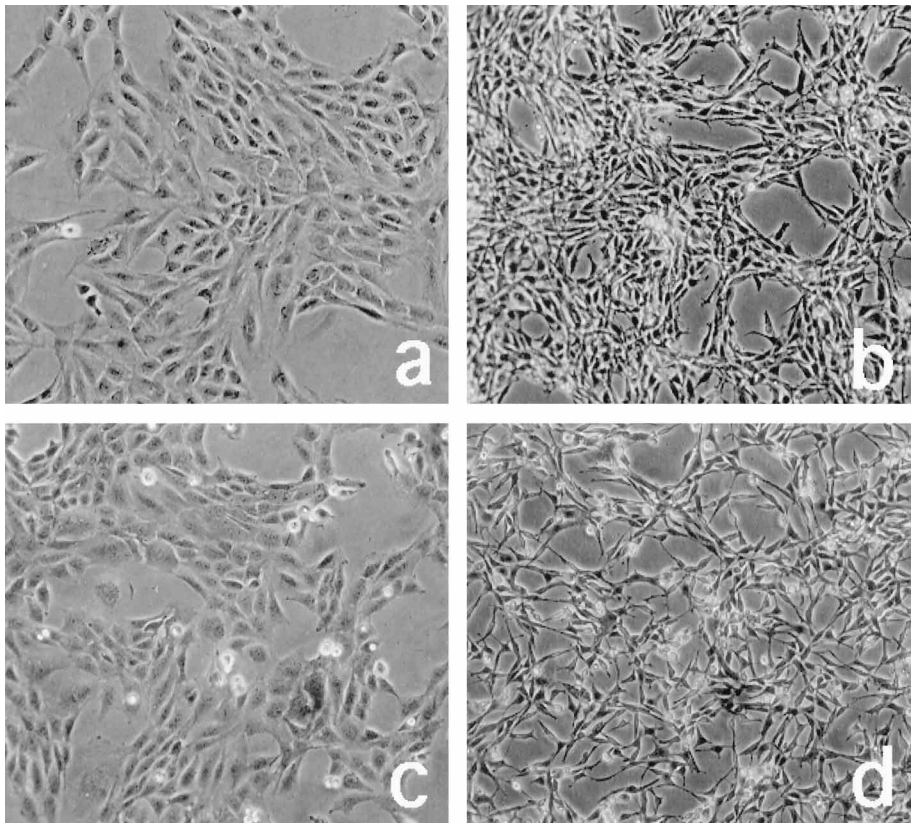

FIG. 7. Differential effects of Cdc42 and Rac1 on Ras transformation. Phase-contrast micrographs showing morphologies of Rat1 cells expressing vector (a), V12-H-Ras (b), V12-H-Ras and N17-Cdc42 (c), and V12-H-Ras and N17-Rac1 (d). (e) Low (0.5%)-serum growth curves of the cell lines analyzed. The various lines were plated at 10^5 cells/well in six-well plates. Data shown are representative of two independent clones. Error bars indicate the spread of data from two independent experiments performed in duplicate.

NIH 3T3 fibroblasts grew much better in suspension than lbc-transfected cells (43). Whereas lbc acts specifically on Rho (56), dbl functions as an exchange factor for Cdc42 as well as Rho (12), further supporting a role for Cdc42 in anchorage-independent growth.

The involvement of Cdc42 in Ras transformation is also consistent with Cdc42 acting downstream of Ras. Ras has been shown to bind to RalGDS and stimulate RalGDS-mediated nucleotide exchange activity on Ral (15, 21, 46, 51, 55), consistent with a role for RalGDS in Ras transformation (51, 54). Activated Ral, in turn, has been shown to bind to Ral-BP1, which possesses GAP activity toward Cdc42 (4, 18, 33). Sequestration of Ral-BP1 away from Cdc42 might be predicted to cause an increase in Cdc42-GTP levels. One pathway mediating activation of Cdc42 by Ras therefore could consist of RalGDS, Ral, and Ral-BP1. We have investigated the possibility of a direct pathway leading from Ras to Cdc42, using filopodium formation as a readout for Cdc42 activation. However, we could not observe any increase in the formation of filopodia after microinjection of D12-H-Ras proteins in either *Xenopus* XTC fibroblasts or porcine aortic endothelial cells, although D12-H-Ras did induce lamellipodia in these conditions (8a).

A third possible mechanism for the role of Cdc42 in Ras transformation is that Cdc42 controls an autocrine loop which is activated by oncogenic Ras. A Cdc42-mediated autocrine loop would also be consistent with the inhibition of RafCAAX-induced focus formation by N17-Cdc42.

The observation that Cdc42 is necessary for Ras transformation, together with previous findings that Rac and Rho are also essential for Ras transformation (19, 36–38), raises the question as to whether the pathways governed by these three GTPases function independently of each other in the control of cell proliferation. Although it has been shown that Rac can function downstream of Cdc42 in a cascade to control the organization of the actin cytoskeleton (24, 30), our data indicate that the pathways driven by these GTPases in the control of Ras transformation are likely independent of each other. First, the role of Cdc42 in transformation appears to be largely independent of Rac: N17-Rac1, at expression levels which inhibit Cdc42-dependent reorganization of the actin cytoskeleton, does not have a significant effect on anchorage-independent growth of V12-Cdc42-expressing cells. Second, some of the properties of the lines coexpressing oncogenic Ras and

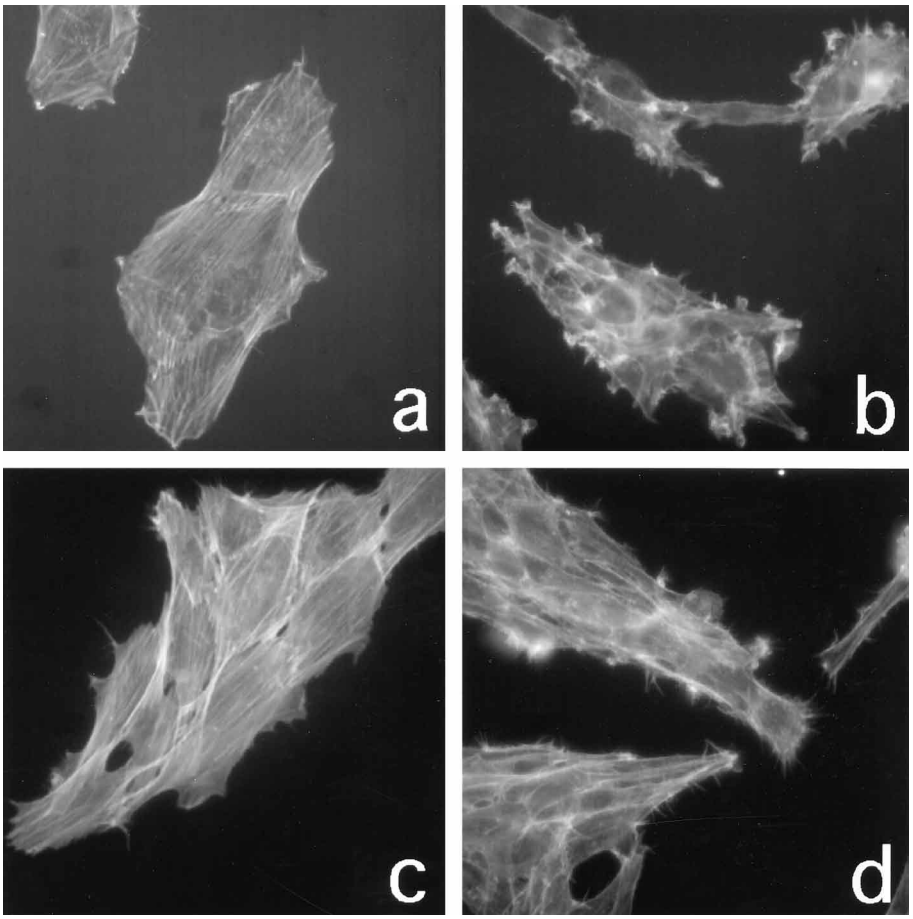

FIG. 8. Dissociation of filopodium formation and anchorage-independent growth. Immunofluorescence micrographs show FITC-phalloidin staining of Rat1 cells expressing vector (a), V12-H-Ras (b), V12-H-Ras and N17-Cdc42 (c), and V12-H-Ras and N17-Rac1 (d).

dominant negative Cdc42 or Rac are clearly distinct. Whereas anchorage-independent growth is abolished in both types of lines, Ras-induced morphological changes were strongly reverted by N17-Cdc42 but not by N17-Rac1. In addition, N17-Rac1 strongly inhibited low-serum growth in oncogenic Ras-expressing cells, whereas N17-Cdc42 had only a small effect on this growth characteristic. The distinct roles played by Rac and Cdc42 in Ras transformation are also reflected in the difference in growth properties of the lines expressing activated forms of Rac and Cdc42 in untransformed Rat1 fibroblasts: whereas lines expressing V12-Rac1 can grow in soft agar and show enhanced growth in low serum (37), lines expressing V12-Cdc42, while being able to grow in soft agar, do not have a diminished requirement for serum.

The relationship between the Rac and Rho pathways in the control of cell transformation remains to be clarified. Earlier observations that Rho can act downstream of Rac in the control of stress fiber formation induced by growth factors and oncogenic Ras (41) suggested that Rho could also act downstream of Rac in a pathway that controls cell proliferation (38). However, the observation that N19-RhoA inhibits RafCAAX focus formation, whereas N17-Rac1 does not, suggests that Rho could also function in a signaling cascade which is distinct from the Rac pathway and could mediate an autocrine loop which is necessary for transformation by Ras and RafCAAX. Clearly however, the pathways controlled by Cdc42, Rac, and Rho are distinct from the ERK kinase cascade, which is also essential for Ras transformation (7, 8, 29).

The observation that multiple pathways are necessary for Ras transformation suggests that each of these pathways may deliver a distinct contribution to the transformation process. In line with this, we find that in Ras-transformed Rat1 fibroblasts, Cdc42 is specifically involved in morphological transformation and Rac in serum-independent growth. The existence of multiple independent signaling cascades which are essential for Ras transformation is also consistent with recent results obtained with three different Ras effector domain mutants, showing that coexpression of any pair of these mutants leads to strong synergism in focus formation (20, 53).

The pathways downstream of Cdc42 which mediate its role in transformation remain to be identified. Cdc42 has been shown to control the formation of actin-linked cytoskeletal structures such as filopodia and focal complexes (24, 30). However, we could not establish any correlation between filopodium formation and anchorage-independent growth, suggesting that anchorage independence and filopodium formation are controlled by Cdc42 via independent pathways. Cdc42 has also been shown to regulate the $p70^{S6k}$, JNK, and p38 kinases (6, 7, 29) and serum response factor transcriptional activation (14), but the roles of these activities in transformation remain to be determined.

The earlier observation that Cdc42 is essential and sufficient

for DNA synthesis in Swiss 3T3 fibroblasts (32) appears to be at variance with our results that modulation of Cdc42 activity has hardly any effect on mitogenicity. One explanation for this discrepancy might be that different cell lines, conditions, and assays were used in the two studies. Moreover, entry into S phase may not be sufficient to allow for growth in low serum.

The many different signaling pathways activated by Rho family GTPases are likely to branch out at the level of these GTPases themselves (16, 25, 52). This is consistent with the existence of a large number of putative effectors for these GTPases, which include members of the PAK family of serine/threonine kinases, $p70^{S6k}$ kinase, IQGAP1, the Wiskott-Aldrich syndrome protein, and several other proteins which contain a conserved Cdc42 or Rac interaction binding domain (39). The potential involvement of any of these Cdc42 effectors in cell proliferation remains to be established.

In summary, this study has identified a novel role for Cdc42 in cell growth and Ras transformation. These findings have provided new insights into the mechanisms underlying Ras transformation and suggest that the elucidation of the Cdc42 signaling cascade involved in cell proliferation will lead to the identification of novel targets for cancer therapy.

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