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A Role of STAT3 in Rho GTPase-regulated Cell Migration and Proliferation*

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Rho family GTPases and STAT3 act as mediators of cytokine and growth factor signaling in a variety of cellular functions involved in inflammation, tumorigenesis, and development. In the course of searching for their functional connections, we found by using STAT3 knock-out mouse embryonic fibroblasts that RhoA, Rac1, and Cdc42 could cause nonspecific activation of STAT3 promoter-driven luciferase reporter in the absence of STAT3, raising concerns to a body of literature where STAT3 was associated with Rho GTPases based on the reporter system. We also found that although active RhoA, Rac1, and Cdc42 could all mediate Ser-727 and Tyr-705 phosphorylation and nuclear translocation of STAT3, the Rho GTPases were able to induce STAT3 activation independently of the interleukin-6 autocrine pathway, and active RhoA, Rac1, or Cdc42 could not form a stable complex with STAT3 as previously suggested, indicating an unappreciated mechanism of STAT3 activation by the Rho GTPases. The RhoA-induced STAT3 activation partly depended on Rho-associated kinase (ROK) and involved multiple effector signals as revealed by the examination of effector domain mutants of RhoA. Genetic deletion of STAT3 led to a loss of response to RhoA in myosin light chain phosphorylation and actin stress fiber induction but sensitized the cells to RhoA or ROK-stimulated cell migration. STAT3 was required for the RhoA-induced NF-κB and cyclin D1 transcription and was involved in NF-κB nuclear translocation. Furthermore, loss of STAT3 expression inhibited RhoA-promoted cell proliferation and blocked RhoA or ROK induced anchorage-independent growth. These phenotypic changes in STAT3$^{-/-}$ cells could be rescued by reconstituting STAT3 gene. Our studies carried out in STAT3 null cells demonstrate unambiguously that STAT3 represents an essential effector pathway of Rho GTPases in regulating multiple cellular functions including actin cytoskeleton reorganization, cell migration, gene activation, and proliferation.

Rho family GTPases act as intracellular molecular switches cycling between the active, GTP-bound state and the inactive, GDP-bound state in response to a large number of mitogenic or cytokine signals (1–3). Upon activation, the Rho family members RhoA, Rac1, and Cdc42 each can turn on a distinct panel of effectors (4, 5) to regulate several cell functions relevant to inflammation, tumorigenesis, and development including cytoskeleton reorganization, cell migration, cell cycle progression, and proliferation. In particular, on route to stimulate cell growth, the Rho GTPases have been shown to promote G_1- to S-phase cell cycle transition (6) in part through modulation of key cell cycle machineries, such as cyclin D1 and NF-κB, at the transcription level (7, 8). Moreover, in the regulation of cell morphology and motility, Rho GTPases have emerged as important signal transducers to control actin cytoskeleton structure, focal adhesion complex, cell polarity as well as cell-cell communication (2, 5, 9). Studies of the past decade have also shown that these Rho GTPases and their signaling components are intimately associated with human disease development; they have been suggested to serve as candidate targets for future pharmacological interventions (10, 11).

Signal transducers and activators of transcription (STATs)[1] were discovered as latent cytoplasmic transcription factors that are activated by many cytokines and growth factors (12). Among seven mammalian STAT genes identified, STAT3 is ubiquitously expressed and appears to have important and unique functions since STAT3 gene targeting leads to early embryonic lethality (13). Cell stimulation by cytokine and growth factors such as interleukin-6 (IL-6), platelet-derived growth factor, and granulocyte colony-stimulating factor can activate STAT3 by tyrosine and serine residue phosphorylation to induce STAT3 dimerization, and the activated STAT3 translocates from cytosol to cell nucleus to mediate transcription of a number of STAT3-responsive genes. It is well established that STAT3 is involved in the control of essential cellular processes such as cell differentiation and proliferation, and growing evidence draws a correlation between abnormal STAT3 regulation and oncogenic transformation (14). Constitutively active STAT3 occurs with a high incidence in a number of human tumor types (15, 16) and displays by itself a weak oncogenic activity in fibroblasts (14).

A few recent studies have suggested a functional link between Rho GTPases and STAT3 (17–19). In one study it was reported that active Rac1 can directly interact with STAT3 in a yeast two-hybrid system and in co-expression/co-immunoprecipitation assays, leading to STAT3 activation (17). In another work it was shown that Rac1 could indirectly activate STAT3 activity through autocrine induction of IL-6, which in turn caused STAT3 activation (18). A third study suggested that constitutively active RhoA, but not Rac1, could stimulate

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[1] The abbreviations used are: STAT, signal transducer and activator of transcription; CS, calf serum; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; WT, wild type; TRITC, tetramethylrhodamine isothiocyanate; KO, knock-out cells; ROK, Rho-associated kinase; mDia, mammalian Diaphanous; IL-6, interleukin; HA, hemagglutinin; MEF, mouse embryonic fibroblast; DAPI, 4,6-diamidino-2-phenylindole.

STAT3 transcription activity in a transient expression system (19). Although these results raise interesting possibilities of potential signaling cross-talk and interdependence between the Rho GTPases-controlled signaling cascades and the STAT3-mediated transcriptional events, the controversial nature of the reported observations has stalled the Rho GTPase research field in incorporating STAT3 to the Rho GTPase-signaling paradigm.

To clarify the functional relationship between Rho GTPases and STAT3, we have attempted to assess the involvement of STAT3 in Rho GTPase-regulated cell functions and pathways by using the STAT3 knock-out mouse embryonic fibroblast cells in the present studies. We found unexpectedly that the widely used STAT3-luciferase reporter gene constructs could produce a strong reporter signal in the presence or absence of STAT3, raising concerns to a body of literature where STAT3 was associated with Rho GTPases based on the reporter system. In addition, we found that although active RhoA, Rac1, and Cdc42 could all mediate Ser-727 and Tyr-705 phosphorylation and nuclear translocation of STAT3, the Rho GTPases were able to induce STAT3 activation independently of the IL-6 autocrine pathway, and active Rac1, RhoA, or Cdc42 could not form a stable complex with STAT3 as previously suggested. We show that the RhoA-induced STAT3 activation involves multiple effector signals including ROK. Moreover, we demonstrate that STAT3 is required for the RhoA-induced NF-κB and cyclin D1 transcription, cell proliferation, and transformation as well as actin stress fiber formation and migration. These studies carried out in STAT3 null cells unambiguously establish that STAT3 represents an essential effector pathway of Rho GTPases in regulating multiple cellular functions.

EXPERIMENTAL PROCEDURES

cDNA Constructs—The constitutively active mutants of RhoA, Rac1, and Cdc42 (L63RhoA, V14RhoA, L61Rac1, V12Rac1, L61Cdc42, and V12Cdc42) and the effector domain mutants of RhoA in the constitutively active backbone (L63RhoA-F39V, L63RhoA-E40T, L63RhoA-E40L, and L63RhoA-Y42C) were generated by site-directed mutagenesis based on oligonucleotide-mediated PCR as described (20–22). For transient expression, the mutants were cloned into the pKH3 mammalian expression vector in-frame with a triple-hemagglutinin (HA_3) tag at the N termini (23). For retroviral expression, cDNAs encoding the respective mutants and ROK were ligated into the BamHI and EcoRI sites in-frame with the HA_3 tag at the 5′ end of the retroviral vector MIEG3 that expresses enhanced green fluorescent protein (EGFP) bicistronically (22).

Cell Culture and Retroviral Transduction—Wild type and STAT3 deficient MEFs were generated as previously described (24). The STAT3 floxed/floxed (wild type) MEFs were derived from 14-day-old STAT3 floxed/floxed mouse embryos and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (Invitrogen) and immortalized according to Todaro and Green (25). To generate the STAT3 knock-out MEFs, the STAT3 floxed/floxed MEFs were infected with a recombinant adenovirus expressing the Cre recombinase (26). Individual clones were isolated from the infected pool by limited dilution and were genotyped by PCR (27). To generate the STAT3-reconstituted $STAT3^{-/-}$ cells, the $STAT3^{-/-}$ MEFs were stably transfected with pZeo-STAT3 and selected with zeocin (400 μg/ml, Invitrogen). Individual clones were characterized for comparable STAT3 expression to wild type MEFs and were cultured in DMEM with 10% calf serum, 4.5 mg/ml D-glucose, 4.5 mg/ml L-glutamine, and 10 μg of gentamicin/ml.

Recombinant retroviruses were produced using the Phoenix cell packaging system by transient expression of relevant cDNAs in the MIEG3 retroviral vector containing bicistronically expressed EGFP (22). The MEF cells were infected with the respective retroviruses and harvested 48–72 h post-infection. The EGFP-positive cells were isolated by fluorescence-activated cell sorting.

Luciferase Reporter Assay—Two luciferase reporter constructs for STAT3 used in the studies were as described before (APRE-Luc (28–30)) or were obtained from BD Biosciences (pSTAT3-TA-Luc). The NF-κB reporter construct was obtained from Stratagene, whereas the cyclin D1 reporter construct was described previously (8, 22). To probe STAT3, NF-κB, or cyclin D1 gene induction, the luciferase reporter constructs fused with the promoter sequences of the respective genes were transiently co-expressed with the respective small GTPases and cDNA encoding β-galactosidase. Transient transfection of these reporter plasmids was carried out by using FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer's protocols. Twenty-four hours before harvesting, the cells were switched to a medium containing 0.5% serum. Analysis of luciferase and β-galactosidase activities of the transfected cells was performed by using a luciferase assay kit (Promega). Transfection efficiencies were routinely corrected by obtaining the ratio of the luciferase and the β-galactosidase activities observed in the same sample as previously described (31).

Immunofluorescence—Cells grown on cover glasses were fixed with 3.7% formaldehyde in phosphate-buffered saline for 15 min and washed with phosphate-buffered saline once followed by permeabilization with 0.1% Triton X-100 for 20 min. The cells were then blocked with 2% bovine serum albumin for 20 min. For actin staining, the cells were incubated with rhodamine-conjugated phalloidin. For STAT3 or NF-κB staining, the cells were labeled with anti-STAT3 polyclonal antibody (Cell Signaling) followed by incubation with a TRITC-conjugated anti-rabbit secondary antibody or with anti-p65 NF-κB monoclonal antibody (Sigma) followed by a TRITC-conjugated anti-mouse antibody. Cell nuclei were labeled with DAPI for 10 min. The stained cells were mounted onto slides in Aqua-mount and viewed with a Zeiss LSM510 confocal microscopy or a Leica fluorescence microscopy equipped with deconvolution software (Improvision, Inc.).

Immunoblotting—Whole cell lysates were prepared by extraction of the cells with a lysis buffer containing 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.2% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, 10 μg of aprotinin/ml, and 0.5 mM dithiothreitol for 30 min. The nuclear proteins were purified by the method described before (8). Briefly, cells were washed in a hypotonic buffer (25 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 5 mM KCl) and lysed in hypotonic buffer containing 0.25% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, and 10 μg of aprotinin/ml for 30 min. The lysates were centrifuged at 500 × *g* for 5 min. The nuclear pellet was washed with hypotonic buffer containing 2 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, and 10 μg of aprotinin/ml, resuspended in a solution containing 20 mM Tris-HCl (pH 8.0), 0.42 M NaCl, 1.5 mM $MgCl_2$, and 25% glycerol, vortexed, and incubated at 4 °C for 30 min. The extracts were centrifuged at 900 × *g* for 5 min, and the supernatants were taken as the nuclear protein lysates. Protein contents in the whole-cell lysates and nuclear lysates were normalized by the Bradford method. The lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The ectopic expression of the constitutively active forms of Rac1, RhoA, and Cdc42 were probed by using an anti-HA antibody (Roche Applied Science). Phosphotyrosine 705 and phosphoserine 727 of STAT3 and total STAT3 from the cell extracts were probed by using the anti-phospho-Tyr-705, anti-phospho-Ser-727 and anti-STAT3 antibodies, respectively (Cell Signaling).

Wound Healing and Transwell Migration Assays—For wound healing assays cells were plated at 2×10^6/dish density in 60-mm diameter dishes. A plastic pipette tip was drawn across the center of the plate to produce an ~1-mm-wide wound area after the cells have reached confluency. After 12 h in DMEM supplemented with 0.5% calf serum (CS), cell movement into the wound area was examined under a phase contrast microscope. The distances between the leading edge of the migrating cells and the edge of the wound were measured (21).

Cell migration was also assayed by using a Transwell plate inserted with a 6.5-mm polycarbonate membrane (8.0-μm pore size; Costar Inc.) (21). Briefly, 5×10^4 cells were suspended in 0.2 ml of culture medium and were added to the upper chamber. 10% fetal calf serum in DMEM was used as chemoattractant in the lower chamber. The cells were incubated for 16 h in a humidified CO_2 incubator at 37 °C. Cells that traversed the 8.0-μm membrane pores and spread to the lower surface of the membrane were stained with 5% Giemsa solution and were counted in six different fields. Each experiment was carried out in triplicate, and error bars represent the mean S.E.

Cell Proliferation Assay—Cell growth was measured by tracing [^{3}H]thymidine incorporation. Cells were cultured in a medium containing 2% CS for the assays. The cell cultures were assayed at 0, 1, 2, and 3 days by the addition of 1 μCi of [^{3}H]thymidine/ml to the medium followed by an incubation for 4 h at 37 °C. The radioisotope chased cells were harvested by trypsinization, and [^{3}H]thymidine incorporated into the cells was quantified by liquid scintillation counting.

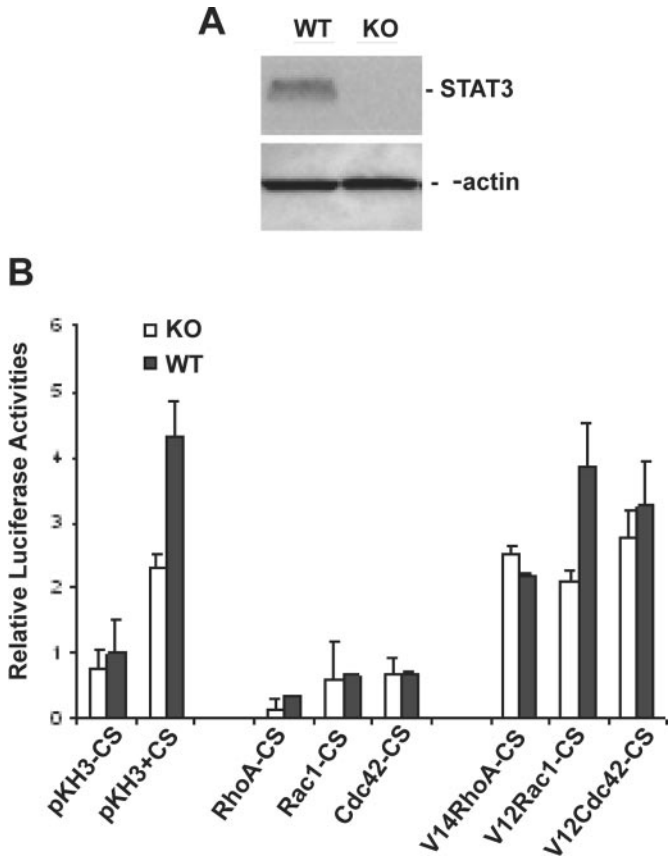

FIG. 1. **The STAT3 promoter driven luciferase reporter system can be nonspecific.** *A*, lysates containing 50 μg of proteins from WT or STAT3$^{-/-}$ cells were probed by anti-STAT3 antibody. Anti-β actin blotting was carried out in parallel. *B*, WT and STAT3$^{-/-}$ cells were transiently transfected with the STAT3-luciferase reporter plasmid APRE-Luc with pKH_3 alone or pKH3 containing V14RhoA, V12Rac1, V12Cdc42, RhoA, Rac1, or Cdc42 cDNAs in the presence of β-galactosidase-expressing vector. In the presence or absence of 10% CS, cell lysates were prepared 48 h after transfection for the measurement of luciferase activity, β-galactosidase activity, and protein concentration. The data were normalized by β-galactosidase expression and are means ± S.E. They are representative of three independent experiments.

Cell Transformation Assay—To determine the transforming potential of the STAT3 deficient or wild type (WT) cells transduced with active RhoA mutant, ROK, or EGFP, 10,000 cells were suspended in 10% CS supplemented DMEM containing 0.3% agarose and were plated on top of a solidified medium containing 0.6% agarose. The cells were fed weekly by the addition of 1 ml of DMEM supplemented with 10% CS. Three weeks after plating the colony numbers were scored, and the foci morphologies were recorded under a phase microscope (22).

RESULTS

Active RhoA, Rac1, and Cdc42 All Can Mediate STAT3 Activation in MEFs—Previously by using a STAT3-responsive luciferase reporter system, it has been shown that constitutively active Rac1 mutant was capable of activating STAT3 transcriptional activity in Cos-1 and Rat-1 cells (17, 18). However, in transient transfection experiments carried out in HEK 293T cells, constitutively active RhoA, but not Rac1 or Cdc42, was shown to activate a STAT3-responsive reporter (19). To clarify whether and which Rho GTPases could mediate STAT3 activation, we have utilized STAT3 knock-out MEF cells (KO) that have been genetically deleted of the STAT3 gene product (Fig. 1*A*) in the present studies to determine the functional relationship between the active Rho GTPases, RhoA, Rac1, and Cdc42, and STAT3. Unexpectedly, upon co-transfection of a STAT3-responsive luciferase reporter construct (APRE-Luc; Refs. 28–30) with the constitutively active mutant of RhoA, Rac1, or Cdc42 (V14RhoA, V12Rac1, or V12Cdc42, respectively), we observed a ~2.5-fold induction of the STAT3 reporter activity by V14RhoA or V12Rac1 and a ~3-fold induction by V12Cdc42 over the respective controls in the STAT3 knock-out cells, similar to that observed in the wild type (WT) cells (Fig. 1*B*). Co-expression of the wild type Rho GTPases did not further enhance the reporter activity (Fig. 1*B*). To verify whether the apparent lack of specificity of the reporter system was due to the particular reporter construct tested, we examined another STAT3 reporter system containing four copies of the STAT3 enhancer element that has been widely in use (pSTAT3-TA-Luc, BD Biosciences) and obtained similar results (data not shown). These data indicate that the STAT3-responsive reporter systems that have been believed to be specific to STAT3 activity could have nonspecific effects caused by other STAT3-related transcription factors, raising concerns to a body of literature where STAT3 was associated with Rho GTPase activities based on the use of such reporters.

STAT3 in quiescent cells resides in the cytoplasm in a latent state. Upon activation it translocates to the nucleus after phosphorylation-dependent dimerization (12, 13). To circumvent the potential problems caused by using the nonspecific STAT3 transcription reporter systems, as alternative approaches we have adopted immunofluorescence and anti-phospho-STAT3 Western blotting to track STAT3 activation state by its intracellular localization and by its phosphotyrosine 705 (Try(P)-705) and phosphoserine 727 (Ser(P)-727) statuses, events that are known to be associated with STAT3 activation. We used recombinant retrovirus transduction to introduce various constitutively active Rho GTPases (L63RhoA, L61Rac1, or L61Cdc42) together with an EGFP marker or EGFP alone to wild type cells. As shown in Fig. 2*A*, in the serum-starved cells expressing the active RhoA, Rac1, or Cdc42 mutant, STAT3 was found to be co-localized with the nucleus as revealed by anti-STAT3 and DAPI staining, similar to that observed in the CS-stimulated cells, whereas in EGFP-expressing cells STAT3 appeared almost exclusively localized in the cytosol under serum-free conditions. A quantification of the nuclear translocation of STAT3 indicated that the RhoA-, Rac1-, or Cdc42-stimulated effect was comparable with that induced by serum (>85%, Fig. 2*A*, *lower panel*). Anti-Tyr(P)-705 or anti-Ser(P)-727 of STAT3 Western blotting revealed that in cells expressing constitutively active RhoA, Rac1, or Cdc42, Tyr-705, and Ser-727 residues were phosphorylated similarly as by serum stimulation (Fig. 2*B*; data not shown). The expression of the HA-tagged RhoA, Rac1, or Cdc42 mutant was revealed by anti-HA Western blotting (data not shown). Immunofluorescent staining of the active RhoA-, Rac1-, or Cdc42-expressing cells by anti-Tyr(P)-705 or anti-Ser(P)-727 of STAT3 also showed that the Rho GTPases potently stimulated Tyr-705 and Ser-727 phosphorylation that accompanied STAT3 nuclear translocation (Fig. 2*C*). Both events of phosphorylation at Tyr-705 and Ser-727 were closely associated with the STAT3 nuclear localization pattern induced by the active Rho GTPase expressions (Fig. 2*D*; data not shown). Together these results demonstrate unambiguously that RhoA, Rac1, and Cdc42 can all mediate STAT3 activation by means of Tyr-705 and Ser-727 phosphorylation and nuclear translocation.

Rho GTPases Can Induce STAT3 Nuclear Translocation Independently from Autocrine IL-6—So far the available data linking Rho GTPase pathways to STAT3 activation came mostly from two studies. By using yeast two-hybrid binding and co-expression/co-immunoprecipitation approaches one study suggested that active Rac1 could directly interact with STAT3 and caused its activation (17). Despite repeated attempts using Cos-7, HEK 293T, or NIH 3T3 as host cells, we

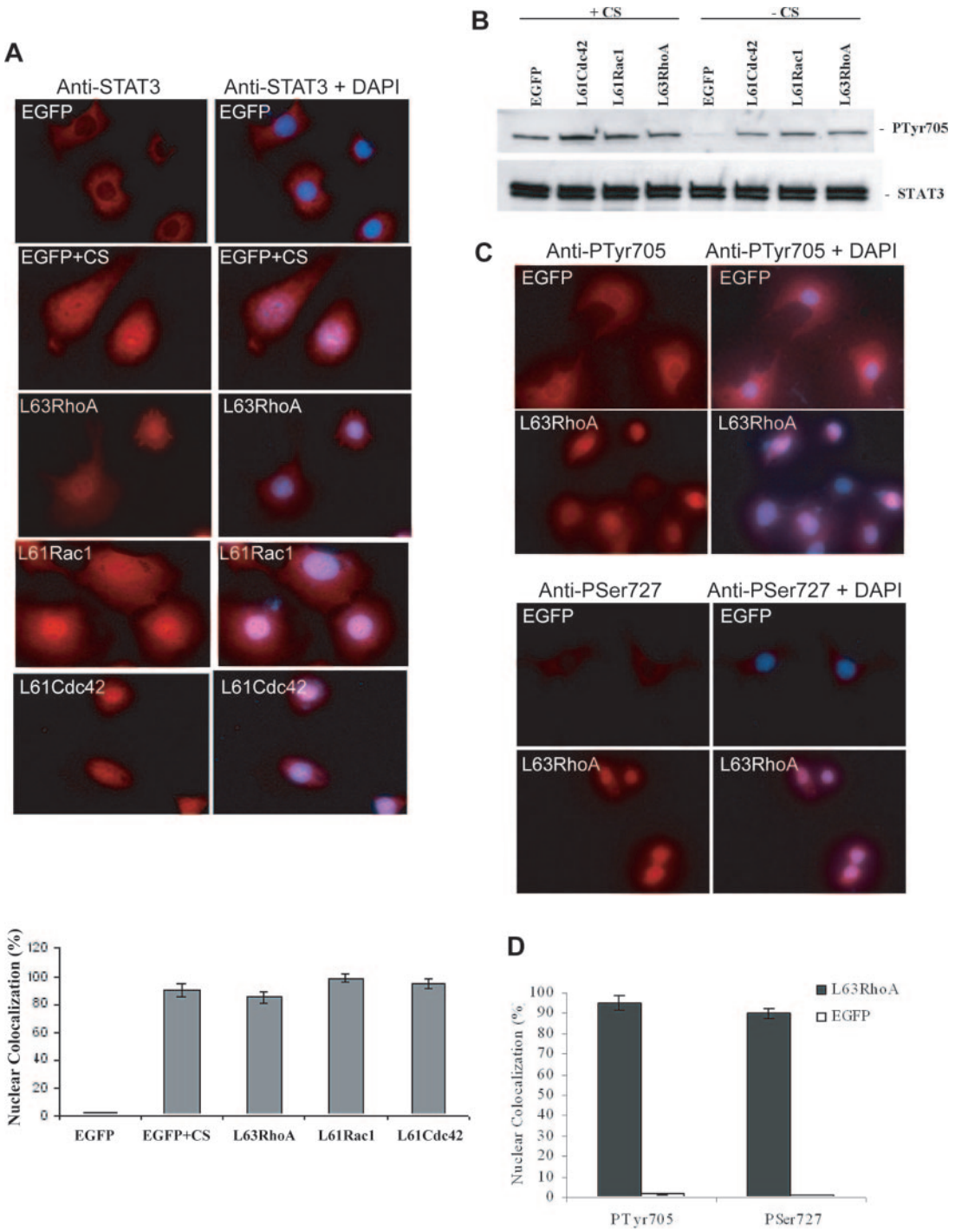

FIG. 2. **RhoA, Rac1, and Cdc42 all can activate STAT3 in cells.** *A*, RhoA, Rac1, and Cdc42 can all stimulate STAT3 nuclear translocation. WT MEFs infected with retrovirus expressing EGFP together with L63RhoA, L61Rac1, or L61Cdc42, or EGFP alone were fluorescence-activated cell sorting-isolated for EGFP expression and were serum-starved for 24 h. The cells were then fixed and stained with anti-STAT3 antibody and TRITC-labeled secondary antibody as well as with DAPI. The percentage of cells displaying a STAT3 staining co-localized with DAPI was quantified. *B*, RhoA, Rac1, and Cdc42 stimulate Tyr-705 phosphorylation of STAT3. Lysates from the respective Rho GTPase mutant-expressing cells were probed by anti-STAT3 and anti-phospho-Tyr-705 STAT3 antibodies. *C*, WT MEFs expressing EGFP or L63RhoA were analyzed by immunofluorescence for STAT3 Tyr(P)-705 or Ser(P)-727 after overnight serum starvation. Cell nuclei were stained with DAPI. *D*, the number of cells displaying Tyr(P)-705 or Ser(P)-727 colocalization with nuclei was quantified under a fluorescence microscope. The data are representative of two independent experiments.

have failed to detect stable binding interaction of exogenously expressed STAT3 with constitutively active L61Rac1, L63RhoA, or L61Cdc42 in the respective cell immunoprecipitates even when the exogenous STAT3 and respective Rho GTPase mutants were at least 5-fold (>10-fold in Cos-7 or HEK 293T cells) over that of the endogenous STAT3 or Rho proteins (data not shown). Another study showed that in rat-1 fibroblasts Rac1-mediated STAT3 activation was through an indirect autocrine induction of IL-6, which in turn stimulated STAT3 activation (18). In wild type MEFs, as shown in Fig. 3, we observed that STAT3 was efficiently translocated to the nucleus upon IL-6 stimulation, whereas it stayed mostly in the cytosol without IL-6 stimulation. The addition of neutralizing IL-6 receptor antibody (*IL-6 R Ab*) to the cells prevented STAT3

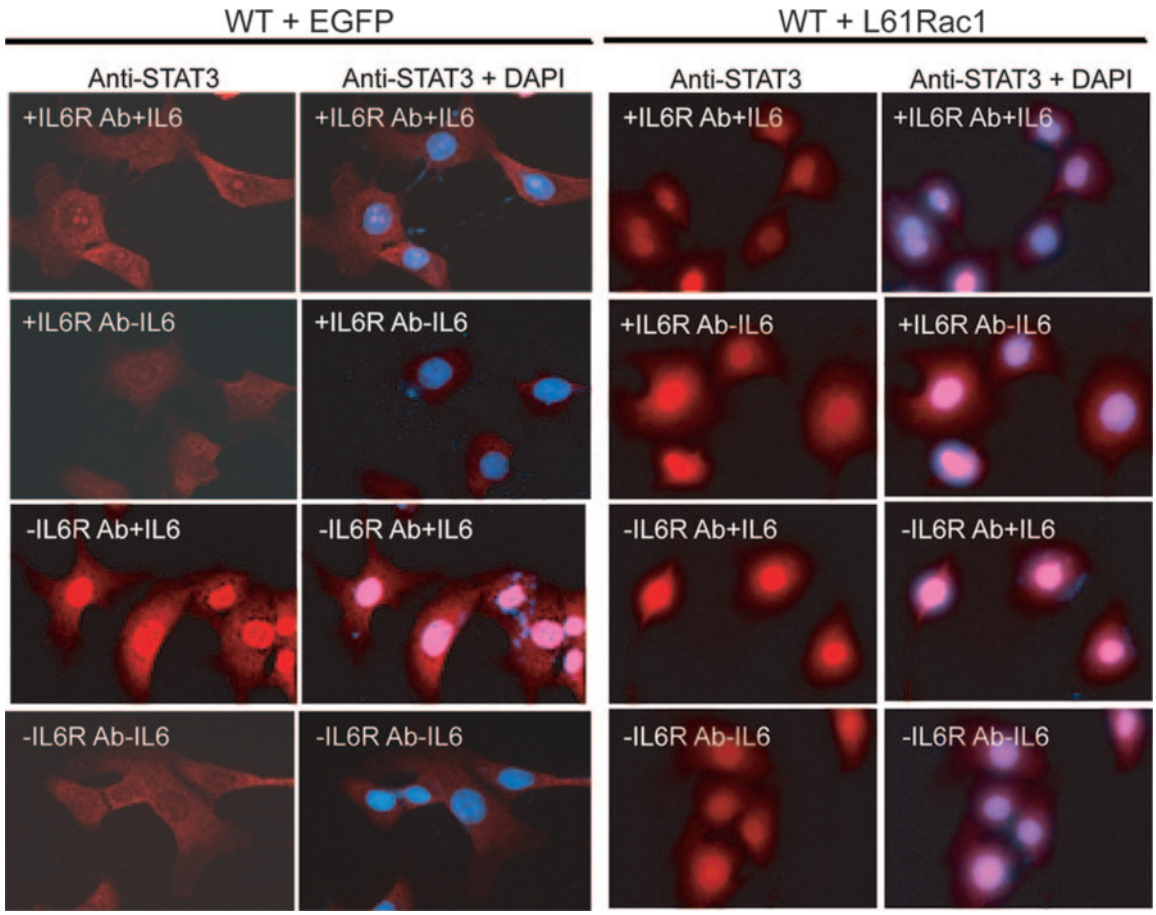

FIG. 3. **The Rho GTPase-mediated STAT3 nuclear translocation is independent of the autocrine IL-6.** EGFP- and L61Rac1-expressing WT MEF cells were plated on cover-glass and cultured in 10% serum for 24 h. The cells were serum-starved for 24 h and cultured in the presence or absence of 10 μg/ml of neutralizing anti-IL-6 receptor antibody (*IL-6 R Ab*) for 18 h. The cells were treated with IL-6 (100 ng/ml) or phosphate-buffered saline for 15 min before fixation.

activation by IL-6. However, the neutralizing IL-6 receptor antibody did not affect L61Rac1 or other active Rho GTPase-induced STAT3 nuclear translocation (Fig. 3; data not shown), suggesting that the Rho GTPases-mediated STAT3 activation can take an alternative route from that of Rho to IL-6 to STAT3 autocrine loop. These data point to a previously unappreciated mechanism of STAT3 activation by the Rho GTPases.

Multiple Pathways Regulated by RhoA Contribute to STAT3 Activation—To begin to understand how individual Rho GTPases activate STAT3, we focused on the RhoA downstream signals that might impact on STAT3 nuclear translocation. In L63RhoA-expressing WT cells, STAT3 was constitutively localized in the nucleus compared with EGFP expressing cells in which STAT3 was localized in the cytosol in the absence of serum (Fig. 4*A*). Pharmacological inhibition of one of the RhoA effectors, ROK, by Y27632 led to a partial inhibition of STAT3 nuclear localization (Fig. 4*A*), whereas active ROK expression itself caused a partial nuclear translocation of STAT3 that was reversible by the treatment with Y27632 (Fig. 4*B*). Blockade of two of the downstream components of ROK, c-Jun N-terminal kinase (JNK) and myosin light chain kinase (MLCK) (33, 34), by their respective inhibitors, SP600125 and ML-7, at a dose effective in inhibiting JNK and MLCK caused no detectable effect on STAT3 localization pattern (Figs. 4, *A–C*), suggesting that STAT3 regulation by RhoA-ROK involves the addition effectors.

To confirm the contribution of ROK and to assess the involvement of additional effectors of RhoA in STAT3 regulation by RhoA, we next examined a set of effector domain mutants of RhoA for their ability to induce STAT3 nuclear translocation. As previously described (22, 35) and illustrated in Fig. 5*A*, the E40L mutant is defective for ROK recognition but retains strong protein kinase N and mDia binding; F39V is active for binding to ROK, and mDia but fails to interact with protein kinase N; E40T is capable of binding to protein kinase N and mDIA strongly but only weakly interacts with ROK; Y42C retains strong ROK binding but is weakened for mDia coupling and defective in protein kinase N binding. Fig. 5*B* shows the STAT3 nuclear localization induction profiles by the respective effector domain mutants of RhoA made in the constitutively active L63RhoA backbone. Although the E40T mutant that retains binding activity to the three effectors displayed the strongest STAT3 induction activity that was indistinguishable from that of L63RhoA, F39V, Y42C, and E40L were partially active in inducing STAT3 nuclear translocation in a decreasing order. Interestingly, the RhoA mutants that have lost protein kinase N binding activity (F39V and Y42C) remained active, thus excluding a protein kinase N contribution to RhoA-mediated STAT3 activation. The E40L mutant that lacks the ability of ROK binding could still partially mediate STAT3 nuclear translocation, suggesting that ROK-independent pathways downstream of RhoA is involved. Furthermore, mDia or other RhoA effectors sharing a similar effector mutant binding pattern as mDia may play an important role in mediating STAT3 nuclear translocation, since Y42C, whose effector binding profile is similar to F39V except that its ability to bind mDia is reduced, appeared to be less active than F39V. From these data we conclude that RhoA activates STAT3 by multiple effector pathways such as ROK and mDia but not protein kinase N.

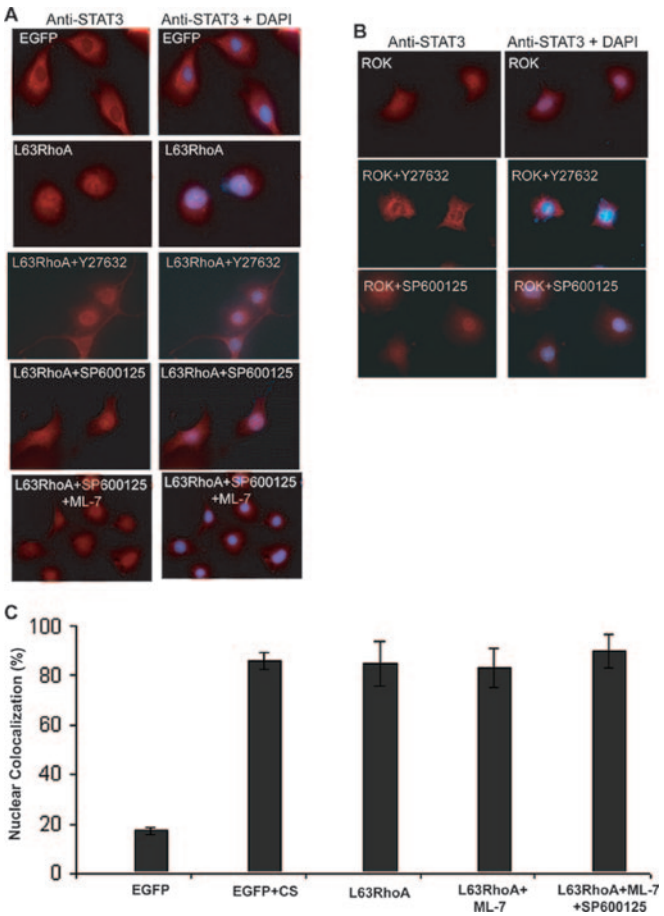

FIG. 4. **RhoA regulates STAT3 partly through ROK and independently of c-Jun N-terminal kinase or myosin light chain kinase.** *A* and *B*, WT MEFs expressing EGFP, EGFP/L63RhoA, or EGFP/ROK were cultured in serum-free conditions overnight. The cells were treated for 2 h with 20 μM ROK inhibitor Y27632, 20 μM c-Jun N-terminal kinase inhibitor SP600125, 10 μM myosin light chain kinase inhibitor ML-7, or phosphate-buffered saline. Cellular STAT3 and nuclei were visualized by anti-STAT3 and DAPI staining, respectively. *C*, RhoA-induced STAT3 nuclear translocation was insensitive to myosin light chain kinase and c-Jun N-terminal kinase inhibition. WT cells expressing EGFP or L63RhoA were cultured in 10% CS before serum withdrawn for 24 h. The EGFP cells were stimulated with 10% CS for 30 min, and the L63RhoA-expressing cells with or without treatment with 10 μM ML-7 and/or 20 μM SP600125 for 30 min were fixed and stained for STAT3 and DAPI to reveal nuclear localization.

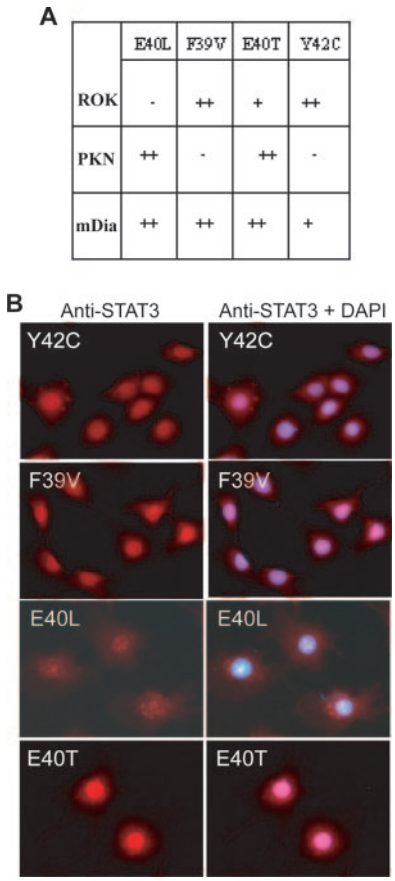

FIG. 5. **Multiple effector pathways regulated by RhoA contribute to STAT3 nuclear translocation.** *A*, the effector domain mutants of RhoA allow selective uncoupling of RhoA with ROK, proteinkinase N (*PKN*), and/or mDia. *B*, WT MEFs expressing EGFP and the respective effector domain mutants made in the L63RhoA backbone were stained with anti-STAT3 antibody and TRITC-labeled secondary antibody after serum starvation, and cell nuclei were stained with DAPI. The data are representative of two independent experiments.

STAT3 Is Required for RhoA- and ROK-mediated Actin Stress Fiber Formation and Cell Migration—Rho GTPases are key regulators of actin cytoskeleton and cell morphology (1, 2). In contrast, little information is available for the role of STAT3 in actin-based cell biology. We next asked if STAT3 could be a part of RhoA-regulated cell machinery of actin reorganization by examining the STAT3$^{-/-}$ cells. Comparison of the actin structures of the L63RhoA-expressing WT-, STAT3$^{-/-}$ (KO)-, and STAT3-reconstituted MEFs led to the observation that, whereas active RhoA was able to induce abundant actin stress fibers in WT and reconstituted cells under serum-free conditions, it failed to stimulate stress fiber formation in the KO cells (Fig. 6*A*). Constitutive expression of ROK that was active in actin stress fiber induction by itself also failed to induce actin stress fiber formation in the KO cells (data not shown). One of the critical links from RhoA and ROK to actin bundling activity has been proposed to be myosin light chain kinase (2). Western blotting of WT cells expressing constitutively active RhoA mutants showed a significant increase in phospho-myosin light chain 2 compared with WT cells expressing only EGFP (Fig. 6*B*). However, STAT3$^{-/-}$ cells were unresponsive to L63RhoA in myosin light chain phosphorylation, similar to the MCLK inhibitor ML-7-treated WT cells (Fig. 6*B*). These results suggest that STAT3 is required for RhoA-ROK-mediated actin cytoskeleton reorganization by mediating myosin light chain kinase regulation by RhoA and ROK.

It is well established that increased RhoA activity could reduce cell motility in part due to actin stress fiber induction and the associated focal adhesion complex formation in fibroblasts (2, 36). Given the fact that the STAT3-deficient MEFs were unable to form stress fibers in response to active RhoA, we reasoned that cell migration rate could be affected in the KO cells. In a wound healing assay in which a scratch of wound was introduced to cell monolayer by a pipette tip and the cells were allowed to migrate to the wound gap, we observed that KO cells were able to move faster to the open wound than WT cells when only EGFP was expressed (Fig. 6*C*). Overexpression of L63RhoA or ROK in WT cells did not increase the migration rate, whereas expression of either L63RhoA or ROK was able to significantly accelerate KO cell migration in a 12 h period (Fig. 6*C*), suggesting that loss of STAT3 sensitizes the cells for the induction of migration by RhoA or ROK. To further determine the role of STAT3 in the regulation of directional cell migration, we measured the cell migration rates using Transwell chambers. As shown in Fig. 6*D*, WT cells expressing EGFP only, active RhoA mutant, or ROK did not display detectable difference in movement toward a serum gradient. However, the STAT3-deficient cells expressing either active RhoA or ROK

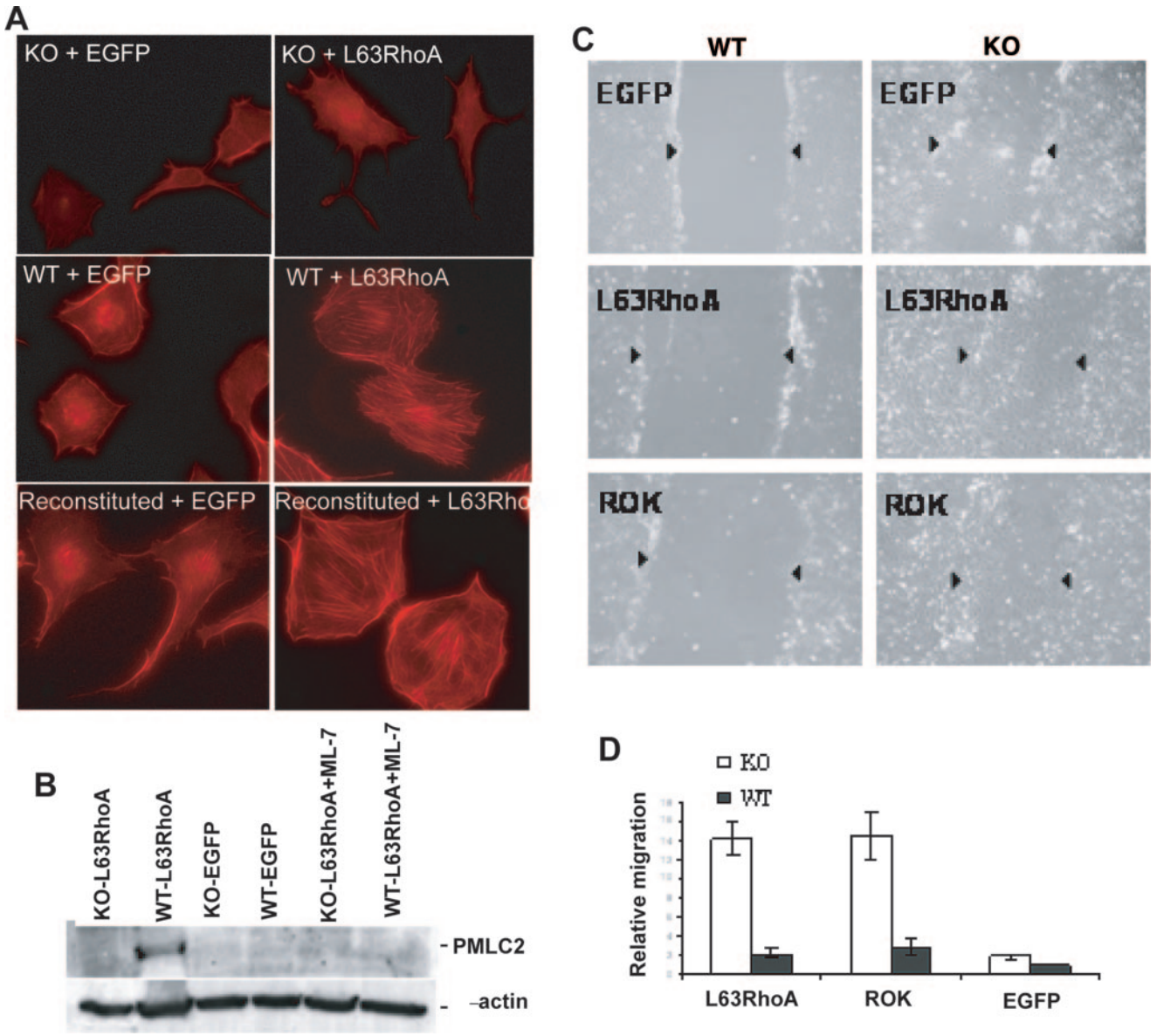

FIG. 6. **STAT3 is essential for RhoA- and ROK-mediated stress fiber formation and cell migration.** *A*, STAT3 is required for RhoA induced actin stress fiber formation. WT-, STAT3 KO-, or STAT3-reconstituted MEFs expressing EGFP or EGFP together with L63RhoA were stained for F-actin by using rhodamine-conjugated phalloidin under serum-free conditions. *B*, STAT3 knock out causes a loss of myosin light chain phosphorylation. WT or STAT3$^{-/-}$ cells with or without ML-7 treatment were subjected to anti-phospho-myosin light chain (*PMLC2*) and anti-β-actin blotting. *C*, STAT3 deficiency sensitizes the cells for RhoA- and ROK-mediated wound healing migration. WT and KO cells expressing EGFP or EGFP with the indicated RhoA/ROK construct were cultured to confluency before a scratch was made on the cell surface using a pipette tip. *Black arrows* indicate the time when the wound was introduced, and cell migration into the wound is shown after 12 h of culturing in 0.5% CS. *D*, STAT3 deficiency sensitizes cells for RhoA and ROK-mediated Transwell migration. WT and KO cells expressing EGFP or EGFP together with RhoA or ROK were subjected to a 10% fetal calf serum-induced Transwell migration assay. Cells that had migrated toward the serum were quantified 24 h after plating. Cell numbers were normalized to that of WT cells. Data are representative of three independent experiments.

were able to migrate 10–12-fold faster than WT cells. We also noticed an ~1-fold increase in the basal migration rate of the STAT3-deficient cells compared with WT cells (Fig. 6*D*). These results strongly suggest that in fibroblasts STAT3 functions as an important mediator of RhoA-regulated cell migration, serving in a negative manner to inhibit cell movement induced by RhoA. It is also of interest to note that in different genetic contexts (*e.g.* STAT3 gain or loss of function) RhoA may either stimulate or inhibit fibroblast migration.

STAT3 Is Involved in NF-κB and Cyclin D1 Regulation by Rho GTPases—In addition to the regulation of actin cytoskeleton, Rho GTPases have been implicated as important regulators of gene transcription (2, 5). Next we examined if STAT3, as a transcription factor itself, might be involved in mediating Rho GTPase signals to the transcriptional machinery. As shown in Fig. 7*A*, STAT3 KO cells were unresponsive to the active RhoA-, Rac1-, or Cdc42-stimulated cyclin D1 transcription in a luciferase-based reporter assay, whereas in WT- or STAT3-reconstituted cells, the cyclin D1 luciferase activity was significantly up-regulated by all three GTPases. Similarly, although all three Rho GTPases were able to activate NF-κB transcription activity in the WT- or STAT3-reconstituted KO cells, they were ineffective in inducing a luciferase-based NF-κB transcription reporter construct in the KO cells (Fig. 7*B*). Immunofluorescent tracking of one of the major subunit of NF-κB, p65, further provided evidence that although active RhoA, Rac1, or Cdc42 could effectively induce NF-κB nuclear translocation in WT cells, the active Rho proteins could not stimulate NF-κB relocation from the cytosol to the nucleus in KO cells (Fig. 7*C*; data not shown). These results clearly indi-

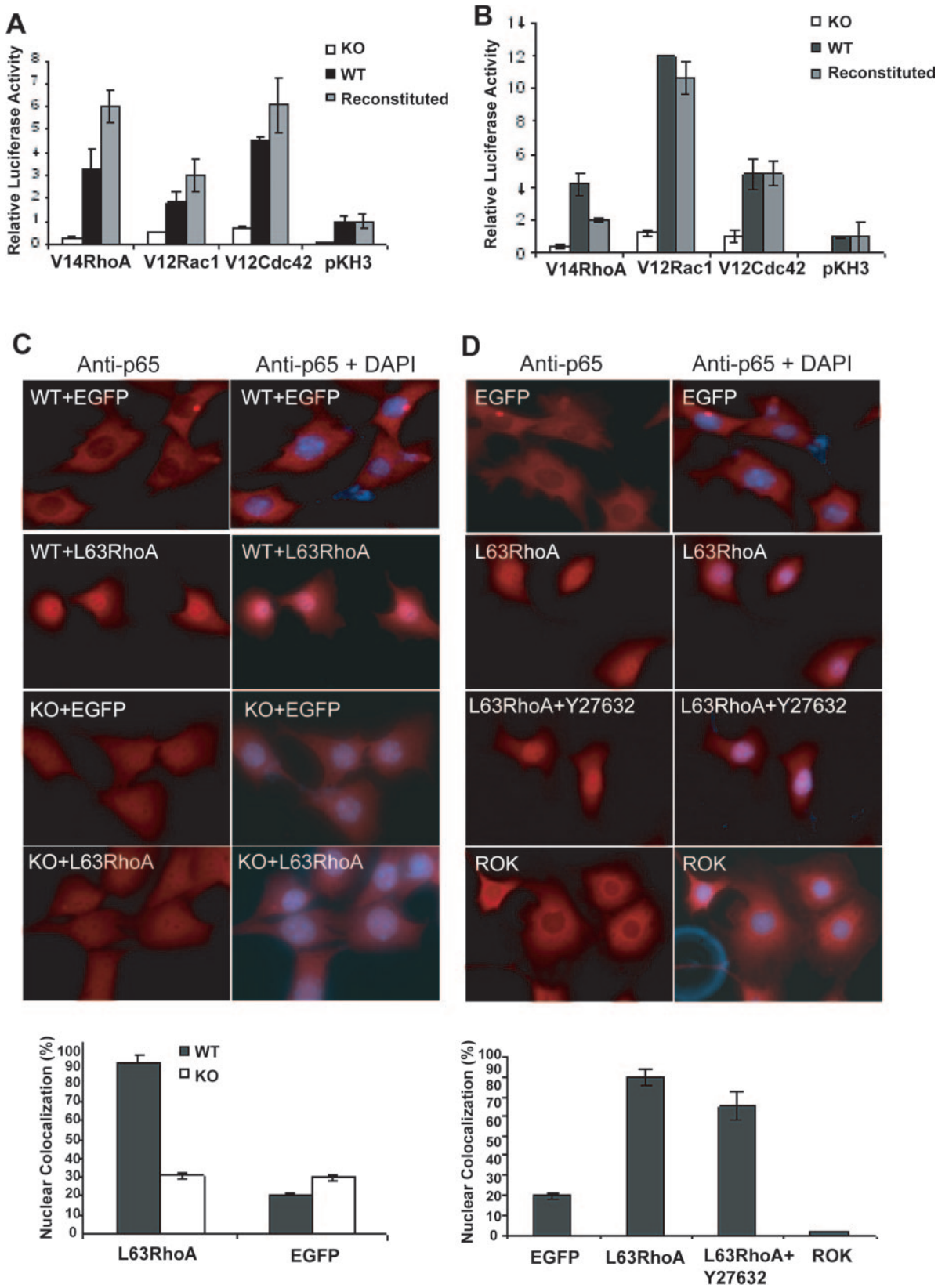

FIG. 7. **STAT3 is required for Rho GTPase-induced cyclin D1 and NF-κB transcription and NF-κB nuclear translocation.** *A* and *B*, the cyclin D1 (*A*) or NF-κB (*B*) promoter-driven luciferase reporter plasmid was transiently transfected into WT-, KO-, and STAT3-reconstituted KO MEFs together with vectors expressing various constitutively active Rho GTPases and β-galactosidase. 30 h post-transfection followed by a 12-h starvation period, the cells were lysed and analyzed for the luciferase activity, β-galactosidase activity, and protein concentration. The luciferase activities were expressed as the -fold of activation relative to the activity induced by the empty vector alone in WT cells and normalized to the internal transfection control of β-galactosidase activity. *C*, a role of STAT3 in RhoA-mediated NF-κB nuclear translocation. The WT and KO MEFs were serum-starved overnight and co-stained with anti-p65 antibody and DAPI. The number of cells showing a co-localization pattern in the nucleus was quantified under a microscope. The data are representative of two independent experiments. *D*, RhoA-induced NF-κB nuclear translocation is independent of ROK. WT MEFs expressing EGFP, EGFP/L63RhoA, or EGFP/ROK were serum-starved overnight and treated with or without 20 μM ROK inhibitor Y27632. Cellular NF-κB localization was visualized by anti-p65 antibody.

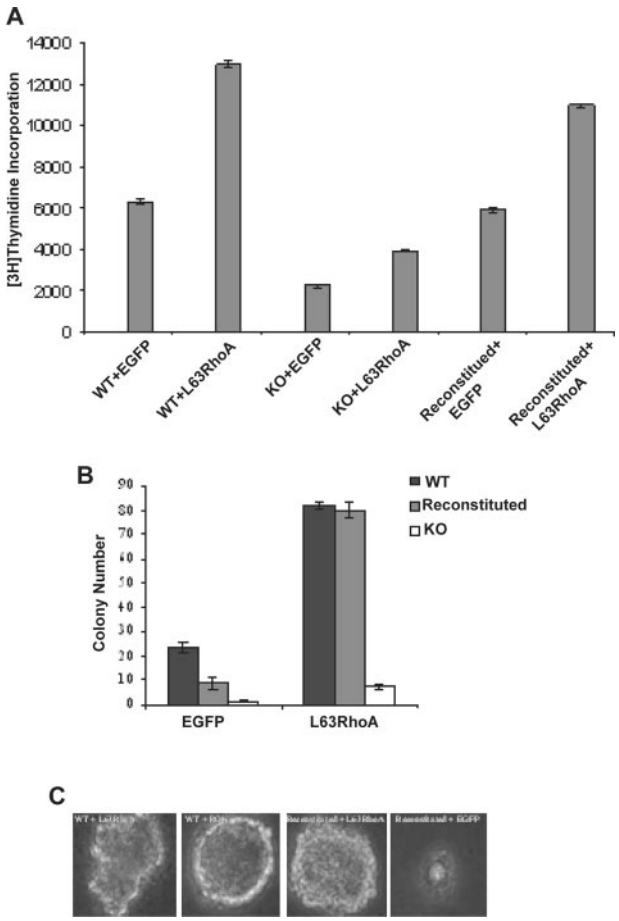

FIG. 8. **STAT3 is required for RhoA-mediated cell proliferation and transformation.** *A*, WT, KO, or KO reconstituted with STAT3 were transduced with EGFP- or L63RhoA/EGFP-expressing retrovirus, and the EGFP-positive cells were isolated by fluorescence-activated cell sorting. 5×10^3 cells of the indicated genotypes were plated in 1 ml of culture medium containing 10% calf serum on 24-well plates. The incorporation of [^{3}H]thymidine was measured at the indicated times. Data are representative of three independent experiments. *B*, 1×10^4 WT-, KO-, or STAT3-reconstituted KO MEFs expressing EGFP, EGFP/L63RhoA, or EGFP/ROK were cultured on 0.3% soft agar for 2 weeks before scoring for foci colonies. The data are representative of two independent experiments. *C*, the morphologies of colonies formed by various cells transduced with active RhoA, ROK, or EGFP alone were examined under a phase microscope.

cate that the Rho GTPases require STAT3 for cyclin D1 and NF-κB transcriptional activation as well as for NF-κB nuclear translocation.

A recent report showed that ROK was able to positively modulate the transcriptional activity of NF-κB (37). Surprisingly, in WT cells we observed that the active RhoA-induced NF-κB nuclear translocation was not significantly affected by treatment with the ROK inhibitor, Y27632 (Fig. 7*D*). Furthermore, ROK expression alone in the cells appeared to inhibit basal NF-κB nuclear translocation (Fig. 7*D*). It is, therefore, possible that the RhoA-ROK branch of RhoA signals may actually produce a negative effect on the net NF-κB regulation by RhoA. This interpretation was confirmed by using the panel of RhoA effector mutants (data not shown).

STAT3 Is Required for RhoA-mediated cell Proliferation and Transformation—Both RhoA and STAT3 are known to have a crucial role in the control of cell proliferation, and each can confer weak transforming activity in fibroblasts upon activation (14, 22). To determine whether STAT3 is involved in RhoA-mediated cell growth, we compared the cell proliferation and transformation properties of WT and STAT3 KO MEFs in the presence or absence of constitutively active RhoA mutant. As shown in Fig. 8*A*, WT cells were able to proliferate three times faster than the KO, consistent with a role of STAT3 in cell proliferation. WT cells expressing active L63RhoA mutant appeared to proliferate twice as fast as WT cells expressing EGFP, but L63RhoA could only marginally stimulate the KO cell proliferation. When the STAT3 gene was reintroduced into the KO cells, active RhoAL63 regained the ability to stimulate the cell proliferation to an extent comparable with WT cells (Fig. 8*A*). These results indicate that active RhoA depends on the presence of STAT3 in promoting cell proliferation.

To examine the consequences of STAT3 deficiency on RhoA-induced cell transformation, WT-, KO-, and STAT3-reconstituted KO cells expressing active RhoA mutant or EGFP alone were plated on soft-agar surface, and their ability to grow to anchorage independent colonies was evaluated. As shown in Fig. 8*B*, WT- and STAT3-reconstituted KO cells expressing L63RhoA were able to form a significant number of colonies, although the KO cells displayed an ~10-fold reduction of colony-forming activity under L63RhoA induction. Similar observations were made for ROK-induced colony formation (data not shown). Interestingly, although the colony numbers of WT and reconstituted cells induced by L63RhoA were similar to that induced by ROK, the morphology of active RhoA-induced colonies was distinct from those induced by ROK with a rugged, blebbing surface of cell clusters compared with a smooth, round cluster of ROK-expressing cells (Fig. 8*C*). These results demonstrate that RhoA-mediated cell transformation is dependent on STAT3 signals.

DISCUSSION

Both Rho family GTPases and STAT3 regulate cell proliferation and gene induction, and their activating mutants are known to be oncogenic (5, 14). The intense search for their functional connection has led to a number of reports suggesting that STAT3 mediates certain aspects of Rho GTPase signaling (17–19). However, this issue remains controversial due to the apparent differences in the observations that active Rac1 could directly bind to and activate STAT3 (17) or indirectly activate STAT3 through autocrine induction of IL-6 (18) and that active RhoA but not Rac1 could stimulate STAT3-responsive gene inductions (19). By utilizing the recently available STAT3 knock-out cells that enable us to examine the functional contribution by STAT3 to Rho GTPase signaling events in the STAT3 null genetic background, we started the present work with an open mind, trying to confirm some of the previous findings and/or to sort out the apparent conflicts among the related reports of this subject. Unexpectedly, our studies in the STAT3$^{-/-}$MEF cells strongly indicate that the experimental approaches adopted by a large body of literature using the STAT3-promoter reporter systems were flawed due to the nonspecific nature of the reporter systems, possibly attributed to the similarity of the STAT3-promoter sequences with that of other closely related STAT family member or transcription factors. Because the STAT3-reporter system is widely used in the STAT3 functional assays and has provided crucial support for previous studies of the Rho-STAT3 connection (17–19), this observation added further to the controversy and prompted us to re-examine the interdependence of RhoA, Rac1, and Cdc42 on STAT3 in order to clearly define their relationships.

It is well established that quiescent STAT3 exists mostly in the cytosol whereas activated STAT3 is tyrosine-phosphorylated at Tyr-705 and Ser-727 form homo- or heterodimers and translocate to the nucleus to induce gene transcription (12). Taking advantage of the STAT3 phosphorylation and intracellular localization status associated with its activation state, we demonstrate that active RhoA, Rac1, and Cdc42 can indeed

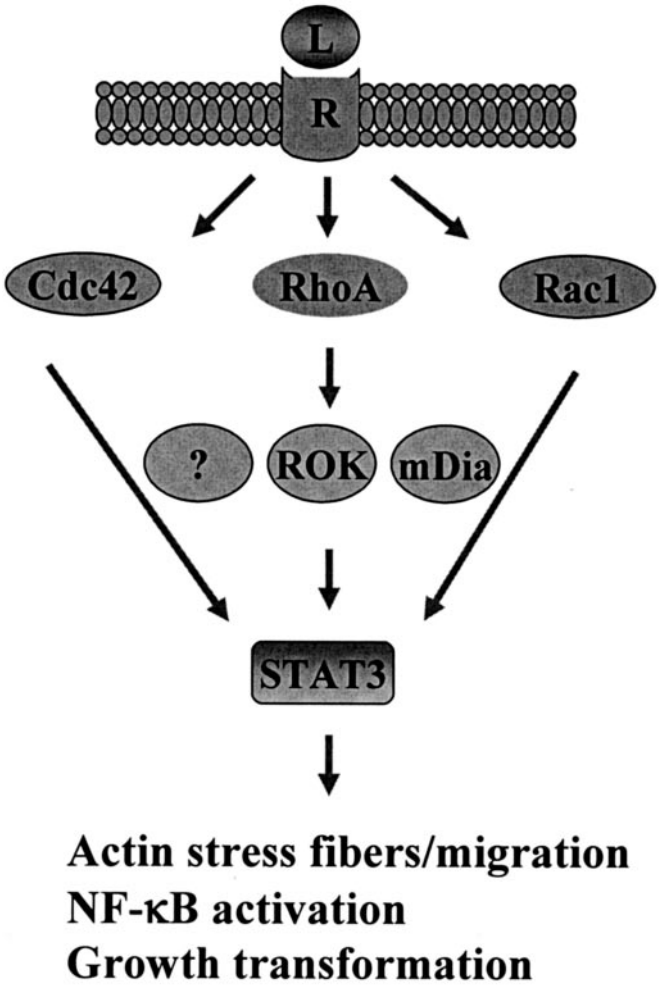

FIG. 9. **A model of the role of STAT3 in the Rho GTPase-mediated cell functions.** Many cell surface receptors stimulate RhoA, Cdc42, and/or Rac1 to elicit effects on actin organization, cell migration, gene transcription, and cell proliferation. Each of these Rho GTPases may rely on STAT3- and/or STAT3-regulated genes to achieve these cellular responses.

activate STAT3 in fibroblast cells by virtue of induction of Tyr-705 and Ser-727 phosphorylation as well as nucleus translocation of STAT3. However, upon examination of the mechanism involved, we were unable to reproduce the previously reported direct physical interaction between constitutively active Rac1 and STAT3 (17) nor between active RhoA or Cdc42 and STAT3, consistent with the observations of another group (18). One other unexpected finding is that the RhoA-, Rac1-, or Cdc42-induced STAT3 nucleus translocation appeared to be independent of the IL-6 receptor pathway in MEFs, a result differing from the reported Rac1-IL-6-STAT3 autocrine loop in rat-1 cells (18). These results suggest that RhoA, Rac1, and Cdc42 can each activate STAT3 by previously unappreciated mechanisms.

To begin to dissect the mechanism by which the Rho GTPases modulates STAT3 activity, we have examined the involvement of a few candidate effector pathways of RhoA signaling. Both pharmacological inhibitor administration and RhoA effector domain mutant expression led to a consistent conclusion that although the ROK pathway by itself can partially activate STAT3, a participation of other effectors such as mDia is required to fully promote the RhoA-mediated STAT3 activation. Along this line, we have excluded the possible involvement of downstream components of RhoA signaling including myosin light chain kinase and c-Jun N-terminal kinase. Although we have not attempted to dissect the pathways controlled by Rac1 or Cdc42 leading to STAT3 activation, given the complexity of the immediate effector networks controlled by each (4), it is likely that these Rho proteins also engage multiple effectors to regulate STAT3.

By examining the STAT3$^{-/-}$ MEF cell behaviors, we have demonstrated that STAT3 is an essential effector for a variety of Rho-mediated cell functions (Fig. 9). In particular, we show that genetic deletion of STAT3 leads to a loss of response to RhoA-induced actin stress fiber formation and sensitizes the cells to RhoA- or ROK-stimulated cell migration. This is somewhat surprising since STAT3 is not known for its role in actin cytoskeleton regulation and in keratinocytes STAT3 deficiency appeared to inhibit epithelial cell migration (38). It suggests the importance to examine the functional contributions of STAT3 in the context of Rho GTPase signaling and may also reflect the differences of Rho-STAT3 signals to regulate cell migration in fibroblasts and keratinocytes. Another important finding is that STAT3 is required for the RhoA-induced NF-κB and cyclin D1 transcription and is involved in NF-κB nuclear translocation. Furthermore, loss of STAT3 expression inhibits RhoA-promoted cell proliferation and blocks RhoA- or ROK-induced anchorage-independent growth. By adding back the STAT3 gene to the STAT3$^{-/-}$ cells, these phenotypic changes were readily rescued. These observations provide an important link of Rho GTPase signaling to the nucleus, since although the relationship between Rho GTPase activation and transcription/growth regulation has been extensively studied in the last decade, the events involved in the regulation of some of the transcription factors, such as NF-κB, remain unclear (39). By putting STAT3 activation as an intermediate step before Rho-mediated NF-κB and cyclin D1 activation and cell proliferation, we have progressed a step closer to the appreciation of the interrelationship of Rho GTPases-regulated transcription events that are the key for the cell cycle and/or survival signals. Paradoxically, our observations that NF-κB nuclear translocation and cyclin D1 transcription are dependent on the presence of STAT3 gene while filling up a gap in the Rho GTPase signaling cascades (Fig. 9) raise more questions on how these transcriptional machineries are interconnected and how they are coordinately involved in the cell growth control.

More detailed mechanisms of STAT3 activation by each Rho GTPase as well as the molecular contribution of STAT3 to the proliferation and migration regulation need to be further determined. Because RhoA, Rac1, and Cdc42 can all stimulate STAT3 Tyr-705 and Ser-727 phosphorylation, it is likely that downstream effectors of each Rho GTPase could engage both tyrosine kinases such as JAK2 and Src (18) and Ser/Thr kinases such as PAK and ROK to elicit the phosphorylation events. By using the c-Jun N-terminal kinase and extracellular signal-regulated kinase (ERK)-specific pharmacological inhibitors, we were able to exclude the potential contribution by c-Jun N-terminal kinase, ERK1/2, or p38 mitogen-activated protein kinase (Fig. 4; data not shown). Whether the Ser-727 phosphorylation is essential to STAT3 activation remains controversial, but it is well established that phosphorylation of Tyr-705 is required for STAT3 nuclear translocation and activation (12). From our data it is expected that the activated STAT3 would turn on specific transcriptional events and target genes that are integral parts of the cell migration and proliferation machinery of the Rho GTPase signaling paradigms. One way to further map out the involvement of specific STAT3 functions in the context of Rho-mediated migration and/or proliferation could come by reconstituting point mutants of STAT3 with well defined functions combined with examining the STAT3 responsive target genes by a micro-array approach (32).

Identification of such STAT3 functions and targets may help define the essential components of Rho GTPase-signaling cascades and reveal the critical link of the signaling networks controlled by these two important classes of intracellular signal molecules.

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A Role of STAT3 in Rho GTPase-regulated Cell Migration and Proliferation

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