Oncogenic Ras Leads to Rho Activation by Activating the Mitogen-activated Protein Kinase Pathway and Decreasing Rho-GTPase-activating Protein Activity

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Transformation by oncogenic Ras requires signaling through Rho family proteins including RhoA, but the mechanism(s) whereby oncogenic Ras regulates the activity of RhoA is (are) unknown. We examined the effect of Ras on RhoA activity in NIH 3T3 cells either stably transfected with H-Ras(V12) under control of an inducible promoter or transiently expressing the activated H-Ras. Using a novel method to quantitate enzymatically the GTP-bound to Rho, we found that expression of the oncogenic Ras increased Rho activity 2-fold. Increased Rho activity was associated with increased plasma membrane binding of RhoA and decreased activity of the Rho/Ras-regulated p21WAF1/CIP1 promoter. RhoA activation by oncogenic Ras could be explained by a decrease in cytosolic p190 Rho-GAP activity and translocation of p190 Rho-GAP from the cytosol to a detergent-insoluble cytoskeletal fraction. Pharmacologic inhibition of the Ras/Raf/MEK/ERK pathway prevented Ras-induced activation of RhoA and translocation of p190 Rho-GAP; expression of constitutively active Raf-1 kinase or MEK was sufficient to induce p190 Rho-GAP translocation. We conclude that in NIH 3T3 cells oncogenic Ras activates RhoA through the Raf/MEK/ERK pathway by decreasing the cytosolic activity and changing the subcellular localization of p190 Rho-GAP.

Proteins of the Ras superfamily, including the Ras and Rho families, cycle between active GTP- and inactive GDP-bound forms and function as essential switches in signal transduction pathways that regulate cell growth, differentiation, and survival (1). Activating mutations in H-, K-, and N-Ras are found in up to 30% of all human cancers; in cancers with wild type Ras, overexpression of growth factor receptors frequently leads to activation of the Ras/Raf/MEK/ERK pathway, suggesting an important contribution of Ras functions to the development of human cancers (1–3). Although there are no reports of activating mutations of Rho proteins in human tumors, several Rho proteins are overexpressed in tumors, and Rho family activating guanine nucleotide exchange factors (GEFs) have been isolated in screens for transforming genes, suggesting a role of Rho proteins in tumorigenesis (4).

Members of the Ras family regulate the actin cytoskeleton, thereby affecting cell morphology and motility; in addition, they modulate gene expression, cell cycle progression, and cell survival (1, 4, 5). RhoA, B, and C and Rac1 play critical roles in cell transformation induced by activated, oncogenic Ras, with dominant negative Rho and Rac1 constructs inhibiting Ras-induced transformation and constitutively active constructs inducing anchorage-independent growth and other features of the transformed phenotype (4, 6–9). The requirement of Rho for Ras-induced transformation exists in part because Ras and Rho play opposing roles in control of the cyclin-dependent kinase inhibitor p21WAF1/CIP1, with Ras inducing and Rho inhibiting p21WAF1/CIP1 transcription; thus increased Rho activity actually blocks cell cycle progression when Rho signaling is inhibited by C3 exoenzyme (8, 10, 11).

Activated, oncogenic Ras may regulate RhoA and Rac1 activities, but the effects of Ras appear to be cell type-specific, vary with the Ras subtype, and depend on the kinetics and duration of Ras activation (8, 12–18). Microinjection or transient transfection of oncogenic H-Ras(V12) into Swiss 3T3 cells leads to acute cytoskeletal changes, suggesting a hierarchal system with Ras activating Rac (causing membrane ruffling) and Rac in turn activating RhoA (causing induction of stress fibers); however, these studies were performed before direct measures of Rac and Rho/GTP levels were available (12, 13). Ras activation of Rac can occur through the Raf effector phosphatidylinositol 3-kinase, with increased phosphoinositides activating a multimolecular complex including a Rac-activating GEF (19–21). How Rac activation can lead to activation of RhoA is less clear, but it may involve Rac activation of phospholipase A2, subsequent arachidonic acid and leukotriene production, at least in Swiss 3T3 cells (22).

In contrast to the acute response to oncogenic Ras, studies in Ras-transformed cell lines have produced conflicting results, with some studies reporting decreased Rac and increased RhoA activation compared with nontransformed cells, and others reporting increased activation of both GTPases or no changes (8, 14–18). In v-H-Ras-transformed MDCK cells, decreased Rac guanine nucleotide exchange factor; GST, glutathione S-transferase; GTPyS, guanosine 5′-O-(thiotriphosphate); LTR, long terminal repeat; Lue, luciferase; MAP, mitogen-activated protein; MDCK, Madin-Darby canine kidney; RBD, Rho binding domain; RSV, Rous sarcoma virus; TK, thymidine kinase; BXB, catalytic domain of Raf-1 kinase.
activity appeared to be secondary to transcriptional down-regulation of the Ras-specific GEF Tiam1; the mechanism for increased RhoA activation was not elucidated, but the effect of oncogenic Ras on RhoA and Rac activity was mimicked by stable transfection of constitutively active Raf (16). H-Ras(V12)-transformed Swiss 3T3 cells also demonstrated decreased Rac and increased Rho activity compared with untransformed cells, but short term expression of a constitutively active Raf in Swiss 3T3 cells did not lead to elevation of RhoA activity; elevated RhoC-GTP levels were only seen after prolonged (>4 weeks) culture of the active Raf-overexpressing cells, suggesting that they were a consequence of selection rather than direct signaling (8). In HT1080 human fibrosarcoma cells containing oncogenic N-Ras, both Rac and RhoA activity were increased compared with cells lacking the mutant N-Ras; Rac and RhoA activities were also increased when cells lacking the mutant N-Ras were stably transfected with constitutively active Raf or MEK (18). In K-Ras(V12)-transformed normal rat kidney cells, no significant change of RhoA activity was observed compared with untransformed cells (23). Several older studies reported loss of stress fibers in Ras-transformed Rat1 cells, with restoration of stress fibers upon transfection of constitutively active RasA, suggesting loss of Rho activity in the Ras-transformed cells (7, 24); others reported increased stress fibers in Ras-transformed breast cancer cells and NIH 3T3 cells without direct measurement of Rho activity (14, 15).

Because the mechanism of Rho regulation by Ras is not clear, we decided to examine the effects of oncogenic Ras on the activation state of RhoA in NIH 3T3 cells stably expressing H-Ras(V12) under control of an inducible promoter (LTR-H-Ras(A) cells (25)); these cells have low basal and high induced levels of H-Ras(V12) and allowed us to study short term effects of Ras activation avoiding complex genetic changes that may occur during long term culture of Ras-transformed cells. Using two different methods to assess Rho activation, we found that induction of H-Ras(V12) in LTR-H-Ras(A) cells or transient transfection of H-Ras(V12) into wild type NIH 3T3 cells caused an approximate 2-fold increase in Rho-GTP levels. Concomitant with Rho activation, we found increased RhoA translocation to membranes and decreased activity of a p21WAF1/CIP1 promoter with Rho activation, we found increased RhoA translocation to membranes and decreased activity of a p21WAF1/CIP1 promoter with Rho activation, we found increased RhoA translocation to membranes and decreased activity of a p21WAF1/CIP1 promoter

Measurement of Absolute Amounts of GTP and GDP Bound to Rho—This method is a modification of a procedure we have used previously to measure GTP, and GDP, bound to Ras, Rap1, and Rho (2, 26, 38–40). Cells grown on a 100-mm plate under the conditions described in the figure legends were extracted quickly in situ by washing once with ice-cold Tris-buffered saline, pH 7.4, and adding lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 1% CHAPS, 200 mM NaCl, 1 mM MgCl2, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. After a 1-min incubation on ice, the lysed cells were scraped with a rubber policeman, transferred to a microcentrifuge tube, and subjected to vortexing for 10 s. Cell extracts were centrifuged at 10,000 g for 2 min, and a portion of the supernatant was added to tubes containing 10 mM MgSO4 and 30 μg of GST-tagged Rhotekin RBD bound to glutathione-Sepharose beads; these samples were used for measuring GTP bound to Rho (“unloaded” samples). The remaining supernatant was added to tubes containing 10 μM GTP, 10 μM EDTA, and 50 μg of GST-tagged RBD on glutathione beads, allowing the free GTP to exchange for GDP bound to Rho, thus converting all of the Rho to the GTP-bound state (“loaded” samples). After gentle shaking for 1 h at 4 °C, the beads with Rho-GTP bound to the Rhotekin RBD were washed four times with 50 mM Tris-HCl, pH 7.4, 2% Nonidet P-40, 500 mM NaCl, 10 mM MgSO4, and twice with 20 μM Tris-PiO4, pH 7.4, 5 mM MgSO4, 1 mM EDTA, 10 μM dithiothreitol, 2 mM EDTA (TDE buffer). We have shown previously >95% recovery of GTP under these conditions (26). GTP eluted from the unloaded and loaded samples was measured in a coupled enzymatic assay by conversion to ATP in the presence of ADP and nucleoside diphosphate kinase (26); the resulting ATP was measured by the firefly luciferase method in a photon-counting luminometer (MGM Instruments, Hamden, CT). This method is sensitive to 1 fmol of GTP and is quantitative because the second reaction is irreversible, from light generation, allowing both reactions to go to completion (26).

Assessment of Rho-bound GTP by Western Blotting—Cells were extracted and processed as described above for the unloaded samples except the magnesium concentration in the initial lysis buffer was increased to 10 mM; Rho-GTP isolated by binding to the Rhotekin RBD-coated beads was quantitated by Western blotting using a Rho-specific antibody (Santa Cruz Biotechnology), as described by Ren et al. (33). Measurement of the Activation State of EE Epitope-tagged RhoA Expressed in Cells—Cells transfected with EE epitope-tagged RhoA constructs were extracted in situ in 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 500 mM NaCl, 10 mM MgCl2, 0.5% deoxycholate, 0.05% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin (RIPA buffer). After centrifuging the extracts, supernatants were split in half and added to tubes containing protein G-agarose beads coated with either a mouse monoclonal anti-EE antibody or control mouse IgG. The tubes were shaken gently for 1 h at 4 °C, and the beads were washed four times with RIPA buffer, and twice with 20 μM Tris-PiO4, pH 7.4, 5 mM MgSO4. GTP and GDP were released from Rho by heating the beads for 3 min at 100 °C in 5 mM Tris-PiO4, pH 7.4, 2 mM dithiothreitol, 2 mM EDTA (TDE buffer). We have shown previously >95% recovery of GTP under these conditions (26).

Assessment of p21WAF1/CIP1 Promoter Activity—Plasmids were transfected into 8 × 106 cells in a 24-well plate culture. In 24 h later the cells were transfected with 300 ng of DNA using Polyfect (Invitrogen) according to the manufacturer’s recommendation. All cells received 25 ng of p21-Luc, and as indicated some cells additionally received 50 ng pEF-C3exo or 100 ng of pCDNA3-EE-RhoA(16L). Cells were treated for 24 h with 1 μM dexamethasone, and luciferase activity was measured in cell extracts as described previously (41). We did not include an internal control vector because all four genes were transfected, i.e., pRSV-bgalactosidase, pCMV-bgalactosidase, pSV40-bgalactosidase, and pCV-bgalactosidase.
and pTRC-β-galactosidase, demonstrated some increase in transcription when LTR-H-Ras(A) cells were treated with dexamethasone.

**Assessment of Rac Activation**

Subconfluent cells grown on two 150-mm plates were extracted by incubating for 2 min in cold RIPA buffer, and extracts were centrifuged at 10,000 × g for 2 min. The p21Rac(CDC42)-binding domain of human PAK-1 was used to isolate Rac-GTP, and the amount of Rac-GTP bound to the beads was quantitated by Western blotting with a mouse monoclonal anti-Rac antibody as described previously (42), using an assay kit from Upstate Biotechnology.

**Assessment of Subcellular Localization of RhoA, Rho-GAP, and Ras-GAP**

Cells grown on 150-mm plates were extracted by Dounce homogenization in 10 mM Hepes, pH 7.5, 2 mM EDTA, 1 mM MgCl₂ (HEM buffer). The resulting cell homogenate was centrifuged at 500 × g for 5 min to remove nuclei and subcellular organelles, and the supernatant was centrifuged at 37,000 × g for 10 min. The supernatant and pellet from the second centrifugation are referred to as cytosol and membranes, respectively, with the membrane preparation washed twice in HEM buffer to remove contaminating cytosol. Protein concentrations were determined according to Bradford (43), and equal amounts of protein from each preparation (30 µg of homogenate, 20 µg of cytosol, and 40 µg of membranes) were subjected to SDS-PAGE/Western blotting using mouse monoclonal anti-Rho-GAP (Santa Cruz Biotechnology, 1:1,000) and anti-Ras-GAP (Sigma, 1:500) antibodies, or a rabbit polyclonal mouse monoclonal anti-Rho-GAP antibody (Santa Cruz Biotechnology, 1:1,000). Triton X-100-insoluble cytoskeletal fractions were prepared as described previously (44). Cells were washed in phosphate-buffered saline and extracted in situ for 40 s at room temperature in 50 mM Na-Hepes, pH 6.4, 3 mM EGTA, 5 mM MgCl₂, 0.5% Triton X-100. The detergent-insoluble supernatant was removed; the detergent-insoluble material was scraped off with a rubber policeman in the presence of phosphate-buffered saline and protease inhibitor mixture, and centrifuged for 10 min at 300,000 × g at 4 °C. Pellets were resuspended in SDS-sample buffer and analyzed by SDS-PAGE/Western blotting using the monoclonal anti-Rho-GAP antibody and an actin-specific antibody (C2, Santa Cruz Biotechnology, 1:3000 dilution).

**Assessment of Rho/Rho-GDI Association**

Cytosolic extracts were prepared as described above and subjected to immunoprecipitation using a rabbit polyclonal anti-Rho-GDI antibody or control rabbit IgG. Immunoprecipitates were collected on protein G-agarose beads and analyzed by SDS-PAGE/Western blotting using a mouse monoclonal anti-Rho-GAP antibody and the rabbit polyclonal anti-Rho-GDI antibody (both from Santa Cruz Biotechnology, used at 1:1,000).

**Measurement of Rho-GEF Activity**

Cells grown on 100-mm plates were extracted by sonication in 10 mM Hepes, pH 7.4, 1 mM EDTA. Bacterially expressed GST-RhoA was purified on glutathione-Sepharose beads and was loaded with [γ-32P]GTP (specific activity of 11.7 Ci/mmol) by a 30-min incubation at 37 °C in 50 mM Hepes, pH 7.5, 5 mM EDTA, as described previously (45). The [γ-32P]GTP-loaded RhoA was incubated with cell extracts in the presence of 1% Triton X-100, 250 or 500 ng of GST-RhoA preloaded with [γ-32P]GTP (specific activity 6,000 Ci/mmol) was added to initiate the reaction (45).

**Assessment of Ras Activity**

Cytosolic extracts were prepared and subjected to immunoprecipitation with either the anti-Rho-GAP or anti-Ras-GAP antibodies as described above. Immunoprecipitates were analyzed by SDS-PAGE/Western immunoblotting using the same antibodies. The amount of phosphorylated Rho-GAP was determined by probing the blots with an anti-phosphotyrosine-specific antibody (Santa Cruz Biotechnology, 1:500).

**Assessment of MAP Kinase Activation**

MAP kinase activity was assessed by Western blotting using a phospho-ERK-specific antibody that recognizes a dually phosphorylated peptide sequence corresponding to Thr185 and Tyr202 of p42 MAP kinase, as described previously (27).

**RESULTS**

**Quantitative Measurement of Rho Activation—**As part of these studies, we developed a new quantitative method to assess Rho activation by measuring absolute amounts of GTP and of total nucleotides i.e. the sum of GTP plus GDP, bound to Rho. To measure Rho-GTP, it was isolated from cell extracts according to the method of Ren et al. (36) using glutathione-agarose beads coated with a GST-Rhotekin RBD fusion protein; however, instead of assessing the Rho-GTP semi quantitatively by Western blotting, we eluted GTP from Rho and measured it in a coupled enzymatic assay as described previously for measuring GTP bound to other Ras-related proteins (26, 38–40). To measure total nucleotides bound to Rho, we converted Rho-GDP to Rho-GTP by incubating a separate aliquot of extract in the absence of magnesium and in the presence of 100 µM GDP; under these conditions, Rho-GDP is converted rapidly to Rho-GTP (46), and the latter was measured as just described.

In extracts prepared from logarithmically growing NIH 3T3 cells, the assay yielded a linear response over a 5-fold range of cellular protein for both unloaded samples, i.e. those in which Rho-GTP was measured directly (Fig. 1a, and for loaded samples, i.e. those in which total nucleotides bound to Rho were measured after converting Rho-GDP to Rho-GTP (Fig. 1c). Because the graphs obtained with unloaded and loaded samples overlap, the assay was linear over more than a 5-fold range, and the data allow calculation of Rho activation, i.e. [Rho-GTP/(Rho-GTP + Rho-GDP)] × 100. Thus, the two data points marked with an asterisk and a pound sign in Fig. 1, a and c, yielded a Rho activation of 4.1%, and the data point marked with a double dagger yielded a Rho activation of 3.2%. Rho activation can also be calculated from the slopes of the lines in Fig. 1, a and c (181 and 4,887 fmol of GTP/mg of protein, respectively), yielding a Rho activation state of 3.7%. Similar results were found in two other independent experiments, and experiments performed with suspension cells (HL-60 human leukemia cells) also yielded a linear response over a 5-fold range of cell number. For comparison, Rho-GTP bound to the Rhotekin RBD-coated beads was also assessed by Western blotting using a RhoA-specific antibody; within the limits of the method, a linear response was obtained for both unloaded and loaded samples (Fig. 1, b and d). The enzymatic method detected Rho-GTP with slightly higher sensitivity compared with the Western blot method; we define our limit of detection as the amount of Rho-GTP which produces a signal greater than 2-fold above background.
Fig. 1. Quantitative measurement of Rho activation. Varying numbers of logarithmically growing NIH 3T3 cells cultured in DMEM supplemented with 10% FBS were extracted in situ. From one aliquot of the extracts, Rho-GTP was isolated directly on glutathione-agarose beads using GST-tagged Rhotekin RBD (unloaded samples, a and b); to another aliquot of the extracts 10 mM EDTA plus 10 μM GTP were added prior to adding the Rhotekin RBD (loaded samples, c and d). GTP eluted from the washed beads was measured in a coupled enzymatic assay as described under “Experimental Procedures” (a and c), or RhoA protein isolated on the beads was assessed by Western blotting (b and d) with lanes 1–5 corresponding to the five protein concentrations in a and c. The data in a and c are the means of duplicates of a representative experiment, with similar results found in two other independent assays; the asterisks, pound sign, and double dagger indicate paired unloaded and loaded samples.

Rho Activation under Different Harvesting and Culture Conditions and Activation Levels of Transfected Wild Type and Constitutively Active RhoA Constructs—Because Rho activation levels can change in response to shear stress, changes in integrin ligation, and cell density (36, 47, 48), we studied the effect of different cell harvesting methods on Rho activation in NIH 3T3 cells. We found that scraping cells with a rubber policeman from tissue culture dishes followed by centrifugation and extraction yielded Rho activation levels 1.7 ± 0.3-fold higher than directly lysing cells on culture plates (data are the means ± S.D. of three independent experiments performed in duplicate, p < 0.05). These results are consistent with the findings of others that Rho can be activated by mechanical stimuli (47). In all subsequent experiments, cells were extracted rapidly by lysis in situ. When we extracted cells from subconfluent and confluent cultures, we found no dependence of the Rho activation state on cell density, as described by others for NIH 3T3 cells (48).

After 24 h of serum starvation, we found a Rho activation level of 4.8 ± 1.1% in wild type NIH 3T3 cells; this level increased to 14.4 ± 0.5% and 7.9 ± 2.5% after 1 and 3 min of serum stimulation, respectively, yielding a 3-fold increase in Rho activation at 1 min (Fig. 2a). Assessment of Rho activation by Western blotting also yielded an approximate three-fold increase after one minute of serum stimulation (Fig. 2b), thus confirming that the two methods provide similar results. Previous workers reported a 2–6-fold increase in RhoA activation during serum stimulation of Swiss 3T3 cells (90).

We modified the enzymatic assay to quantitate the activation state of transfected RhoA constructs. We transfected NIH 3T3 cells with EE epitope-tagged constructs of wild type RhoA, RhoA(63L), and RhoA(14V), isolated the expressed protein from cell extracts using an anti-EE epitope antibody, and measured GTP and total nucleotides bound to the isolated RhoA as described under “Experimental Procedures.” We found activation states of 3.6 ± 0.8%, 64 ± 5%, and 83 ± 4% for wild type RhoA, RhoA(63L), and RhoA(14V), respectively (Fig. 2c; compare RhoA(wt), open bar on the far left, with RhoA(63L) and (14V), closed bars). When we cotransfected the constitutively active DN-p115-Rho-GEF with wild type RhoA, we found that Rho activation increased to 15.1 ± 2.2% (Fig. 2c). Although this increase in Rho activation is similar to that observed in serum-stimulated cells (Fig. 1b), it is considerably less than the activation state of the constitutively activated, GTPase-deficient forms of RhoA. Because it seemed possible that the lower activation level of wild type RhoA, compared with the constitutively activated RhoA, was from its higher intrinsic GTPase activity leading to a loss of GTP during immunoprecipitation, we performed experiments in which we added soluble Rhotekin RBD to cell extracts to protect Rho-GTP; binding of the RBD of Rhotekin to Rho inhibits both intrinsic and GAP-enhanced Rho GTPase activity (49). We found no effect of the peptide on Rho activation, either at basal levels or in the presence of p115-Rho-GEF (Fig. 2c), suggesting little loss of GTP during the assay procedure. In Fig. 2d we show the expression of the three RhoA constructs, with wild type EE-RhoA and RhoA(14V) expressed at somewhat higher and RhoA(63L) at somewhat lower levels, than endogenous RhoA, considering a transfection efficiency of about 50%.

Rho Activation in NIH 3T3 LTR-H-Ras(A) Cells and NIH 3T3 Cells Transiently Transfected with Wild Type and Activated Ras—Rho proteins are required for Ras-induced transformation, but there are conflicting data concerning whether Ras signaling directly causes Rho activation or whether some changes in Rho activity occur only during the selection of Ras-transformed cells (5, 8, 16, 18, 23). Some of the variation in results may be the result of cell type-specific differences, but some may also be methodologic in origin. To address the latter specifically, we used two different methods to assess Rho activation, i.e. the quantitative enzyme-based method and Western blotting, and we performed the studies under two different conditions: (i) in NIH 3T3 cells stably expressing activated Ras under an inducible promoter (LTR-H-Ras(A) cells) and (ii) in NIH 3T3 cells transiently transfected with wild type Ras and Ras(12V) expression vectors.

LTR-H-Ras(A) cells express H-Ras(V12) under control of the dexamethasone-inducible mouse mammary tumor virus long terminal repeat and exhibit a transformed phenotype strictly dependent on the presence of hormone in the growth medium (50). The addition of dexamethasone leads to a significant increase in Ras expression at 8 h with peak expression at 24 h; this is associated with a dramatic change in cell morphology and acquisition of anchorage-independent growth (50). Tumor formation in nude mice does not require dexamethasone treat-
Ras Activates Rho

Fig. 2. Serum stimulation of endogenous Rho activity and activation levels of transfected wild type and constitutively active RhoA constructs. a and b, NIH 3T3 cells were serum starved for 36 h in DMEM containing 0.1% FBS and 0.1% bovine serum albumin followed by stimulation with 10% FBS for the indicated time. Cells were extracted in situ, and Rho-GTP was isolated by RBD-Rhotekin pulldown as described under “Experimental Procedures.” In a, the amount of GTP and total nucleotides bound to Rho was measured enzymatically as described in the legend to Fig. 1. In b, the amount of RhoGTP bound to the beads was quantitated by Western immunoblotting using a mouse monoclonal anti-RhoA antibody (upper blot); to demonstrate the input of total RhoA protein, 5% of cellular lysate was analyzed by Western blotting using the same antibody (lower blot). c, NIH 3T3 cells grown in six-well culture dishes were transiently transfected with 400 ng of EE epitope-tagged RhoA constructs (wild type RhoA (Rho wt) and mutant RhoA(V14L) or RhoA(V14)) as described under “Experimental Procedures.” Some experiments with wild type Rho included 500 ng of an expression vector encoding constitutively active ΔN-p115-RhoGEF. Cells were extracted 36 h later, and Rho proteins were isolated on protein G-agarose beads using an anti-EE antibody. To some samples 80 μg of soluble Rhotekin RBD peptide was added immediately after extraction. Rho activation was measured enzymatically as described in the legend to Fig. 1. d, NIH 3T3 cells were transfectared as described in c, and whole cell extracts were analyzed by SDS-PAGE/Western blotting using a monoclonal antibody specific for RhoA. Lane 1, empty vector; lane 2, EE-RhoA(wt); lane 3, EE-RhoA(V14L); lane 4, EE-RhoA(V14). Transfection efficiency was about 50%. Reprobing the blot with an anti-EE epitope antibody confirmed that the EE-tagged RhoA proteins migrate with a higher apparent molecular weight than endogenous RhoA (not shown); the 63L and V14 point mutations are known to alter the migration of RhoA differently (73). The data in a and c are the means ± S.D. of at least three independent experiments performed in duplicate.

Fig. 3. Measurement of Rho activation in LTR-H-Ras(A) cells and in wild type NIH 3T3 cells transiently transfected with H-Ras(V12). Wild type NIH 3T3 cells (NIH 3T3 wt) and stably transfected NIH 3T3 cells expressing H-Ras(V12) under control of the inducible murine mammary tumor virus promoter (LTR-H-Ras(A)) cells were cultured in the absence or presence of 1 μm dexamethasone (Dex) for 24 h; some of the LTR-H-Ras(A) cultures were also treated with the MEK inhibitor U0126 (20 μm) for 24 h as indicated. a, Rho activation was measured by a quantitative enzymatic assay as described in the legend to Fig. 1; open bars, without dexamethasone; filled bars, with dexamethasone. Rho activation in the absence of drugs was assigned a value of 1 for both cell types. For the LTR-H-Ras(A) cells, the asterisk indicates a statistically significant difference (p < 0.01) between the absence and presence of dexamethasone, and the pound sign designates a statistically significant difference (p < 0.05) between the absence and presence of U0126 in dexamethasone-treated cells. b, Rho-GTP (top blot), total cellular RhoA (middle blot), and Ras (bottom blot) were assessed by Western immunoblotting as described in Fig. 2c; for total RhoA and Ras, 5% of the cellular lysate was used. Lanes 1 and 2 are wild type NIH 3T3 cells cultured in the absence and presence of dexamethasone, and lanes 3–6 are LTR-H-Ras(A) cells cultured in the absence and presence of dexamethasone and U0126 as indicated. c, wild type NIH 3T3 cells were transiently transfected with EE epitope-tagged RhoA(wt) as described in the legend to Fig. 2c and were cotransfected with either 700 ng of empty vector or expression vectors for wild type Ras (Ras wt) or Ras(12V). During the last 24 h before harvest, 20 μm U0126 was added as indicated. Rho activation was measured as described in Fig. 2c. The asterisk indicates a significant difference (p < 0.05) between the absence and presence of U0126.

measurement, presumably because of endogenous glucocorticoid hormones in the animals (50). When we induced oncogenic Ras expression in LTR-H-Ras(A) cells by treating the cells with 1 μm dexamethasone for 24 h, we found a 2-fold increase in Rho activation using the quantitative enzymatic method to measure GTP bound to Rho; treating wild type NIH 3T3 cells with dexamethasone had no effect on Rho-GTP levels (Fig. 3a; p < 0.05 for the difference in Rho activation for LTR-H-Ras(A) cells
in the absence and presence of dexamethasone). Confirming these results, dexamethasone induced a similar approximate 2-fold increase in Rho-GTP in LTR-H-Ras(A) cells when assessed by the immunoblot method (Fig. 3b, top panel, shows lanes 3 and 4), whereas no increase in Rho-GTP was observed in wild type NIH 3T3 cells (Fig. 3b, top panel, lanes 1 and 2). Dexamethasone had no effect on total RhoA expression in either cell type, measured either by Western blotting (Fig. 3b, middle panel) or by determining total nucleotides bound to Rho (not shown).

As expected, dexamethasone increased Ras expression in LTR-H-Ras(A) cells but had no effect on Ras levels in wild type cells (Fig. 3b, bottom panel, shows a Western blot). Measuring total nucleotides bound to Ras (26), we found that dexamethasone induced a 2.2-fold increase in total Ras protein in the LTR-H-Ras(A) cells, and the activation state of Ras increased from 7.1 to 24.7% in the absence and presence of dexamethasone, respectively, with no effect of dexamethasone on Ras activity or expression levels in wild type NIH 3T3 cells (Table I). The degree of Ras activation in dexamethasone-treated LTR-H-Ras(A) cells is similar to the degree of Ras activation found in human pancreatic tumor cells containing a mutated K-Ras allele, or in HL-60 cells containing a mutated N-Ras (51). Because the antibody Y13259 used for immunoprecipitation of Ras recognizes all three Ras isoforms, the Ras activation measured in Table I represents an average activation state of H-, K-, and N-Ras, with the mutant H-Ras(V12) probably contributing only about half of total Ras in dexamethasone-treated LTR-H-Ras(A) cells.

To be sure that the increased Rho activity in dexamethasone-treated LTR-H-Ras(A) cells was not influenced by clonal selection of the stably transfected cells, we performed transient transfection experiments in wild type NIH 3T3 cells and found a 2.1-fold increase in Rho activation in cells expressing Ras(12V) compared with cells transfected with empty vector (Fig. 3c; p < 0.05 for the difference between control and Ras(12V)-expressing cells). Rho activation required activated Ras because there was only a minimal increase in Rho activation which did not reach statistical significance in cells transfected with wild type Ras (Fig. 3c). The effects of the drug U0126 on Rho activation (Fig. 3, a, b, and c) will be presented later.

Effect of Ras-induced Rho Activation on the p21WAF1/CIP1 Promoter—To determine whether the observed Ras activation of Rho in NIH 3T3 cells had downstream effects, we studied the activity of the p21WAF1/CIP1 promoter in the absence and presence of dexamethasone in LTR-H-Ras(A) cells. The p21WAF1/CIP1 promoter was chosen because it is regulated coordinately by both Ras and Rho, with Ras activating the promoter and Rho inhibiting it (10). We transfected LTR-H-Ras(A) cells with a p21-Luc construct and found no significant effect of dexamethasone (Fig. 4, cells cotransfected with empty vector). However, when these cells were cotransfected with an expression vector for C3 exoenzyme, which ADP ribosylates and inhibits RhoA (52), there was a 1.7-fold increase in luciferase activity in the absence of dexamethasone and more than a 3-fold increase in the presence of dexamethasone (Fig. 4), the difference between the absence and presence of dexamethasone was statistically significant (p < 0.05). The increase in p21WAF1/CIP1 promoter activity in the presence of C3 exoenzyme was likely secondary to Rho inhibition, and the further increase with dexamethasone was the effect of activated Ras on the promoter free of inhibition by Rho. We demonstrated RhoA inhibition of the p21WAF1/CIP1 promoter by expressing a constitutively active Rho (RhoA63L; Fig. 4); the RhoA(63L) effect was dominant over that of activated Ras, possibly because of high expression levels of the transiently transfected construct and the extremely high activation level of the mutant RhoA (Fig. 2d). We conclude that RhoA activation by Ras is sufficient to prevent stimulation of the p21WAF1/CIP1 promoter by Ras.

**Table I**  
**Ras activation state and Ras expression in wild type NIH 3T3 and LTR-H-Ras(A) cells**

| Cell type and treatment | Ras activation | Total nucleotides bound to Ras |
|-------------------------|----------------|-------------------------------|
| Wild type NIH 3T3 (−Dex) | 1.9 ± 1        | 2.44 ± 0.48                   |
| Wild type NIH 3T3 (+Dex) | 2.1 ± 0.9      | 2.28 ± 0.70                   |
| LTR-H-Ras(A) cells (−Dex) | 7.1 ± 2.2       | 2.03 ± 0.42                   |
| LTR-H-Ras(A) cells (+Dex) | 24.7 ± 5.6      | 4.46 ± 1.28                   |

$^a$ F. C. von Lintig and G. R. Boss, unpublished observation.

**Fig. 4. Effect of Ras-induced Rho activation on the p21WAF1/CIP1 promoter.** LTR-H-Ras(A) cells were cotransfected with p21-Luc (a luciferase reporter gene under control of the p21WAF1/CIP1 promoter) and empty vector, expression vectors encoding C3 exoenzyme, or activated RhoA (RhoA63L) as described under “Experimental Procedures.” After a 24-h culture in DMEM containing 10% FBS in the absence (open bars) or presence of 1 μM dexamethasone (filled bars), luciferase activity was measured as described under “Experimental Procedures.” The luciferase activity obtained in untreated cells transfected with empty vector was assigned a value of 1; the data are the means ± S.D. of at least three independent experiments performed in duplicate.
methasone-induced increase in Rac1 may limit the increase in RhoA activity observed in LTR-H-Ras(A) cells.

Effect of Oncogenic Ras on the Subcellular Localization of RhoA, RhoA Association with Rho-GDI, and on Rho-GEF Activity—Changes in the distribution of RhoA between membrane and cytosol have been used as indication for Rho activation because increased membrane association of RhoA occurs when RhoA is activated by the addition of GTPyS to cell lysates (52). We assessed the amount of RhoA associated with membrane fractions in wild type NIH 3T3 cells and LTR-H-Ras(A) cells in the absence and presence of dexamethasone. We found no change in the amount of RhoA in membranes of wild type cells, but there was an approximately 2-fold increase in membrane association of RhoA in dexamethasone-treated LTR-H-Ras(A) cells (Fig. 6a, upper blots). Wild type NIH 3T3 cells appeared to express slightly higher amounts of RhoA protein; we do not know the significance of this finding because it may simply reflect the effects of clonal evolution in culture. The increased membrane association of RhoA in dexamethasone-treated LTR-H-Ras(A) cells correlated well with the 2-fold increase in Rho activity found under these conditions (Fig. 3a); because the increase in membrane-bound RhoA represented only a small fraction of total cellular RhoA, it is not surprising that we did not detect any significant change in the amount of RhoA in the cytosol (Fig. 6a, lower blots; ~2.5% of the cytosolic fraction compared with 50% of the membrane fraction was loaded on the gels).

Rho-GDI forms a cytosolic complex with Rho-GDP; Rho-GDI prevents Rho activation by Rho-GEFs and serves to regulate membrane association/dissociation of Rho (56). We hypothe-

**Fig. 5. Effect of oncogenic Ras on Rac-GTP Levels.** LTR-H-Ras(A) cells (a) and wild type NIH 3T3 cells (b) were cultured for 24 h in DMEM containing 10% FBS in the absence or presence 1 μM dexamethasone (Dex) as indicated. Cells were extracted, and Rac-GTP was isolated using the CDC42/Rac binding domain of Pak bound to glutathione beads as described under “Experimental Procedures.” Rac-GTP bound to the beads (left panels) and total Rac present in 5% of the input cell lysate (right panels) were detected by Western blotting using a Rac-specific antibody. Ctrl refers to cell lysates incubated with GST-loaded glutathione beads lacking the CDC42/Rac binding domain of Pak. c, Rac-GTP was isolated on beads and detected as described above; the Western blots for Rac-GTP were scanned, and the band intensity obtained with untreated cells was assigned the value of 1. The data represent the means ± S.D. of five independent experiments.

**Fig. 6. Effect of oncogenic Ras on the subcellular location of RhoA, RhoA association with Rho-GDI, and on Rho-GEF activity.** Wild type (WT) NIH 3T3 cells (a and c) and LTR-H-Ras(A) cells (a–c) were cultured for 24 h in DMEM containing 10% FBS; as indicated, some cells were treated with 1 μM dexamethasone (Dex), a, cells were extracted in hypotonic lysis buffer by Dounce homogenization, and cytosol and membrane fractions were generated by differential centrifugation as described under “Experimental Procedures.” The two fractions were subjected to SDS-PAGE/Western blotting using a mouse monoclonal anti-RhoA antibody. b, cytosolic extracts were prepared and subjected to immunoprecipitation using either rabbit control immunoglobulin (C, lane 1) or a rabbit polyclonal anti-Rho-GDI antibody (lanes 2–5) as described under “Experimental Procedures.” Immunoprecipitates were analyzed by SDS-PAGE/Western blotting using a mouse monoclonal anti-RhoA antibody (upper blot) and the rabbit anti-Rho-GDI antibody (lower blot). For comparison, 5% of the cytosolic extracts were analyzed in parallel (lanes 6–10). c, In the left panel, LTR-H-Ras(A) cells were extracted in a buffer containing 1% Triton X-100, the extracts were centrifuged, and to the supernatants were added 1 mM GTP and GST-tagged RhoA preloaded with [3H]GDP that was bound to glutathione beads. After 10 and 20 min, the reaction was stopped, and radioactivity remaining on the washed beads was quantitated by liquid scintillation counting as described under “Experimental Procedures.” The data are expressed as the percent increase in [3H]GDP exchange compared with control beads incubated with extract buffer. In the right panel, wild type NIH 3T3 cells were transfected with either empty vector or a vector encoding constitutively active AN-p115-Rho-GEF; Rho-GEF activity was quantitated as described for the left panel, and the activity found in cells transfected with empty vector was assigned a value of 1. The data are the means ± S.D. of three independent experiments performed in duplicate.

sized that Ras-induced Rho activation in LTR-H-Ras(A) cells could be from a decreased Rho/Rho-GDI interaction. We assessed the amount of Rho complexes with Rho-GDI in LTR-H-Ras(A) cells by coimmunoprecipitation and found no change in the amount of RhoA in Rho-GDI immunoprecipitates when the cells were treated with dexamethasone (Fig. 6b). The reciprocal experiment, i.e. assessing the amount of Rho-GDI in RhoA immunoprecipitates, also yielded no difference in the absence or presence of dexamethasone (not shown).

Another possible mechanism for Rho activation by Ras could be through an increase in Rho-GEF activity. Because there are
Effect of Oncogenic Ras on Rho-GAP Activity and Subcellular Localization—Because we found no effect of oncogenic Ras on Rho/Rho-GDI association and Rho-GAP activity, we hypothesized that Ras could activate Rho through a decrease in Rho-GAP activity and/or a change in the subcellular location of a Rho-GAP. A likely candidate for regulation by Ras is p190 Rho-GAP because this protein associates with p120 Ras-GAP in a reversible, tyrosine phosphorylation-dependent manner, and several observations suggest that the p190 Ras-GAP complex may function to regulate actin cytoskeletal dynamics through RhoA (59, 60, 60–63).

We measured p190 Rho-GAP activity in immunoprecipitates prepared from cytosolic extracts of cells by following the hydrolysis of [$\gamma$-$^{32}$PO$_4$]GTP preloaded on RhoA (62). We found significantly less activity in extracts from dexamethasone-treated LTR-H-Ras(A) cells compared with extracts from non-treated cells ($p < 0.05$), whereas dexamethasone had no effect on p190 Rho-GAP activity in wild type NIH 3T3 cells (Fig. 7a shows results for LTR-H-Ras(A) cells; at 12 min, RhoA incubated with p190 Rho-GAP immunoprecipitates from dexamethasone-treated cells had 24% more bound GTP remaining compared with RhoA incubated with immunoprecipitates from untreated cells).

The basis for decreased cytosolic p190 Rho-GAP activity during expression of oncogenic Ras could be a decrease in the amount of total cellular p190 Rho-GAP, a shift in subcellular localization, or a decrease in the specific activity of the protein, e.g. because of decreased tyrosine phosphorylation (59, 64). We observed a significant decrease in the amount of cytosolic p190 Rho-GAP in dexamethasone-treated LTR-H-Ras(A) cells with collecting proteins on nitrocellulose filters; radioactivity bound to RhoA was measured by liquid scintillation counting. The data are the means ± S.D. of five independent experiments performed in duplicate and are expressed as the percent of GTP bound to RhoA remaining at the indicated time. Note the intrinsic GTPase activity of RhoA measured in the presence of control IgG immunoprecipitate (open circles). b, cells were cultured in the absence (open bars) or presence (filled bars) of dexamethasone, and cytosolic extracts were prepared as described above. Equal amounts of extract protein were analyzed by SDS-PAGE/Western blotting using the anti-p190 Rho-GAP antibody, and signal intensities were determined by scanning autoradiographs within the linear range of exposure. The amount of p190 Rho-GAP detected in untreated cells was assigned a value of 100%. The data represent the means ± S.D. of six independent experiments. c, cells were extracted, and subcellular fractions were generated as described in Fig. 6a. Equal amounts of extract protein were analyzed by SDS-PAGE/Western blotting using mouse monoclonal antibodies specific for p190 Rho-GAP and p120 Ras-GAP. d, cells were extracted in situ by incubation in a Triton X-100-containing buffer; the remaining Triton-insoluble cytoskeletal structures were collected and solubilized in SDS-sample buffer as described under “Experimental Procedures.” The Triton-insoluble fractions from equal numbers of cells were analyzed by Western blotting using a p190 Rho-GAP-specific antibody (upper blot). Duplicate samples were blotted with an actin-specific antibody (lower blot). Results similar to those shown in c and d were obtained in three other experiments.
no effect of dexamethasone in wild type NIH 3T3 cells (Fig. 7c, cytosol, shows a representative experiment, and Fig. 7b summarizes the results of eight independent experiments). We found no change in total cellular p190 Rho-GAP when LTR-H-Ras(A) cells were treated with dexamethasone (Fig. 7c, Homogenate), and there was only a slight increase in membrane-associated p190 Rho-GAP in response to dexamethasone which was not seen in wild type NIH 3T3 cells (Fig. 7c, Membrane). To determine whether the decrease in cytosolic Rho-GAP may be the result of a shift into a cytoskeletal compartment, we extracted cells in Triton X-100-containing lysis buffer and examined the detergent-insoluble cytoskeletal fraction (44). Dexamethasone treatment of LTR-H-Ras(A) cells, but not of wild type NIH 3T3 cells, induced a significant increase in the amount of p190 Rho-GAP associated with the Triton-insoluble cytoskeletal fraction (Fig. 7d).

We simultaneously assessed p120 Ras-GAP expression and found that total and cytosolic p120 Ras-GAP were unaffected by dexamethasone in both LTR-H-Ras(A) and wild type NIH 3T3 cells but that dexamethasone induced a shift of p120 Ras-GAP to the membrane as has been described under other conditions of Ras activation (Fig. 7c and Ref. 65).

Effect of Oncogenic Ras on the Interaction of p190 Rho-GAP with p120 Ras-GAP and on p190 Rho-GAP Phosphorylation—Because the association of p190 Rho-GAP with p120 Ras-GAP is regulated by tyrosine phosphorylation and may influence the specific Rho-GAP activity of p190 (60, 62), we examined the effect of oncogenic Ras expression on p190 tyrosine phosphorylation and association with p120. We immunoprecipitated p190 Rho-GAP from cytosolic extracts and assessed the immunoprecipitates for the presence of p120 Ras-GAP; we also performed the reciprocal experiments, immunoprecipitating Ras-GAP and looking for Rho-GAP. As would be expected from the decrease in cytosolic p190 Rho-GAP, we found less p190 in anti-Rho-GAP immunoprecipitates from dexamethasone-treated LTR-H-Ras(A) cells compared with nontreated cells (Fig. 8a, compare lanes 4 and 5 with lanes 2 and 3). Dexamethasone treatment appeared to cause a proportional decrease in the amount of p120 Ras-GAP in the anti-Rho-GAP immunoprecipitates, without causing any changes in cytosolic p120 Ras-GAP (Fig. 8a, compare lanes 4 and 5 with lanes 2 and 3, and lanes 8 and 9 with lanes 6 and 7). We also found about 70% less p190 Rho-GAP in anti-Ras-GAP immunoprecipitates of dexamethasone-treated cells, even though the amount of Ras-GAP in the immunoprecipitates was constant in the presence and absence of dexamethasone (Fig. 8b). In parallel experiments, we found no effect of dexamethasone on the amount of p190 Rho-GAP/p120 Ras-GAP association in wild type NIH 3T3 cells (data not shown). We conclude that induction of oncogenic Ras decreases the amount of p190 Rho-GAP/p120 Ras-GAP complex present in the cytosol of LTR-H-Ras(A) cells in proportion to the decrease in cytosolic p190 Rho-GAP.

The amount of tyrosine-phosphorylated p190 Rho-GAP, both in anti-Rho-GAP and anti-Ras-GAP immunoprecipitates, was not decreased in dexamethasone-treated LTR-H-Ras(A) cells, suggesting that the remaining p190 in dexamethasone-treated cells may actually be slightly more tyrosine-phosphorylated compared with untreated cells (Fig. 8c shows anti-Rho-GAP immunoprecipitates). Thus, the decrease in the amount of p190 Rho-GAP/p120 Ras-GAP complexes appeared to be secondary to the shift in p190 Rho-GAP location and was not associated with a detectable decrease in p190 Rho-GAP phosphotyrosine.

Effect of the Raf1/MEK/ERK MAP Kinase Pathway on Ras-induced Rho Activation—Multiple effector pathways contribute to transformation by oncogenic Ras, but the function of the Raf/MEK/ERK pathway is critical because activating mutants of Raf-1 and MEK are necessary and sufficient for transformation of rodent fibroblasts (34, 66). We examined whether RhoA activation by oncogenic Ras required activation of the Raf/MEK/ERK pathway by using the specific MEK inhibitor U0126 (28). We found that dexamethasone treatment of LTR-H-Ras(A) cells led to activation of the MAP kinases ERK-1/2, as demonstrated by Western blotting using specific antibodies for p190 Rho-GAP (a c) or p120 Ras-GAP (b). a, Anti-Rho-GAP immunoprecipitates (lanes 2–5) or 5% of the cytosolic input (lanes 6–9) were analyzed by SDS-PAGE/Western blotting using anti-Rho-GAP and anti-Ras-GAP antibodies; lane 1 shows immunoprecipitates obtained with control mouse IgG. b, anti-Ras-GAP immunoprecipitates were analyzed by Western blotting using the same antibodies as in a. Rho-GAP and Ras-GAP bands were analyzed by scanning densitometry, and the ratio of Rho-GAP to Ras-GAP found in untreated cells was assigned a value of 1. The amount of immunoprecipitated Ras-GAP was the same in the absence and presence of dexamethasone. The data are the means ± S.D. of two experiments performed in duplicate and scanned at two different exposures. c, anti-Rho-GAP immunoprecipitates were analyzed by Western blotting using a phosphotyrosine-specific antibody (upper panel) or an anti-Rho-GAP antibody (lower panel). Results similar to those shown in a and c were obtained in two other experiments.
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cytoskelton-associated p190 Rho-GAP (Fig. 9c).

To determine whether activation of the Raf/MEK/ERK pathway is sufficient to induce translocation of p190 Rho-GAP from the cytosol to the cytoskeleton, we used constitutively active constructs of Raf-1 kinase (BXB) and MEK (MEK(HRU218,Asp222)) (27, 34). In wild type NIH 3T3 cells transfected with HA-tagged p190 Rho-GAP, significantly more HA-tagged Rho-GAP was found in the detergent-insoluble cytoskeletal fraction when cells were cotransfected with BXB than when cotransfected with empty vector (Fig. 9d). Similar results were found when endogenous p190 Rho-GAP was examined in the cytoskeletal fraction of cells transfected with BXB or activated MEK(E218,D222); however, the observed increases in cytoskeletal p190 Rho-GAP were less pronounced because the transfection efficiency was only about 30% (Fig. 9e). Thus, the Ras/Raf/MEK/MAP kinase pathway appears to regulate RhoA activity by regulating the translocation of p190 Rho-GAP from the cytosol to a detergent-insoluble cytoskeletal fraction.

DISCUSSION

We found that increasing the amount of activated, oncogenic H-Ras in NIH 3T3 cells increased RhoA activity. We used two different approaches, i.e. transient transfection and stable transfection of an H-Ras(V12) construct under conditional promoter control, and we measured Rho activation by two different assays, i.e. Rhotekin pulldown with Western blotting for RhoA and enzymatic quantitation of GTP bound to Rho. The mechanism of RhoA activation by Ras involved redistribution of p190 Rho-GAP from the cytosol to a detergent-insoluble cytoskeletal fraction with decreased cytosolic Rho-GAP activity; the Ras-induced translocation of p190 Rho-GAP and increase in RhoA activity were prevented by pharmacologic inhibition of the Raf/MEK/ERK pathway. The enzymatic assay for measuring Rho activation is based on a similar assay we have used for measuring Ras, Rap1, and Rheb activation (26, 38–40) and has several noteworthy features: (i) it is quantitative and highly sensitive; (ii) it provides a measure of the total amount of intracellular Rho; (iii) it can be used for Rho expressed from transfected Rho constructs; and (iv) it can be used on human tumor samples that may have large variations in the amount of Rho present (4, 67). Using the enzymatic assay, we have measured Ras activation in several different human cancers (2, 68, 69), and work is in progress to measure Ras and Rho activation in breast and prostate cancers. A potential problem is that Rhotekin interacts with RhoA and C and may bind weakly to RhoB (49); thus measuring GTP eluted from Rhotekin-bound Rho may provide a mean activation of all three Rho subtypes. However, for the present studies this was not a significant concern because NIH 3T3 cells contain predominantly RhoA, with much less RhoB and C (37).

While the present work was in progress, several groups reported increased Rho-GTP levels in different types of Ras-transformed cells, although one group did not confirm this (8, 16, 18, 23). Our work differs from these published results because we purposefully avoided comparing Rho activities in cell lines with different clonal origins, which may differ because of secondary genetic changes that accumulate during selection in culture. Sahai et al. (8) stably transfected Swiss 3T3 cells with H-Ras(V12) and found higher RhoA activity and lower Rac activity in transformed versus untransformed clones. The increased RhoA activity appeared to be the result of long term selection of the Ras-transformed clones rather than direct Ras signaling because Swiss 3T3 cells stably transfected with a hormone-inducible, constitutively active Raf-1 construct demonstrated an increase in Rho-GTP only after more than 4 weeks of selection in the presence of the hormone; no change in RhoA activity after a 6-h induction of active Raf-1 was observed, even
though activation of the Raf/MEK/ERK pathway was demonstrated (8). Zondag et al. (16) transformed MDCK cells with v-H-Ras or constitutively active Raf and found similarly increased RhoA and decreased Rac1 activity compared with untransformed cells. In both Ras- and Raf-transformed Swiss 3T3 and MDCK cells, pharmacologic inhibition of MEK with U0126 or PD98059 was without effect on RhoA and Rac1 activities, further strengthening the view that selective pressures rather than direct signaling events determined the levels of RhoA and Rac1 activity observed in these studies (8, 16). In contrast, we found that relatively short term (24–36 h) expression of oncogenic H-Ras increased RhoA and Rac1 activity in NIH 3T3 cells, with U0126 preventing this effect at concentrations at which it prevented activation of the Raf/MEK/ERK pathway. Our results are more compatible with the results of Gupta et al. (18), who found increased RhoA and Rac1 activities in human fibrosarcoma cells expressing mutant N-Ras and in wild type Ras-containing cells stably transfected with constitutively active Raf or MEK compared with wild type Ras-containing cells transfected with empty vector. These authors also found a partial decrease of RhoA and Rac1 activity when the hyperactive Ras/MEK/ERK pathway in mutant N-Ras-containing cells was blocked by transfection of dominant negative Raf and MEK constructs (18). Interestingly, treatment of v-H-Ras-transformed MDCK cells with the drug INd 12 inhibited activity of the Raf/MEK/ERK pathway and restored the activity of RhoA and Rac1 to that found in untransformed MDCK cells (17). Taken together, these results suggest that the increased RhoA activity found in Ras-transformed cells is, at least in part, a consequence of increased Raf/MEK/ERK signaling.

Although our work does not exclude the possibility that RhoA activation by oncogenic Ras involves activation of one or multiple Ras-GEFs, we did not find any detectable change in overall cellular Ras-GEF activity. However, we observed a significant decrease in cytosolic p190 Rho-GAP activity which likely, at least partially, accounts for the increased Rho-GTP levels in cells expressing H-Ras(V12). In murine fibroblasts, p190 Rho-GAP accounts for the majority of Rho-GAP activity in cell lysates, and inhibition of Rho-GAP activity is sufficient for induction of RhoA-mediated actin reorganization (70). We found that expression of oncogenic Ras in LTR-H-Ras(A) cells induced the translocation of p190 Rho-GAP from the cytosol to a detergent-insoluble cytoskeletal fraction. Similarly, integrin-mediated cell-substrate interaction has been shown to result in p190 Rho-GAP recruitment to the cytoskeleton at 1–2 h after plating NIH 3T3 cells on fibronectin, and this change in subcellular localization of Rho-GAP occurs at a time when stimulation of RhoA activity by prolonged integrin engagement has been explained by the finding that MEK inhibitors prevent translocation of the RhoA effector ROCK from the cytosol to a detergent-insoluble cytoskeletal fraction in Ras-transformed cells (8). The mechanism by which prolonged activation of the Raf/MEK/ERK pathway leads to translocation of ROCK has not been resolved, but it might be similar to the mechanism by which oncogenic Ras leads to the redistribution of p190 Rho-GAP.

Our analysis of p21Val12C1IP1 promoter activity in LTR-H-Ras(A) cells demonstrated that Rho activation by Ras had a physiological effect with downstream changes in gene transcription. The opposing function of Ras and Rho in the regulation of the p21Val12C1IP1 promoter appears to be important for Ras transformation because when Rho signaling is blocked, oncogenic Ras induces cell cycle arrest in p21Val12C1IP1-expressing, but not in p21Val12C1IP1-deficient cells (10). In pancreatic carcinoma cells expressing oncogenic Ras, a dominant negative RhoA, but not Rac1, was able to activate the p21Val12C1IP1 promoter, whereas a constitutively active RhoA suppressed it, similar to our findings in LTR-H-Ras(A) cells (72).

In conclusion, our work demonstrates direct regulation of RhoA activity by oncogenic Ras and offers an explanation for the increased RhoA activation found in Ras-transformed cells. Further work is needed to understand the mechanism(s) whereby the Ras/Raf/MEK pathway induces redistribution of p190 Rho-GAP and ROCK (8) from the cytosol to a cytoskeletal fraction.

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