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The Hematopoiesis-Specific GTP-Binding Protein RhoH Is GTPase Deficient and Modulates Activities of Other Rho GTPases by an Inhibitory Function

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The Rho subfamily of small GTP-binding proteins mediates many fundamental cellular functions. The commonly studied members (Rho, Rac, and CDC42) regulate actin reorganization, affecting diverse cellular responses, including adhesion, cytokinesis, and motility. Another major function of the Rho GTPases is their role in regulating transcriptional factors and nuclear signaling. RhoH is encoded by a hematopoiesis-specific Rho-related gene recently identified in a fusion transcript with bcl6 in lymphoma cell lines. Significantly, translocations and a high frequency of RhoH mutation have been detected in primary lymphoma cells. We show here that RhoH functions differently from other Rho GTPases. RhoH exerts no significant effect on actin reorganization. However, RhoH is a potent inhibitor of the activation of NFκB and p38 by other Rho GTPases. This property, together with the differential expression of RhoH in the Th1 subset of T cells, suggests a role for RhoH in the functional differentiation of T cells. RhoH has different amino acids in two highly conserved residues critical for GTPase activity. Consequently, RhoH is GTPase deficient, remaining in a GTP-bound activated state without cycling. Reduction of RhoH levels in T cells augments the response to Rac activation. Furthermore, RhoH is dramatically down regulated after phorbol myristate acetate treatment and in Th1 cells after activation by anti-CD3. Hence, a mechanism for regulation of RhoH function is likely to exist at the transcriptional level. The inhibitory function of RhoH supports a model in which Rho GTPases with opposing functions may compete to modulate the final outcome of a particular GTPase-activated pathway.

The Ras superfamily of small GTP-binding proteins constitutes a large family of regulatory proteins that perform an extensive repertoire of cell functions (6, 26). Within this family, the Rho subfamily has emerged as a group of proteins that participate in many critical and fundamental cellular functions (8, 60). At least 15 related members have been identified that exhibit both distinctive and overlapping functions. Many of the Rho GTPases exert a dominant effect on actin polymerization but with different morphological consequences (20). While RhoA induces stress fiber formation (50), RhoE (15, 19) and RhoD (41, 58) inhibit the formation or cause the disassembly of stress fibers. Injection of the dominant active form of RacV12 into cells is sufficient to induce membrane ruffling, formation of lamellipodia (51), and subsequent stress fiber formation, while expression of activated CDC42 induces the formation of filopodia, followed by the formation of lamellipodia and membrane ruffles (42). Constitutively active RhoG produces cytoskeletal changes similar to those elicited by simultaneous activation of Rac1 and CDC42 (18, 61).

A large body of literature has shown that another major activity of the Rho family members is their role in regulating nuclear signaling and the activation of several families of key transcriptional factors that regulate gene expression and cell growth (60). RhoA, Rac1, and CDC42 activate the nuclear transcription factor κB (NF-κB) (46) and the serum response factor transcription factors (24). Various inflammatory cytokines and stresses, such as UV radiation, heat shock, and gamma radiation, activate the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway and the reactivating kinase p38 (14, 29). Evidence that activation of these factors is mediated by Rho GTPases has been found in various cell types. Expression of constitutively active mutant forms of Rac and CDC42 in NIH 3T3, HeLa, and Cos cells elicits stimulation of JNK and p38 activity (3, 10, 39). Others have reported that in human kidney 293T cells, CDC42 and Rho protein, but not Rac, can induce activation of JNK (57). Activation of these transcription factors and kinases, in turn, regulates genes that promote cell growth. Finally, Rho proteins are also required for progression through the G_1 phase of the cell cycle, partly through regulation of cyclin D1 expression (44).

A biochemical property common to the GTP-binding proteins is that they are able to bind to and hydrolyze GTP, thus providing the mechanistic basis for their unique ability to switch between an inactive GDP-bound state and an active GTP-bound state (6, 26, 60). This property is determined by highly conserved residues that first became evident through the identification in tumors of mutations that render the protein GTPase deficient. Replacement of highly conserved amino acids, including ras amino acids 12, 59, and 61, resulted in constitutively activated and transforming ras proteins (13). Mutations in analogous residues in Rac, Rho, and CDC42 render them GTPase deficient, resulting in constitutively activated GTP-bound protein (17, 23, 54, 62, 65). Recently, mu-

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tagenesis experiments have shown that with mutations in Rho, Rac, and CDC42 that convert the proteins into rapid cyclers (35), the GTPases become strongly transforming. These findings underscore the importance of the cycling function of the GTPase. In contrast, other Rho GTPases have been identified in which the wild-type forms contain replacements of those residues critical for GTPase activity that render them naturally GTPase deficient (15, 19, 21, 66). For example, the Rnd proteins Rnd1, -2, and -3 (also known as RhoE) constitute an unusual subgroup of Rho GTPases that have very low GTPase activity and exist in constitutively active GTP-bound form in cells (43).

In this communication, we report functional studies of a novel Rho family member. RhoH/TTF was recently identified as a fusion transcript with bcl6 in lymphoma cell lines (11, 12). The novel cDNA, named TTF (for translocation three four), was found to be a member of the Rho GTPase subfamily of proteins, and because the gene is expressed in hematopoietic cell lines only, it was renamed RhoH. Nonrandom chromosomal translocations involving RhoH have been identified in primary lymphoma cases and multiple myeloma (47). More recently, a high frequency of mutations of the gene for RhoH has been found in certain subtypes of primary lymphomas (45). The exact role of RhoH in the pathogenesis of lymphoma has not been clarified, and the extent of RhoH involvement in hematological malignancies also remains to be fully revealed. Furthermore, the functional and biochemical properties of RhoH are completely unknown. We show here that RhoH displays several characteristics different from those of other Rho family members. Unlike many other Rho family members, RhoH appears to have no significant effect on actin reorganization, as tested here in nonhematopoietic cells. While most other Rho-related proteins are strong activators of several transcriptional pathways, RhoH inhibits the activation of NFκB and p38. We demonstrated that wild-type RhoH has no GTPase activity and appears to remain in a GTP-bound state only. This unusual property raises the important question of how the activity of such a protein is regulated. Here we present evidence that RhoH is transcriptionally regulated and that modulation of RhoH mRNA levels can alter the effective activities of other Rho GTPases. Finally, we demonstrated that in the Th1 subtype of T-helper cells, RhoH is expressed at a higher level than in the Th2 subpopulation, indicating a role for RhoH in the specification of lymphocyte functional development.

MATERIALS AND METHODS

Cell cultures. The 293 (human embryo kidney), NIH 3T3, and MDCK (Madin-Darby canine kidney) cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (HyClone, Denver, Colo.). The Jurkat cell line was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Th1 and Th2 cells were derived as previously described (34, 36). Briefly, naive $CD4^+$ $CD62L^+$ $CD44^{low}$ T cells were sorted on a Becton Dickinson FACStar sorter. These $CD4^+$ T cells were cultured on 24-well plates precoated with anti-CD3 (10 μg/ml) and anti-CD28 (5 μg/ml) in medium containing interleukin-12 (IL-12; 1.5 ng/ml), human IL-2 (20 U/ml), and anti-IL-4 (10 μg/ml) for 4 days to generate Th1 cells. To generate Th2 cells, culture medium was supplemented with IL-4 (3,000 U/ml), human IL-2 (20 U/ml), and anti-gamma interferon (IFN-γ) antibody (10 μg/ml) were added. For activation, these cells were washed and restimulated with plate-bound anti-CD3 antibodies (10 μg/ml) for 4 h.

Derivation of RhoH cDNA. Using published sequences, we generated PCR primers to obtain full-length human RhoH cDNA by reverse transcription-PCR. Total RNA from normal human bone marrow was used to derive the PCR product. Several clones were obtained and sequenced, all of which had sequences identical to the published data. One clone was selected to construct the expression vector used to perform subsequent experiments.

Reagents. Tumor necrosis factor alpha (TNF-α) was purchased from GIBCO; anti-hemagglutinin (HA), anti IκB-α, and anti-phosphorylated IκB-α antibodies were purchased from Santa Cruz Biotech. ERK1/ERK2, JNK/SAPK, and P38 antibodies and antibodies against the respective phosphorylated proteins were purchased from New England BioLabs. An NF-κB luciferase reporter (3X-κB-L) plasmid (40) and an IκB-kinase β (IKKβ) expression vector (pcDNA3IKKβD9) (52) were kindly provided by George Mosialos (Harvard Medical School). The pGST-PBD bacteria were obtained from K. O'Connor (Harvard Medical School). Glutathione *S*-transferase (GST)-Rac1 and GST-Rho GAP p50 fusion proteins were purchased from Cytoskeleton Inc. GST-RhoA and GST-RhoH fusion vectors were constructed by cloning the human RhoA and RhoH cDNAs in frame with GST in the pGEX-4T-1 vector. HA-tagged RhoH was cloned in frame into the HA-pCGNM2 vector. An antisense RhoH (αs-RhoH) vector was constructed by ligating a 300-bp fragment of RhoH cDNA from nucleotide 26 to nucleotide 326 (11, 12) in the reverse orientation into the pcDNA3 vector.

Northern blot analysis. Total RNA was extracted from normal mouse tissues or human cell lines using Tri Reagent (Molecular Research Center, Inc.). A 20-μg sample of total RNA was loaded per lane and resolved on a 1.2% agarose-formaldehyde gel, transferred to Hybond-N nylon membrane (Amersham, Arlington Heights, Ill.), and hybridized with a ^{32}P-radiolabeled RhoH cDNA probe under standard high-stringency conditions. Filters were washed under high-stringency conditions, and ethidium bromide staining was used to monitor RNA integrity and constancy of loading.

Immunofluorescence analysis. MDCK cells were plated onto 2% gelatin-coated coverslips the day before transfection. HA-tagged RhoH vector was transiently transfected into cells with Lipofectamine Plus reagent (GIBCO/BRL, Gaithersburg, Md.) in accordance with the manufacturer's instructions. Cells were starved for 48 h, restimulated with 10% serum, and examined at different time points after stimulation. Cells were fixed for 10 min with 3% paraformaldehyde in phosphate-buffered saline (PBS) and washed with 0.1% NP-40–PBS buffer. Nonspecific sites were blocked with 5% skim-milk in PBS for 30 min. The cells were then incubated with anti-HA monoclonal antibody for 1 h at room temperature. Cells were washed three times and then incubated with fluorescein isothiocyanate-conjugated anti-mouse (Santa Cruz) secondary antibody and rhodamine-conjugated phalloidin to stain actin. After three washes, the coverslips were inverted, mounted on slides, and sealed with nail polish. Pictures were taken by confocal microscopy.

Immunoprecipitation and Western blot analysis. Each myc-tagged GDP dissociation inhibitor alpha (GDIα), GDIβ, and GDIγ expression plasmid was cotransfected with HA-tagged RhoH vector into 293 cells, respectively. After 24 h, cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per ml, and 5% glycerol. Whole-cell lysates were incubated with normal immunoglobulin G or HA monoclonal antibody (Santa Cruz Biotech, Santa Cruz, Calif.) at 4°C for 4 h. Preequilibrated protein A-Sepharose beads were then added. The Sepharose beads were washed three times with the same buffer as described above to remove the nonspecifically bound proteins. Bound proteins were eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer and resolved by SDS–10% polyacrylamide gel electrophoresis (PAGE). The proteins were transferred onto nitrocellulose membranes, and the membranes were blocked and probed with a 1:1,000 dilution of anti-myc polyclonal antibody (Santa Cruz Biotech) for 1 h at room temperature. Immunoreactive bands were visualized with a horseradish peroxidase-conjugated secondary antibody and the enhanced-chemiluminescence system (Amersham Pharmacia Biotech).

Luciferase assay. 293 cells (10^6/well) were seeded in six-well tissue culture plates 12 h before transfection. Transient transfections were carried out using Lipofectamine Plus or Cytofectene Transfection Reagent (Bio-Rad) in accordance with the manufacturer's recommendations. Jurkat cells were transfected before plating on a six-well plate at a density of 10^6/well. For each transfection, 100 ng of plasmid DNA for the luciferase reporter and 10 ng of internal control plasmid pRL-CMV were used. For NF-κB response, 1 μg of an NF-κB reporter plasmid (3X κB-L) was used; for p38, the CHOP-luciferase assay kit vectors (Stratagene) were used. Cells were transfected with the indicated plasmids. At 24 h posttransfection, cells were harvested or treated with TNF-α (10 ng/ml) as indicated before harvesting. Cell lysates were assayed for luciferase activity by using the dual luciferase assay kit (Promega) in accordance with the manufacturer's directions.

Guanine nucleotide-binding assay. Fusion vectors GST-RhoA and GST-RhoH were expressed in *Escherichia coli* BL21 bacteria, and the fusion proteins were extracted and purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). Expression of GST-RhoH and GST-RhoA proteins was confirmed by SDS-PAGE, followed by Coomassie blue staining. A 10-μg sample of recombinant protein was loaded with [γ-^{32}P]GTP (10 μCi; 6,000 Ci/mmol; Dupont NEN) in assay buffer (50 mM Tris-HCI [pH 7.6], 50 mM NaCl, 5 mM EDTA,5 mM DTT) at 30°C for 10 min. An excess of unlabeled GTP (2 mM) was then added to the loaded protein in assay buffer containing either 5 mM EDTA (low Mg^{2+}) or 5 mM MgCl (high Mg^{2+}) at 30°C for the indicated times. Samples were filtered through prewetted 25-mm-diameter nitrocellulose membranes (BA85; 0.45-μm pore size; Schleicher & Schuell), and the membranes were washed three times with 3 ml of ice-cold assay buffer and allowed to dry in air. The amount of radioactive nucleotide remaining on the GTPase was determined by scintillation counting.

GTPase and GAP assays. To measure GTPase activity in a filter-binding assay, [γ-^{32}P]GTP-loaded proteins, prepared as already described, were incubated in GTP hydrolysis buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, 10 μM GTP, and 1 mM DTT at 37°C for the indicated time and subjected to a filter-binding assay to quantify GTP hydrolysis. GTPase-activating protein (GAP) assays were performed with Rho GAP p50 (Cytoskeleton, Inc.) in a filter-binding assay as previously described (49). GST-RhoA, RhoH, and Rac1 were purified from *E. coli*. Purified GTPases (0.5 to 5 μg of each) were loaded with [γ-^{32}P]GTP as already described, and then $MgCl_2$ was added to a final concentration of 10 mM. GTPases were then incubated in 100 μl of a solution containing 50 mM Tris (pH 7.5), 500 mM NaCl, 10 mM $MgCl_2$, 1 mg of BSA per ml, and 5 mM DTT with or without 100 ng of Rho GAP p50 for 10 or 20 min at 25°C. Samples were brought to 500 μl with 50 mM Tris (pH 7.5)–10 mM $MgCl_2$–1 mM DTT and filtered through nitrocellulose. Filters were washed with 10 ml of the same solution, and the amount of radioactivity remaining on the filters was determined.

PAK-binding domain affinity precipitation of Rac. Precipitation of GTP-Rac was performed as previously described (7). Briefly, GST-PBD fusion protein expressed in *E. coli* was isolated with glutathione-Sepharose beads and the beads were washed several times in PBS–10 mM DTT–1% Triton X-100 buffer. Cells from a six-well plate were lysed in 150 μl of lysis buffer. Clarified lysates were combined with 20 to 30 μl of beads and 300 μl of binding buffer (25 mM Tris-HCl [pH 7.5], 1 mM DTT, 30 mM $MgCl_2$, 40 mM NaCl, 0.5% NP-40) and incubated for 30 min at 4°C with agitation. The beads were washed several times in binding buffer and finally suspended in 20 μl of Laemmli sample buffer. Proteins were separated by SDS–12% PAGE, transferred to nitrocellulose membrane, and blotted using specific Rac1 antibody.

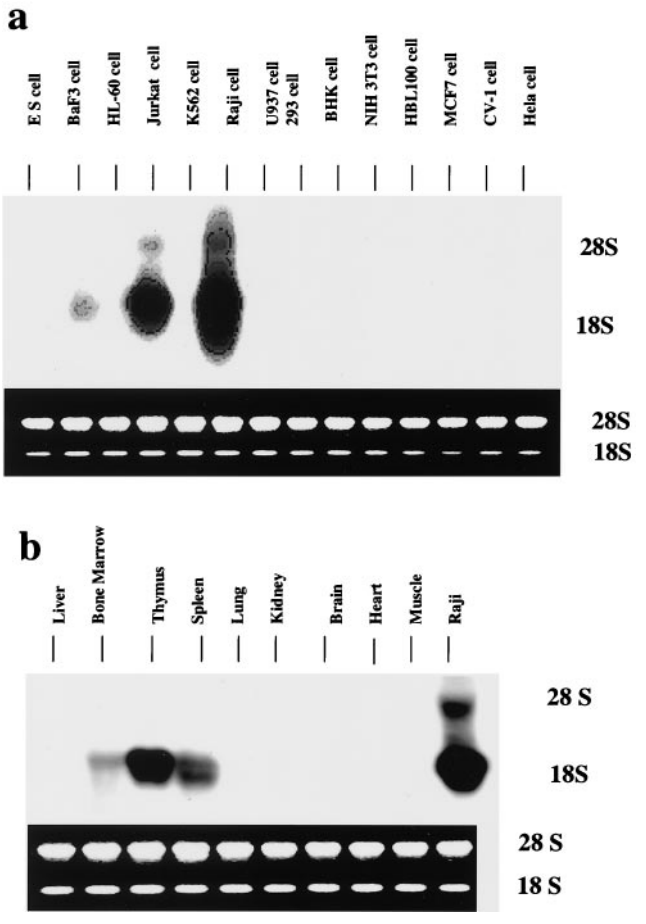

FIG. 1. RhoH is a hematopoiesis-specific gene. (a) Expression in cell lines. RhoH is expressed at high levels in T (Jurkat) and B (Raji) cell lines and at low levels in the mixed lymphoid-erythroid line BaF3. In myeloid (HL60 and U937) and erythroid (K562) cell lines, RhoH is not detectable. All nonhematopoietic cells lines, including undifferentiated embryonal stem (ES) cells, are negative for RhoH. (b) Expression in tissues. mRNA was seen at high levels in the thymus, was less abundant in the spleen, and was lowest in abundance in bone marrow. In all other tissues, RhoH mRNA was nondetectable.

RESULTS

RhoH is a hematopoiesis-specific protein. The human RhoH cDNA was used to probe total RNAs from cell lines and murine tissues. Northern blot analysis of human hematopoietic cell lines (Fig. 1a) confirmed that RhoH is expressed at high levels in T (Jurkat) and B (Raji) cell lines. In myeloid (HL60 and U937) and erythroid (K562) cell lines, RhoH is not detectable. All nonhematopoietic cells lines, including undifferentiated embryonal stem, kidney (293 and BHK), ovarian (HeLa), breast (MCF-7 and HBL100), and fibroblast (3T3) cells, do not express RhoH. Northern blot analysis of normal tissues revealed that RhoH is expressed only in hematopoietic cells (Fig. 1b). A 2.5-kb transcript is present at very high levels in the thymus, less abundant in the spleen, and least abundant in the bone marrow. In all of the other nonhematopoietic tissues examined, RhoH mRNA was nondetectable.

RhoH has no obvious effect on actin reorganization. Since it is rather difficult to assess cytoskeletal changes in hematopoietic cells in suspension, we used the commonly used NIH 3T3 and MDCK adherent cells to test if RhoH can affect cell shape or actin reorganization. The results obtained with the two cell types are comparable, and the results obtained with MDCK cells will be discussed. An HA-tagged RhoH or GST-tagged RhoAV14 expression vector was transfected into MDCK cells. At 24 h after transfection, cells were serum deprived for 24 to 48 h and doubly stained for RhoH or RhoA and actin. RhoH can be seen as a diffusely distributed protein in the cytoplasm (Fig. 2a). Even after 2 days of serum deprivation, MDCK cells still showed minimal residual stress fibers. In cells staining positive for RhoH, the intensity of actin staining was no different (Fig. 2b). In contrast, cells transfected with control RhoAV14 vector (Fig. 2c) showed dense stress fibers and cortical actin staining (Fig. 2d). Serum-starved MDCK cells were then refed with medium supplemented with 10% fetal bovine serum (FBS) or 10 ng of platelet-derived growth factor (PDGF) per ml, and at various time points (15, 30, and 60 min) after stimulation, cells were doubly immunostained for the Rho proteins and actin. After serum stimulation, stress fiber formations were induced in MDCK cells, as shown by a significant increase in stress fiber and actin staining (Fig. 2f). No obvious difference was observed in the morphology and actin

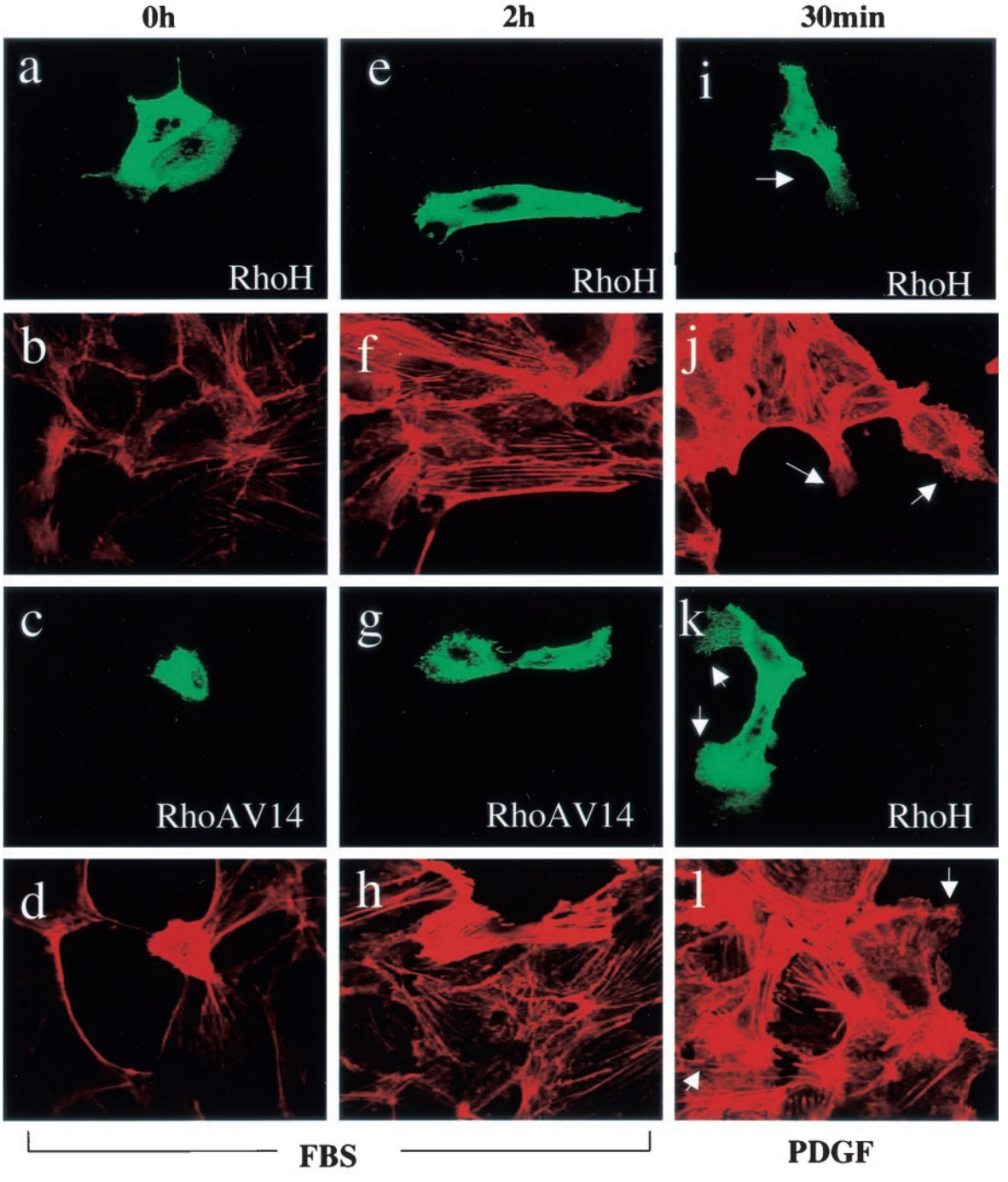

FIG. 2. RhoH has no effect on the cytoskeleton. MDCK cells were transfected with a HA-RhoH or a GST-RhoAV14-expressing vector and, after 24 h of recovery, starved for 48 h. Some cells were fixed and immunostained for RhoH or RhoA (fluorescein isothiocyanate) and actin (rhodamine). Cells were then refed with serum-containing medium (10% FBS) or PDGF (10 ng/ml), fixed at different times after refeeding, and immunostained. (a and b) Starved cells showed minimal stress fibers. RhoH-positive cells did not show any difference in morphology or actin staining pattern. (c and d) RhoAV14-positive cells showed dense stress fibers and cortical actin staining. (e and f) At 2 h after refeeding with 10% FBS medium, increased stress fibers and actin staining were seen in MDCK cells. RhoH-positive cells showed no inhibition of stress fiber formation. (g and h) At 2 h after serum stimulation, RhoV14-positive cells showed even denser actin staining. (i, j, k, and l) At 30 min after restimulation with PDGF, ruffling of cells (arrows) and increased stress fibers were seen. RhoH-positive cells did not show any inhibition of ruffling.

staining of cells that were transfected with and expressing RhoH (Fig. 2e and f). In cells transfected with RhoV14, the staining of stress fibers and cortical actin was even more intense (Fig. 2g and h). In MDCK cells treated with PDGF after starvation, increased ruffling was observed by about 15 to 30 min (Fig. 2j and l). In cells transfected with and overexpressing RhoH (Fig. 2i and k), ruffling induced by PDGF was not inhibited. Together, these results indicate that RhoH does not

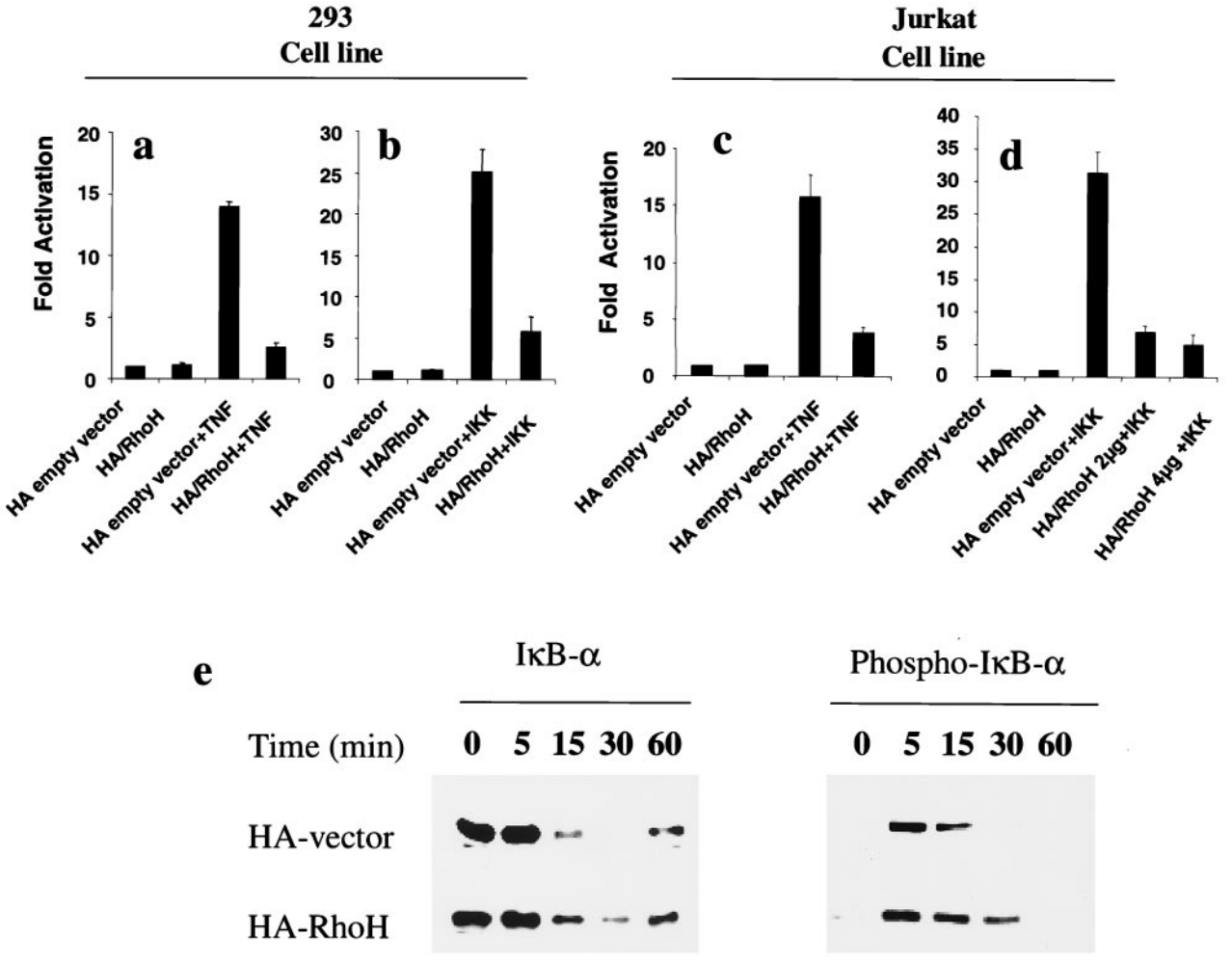

FIG. 3. RhoH inhibits activation of NF-κB. (a and c) An NF-κB–luciferase reporter vector was cotransfected with 2 μg of RhoH into 293 (a) or Jurkat (c) T cells. At 24 h after transfection, cell lysates were evaluated for luciferase activity. RhoH did not induce any increase in luciferase activity above that of an HA-vector control. At 24 h after transfection, cells were treated with TNF. At 6 h after stimulation, maximal luciferase activity (greater than 10-fold induction) was detected in cells transfected with the empty vector, reflecting activation of NF-κB by TNF. In cells transfected with RhoH, NF-κB activation by TNF was almost completely suppressed. (b and d) The pcDNA3IKKβ and NF-κB-reporter vectors were cotransfected with a control vector or RhoH into 293 (b) or Jurkat (d) cells. At 24 h later, cell lysates were evaluated for luciferase activity. IKKβ induced vigorous NF-κB activation (greater than 25-fold induction). In cells transfected with 2 or 4 μg of RhoH, IKK activation of NF-κB was again strongly suppressed with further suppression at a greater RhoH dose. (e) RhoH inhibits IκB degradation but not phosphorylation by IKK. At 24 h after transfection with a control vector or RhoH, 293 cells were treated with TNF. At different times after stimulation, cell lysates were prepared for Western blotting, the level of IκB was measured by anti-IκBα antibody, and the level of phosphorylated IκB was measured by anti-phospho-IκB antibody. Note that in cells transfected with the vector, rapid degradation of IκBα was observed. In cells transfected with RhoH, significantly retarded degradation of IκB was seen and at no time did IκB disappear completely. No difference in phosphorylation of IκB was seen between control vector- and RhoH-transfected cells. Again, there was an obvious retardation of degradation of phosphorylated IκB in RhoH-transfected cells.

appear to exert any significant or obvious effect on actin reorganization, as evaluated with nonhematopoietic cells.

RhoH inhibits activation of NF-κB by TNF and IKKβ. An important example of the involvement of the Rho GTPases in the activities of transcriptional pathways was the demonstration that RhoA, Rac, and CDC42 can activate NF-κB (46) To test for the effect of RhoH on NF-κB, an NF-κB–luciferase reporter vector was cotransfected with pcDNA3-RhoH into 293 or Jurkat cells. At 24 h after transfection, cell lysates were evaluated for luciferase activity. The results obtained with the two cell types were the same. RhoH did not induce any increase in luciferase activity above that of the HA-vector control, indicating that RhoH cannot activate NF-κB (Fig. 3a and c). We then tested whether RhoH can influence the activation of NF-κB by a key physiological stimulus such as TNF. HA-RhoH expression vector DNA was cotransfected with NF-κB reporter vector into 293 or Jurkat cells, and at 24 h after transfection, the cells were treated with TNF-α. We first established that 6 h after TNF stimulation, maximal luciferase activity (greater than 10-fold induction) was detected in cells transfected with the control empty vector, reflecting the activation of NF-κB by TNF. In cells transfected with RhoH, NF-κB activation by TNF was suppressed (Fig. 3a and c) by about 80%. This result was highly reproducible in many exper-

iments, and Fig. 3 shows a typical result. To probe where in the pathway of NF-κB activation RhoH exerts suppressive effect, we tested to see how RhoH affects the activation of NF-κB by IκB kinase. Overexpression of a pcDNA3-IKKβ vector in transfected cells has been shown to induce robust activation of NF-κB (56). The IKKβ vector and the NF-κB reporter vector were cotransfected with a control vector or RhoH, and 24 h later, cell lysates were evaluated for luciferase activity. Overexpression of IKKβ in transfected 293 (Fig. 3b) or Jurkat (Fig. 3d) cells induced a high level of activation of NF-κB. A typical example of greater than 25-fold induction is shown in Fig. 3d. However, in cells cotransfected with 2 or 4 μg of RhoH, IKKβ activation of NF-κB was reduced to about fivefold induction (Fig. 3b and c). A larger dose of RhoH induced a greater degree of suppression. Thus, RhoH is a potent inhibitor of activation of NF-κB by TNF and IKK.

RhoH inhibits IκB degradation. To further test for the mechanism by which RhoH inhibits NF-κB activation, we examined how RhoH affects IκB phosphorylation and IκB degradation.

At 24 h after transfection with a control vector or RhoH, 293 cells were treated with TNF as in previous experiments. At different times after stimulation, cell lysates were prepared for Western blotting and the level of IκB was measured by anti-IκBα antibody while the levels of phosphorylated IκB were measured by anti-phospho-IκB antibody.

In cells transfected with the vector, the emergence of phosphorylated IκB was detected rapidly, within 5 min (Fig. 3e). In cells transfected with RhoH, the same level of phosphorylated IκB-α was detected. Thus, RhoH does not appear to affect the initial phosphorylation of IκB. However, whereas phosphorylated IκB became nondetectable in vector-transfected cells 30 min after TNF treatment, there was clearly a decreased rate of loss of phosphorylated IκB in RhoH-transfected cells, such that phosphorylated IκB could still be detected at 30 min.

This difference was confirmed when the total IκB levels were examined (Fig. 3e). In cells transfected with an empty vector, a rapid onset of degradation of IκBα was observed so that no IκBα was detectable by 30 min. In contrast, in cells transfected with RhoH, there was a significantly retarded degradation of IκB. At 30 min, IκB was still detectable and at no time did IκB completely disappear. Again, this observation was highly reproducible, and Fig. 3e shows a typical result.

RhoH inhibits p38 activation by TNF. Having observed that RhoH affected NF-κB activation in a manner opposite to that of other Rho GTPases, we investigated the effect of RhoH on other transcription pathways that are known to be activated by Rho GTPases.

293 cells were transfected with an empty vector or an RhoH vector and treated with TNF as in previous experiments. The activation of JNK, ERK, and p38 was evaluated by anti-phospho-JNK, -ERK1/ERK2, and -p38 antibodies. Figure 4 shows that the expression of RhoH in 293 cells did not activate ERK, JNK, or p38. Based on the observations of the inhibitory effect of RhoH on NF-κB, we tested how RhoH would affect the activation of these pathways by using TNF as a physiological stimulus. Cells treated with TNF-α showed a clear activation of JNK, ERK, and p38, as measured by the emergence of the phosphorylated proteins in Western blots (Fig. 4). Cells transfected with a control vector showed the same level of activation. Transfection of RhoH did not inhibit the activation of ERK or JNK by TNF (Fig. 4a and b). In contrast, however, the activation of p38 by TNF was significantly inhibited by RhoH (Fig. 4c). When the experiment was repeated with Jurkat T cells, an identical result was observed. Just as in 293 cells, RhoH cannot activate ERKs, JNK, or p38 in Jurkat cells (Fig. 4d, e, and f) but RhoH specifically inhibited activation of p38 by TNF and not activation of JNK or ERK.

RhoH inhibits activities of Rac1, RhoA, and CDC42. The above-described experiments showed that RhoH has a potent inhibitory effect on selected transcriptional pathways whose activation has been shown to be mediated by other Rho GTPases. To see if RhoH can directly interfere or compete with these Rho GTPases, we cotransfected RhoH with constitutively active Rac1L61, RhoAV14, and CDC42V12 in 293 or Jurkat cells. Again, the results obtained with the two cell lines were the same. Transfection of 2 μg of Rac1L61 or RhoAV14 with the NF-κB luciferase reporter plasmid into Jurkat cells showed a vigorous activation of NF-κB (Fig. 5a) similar to that seen with TNF treatment. When 2 μg of RhoH vector was cotransfected with 2 μg of Rac1L61 or RhoAV14, activation of NF-κB was strongly suppressed. Transfection of 4 μg of RhoH inhibited NFκB activation slightly more.

We next utilized the CHOP-luciferase system (Stratagene) to measure p38 activation. Cotransfection of CHOP-luciferase vectors with 2 μg of Rac1L61 or CD42V12 showed a robust activation of p38, whereas transfection of 2 μg of RhoH did not activate p38 (Fig. 5b). However, when 2 μg of RhoH was cotransfected with 2 μg of Rac1L61 or CDC42V12, activation of p38 was dramatically suppressed (Fig. 5b). A larger (4-μg) dose of RhoH further suppressed the activation of p38.

RhoH inhibits Rac at the downstream effectors of Rac. To begin to see where and how RhoH inhibits the activation of p38 by Rac1 and CDC42, we first tested to see if RhoH can inhibit the activation of Rac by competing with or sequestering a Rac exchange factor such as TIAM-1. The fact that RhoH can inhibit the constitutively activated form of Rho GTPases indicated that the inhibition is most likely further downstream. This was confirmed by the PBD assay. Jurkat cells were transfected with either 2 μg of TIAM-1 alone or 1, 2, or 4 μg of RhoH vector DNA. At 24 h later, the level of GTP-bound Rac was assessed by the PBD assay. Figure 6a shows a clear increase in the level of GTP-bound Rac (Fig. 6a) above that in nontransfected control cells. When the cells were cotransfected with RhoH, there was no difference in the level of GTP-bound Rac precipitated. When 4 μg of TIAM-1 was used, a higher level of GTP-Rac was observed that also was not changed by cotransfection of 4 μg of RhoH.

To test if RhoH inhibits downstream Rac effectors, we used an effector known to mediate activation of p38. A constitutively active form of MKK, MKK6glu, was cotransfected with CHOP-luciferase vectors into Jurkat cells. Strong activation of p38 was observed (Fig. 5b). Again, the activation of p38 by MKK6glu was suppressed strongly by cotransfection with 2 or 4 μg of RhoH (Fig. 5b).

RhoH binds GTP only but has no GTPase activity. The biochemical activities of RhoH demonstrated thus far are, unexpectedly, the opposite of those of other frequently studied members of the GTPase family, such as RhoA, Rac, and the CDC42 group. At highly conserved positions (residue 13 and

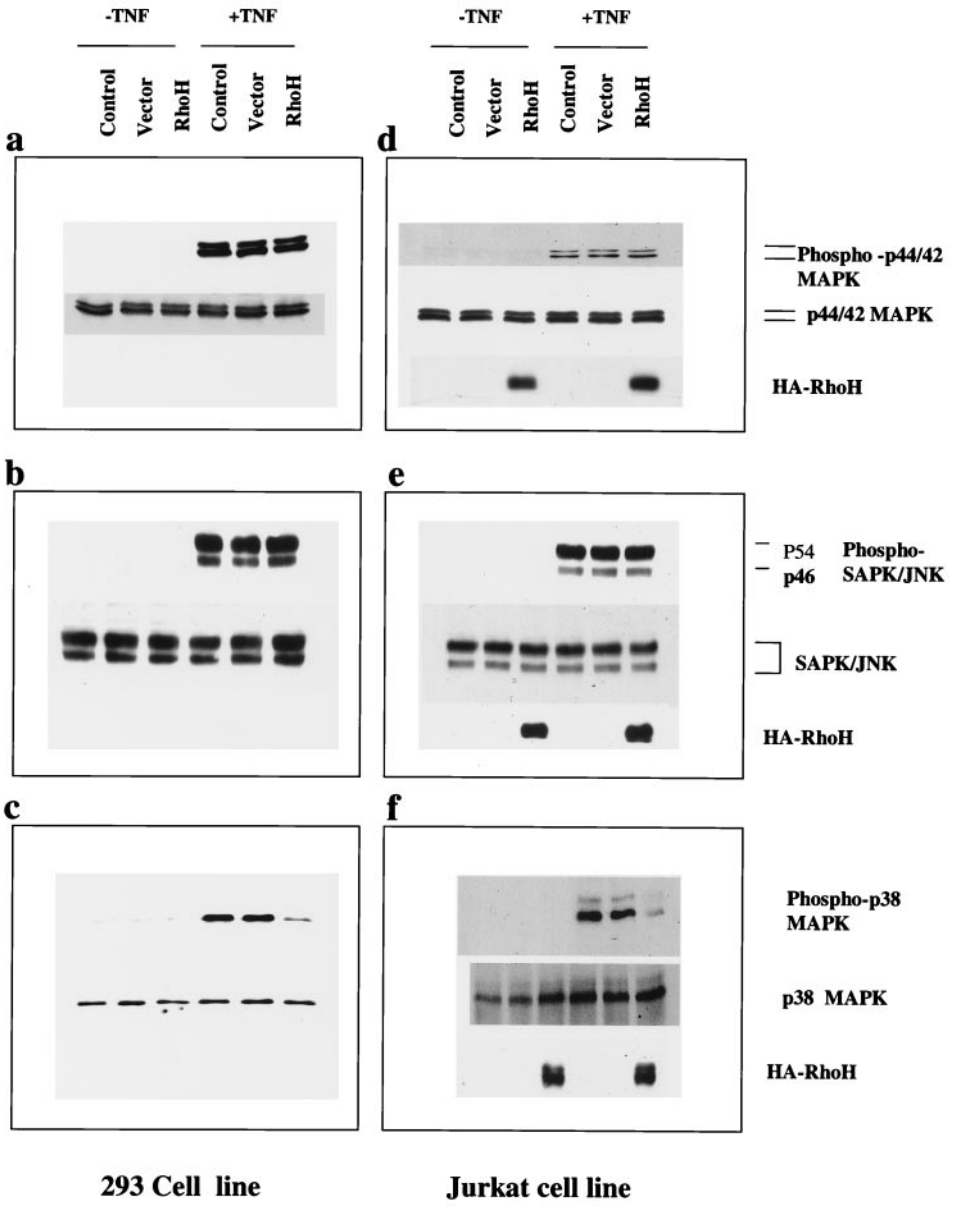

FIG. 4. RhoH inhibits p38 but not ERK or JNK activation. 293 or Jurkat cells were transfected with 2 μg of an empty or RhoH vector. At 24 h after recovery, cells were treated with TNF for 30 min and then harvested for Western blot analysis. Activation of ERK, JNK, and p38 was evaluated by anti-phospho-ERK1/ERK2, -JNK, and -p38 antibodies. The expression level of HA-tagged RhoH protein was revealed by anti-HA antibody, which is shown here only for transfected Jurkat cells (d, e, and f). The results show that RhoH did not activate ERK (a and d), JNK (b and e), or p38 (c and f). Cells treated with TNF showed clear activation of JNK, ERK, and p38, as measured by the emergence of the phosphorylated proteins (+TNF). Cells transfected with the control vector showed the same level of activation as nontransfected cells. RhoH did not inhibit activation of ERK (a and d) or JNK (b and e) by TNF. The amount of phospho-p38 was, however, significantly reduced in RhoH-transfected 293 (c) and Jurkat (f) cells.

62) that are known to be critical for GTP hydrolysis, RhoH differs from the other GTPases (Fig. 7), and Zohn et al. have predicted that RhoH would be GTP deficient (66)

To test for this, GTP binding and hydrolysis by RhoH were analyzed by using GST-RhoH expressed as a bacterial fusion protein. Soluble GST-RhoH fusion protein was expressed at high levels in bacteria and purified on glutathione-agarose beads. Similar to Rac, RhoH binds GTP rapidly upon incubation with radiolabeled nucleotide under nucleotide exchange conditions. A nucleotide dissociation assay revealed that, unlike Rac1 or RhoA, RhoH is highly resistant to exchange for GDP (Fig. 8a). Thus, while RhoH shows specific, high-affinity binding to the GTP form of guanine nucleotide, similar to other small GTPases, RhoH remains only in the GTP-bound state. We then assayed the ability of RhoH to hydrolyze GTP in a filter-binding assay. The results showed that under conditions in which RhoA and Rac hydrolyzed more than 60 to 80% of bound GTP, RhoH did not show any hydrolysis of GTP (Fig. 8b).

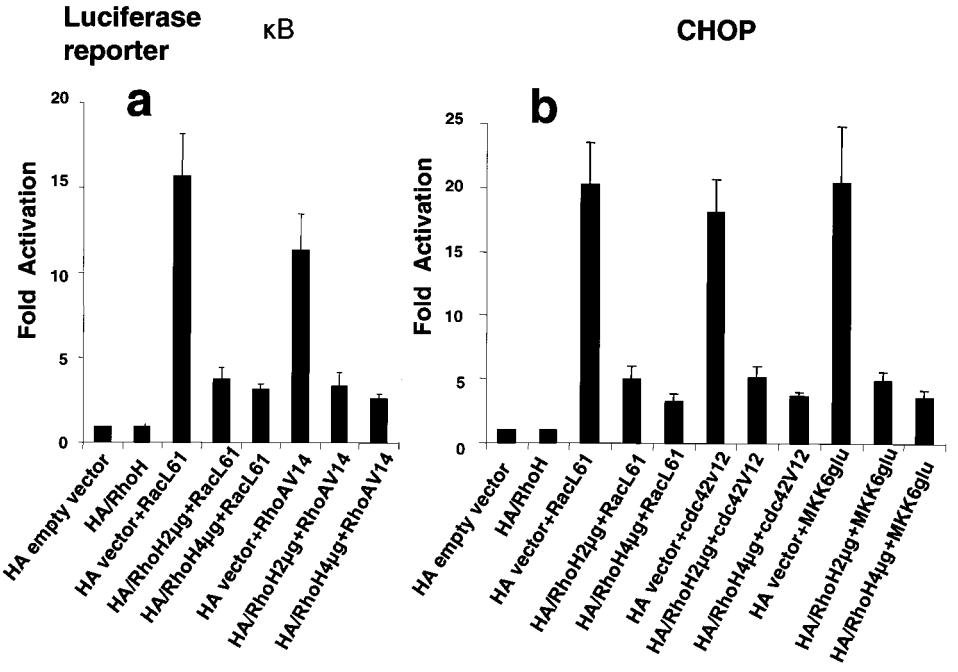

FIG. 5. RhoH inhibits Rac1, RhoA, and CDC42. Jurkat cells were cotransfected by electroporation with HA-RhoH and constitutively activated RacL61, RhoAV14, or CDC42L61 and assayed for an effect on NF-KB activation (a) or p38 activation (b) by using luciferase reporters as indicated. RacL61 and RhoAV14 induced robust activation of NF-κB. Both activations were effectively suppressed by 2 or 4 μg of RhoH. RacL61, CDC4261L, and MKK6glu induced a vigorous activation of p38 (b) in Jurkat cells that was suppressed by RhoH.

RhoH is resistant to Rho GAP. A critical step in the GTPase cycle is the inactivation of activated GTPase by interaction with specific GAPs that catalyze and dramatically augment intrinsic GTPase activity. Each member of the Rho family of proteins responds to one of several Rho-specific GAPs (31, 63). Since it is possible that, in vivo, RhoH has GTPase activity by interaction with a particular Rho GAP, we tested Rho GAP p50, a potent GAP for RhoA, Rac, and CDC42. Incubation of [^{32}P]GTP-loaded RhoH with purified Rho GAP p50 failed to induce detectable GTP hydrolysis by RhoH (Fig. 8C). In the same experiment, Rho GAP p50 strongly enhanced the GTPase activity of RhoA (Fig. 8C) and Rac (data not shown).

RhoH interacts and binds to GDIs. The above-described experiments demonstrated that RhoH exists only as a GTP-bound form and that it is GTPase deficient. This suggests that RhoH is not regulated by cycling of GDP/GTP, as is the case with most other members of the Rho family that are fully functional GTPases. Among the regulators of Rho GTPases are the GDIs. The Rho GDIs inhibit the dissociation of GDP (59) but also bind strongly enough to GTPases to extract them from membrane (33). To see if the GDIs can bind to RhoH and to see if there is any preferential binding to any of the GDIs, we tested for interaction among the three known GDIs for the Rho GTPases. Myc-tagged Rho GDI-α, -β and -γ were cotransfected into 293 cells with HA-tagged RhoH. At 24 h after transfection, cell lysates were prepared from transfected cells and immunoprecipitation was carried out with anti-myc antibody. Immunoprecipitates were separated and transferred to a Western blot. The filter was then probed with anti-HA antibody. Our results showed that RhoH coprecipitated with all three GDIs. Furthermore, there did not appear to be preferential binding between RhoH and the three known Rho GDIs (Fig. 6b).

Reduction of endogenous RhoH mRNA by αs-RhoH augments Rac-mediated lymphocytic activity. Since RhoH appears to have such a strong inhibitory effect on other Rho GTPases and at the same time is constitutively active, we asked if the alteration of the level of endogenous RhoH mRNA can affect the final activity of another Rho GTPase. We examined the effect of modulating RhoH levels on Rac-induced transcriptional activation of IFN-γ in Jurkat cells. Jurkat cells were cotransfected with Rac1L61 and a luciferase reporter vector (IFN-γ–Luc) for IFN-γ transcription as previously described (34). Figure 9 shows that 2 μg of Rac1L61 induced about sevenfold activation of IFN-γ in Jurkat cells. When the cells were cotransfected with a vector expressing αs-RhoH, the level of endogenous RhoH mRNA was reduced proportionately to the dose of antisense vector DNA transfected. Figure 9 shows that a reduction in RhoH mRNA resulted in increased activation of IFN-γ to about 9-fold and with an even greater reduction in RhoH caused by 4 μg of αs-RhoH, IFN-γ activation increased to greater than 11-fold. In contrast, overexpression of αs-RhoH in nonhematopoietic, RhoH-negative 293 cells neither increased nor decreased the activation of IFN-γ.

RhoH is transcriptionally regulated. The modulation of a Rac1-mediated activity simply by alteration of the level of endogenous RhoH mRNA (and hence its protein level) suggests that one way in which RhoH may be regulated is at the transcriptional level. We therefore tested to see if there is any condition under which an alteration of RhoH transcription is induced. Figure 10a shows that upon PMA treatment, the level of endogenous RhoH transcripts in Jurkat cells is dramatically

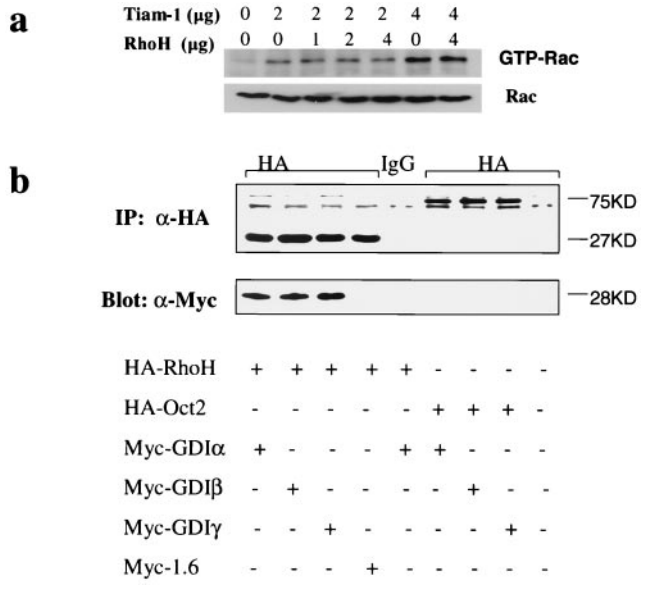

FIG. 6. RhoH does not inhibit exchange factor TIAM-1 and binds to Rho GDP dissociation inhibitors. (a) A 2-μg sample of TIAM-1 was cotransfected with RhoH (1, 2, or 4 μg) by electroporation into Jurkat cells, and 24 h later, the cells were harvested and measured for levels of GTP-Rac with the PBD assay. An increased level of GTP-Rac was seen in TIAM-1-transfected cells. Cotransfection of RhoH did not change the level of GTP-bound Rac. (b) Myc-tagged Rho GDI-α, -β, -γ, and −1.6 (used as a control) were cotransfected into 293 cells with HA-tagged RhoH or HA-tagged Oct2 (used as a control). At 24 h after transfection, cell lysates were prepared from transfected cells and immunoprecipitation (IP) was carried out with anti-HA antibody or immunoglobulin G (used as a control). Immunoprecipitates were separated and transferred to a Western blot. The filter was then probed with anti-HA and anti-myc epitope antibodies. The top of panel b shows the specific immunoprecipitation of HA-RhoH (27 kDa) or HA-Oct2 (75 kDa) protein. Equal levels of protein reflect uniformity of transfection and immunoprecipitation. The lower part of panel b shows the coprecipitation of only Rho GDIs with HA-RhoH. Note the equal levels of Rho GDIs, indicating equal avidity of binding between RhoH and the three different Rho GDIs.

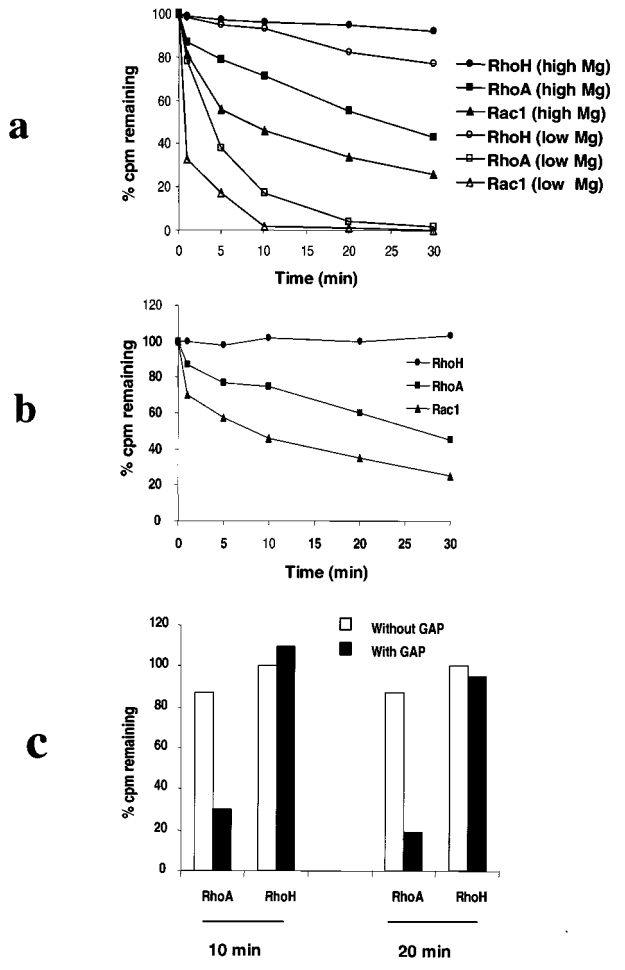

FIG. 8. RhoH binds GTP only but has no GTPase activity. (a) Nucleotide dissociation assay using GST-RhoH, -Rac, and -RhoA fusion proteins. Similar to RhoA and Rac1, RhoH binds GTP rapidly upon incubation with radiolabeled nucleotide under nucleotide exchange conditions. Note that RhoH is almost completely resistant to GDP dissociation compared to Rac and RhoA under both high- and low-magnesium conditions. (b) GTP hydrolysis assay: Under conditions that allow RhoA and Rac to autohydrolyze greater than 80% of bound GTP, RhoH did not show any hydrolysis of GTP. (c) RhoH is resistant to Rho GAP p50. Addition of Rho GAP p50 enhanced the hydrolysis activity of RhoA but had no effect on RhoH.

decreased so that at about 60 to 80 min, the RhoH mRNA is reduced by at least 80% from baseline level. This level of reduction was sustained for the entire period in which the cells were examined, up to 24 h (data not shown). In contrast, there was no significant change in Rac1 transcripts (data not shown) after PMA treatment In TNF-treated cells and there was no significant change in the level of RhoH mRNA, except for a small but consistent transient reduction in RhoH mRNA at about 40 to 60 min after stimulation. Several doses of PMA (20, 50, and 100 ng/ml) or TNF (10, 20, and 50 ng/ml) were tested, and the results were the same.

RhoH is differentially expressed in Th1 and Th2 T cells. To identify the physiological conditions under which a difference

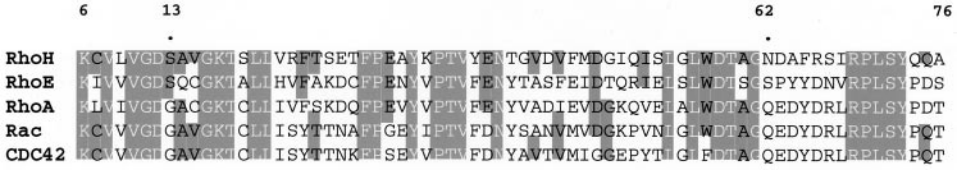

FIG. 7. RhoH has different residues at two key sites that are critical for GTPase activity. Alignment of the amino acid sequences of RhoH, RhoE, RhoA, Rac1, and CDC42 in the GTPase-determining domain. At position 13 of RhoH, a serine is found instead of the glycine at the corresponding position in RhoA, Rac, and CDC 42. At position 62, an asparagine is found in RhoH instead of the glutamine in RhoA, Rac, and CDC42.

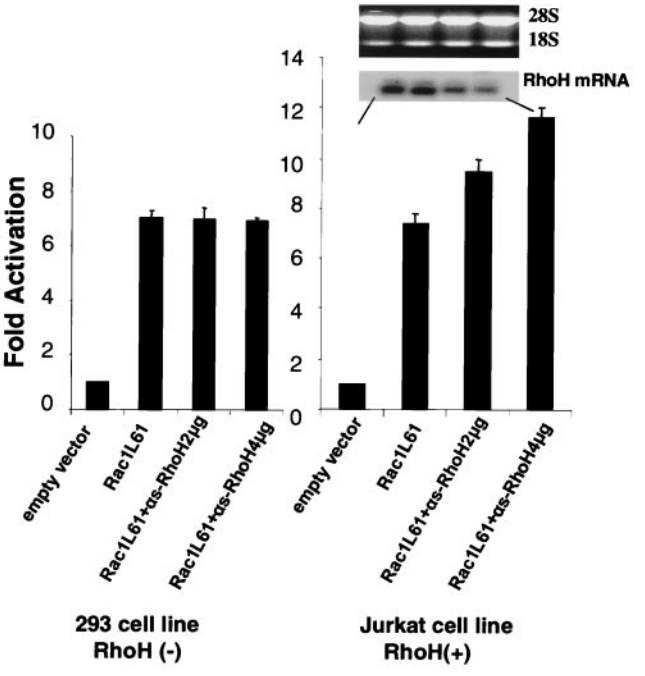

FIG. 9. Antisense blocking of RhoH increases Rac1-induced activity. 293 or Jurkat cells were cotransfected with reporter genes for IFN-γ–Luc (2 μg) and Rac1L61 (2 μg), with or without an αs-RhoH vector (2 or 4 μg). At 24 h after transfection, cell were processed either for luciferase activity measurement or for Northern analysis to assess endogenous levels of RhoH mRNA. (a) In 293 cells, Rac1L61 induced IFN-γ activation and no difference between activity levels was caused by αs-RhoH expression. (b) In Jurkat cells, RacL61 induces an equivalent level of IFN-γ activation. In cells transfected with αs-RhoH, there was an increase in IFN-γ activity proportionate to the reduction in the endogenous RhoH mRNA level. The experiment was repeated two times, and a typical result is shown.

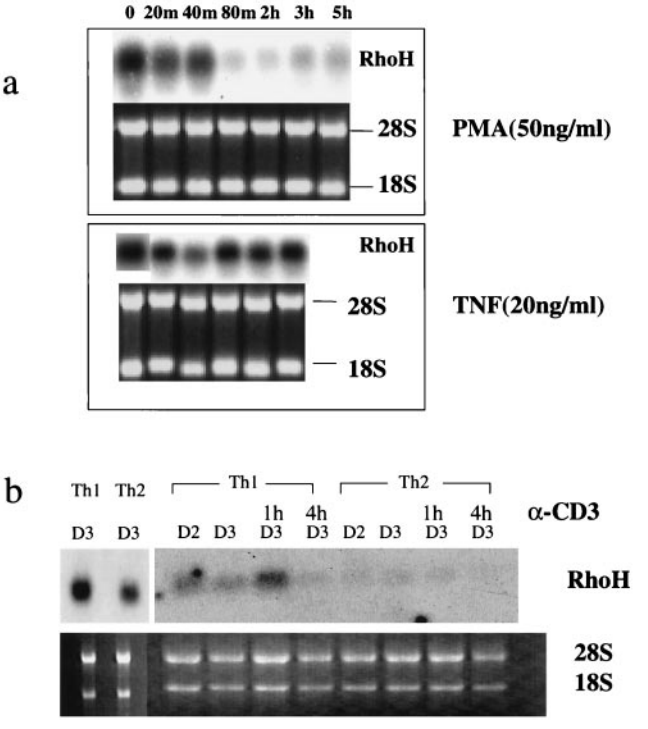

FIG. 10. RhoH is transcriptionally regulated. (a) Jurkat cells were treated with PMA or TNF, and at different time points after stimulation, total RNA was extracted for Northern analysis to assess levels of RhoH mRNA. A 15-μg sample of total RNA was loaded in each lane. PMA was tested at 20, 50, and 100 ng/ml, and TNF was tested at 10, 20, and 50 ng/ml. The results produced by the different treatment dosages are comparable. A typical example is shown. PMA dramatically down regulates RhoH mRNA between 40 and 80 min after treatment. There is little change in RhoH mRNA levels with TNF treatment, except for a small but consistently observed transient decrease at around 40 min. (b) Differential expression in Th1 and Th2 T cells. Naive $CD4^+$ $CD62L^+$ $CD44^{low}$ T cells were separated and treated under Th1 or Th2 differentiating condition as described in Materials and Methods. At days 2 (D2) and 3 (D3) after culture initiation, cells were processed for Northern analysis. (Left) Analysis of 15 μg of total RNA at day 3. The level of RhoH in Th1 cells is about three times as high as that in Th2 cells. (Right) Analysis of 7 μg of total RNAs. Again, the level of RhoH in Th1 cells is higher than that in Th2 cells. At day 3, aliquots of cells were washed and restimulated with anti-CD3. At 1 and 4 h after restimulation, RNA was extracted for Northern analysis. In Th1 cells, down regulation of RhoH was observed by 4 h (the increase at 1 h is likely from a slightly higher level of RNA loading). In Th2 cells, the levels of RhoH remained very low.

in RhoH levels is important, we compared expression levels of RhoH in the two subsets of Th1 and Th2 T-helper cells. Naive $CD4^+$ T cells under different culture conditions differentiate to either Th1 or Th2 cells. Because of the small number of cells in the starting population, we focused on comparing the T cells after they had differentiated. A clear difference in the level of RhoH mRNA was seen by 2 days, when naive cells had become effector Th1 or Th2 cells (Fig. 9b, left, 15 μg of total RNA, and right, 7 μg of total RNA). Northern blot analysis showed that RhoH is expressed at significantly higher levels (at least 2.5- to 3-fold as measured by densitometry and phosphorimaging counts) in the Th1 subset compared to those in Th2 cells. In a second set of experiment, we tested to see the response when day 3 differentiated Th1 or Th2 cells were restimulated with anti-CD3. Total RNAs were collected at 1 and 4 h after restimulation. Interestingly, a significant decrease in RhoH mRNA was observed at 4 h poststimulation (Fig. 9b, right). The level of RhoH in Th2 cells was too low in this experiment to allow any definitive conclusion, but no obvious changes were seen.

DISCUSSION

Most of the Rho GTPases are ubiquitously expressed proteins. Our studies examined, for the first time, the expression of RhoH in normal tissues and showed that RhoH transcripts are detected only in hematopoietic tissues with the highest level of expression in the thymus. In a wide range of nonhematopoietic tissues examined, RhoH transcripts were not detected. From the representative cell lines we have examined, RhoH appears to be preferentially expressed in T and B cells. We cannot rule out the possibility that myeloid cells at later stages of differentiation, such as neutrophils, can express RhoH. However, the much lower level of RhoH mRNA in bone marrow than in the thymus and spleen suggests that RhoH is encoded by a lymphoid tissue-specific gene. Therefore, RhoH very likely has a function of particular relevance to lymphoid cells. In a Northern blot analysis, murine RNA was probed with human cDNA under high-stringency conditions

and the strong signal obtained indicated a very high degree of nucleotide and amino acid similarity between humans and mice.

An extensive body of literature has addressed the involvement of Rho GTPase in the regulation of nuclear signaling, including activation of transcriptional factors involved in stress and inflammatory pathways (30, 39, 46, 55, 60). The precise mechanism by which Rho, Rac, and CDC42 activate NF-κB has not been fully elucidated, but existing evidence suggests that these GTPases do so by inducing or enhancing the phosphorylation of IκB (46). A nodal point at which several pathways that lead to NF-κB activation converge is the phosphorylation of IκB by the IκB kinase (IKK-α, -β, and -ε) complex (4) Phosphorylation of IκB at serine residues leads to ubiquitination of IκB and its degradation in the proteasome complex, resulting in the release of NF-κB and translocation of the p50/p65 dimers to the nuclei (5). RhoH cannot activate NF-κB. Instead, RhoH is a potent suppressor of activation of NF-κB induced by TNF, Rac1, RhoA, and IKK in both hematopoietic and nonhematopoietic cells. Since the inhibitory action of RhoH is directed at or downstream of IKK, it is not surprising that RhoH inhibits the activation of NF-κB by both Rac1 and RhoA. Although we have not tested CDC42, it is most likely that RhoH can also inhibit CDC42-mediated activation of NF-κB. While additional work needs to be done to confirm the data by various other approaches, our results show that suppression by RhoH appears to be due not to inhibition of the phosphorylation of IκB by IKK but rather to retardation of the degradation of both total and phosphorylated IκB. There are a number of ways in which RhoH may cause this effect, including inhibition of the ubiquitination of IκB, release of ubiquitinated IκB from NF-κB dimers, and degradation of free ubiquitinated IκB by proteasomes (4, 5). Further work will attempt to unravel the mechanism involved. Nevertheless, our results show that RhoH acts very differently from the RhoA-Rac-CDC42 group of Rho GTPases and, together with a recent report that RhoB represses NF-κB signaling (16), indicate that the effect of Rho GTPases on this important pathway can be either positive or negative. Since NF-κB regulates the expression of a wide range of genes, including those involved in inflammatory response and regulation of cell death, it is reasonable to construe that RhoH may be an important modulator of these responses in hematopoietic cells.

Further evidence that the inhibitory effect of RhoH on NF-κB activation is a specific function is supported by the next set of experiments examining other transcriptional pathways known to be influenced by RhoA, Rac, and CDC42, namely, the stress and inflammatory response pathways. The ERK kinases or p42/p44 proteins are activated and phosphorylated by mitogenic stimuli. In contrast to p42/p44 mitogen-activated protein kinases, JNKs and p38 are poorly activated by mitogens but strongly activated by inflammatory cytokines and cellular stress. Evidence that these stimuli exert their effects through Rho GTPases is provided by the fact that expression of activated forms of the Rho GTPases has been shown to induce activation of ERK, JNK, and p38 in various cell types (60, 64). The selective inhibition of the TNF-mediated activation of p38 by RhoH but not by JNK or ERK indicates that this is a specific and physiological function of RhoH. This is further supported by the ability of RhoH to suppress p38 activation by Rac1L61 and CDC42L61 equally well in hematopoietic and nonhematopoietic cells.

The mechanism by which RhoH inhibits Rac and CDC42 activation of p38 remains unresolved. Although we have tested only one exchange factor, TIAM-1, it is quite unlikely that RhoH inhibits other Rho GTPases by sequestering their exchange factors. The identity of the downstream effectors that link the Rho GTPases to activation of p38 is not fully understood. An increasing number of the serine/threonine kinases known as PAKs (3) have also been identified as immediate downstream effectors because they bind to Rho GTPase in a GTP-dependent manner and become activated upon binding (3, 28, 38, 65). Further downstream, various kinases, such as the dual-specificity kinase SEK1/MEKK4, activate JNK and p38, and MKK3 and MKK6 specifically activate p38 (48, 53). Here we have shown that RhoH is also able to inhibit MKK6glu activation of p38, indicating that the inhibitory effect of RhoH is likely to be at or downstream of MKK6. Whether RhoH also inhibits other effectors, such as MKK3, remains to be systematically tested.

It was surprising that overexpression of wild-type RhoH was sufficient to induce a potent and specific effect on transcription. In most functional experiments with Rho GTPases, this effect has been best demonstrated by using dominant active or negative mutant proteins since overexpression of the wild-type form alone exerts a weaker or minimal effect. Amino acid substitution of valine for glycine at codon 12 or of leucine for glutamine at codon 61 (Rac1 numbering) has been extensively used to generate constitutively active Rho GTPases (13, 23), and crystal structure studies have confirmed an essential role for both residues in GTP hydrolysis (25, 52). Either mutation alone is sufficient to prevent intrinsic and GAP-induced GTP hydrolysis.

Both residues are replaced in wild-type RhoH, and the vitro GTPase and nucleotide dissociation assays showed that wild-type RhoH is GTPase deficient and exists only in the GTP-bound form. This result explains why the overexpression of wild-type RhoH alone is sufficient to exert the powerful inhibitory effect we had observed in various biochemical studies.

A few small GTPases containing substitutions at one or two of these positions have been found to maintain and exhibit GTPase activity in spite of the difference (37, 49). This implies that a certain degree of structural change within the catalytic region is allowed.

In this regard, RhoH resembles RhoE, which has also been shown to be GTPase deficient. (15). In RhoE, amino acid substitutions exist in three highly conserved positions that are critical for normal GTPase activity and, similar to RhoH, RhoE appears to exist only in the GTP-bound state. Interestingly, it has been shown by microinjection of MDCK cells that RhoE induces complete disappearance of stress fibers, together with cell spreading (19). It was postulated that RhoE inhibits signaling downstream of RhoA. We did not observe any dramatic effect of RhoH on the cytoskeleton or cell morphology in 3T3 and MDCK cells. This may not be universal because the effect of Rho GTPases on actin reorganization can be cell dependent and we cannot entirely rule out the possibility that RhoH has a definitive role in cytoskeletal organization in other cell types. Therefore, whether RhoH has a role in

cytoskeletal changes in hematopoietic cells needs to be further studied.

Another Rho GTPase, RhoD, has been shown to cause disassembly of stress fibers and inhibition of cell motility (58). Introduction of the constitutively active form of RhoD (G26V) into fibroblasts by microinjection or transfection resulted in disassembly of actin stress fibers and focal adhesions. Furthermore, stress fiber enhancement by RhoA or RhoA-activating lysophosphatidic acid was reversed by the transfection of RhoD cDNA. Thus, RhoD, -E, and -H constitute a category of Rho GTPases that either have no effect on actin polymerization or are antagonistic to the effect of RhoA, Rac, or CDC42 on actin.

The C-terminal residues of RhoH, CKIF, represent a typical CAAX motive present in the entire ras superfamily of small GTP-binding proteins. Depending on the identity of the carboxyl-terminal amino acid (X), proteins will be geranylated if X is leucine or phenylalanine. This posttranslational modification is crucial for the localization of both Ras and Rho proteins to the plasma membrane and for their biological activities. Many of the Rho proteins identified thus far end with leucine or phenylalanine and are geranylated (1, 27). Therefore, RhoH is most likely geranylated and, like other Rho GTPases, would have a certain fraction of the protein associating with membrane fractions during biological activities. However, confocal images of immunostained RhoH-transfected cells showed that the protein is diffusely distributed in the cytoplasm. We could not detect any definitive plasma membrane staining. We cannot, however, rule out the possibility that, inside the cell, RhoH is localized to certain membrane compartments. Furthermore, it may be informative to formally demonstrate if RhoH is geranylated.

Analogous to other GTP-binding proteins, the GTP-bound state of RhoH is most likely the active state. The cycling of Rho GTPases between the GDP-bound and GTP-bound states is controlled by three regulatory factors. GDP exchange factors (GEFs) catalyze the release of GDP and replacement with cytosolic GTP (9). Since RhoH exists only in GTP-bound form, GEFs are not likely to be relevant to its function. The down regulation of active Rho GTPases is achieved mainly by specific GAPs that strongly enhance their intrinsic GTPase activities. RhoH is not responsive to Rho GAP p50, which is a GAP for Rho, Rac, and CDC42. About 20 GAPs for Rho GTPases have been identified to date (8, 31, 63). The human genome data have shown that chromosome 2 alone potentially encodes eight GAPs. It is possible that, in some physiological context, RhoH is stimulated to hydrolyze GTP by some GAP. While further tests with other GAPs or with cellular lysates would be useful in resolving this issue, we would argue that since RhoH shows little or no intrinsic GTPase activity, it is likely that RhoH is not regulated by some specific cellular Rho GAP.

A third regulatory protein that is involved in GTPase activity is the GDI (2, 32, 59). The exact in vivo function of Rho GDIs remains unclear. In vitro assays have shown that GDIs inhibit GDP dissociation and compete with GEF for activation of Rho GTPases (2, 32). They also bind to Rho GTPases strongly enough that that they are capable of displacing them from membranes (33). In one of the proposed models for the regulation of GTPases, the GDIs extract the GDP-bound form of GTPases from the membrane after inactivation by hydrolysis and return the GTPases to the cytosolic compartment in a GDI-Rho GTPase complex. Another proposal is that Rho GDIs act as shuttles carrying and targeting Rho GTPases to their site of activity. Finally, Rho GDI-α or Rho GDI-1 has also been shown to be capable of inhibiting GAP activity (22). Of the various functions attributed to the GDIs, the shuttling of GTPases to sites of activity may be relevant to a naturally GTPase-deficient protein like RhoH. Our results showed that after cotransfection of GDIs with RhoH, immunoprecipitation of the GDIs consistently coprecipitated RhoH, indicating that, in vivo, RhoH interacts avidly with the GDIs. Thus, it is possible that the GDIs play a role in the regulation of the cellular function of RhoH.

Most significantly, the selective inhibitory function of RhoH raises the question of how such a protein may compete with other GTPases in the same cell. The fact that RhoH is GTPase deficient and exists only in the GTP-bound form implies that the regulation of the protein must follow a mechanism other than nucleotide cycling. Possible mechanisms for regulating RhoH activity include phosphorylation states, mRNA expression levels, and protein level changes by various proteolysis mechanisms.

We have focused on searching for evidences that expression of RhoH is regulated. Our finding that the RhoH transcript is rapidly down regulated after treatment with a physiological dose of PMA provides clear evidence that RhoH expression is transcriptionally responsive to cell stimulation. The rapid down regulation of the RhoH transcript within 60 min also suggest that active degradation of RhoH mRNA is involved. Further experiments comparing this with the natural half-life of RhoH mRNA should resolve this issue.

A clue to what may be the physiological conditions under which differential expression of RhoH occurs was obtained from our analysis of Th1/Th2 cell differentiation.

Li et al. (34, 36) have shown that Rac2 is preferentially expressed in the Th1 subset of T cells. The authors also showed that Rac2 is required for the production and release of IFN-γ and that dominant negative Rac1N17 almost completely suppresses cytokine release by activated Th1 cells. Significantly, it was shown that this function of Rac2 depends on its ability to activate NF-κB and p38 and that the activation of each alone is not sufficient to induce IFN-γ release. Given our observation that RhoH appears to be expressed only in lymphoid cells and that the biochemical effect of RhoH is the opposite of that of Rac2, we hypothesized that RhoH may be differentially expressed in Th1 and Th2 cells.

The expression of RhoH is clearly differentially regulated between Th1 and Th2 cells. The significantly higher level of RhoH expression in Th1 cells suggests that RhoH has a role in the regulation of the difference in function between Th1 and Th2 cells.

Since RhoH exerts an inhibitory effect on transcription factors in T cells that are activated by Rac2, we postulate that RhoH functionally competes with Rho GTPases such as Rac2 and that, together, the two GTPases modulate T-cell functions such as the secretion of cytokines. Furthermore, the lowering of RhoH mRNA in Th1 cells after restimulation with anti-CD3 indicates that in certain cells, signaling through the T-cell receptor is linked to transcriptional alteration of RhoH.

Here we have obtained the first evidence of how important

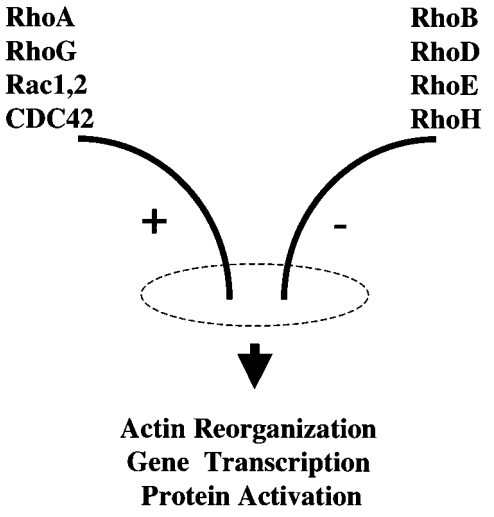

FIG. 11. Model of competing Rho GTPases. Two groups of Rho GTPases that have opposite functions are shown as having a positive or negative effect. The model suggests that some cellular end responses may be modulated by competition between these Rho GTPases.

the transcriptional response of RhoH may be by showing that a reduction of endogenous RhoH in lymphocytes by αs-RhoH overexpression resulted in dose-dependent enhancement of a Rac-induced inflammatory response.

It is possible that a signal that activates Rac proteins is linked to a signal that down regulates RhoH expression in order to remove the inhibitory effect of RhoH. However, the kinetics of activation of Rac is faster than the transcriptional down regulation of RhoH. Therefore, the reduction of RhoH inhibition of Rac would not be synchronous with the activation of Rac, implying that RhoH targets are not immediate Rac effectors but, rather, are further downstream in pathways affected by Rac activation. Whether there is an additional mechanism for the rapid degradation of RhoH protein is an interesting possibility that remains to be tested.

One possibility is that, rather than being linked directly to the rapid on-off activation of other Rho GTPases, RhoH functions as a "thermostat" that sets the levels of response to activation of other Rho GTPases. Another potential hypothesis recognizes the fact that Th1 $CD4^+$ T cells produce large amounts of inflammatory cytokines, including TNF-α, LTα (TNF-β), and IFN-γ. As we have shown, increased expression of RhoH in these cells would reduce the activation of signaling pathways downstream of the TNFs and thus render these cells resistant to the toxic effects of the cytokines they produce.

The possibility that regulation of RhoH does not occur by nucleotide cycling also raises the relevance of binding to GTP. In the context of RhoE, another GTPase-deficient Rho GTPase, it has been suggested that in these noncycling GTP-binding proteins, GTP plays only a structural role, such as coordination of the correct conformation of the protein (15). Whatever the answer, the properties of RhoH serve to further support the notion that nucleotide exchange is not absolutely necessary for the activation of all GTP-binding proteins.

Finally, RhoH and at least three other known Rho GTPases, RhoB (16), RhoD, and RhoE, constitute a category of Rho GTPases having functional effects opposite or antagonistic to those of other Rho GTPases, such as RhoA, Rac, and CDC42. This suggests that another level of regulation of Rho GTPase activity that has to be considered is one in which Rho GTPases with opposing modes of function compete or work together to modulate the final outcome of particular Rho GTPase-activated pathways (Fig. 11). Further work to elucidate the mechanism of RhoH function and additional evidence from other Rho GTPases would help to resolve the validity of such a model involving competing Rho GTPases. Recent work showing the competition between RhoA and RhoE in cell transformation supports such a model of disease development. (21). In this regard, it is pertinent to note that mutations of RhoH have recently been found in as many as 46% of diffuse large-cell lymphomas (45). Most interestingly, all of the mutations are in the first intron of the gene and do not involve the protein coding exon. Whether this translates into abnormal alterations of RhoH expression is currently under investigation.

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