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Overexpression of Wild-Type RhoA Produces Growth Arrest by Disrupting Actin Cytoskeleton and Microtubules

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Abstract We have investigated the role of Rho GTPase in cell growth by generating stable cells that express the wild-type RhoA ($RhoA^{wt}$) under the control of an inducible promoter. Induction of $RhoA^{wt}$ had a biphasic effect on the actin cytoskeleton. At low levels of expression, $RhoA^{wt}$ stimulated the assembly of actin stress fibers without affecting cell growth. At high levels, there was a paradoxical disruption of the actin cytoskeleton accompanied by a growth arrest. Cell cycle analysis revealed a dual block at the G_1/S and G_2/M checkpoints. The G_1/S arrest correlated with the accumulation of $p21^{Cip1}$, resulting in the inhibition of cdk2 activity, whereas the G_2/M block correlated with the loss of microtubules. The cyclin B level and the cdc2 kinase activity, however, were increased, suggesting that the progression through mitosis rather than entry into the G_2/M is defective when $RhoA^{wt}$ is overexpressed. Similar cell cycle defects and the loss of microtubules were observed after a cytochalasin D treatment, indicating that the ability of RhoA to regulate the integrity of actin cytoskeleton may be critical for the cell cycle transition through both the G_1/S and M phase checkpoints. J. Cell. Biochem. 80:229–240, 2000.

Key words: Inducible expression; $p21^{Cip}$; cyclin B; cytochalasin D

The actin cytoskeleton consists of actin filaments and array of actin binding proteins that form discrete subtypes to mediate diverse cellular functions including cell adhesion, membrane trafficking, cell motility, and cytokinesis [Van Aelst and D'Souze-Schorey, 1997; Hall, 1998]. In adherent cells, long bundles of actin filaments form actin stress fibers that traverse the cell between focal adhesion plaques, where the cell makes firm attachment to the extracellular matrix through integrins. In motile cells, a complex meshwork of actin filaments forms membrane ruffles and lamellipodia at the leading edge [Lauffenburger and Horwitz, 1996]. In dividing cells, actin stress fibers and lamellipodia disappear as cells round up and an actin-based contractile ring forms at the cleavage furrow [Glotzer, 1997].

The organization of actin cytoskeleton is regulated by Rho GTPases, which cycle between an active GTP-bound and inactive GDP-bound state [Hall, 1998]. Of the seven known Rho GTPase families, Rho, Rac, and Cdc42 are important for the assembly of actin cytoskeleton. Cdc42 is involved in the induction of actin microspikes and filopodia, fingerlike protrusions that contain a tight bundle of actin filaments. Rac stimulates de novo polymerization of actin at the plasma membrane to produce lamellipodia and membrane ruffling [Ridley et al., 1992]. Rho promotes the formation of focal adhesion plaques and actin stress fibers [Ridley and Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1996]. On activation, Rho translocates from detergent-soluble cytosolic fraction to detergent-insoluble membrane fraction and recruits target proteins by direct protein–protein interaction to the site of actin polymerization [Hall, 1998]. Of the eight proteins known to bind activated Rho, p160Rho kinase [Leung et al., 1996], p140mDia [Watanabe et al., 1997],

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and citron kinase [Maudaule et al., 1998] have been shown to affect the organization of actin cytoskeleton.

Rho GTPases also induce a wide range of cellular responses, including gene induction and cell proliferation. Rho has been shown to activate serum response factor and NF-κB [Hill et al., 1995; Perona et al., 1997]. In quiescent fibroblasts, serum-induced cell cycle entry into the G_1 and subsequent DNA synthesis require Rho [Olson et al., 1995]. Constitutively activated Rho GTPases can stimulate anchorage independent cell growth, whereas their dominant negative forms can block transformation by Ras [Qiu et al., 1995a,b, 1997; Prendergast et al., 1995]. Furthermore, Rho has been shown to be required to suppress $p21^{Cip1}$ in Ras-transformed cells [Olson et al., 1998]. In tumor cells, geranylgeranyltransferase I inhibitor, which inhibits lipid modification of Rho, required for its membrane localization, leads to the accumulation of $p21^{Cip1}$ and a G_0/G_1 block [Vogt et al., 1997; Sepp-Lorenzino and Rosen, 1998].

Although it is not known whether the cell cycle regulation by Rho is dependent on its ability to control actin cytoskeleton, several lines of evidence suggest the importance of cytoskeletal integrity for cell proliferation. When cells are detached from the matrix, they loose actin stress fibers and exit from the cell cycle. The block in cell cycle in the absence of matrix attachment appears to be caused by increased expression of cdk inhibitors, which correlate with lower levels of cyclin E-cdk2 [Fang et al., 1996; Zhu et al., 1996; Bottazzi et al., 1999]. A block in cell cycle at the G_1 transition has also been observed in adherent cells that have been treated with actin depolymerizing drug cytochalasin D (CD) [Bohmer et al., 1996], suggesting that the loss of actin stress fibers may contribute to the cell cycle exit.

In this study, we investigated the role of Rho protein in cell proliferation by generating stable cells expressing the wild-type RhoA ($RhoA^{wt}$) under the control of an inducible promoter. At low levels of $RhoA^{wt}$, there was an increase in the actin stress fibers, consistent with the known functions of RhoA in the assembly of actin cytoskeleton. Surprisingly, at high levels of $RhoA^{wt}$, there was a substantial reduction in the actin stress fibers and growth arrest without the loss of cell-matrix attachment or apoptosis. Cell cycle analysis revealed block at both G_1/S and G_2/M. When $RhoA^{wt}$ induced, there was a progressive accumulation of $p21^{Cip1}$, which would account for the G_1/S block. Furthermore, there was a reduction in microtubules (MTs), which may be responsible for the M-phase block. Inhibition of RhoA with C3 exoenzyme or disruption of actin stress fibers with CD led to a similar reduction in MT, indicating that the defect in MT integrity in the RhoA overexpressing cells may be caused by actin depolymerization.

MATERIALS AND METHODS

Cell Lines and Plasmids

U2OS, Saos, and NIH3T3 were obtained from the American Type Culture Collection. UTA6, a U2OS derivative expressing tetracycline-repressible transactivator, was provided by D. Haber (Massachusetts General Hospital). $RhoA^{wt}$ cDNA was provided by J. S. Gutkind (National Institutes of Health). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. UTA6 cells were maintained in the presence of 1.5 μg/ml tetracycline. Protein expression in UTA6 cells was induced by replacing the tetracycline containing media with fresh DMEM + 10% fetal bovine serum.

Immunoblotting

Fifteen to 30 μg of total cellular protein was fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and immunoblotted with the corresponding antibodies. Anti-flag M2 monoclonal antibody (mAb) was purchased from Kodak Scientific; anti-RhoA (26C4), anti-cyclin D1 (M-20), anti-cdk2(m2)-G, and anti-cdc2p34(c-19), from Santa Cruz; anti-$p21^{WAF1}$ (ab-1) and anti-p53 (ab-6), from Oncogene Research; anti-$p27^{Kip1}$, anti-cyclin B mAb, anti-cyclin A pAb, and anti-cdc2 mAb from Transduction Laboratories.

MTT Cell Proliferation Assays

Cell growth was determined by using a tetrazolium dye–based colorimetric assay [Denizot and Lang, 1986]. Briefly, $RhoA^{wt}$ or the control (pTPH) cells were seeded on 24-well plates and cultured either in the presence or absence of tetracycline. At the indicated time point, the cells were washed once with phosphate-buffered saline and then incubated in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide (MTT) (1 mg/ml) for 3 h at 37°C. The formazan crystals were solubilized in acidified isopropanol (0.04 N HCl) and 1% Triton X-100, and measured at 570 nm.

Cell Cycle Analysis

Cell cycle analysis by DNA content was carried out by staining the nuclear DNA with propidium iodide in a buffer containing 0.1% sodium citrate (wt/vol), 0.3 % Triton X-100, 10 μg/ml propidium iodide, and 2 mg/ml RNase A. The samples were incubated at 4°C for 30 min in the dark and then analyzed by fluorescence activated cell sorter within 1 h. Cell cycle analysis by DNA staining and bromodeoxyuridine incorporation was carried out using an In Situ Cell Proliferation Kit (Boehringer Mannheim), following the manufacturer's instructions. The flow cytometric cell cycle data was analyzed using ModFit software (Becton Dickinson). For cell cycle synchronization, exponentially growing cells were incubated in the complete growth medium containing 150 ng/ml nocodazole. At 16 h, nocodazole was replaced with the complete growth medium to allow the arrested cells to reenter cell cycle.

In Vitro Kinase Assays

Cdk2 and cdc2 kinase assays were carried out following published procedures [Matsushime et al., 1994]. Briefly, cells were incubated in a lysis buffer [50 mM Tris-HCl (pH 7.5), 0.5% NP-40, 150 mM NaCl, 50 mM NaF, 50 mM HEPES, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride, 1 mM $NaVO_3$] for 30 min on ice. Immunoprecipitation was carried out using 500 μg of cleared lysates, 1 μg of polyclonal anti-cdk2 (M2-G) or anti-cdc2p34 (C-19) antibody, and 30 μl protein G/A Agarose beads (50 % wt/vol, Oncogene) for 3 h at 4°C. The beads were washed four times in the lysis buffer, once in 20 mM Tris-HCl (pH 7.5), and then resuspended in 25 μl kinase reaction buffer [20 mM Tris-HCl (pH 7.5), 40 mM HEPES, 4 mM $MgCl_2$, 1 μM ATP, 1 mM dithiothreitol, 10 μg histone type III (Sigma), and 10 μCi ^{32}P γ-ATP (3,000 Ci/mmol)]. After 30 min at 37°C, the reaction was terminated and the samples were equally divided and fractionated on SDS-PAGE. One set was dried and subjected to autoradiography to visualize the incorporation of ^{32}P into histone. The second set was used for immunoblotting with either anti-cdk2 or anti-cdc2 mAb.

Northern Blots

Total RNA samples (10 μg) were separated on a 1.0% agarose-formaldehyde gel and transferred to HyBond N+ (Amersham) for a Northern analysis. A full-length human $p21^{Cip1}$ cDNA was used as a probe to detect the $p21^{Cip1}$ transcripts. A 375-bp probe specific for the 3′ untranslated region of human 14-3-3σ was generated by polymerase chain reaction from EST clone W79136 using the primers 5′-ACAGGGGAACTTTATTGAGAGG-3′ and 5′-AAGGGCTCCGTGGAGAGGG-3′ [Hermeking et al., 1997].

In Situ Immunofluorescence

In situ immunofluorescence for cytoskeleton was carried out following published procedures [Cabral et al., 1983]. Cells were plated on fibronectin-coated glass coverslips and were cultured with or without tetracycline for the indicated time. The glass coverslips were washed three times in a stabilizing buffer (PEM) [80 mM PIPES (pH 6.9), 1 mM EGTA, 1 mM $MgCl_2$] and 4% polyethylene glycol (MW 8,000). The cells were permeabilized in a PEM buffer containing 0.5% Triton X-100 for 2 min and then fixed in a PEM buffer containing 3% formaldehyde and 0.5–1% dimethylsulfoxide for 30 min at room temperature. MT staining was carried out using anti-tubulin α mAb (Sigma) at 1:2,500 dilution for 1 h at 37°C. The actin stress fibers were stained with Texas Red phalloidin (Molecular Probes). The coverslips were mounted in ProLongTM (Molecular Probes) and visualized by confocal microscopy (Zeiss LSM 410).

RESULTS

Overexpression of $RhoA^{wt}$ Disrupts the Actin Stress Fibers

To study the role of actin cytoskeleton in cell growth and cell cycle regulation, we generated stable cells expressing $RhoA^{wt}$ under the control of a tetracycline-inducible promoter [Gossen and Bujard, 1992]. The exogenous RhoA was modified by the addition of FLAG-epitope tag at the NH_2-terminus to distinguish it from the endogenous RhoA (Fig. 1A). U2OS cells are epithelioid in morphology and adopt a cobblestone-like appearance in culture (Fig. 1B; $RhoA^{wt}$ no. 23 +

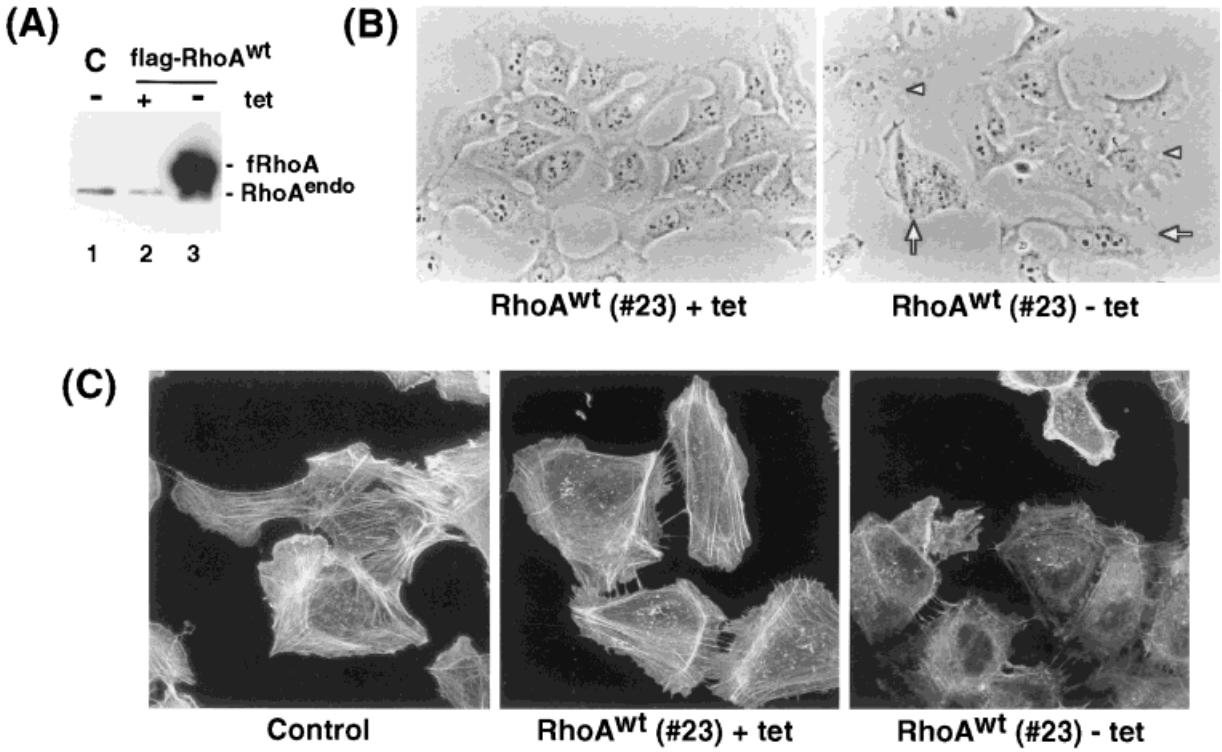

Fig. 1. RhoA^{wt} overexpression disrupts actin stress fibers. **A:** U2OS cells expressing flag-epitope tagged RhoA were plated at low density and incubated for 48 h in medium with (+) or without (−) tetracycline. Equal amounts of protein extracts were analyzed by a Western blot using anti-RhoA (26C4) mAb to detect both the endogenous (RhoA^{Endo}) and transfected RhoA (f-RhoA^{wt}). **Lane 1:** control cells (pTPH vector); **lane 2:** uninduced RhoA^{wt} cells; **lane 3:** induced RhoA^{wt} cells. **B:** The appearance of the RhoA^{wt} cultured (+) or (−) tetracycline is shown. Cells with multiple nuclei (open arrowheads) or large nuclei (open arrows) are indicated. **C:** Cells were plated on fibronectin-coated coverslips and cultured with or without tetracycline for 48 h and then stained with Texas Red phalloidin to visualize the actin stress fibers.

tet). When tetracycline was removed, the cell morphology became more heterogeneous, with a large fraction of the cells displaying a stellate shape resulting from asymmetric cytoplasmic extensions (Fig. 1B; RhoA^{wt} no. 23 − tet). In addition, the RhoA^{wt} overexpressing cells were frequently multinucleated (arrowhead) or contained nuclei that were considerably larger (arrows) than the control cells. Interestingly, fluorescein isothiocyanate–phalloidin staining demonstrated a reduction in the actin stress fibers on induction of RhoA^{wt} (Fig. 1C).

The observed reduction in the actin stress fibers in the RhoA^{wt} overexpressing cells was contrary to the known function of RhoA, which is to stimulate the assembly of actin cytoskeleton. When the level of RhoA^{wt} was controlled by varying the concentration of doxycycline, the assembly of actin cytoskeleton followed a biphasic response where an initial increment at low levels of RhoA^{wt} was followed by the loss of actin stress fibers at high levels of RhoA^{wt} (Fig. 2). Although this result clearly demonstrates that high levels of RhoA^{wt} inhibit the assembly of actin cytoskeleton, the mechanism of this inhibition is not known.

Actin Cytoskeleton Is Required for Cell Cycle Progression Through Both G_1/S and G_2/M

Actin stress fibers can be disrupted by inactivating RhoA with C3 exoenzyme, which ADP-ribosylates RhoA, or by severing actin filaments with CD. Either of these treatments, however, induces apoptosis, making it difficult to examine the role of actin cytoskeleton during the cell cycle. In three independently derived cell lines, the induction of RhoA^{wt} resulted in cytostasis without apoptosis within the first 60 h. After 24 h of induction, there was no appreciable increase in cell numbers on visual inspection or in an MTT cell proliferation assay (Fig. 3A). The growth arrest, however, was re-

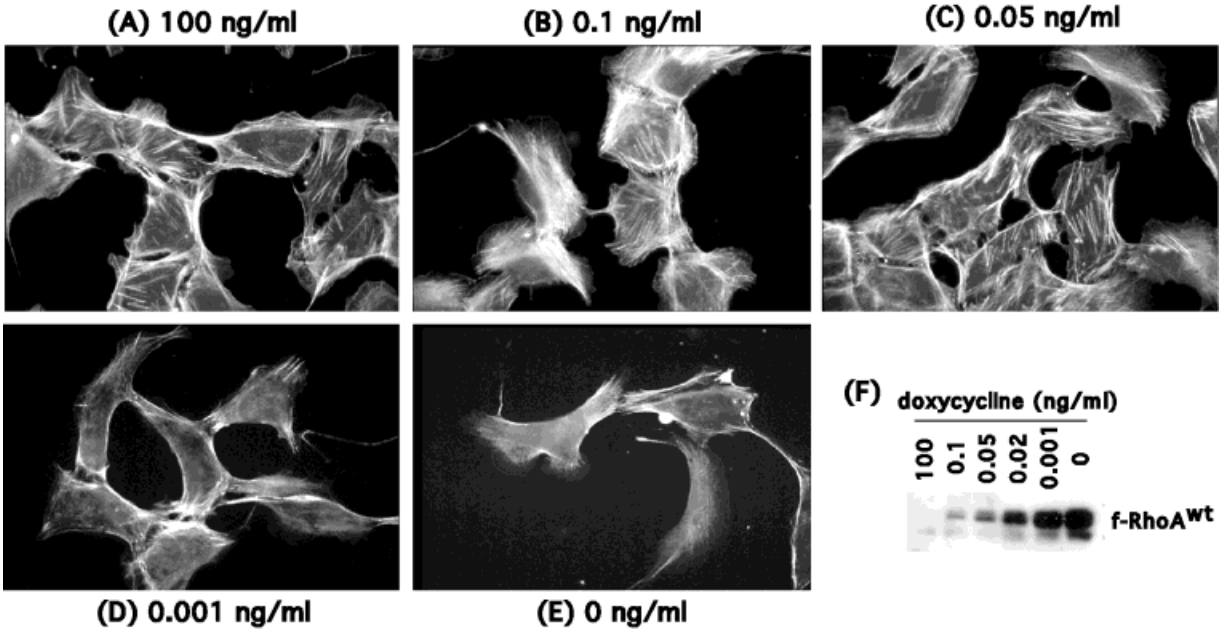

Fig. 2. High levels of $RhoA^{wt}$ disrupts actin stress fibers. $RhoA^{wt}$ was induced to varying degrees by controlling the concentration of doxycyline in the medium. Cell lysates were prepared at 48 h and analyzed by in situ immunofluorescence using Texas Red phalloidin to visualize the actin stress fibers **(A–E)** or by immunoblot using the mAb M2 (Kodak) against the Flag epitope **(F)**.

versible and the arrested cells reentered the cell cycle when the $RhoA^{wt}$ expression was suppressed by the addition of tetracycline to the medium (data not shown).

To determine the specific effects of RhoA on cell proliferation, we performed a cell cycle analysis by flow cytometry using asynchronous cultures maintained in the presence or absence of tetracycline for 60 h. Cells were pulse labeled with bromodeoxyuridine to mark the S-phase cells undergoing DNA replication and were stained with propidium iodide to determine the nuclear DNA content. The most pronounced effect of the $RhoA^{wt}$ overexpression was on S-phase fraction, which was reduced from 26% in the control or uninduced cells to 13% in the induced cells (Fig. 3B). In addition, there was a significant increase in the G_2/M fraction from 24% to 41%, whereas the G_1 content remained relatively constant. There was no appreciable rise in the sub 2N fraction, demonstrating that the majority of the growth-arrested cells do not undergo apoptosis.

To better delineate the effect of RhoA on the cell cycle, the cells were first cultured with or without tetracycline for 24 h and then synchronized in metaphase by a nocodazole treatment. Nocodazole, which reversibly depolymerizes MTs, was removed after 16 h to allow cells to reenter the cell cycle. In the presence of tetracycline, 87% of the nocodazole-treated cells were in the G_2/M fraction (Fig. 3C). These M-phase arrested cells reentered the cell cycle after release from nocodazole, establishing a near baseline G_1, S, and G_2/M distribution by 24 h. The cell cycle reentry of M phase arrested cells were accompanied by a rise in the sub 2N fraction cells, indicative of apoptosis. In the $RhoA^{wt}$ overexpressing cells, the nocodazole treatment promoted a similar accumulation of cells in M phase (Fig. 3D). However, 14 % of the cells overexpressing $RhoA^{wt}$ remained in the G_1 fraction, suggesting the presence of an additional block at the G_1/S. Unlike the control cells, the majority of the $RhoA^{wt}$ cells failed to reenter the cell cycle or undergo apoptosis when released from nocodazole. These studies suggest that when RhoA is expressed at high levels, the cell cycle progression through both G_1/S and G_2/M becomes defective.

Cdk Inhibitor $p21^{Cip1}$ Accumulates in the Absence of Actin Stress Fibers

The entry into G_1/S is tightly controlled by cdks, which in turn is regulated by cyclins and cdk inhibitors [Sherr, 1996]. We next tested

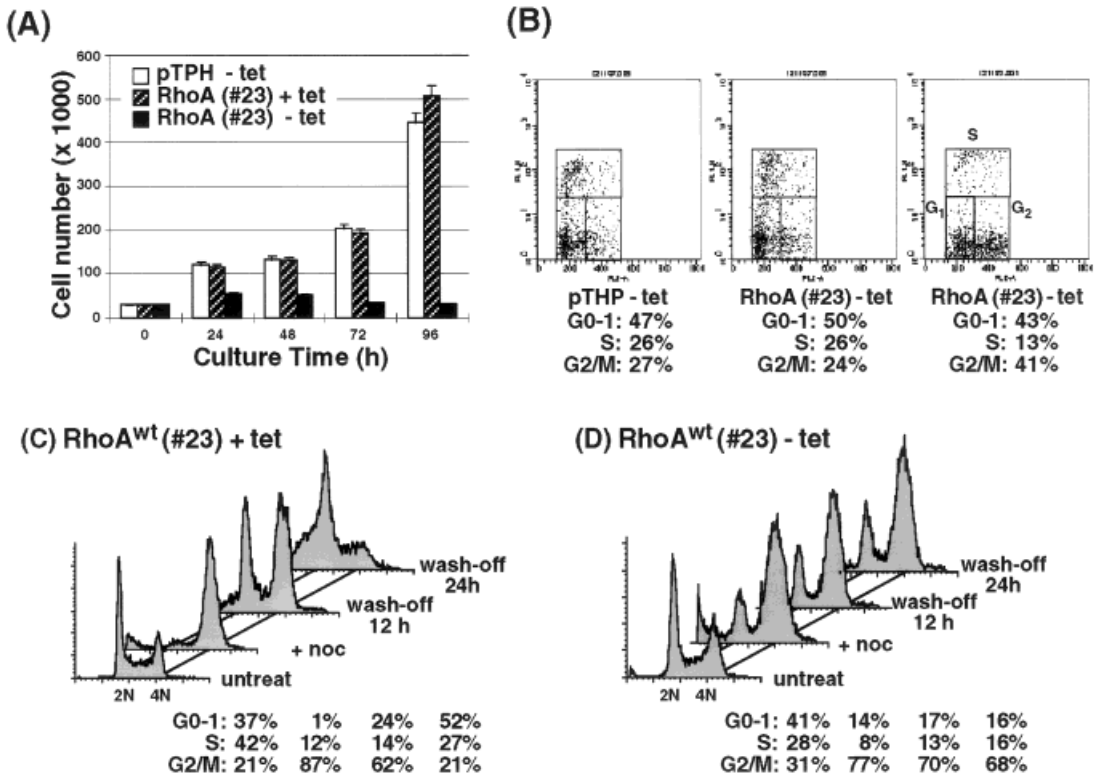

Fig. 3. RhoA^{wt} overexpression results in cell cycle arrest in both G_1 and G_2. **A:** Cells were plated in triplicate in 12-well plates and cultured with (+, uninduced) or without (−, induced) tetracycline for 96 h. At the indicated time points, the cell growth was visually inspected and then quantified by an MTT dye method. pTPH-tet, control cells; Rho^{wt} + tet, uninduced; RhoA^{wt}-tet, induced. **B:** Cells were cultured (+) or (−) tetracycline for 60 h. Bromodeoxyuridine (BrdU) and propidium iodide labeling, and two-dimensional flow were carried out as described in Materials and Methods. The BrdU incorporation (*y* axis), the DNA content (*x* axis), and the windows for G_1, S, and G_2/M fractions are shown. The percentage of cells with G_1 (2N), S, and G_2/M (4N) DNA content are numerated below the flow cytometric cell cycle profiles. **C, D:** Cells cultured in the presence (C) or absence (D) of tetracycline for 24 h were synchronized in G_2/M with nocodazole (150 ng/ml). After 16 h, nocodazole was replaced with the complete growth medium to allow the arrested cells to reenter cell cycle. The nuclear DNA was stained with propidium iodide, and flow cytometric cell cycle analysis was carried out. The DNA content of the cells before the nocodazole treatment (untreat), after a 16-h nocodazole treatment (+ noc), 12 and 24 h after the removal of nocodazole (wash-off) are shown.

whether the cell cycle defect observed in the absence of actin stress fibers is associated with any specific biochemical alterations in the cell cycle regulators. On induction of RhoA^{wt}, an increase in the p21^{Cip1} level became apparent by 48 h and continued to rise (Fig. 4A). These findings indicate that either the RhoA function or the integrity of actin cytoskeleton is required to suppress p21^{Cip1}. As expected from the rise in p21^{Cip1}, the cdk2 activity was decreased in the cells overexpressing RhoA^{wt} (Fig. 4B). The levels of p27^{kip1} and G_1 cyclins A and D_1 all remained constant after the induction of RhoA^{wt} (Fig. 4C).

In quiescent cells, p21^{Cip1} can be stimulated by growth factors in an ERK-dependent manner [Bottazzi et al., 1999]. Because RhoA has been shown to stimulate quiescent cells to enter the cell cycle and suppress the p21^{Cip1} expression in Ras-transformed fibroblasts [Olson et al., 1995, 1998], we next studied whether the overexpression of RhoA^{wt} affected p21^{Cip1} mRNA. A Northern analysis demonstrated that the p21^{Cip1} mRNA level remained constant after the induction of RhoA^{wt}, indicating that the accumulation of p21^{Cip1} does not involve a transcriptional induction (Fig. 4D). Consistently, the p53 level remained unchanged after the RhoA^{wt} induction (Fig. 4C).

Disruption of Actin Cytoskeleton Leads to an M-Phase Block

The G_2/M phase transition is orchestrated by the activation of cdc2 kinase, required for the

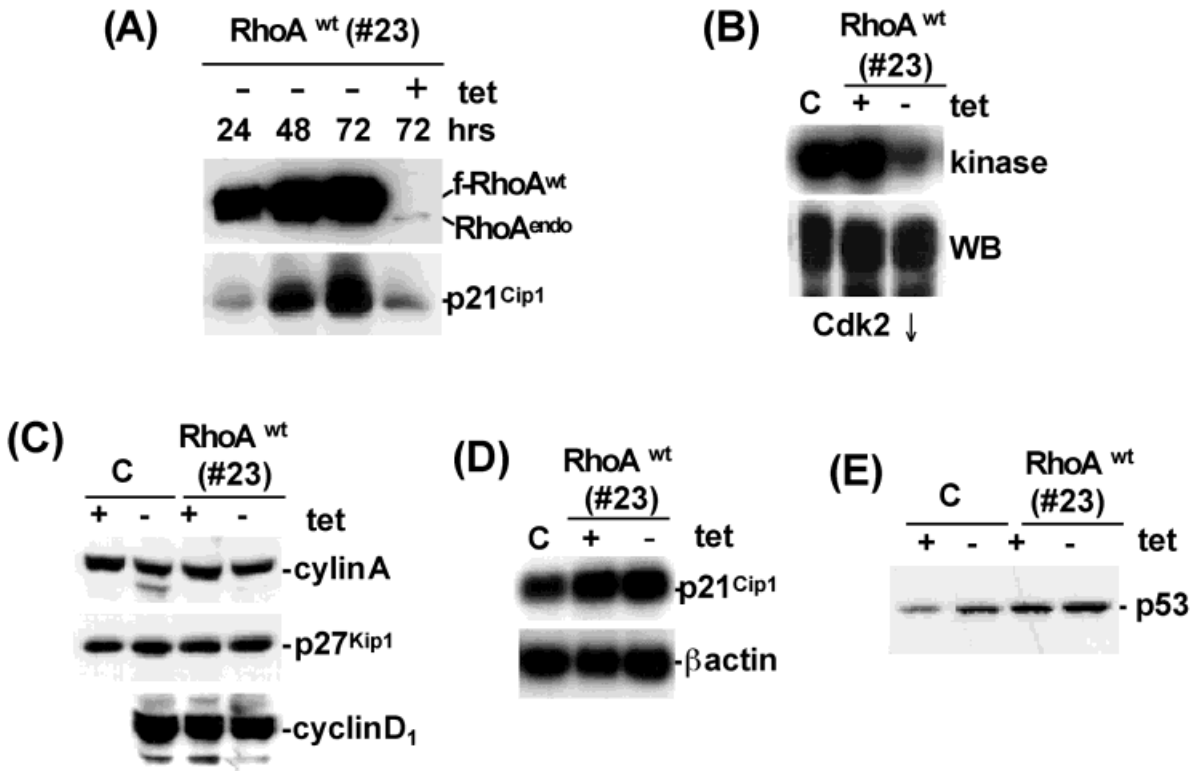

Fig. 4. $p21^{Cip1}$ is induced in the absence of actin cytoskeleton. **A:** $RhoA^{wt}$ cells were plated at equal densities and cultured with (+) or without (−) tetracycline (tet). At the indicated times, cells were harvested and analyzed by immunoblotting using anti-RhoA (26C4) mAb (top) and anti-WAF1 antibody (Ab-1) (bottom). **B:** $RhoA^{wt}$ cells were cultured with (+) or without (−) tetracycline for 72 h. Cdk2 kinase activity was determined as described in Materials and Methods. Histone phosphorylation was visualized by autoradiography (kinase assay). Cdk2 present in the kinase assay was visualized by an immunoblot using the M2 anti-cdk2 mAb. **C:** Twenty micrograms of cell lysates prepared 72 h after the induction of $RhoA^{wt}$ was analyzed by immunoblot using anti-cyclin A, anti-$p27^{Kip1}$, and anti-cyclin D_1 (M-20) antibody. **D:** Ten micrograms of total RNA was analyzed by a Northern blot to determine the expression of $p21^{Cip1}$ (top). The β-actin mRNA level (bottom) verified loading of an equivalent amount of RNA. **E:** Twenty micrograms of cell lysates was analyzed by immunoblot using anti-p53 (ab-6) antibody.

entry into mitosis, and the activation of anaphase promoting complex leading to a rapid destruction of mitotic cyclins and exit from telophase into interphase [King et al., 1996]. The activation of cdc2 kinase requires a progressive accumulation of cyclin B during interphase and the activity of Cdc25 phosphatase, which can be sequestered in an inactive state by 14-3-3 protein [Peng et al., 1997]. In particular, 14-3-3σ isoform, which is transcriptionally regulated by p53, is capable of inducing a G_2/M arrest [Hermeking et al., 1997].

To better delineate the observed defect in the G_2/M transition in the cells overexpressing $RhoA^{wt}$, we measured the levels of cyclin B protein, 14-3-3σ mRNA, and cdc2 kinase activity. In asynchronous cells overexpressing $RhoA^{wt}$, the most notable finding was the accumulation of cyclin B (Fig. 5A). In addition, the 14-3-3σ mRNA level was considerably lower on the induction of $RhoA^{wt}$, suggesting that RhoA overexpression did not prevent entry into mitosis (Fig. 5B). As expected from the elevated cyclin B level, the activity of cdc2 kinase was higher in the $RhoA^{wt}$ cells than in the control (Fig. 5C). These findings indicate that the G_2/M arrest observed in the RhoA overexpressing cells is likely to be caused by a defect at a point subsequent to the entry into mitosis.

Actin Stress Fibers Are Required for the Stability of MTs

Disruption of MT is a known inducer of M-phase checkpoint [Hyman and Karsenti, 1996]. We next investigated whether the disruption of actin cytoskeleton affects the integrity of MTs. In the control cells, a well-organized MT network emanating from a

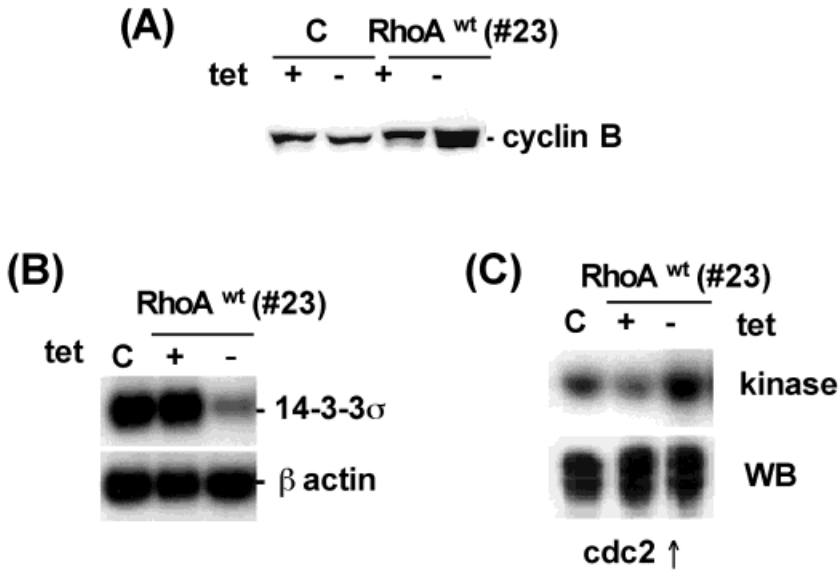

Fig. 5. Absence of actin cytoskeleton leads to a mitotic block. **A:** The control and RhoA^{wt} cells were cultured with (+) or without (−) tetracycline for 72 h. Twenty micrograms of cell lysates was analyzed by immunoblot using anti-cyclin B mAb. **B:** Ten micrograms of total RNA was analyzed by Northern blot to determine the expression of 14-3-3σ (top). The β-actin mRNA level (bottom) verified loading of equivalent amount of RNA. **C:** The RhoA^{wt} cells were cultured with (+) or without (−) tetracycline for 72 h. Cdc2 kinase activity was determined as described in Materials and Methods. Histone phosphorylation was visualized by autoradiography (kinase assay). Cdc2 present in the kinase assay was visualized by immunoblot using anti-cdc2 mAb. WB: Western blot for cdc2.

perinuclear centrosome can be visualized by anti α-tubulin antibody staining in in situ immunofluorescence (Fig. 6A). A similar MT staining pattern is seen in the RhoA^{wt} cells grown in the presence of tetracycline (Fig. 6B). On induction of RhoA^{wt}, there was a substantial reduction in the intensity of MT staining (Fig. 6C). A similar disruption in the MT organization was seen when cells were treated with C3 exoenzyme or CD (Fig. 6D,E), indicating that the actin stress fibers are required for the maintenance of the MT network.

Actin Cytoskeleton Is a Critical Determinant of Cell Cycle Progression

In addition to the well-described role in actin cytoskeleton assembly, RhoA has been implicated in the activation of MAP kinases and nuclear gene transcription, either of which can influence the cell cycle [Hall, 1998]. To test whether the cell cycle defect seen in the cells overexpressing RhoA^{wt} is a consequence of disturbing actin cytoskeleton, we used CD to disassemble actin filaments. When asynchronous U2OS cells were treated with 0.3 μM CD for 6 h, 55% of the cells accumulated in the G_2/M phase (Table I). After an overnight incubation, there was a further increase in the G_2/M to 82%. As in the cells overexpressing RhoA^{wt}, the p21^{Cip1} and cyclin B levels were elevated in the CD treated cells (data not shown).

U2OS cells express functional p53, which have been implicated in the mitotic spindle checkpoint [Cross et al., 1995]. We next examined the effect of CD on three different cell lines with varying p53 status to determine whether the effect of actin cytoskeleton on the cell cycle is mediated through p53. Cell lines included in this study were Saos cells (an osteosarcoma cell line, p53$_{-/-}$), HeLa (an epithelial cell line, p53 is inactive due to the presence of human papilloma virus), and NIH3T3 (a fibroblast cell line). The CD treatment induced a varying degree of block in G_2/M transition in all three cell types, suggesting that the requirement of intact actin stress fibers for the cell cycle progression through G_2/M is independent of p53 (Table I).

DISCUSSION

RhoA is required for the assembly of actin stress fibers and entry of quiescent fibroblasts into the S-phase in response to serum stimulation. The loss of actin stress fibers and growth

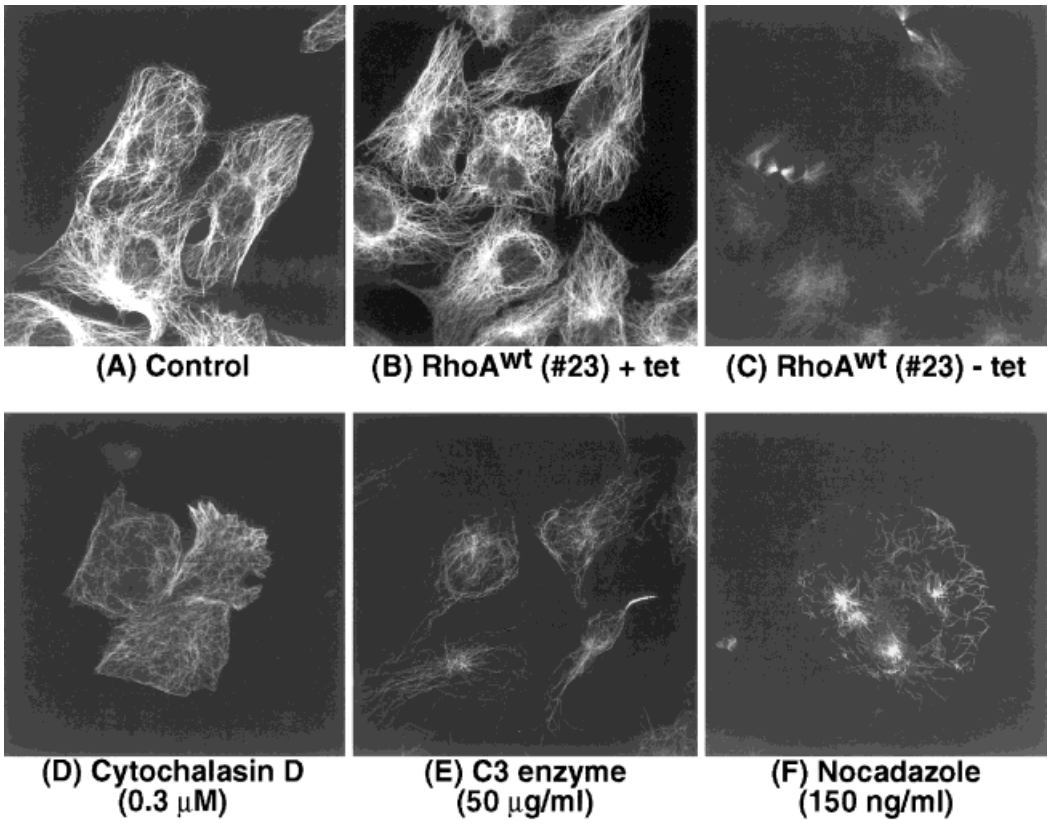

Fig. 6. Actin cytoskeleton is required for the MT stability. **A–C:** Control cells and RhoA^{wt} (no. 23) cells were plated on fibronectin-coated coverslips and cultured with or without tetracycline for 48 h. **D–F:** U2OS cells were plated on fibronectin-coated coverslips and cultured in the presence of cytochalasin D (CD) (0.3 μM); nocodazole (150 ng/ml); or C3 exoenzyme (50 μg/ml) for 16 h. In situ immunofluorescence for MTs was carried out as described in Materials and Methods. Images were captured using a photomultiplier set at standardized parameters using a Zeiss LSM 310.

TABLE I. Cell Cycle Analysis After Cytochalasin D (CD) Treatment

	G_{0-1} (%)	S (%)	G_2/M (%)
U20S, − CD	35	44	21
U20S, + CD, 6 h	5	41	55
U20S, + CD, 18 h	8	9	82
Saos, − CD	48	29	23
Saos, + CD, 18 h	18	18	64
HeLa, − CD	60	27	13
HeLa, + CD, 18 h	31	27	41
NIH3T3, − CD	51	42	7
NIH3T3, + CD, 18 h	62	16	23

Cells were treated with CD (0.3 μM) for the indicated time and then were stained with propidium iodide to determine the DNA content by flow cytometry.

inhibition seen in the RhoA^{wt} overexpressing cells were in contrast to this well-known function of RhoA. We believe the observed phenotype is caused by inhibition rather than stimulation of RhoA because overexpression of activated RhoA^{L63} using a similar tetracycline-inducible system only increased the stress fibers without inducing growth arrest (data not shown).

There are several possibilities to explain why the excess RhoA^{wt} disrupts the function of Rho GTPase. There are seven known families of Rho GTPases and at least five isoforms of Rho (RhoA-E) that control different cellular functions [Van Aelst and D'Souza-Schorey, 1997; Hall, 1998]. First, the overexpression of RhoA^{wt} may have altered the regulation of other Rho GTPases. Inactive GDP-bound Rho GTPases are stabilized by Rho guanine nucleotide dissociation inhibitors (GDI). Activation requires the dissociation of Rho GTPase from this Rho–GDI complex and exchange of GDP with GTP, promoted by guanine nucleotide exchange factor (GEF). Overexpression of RhoA^{wt} may have inappropriately sequestered Rho GEFs, which tend to have a broad spectrum of activity toward more than one Rho GTPases.

Second, the excess amount of $RhoA^{wt}$ may have altered the subcellular distribution of this or related GTPases. Whereas Ras is constitutively in the membrane, inactive GDP-bound Rho GTPases is in the cytosol associated with GDI [Ueda et al., 1990; Kwak et al., 1995]. On activation, the GTP-bound RhoA translocates to plasma membrane, where it is thought to function as a docking site for its downstream effectors [Hall, 1998; Madaule et al., 1998; Dong et al., 1998]. Inappropriate localization or recruitment of downstream effectors could potentially generate a dominant negative phenotype.

The most pronounced effect of $RhoA^{wt}$ overexpression is the reduction in the actin stress fiber and growth arrest. The observed G_1/S arrest in $RhoA^{wt}$ overexpressing cells can be explained by the induction in $p21^{Cip1}$ and decrease in cdk2 activity. In Ras-transformed fibroblasts, RhoA has been shown to be required to regulate $p21^{Cip1}$ expression [Olson et al., 1998]. Prenylation inhibitors that block the covalent linkage of either a farnesyl or geranylgeranyl lipid moiety necessary for the membrane localization of Rho GTPases can also result in the induction of $p21^{Cip1}$ [Vogt et al., 1997; Sepp-Lorenzino and Rosen, 1998]. The regulation of $p21^{Cip1}$ can occur either at the transcriptional level or a posttranslational level. In $RhoA^{wt}$ overexpressing cells, the levels of p53 protein and, more importantly, $p21^{Cip1}$ mRNA did not change (Fig. 4), indicating that posttranslational turnover of $p21^{Cip1}$ may be impaired when $RhoA^{wt}$ is overexpressed.

The M-phase arrest seen in the $RhoA^{wt}$ overexpressing cells correlates with the loss of MT organization. $RhoA^{wt}$ overexpression, C3 exoenzyme, or CD all led to the loss of organized MTs, suggesting that the integrity of stress fibers is required for stable maintenance of MTs. RhoA has been shown to be required for the serum-induced formation of stabilized MTs [Cook et al., 1998]. Conversely, the disruption of MTs with nocodazole has been shown to induce a RhoA-dependent formation of actin stress fibers [Enomoto, 1996; Zhang et al., 1997]. MTs and actin filaments can be crosslinked together by MT-associated proteins [Griffith and Pollard, 1978; Sattilaro et al., 1981]. One possibility, therefore, is that the absence of actin stress fibers may have directly destabilized MTs by preventing this stabilizing interaction between MTs and actin filaments. A second possibility is that the loss of MT organization is a consequence of cyclin B accumulation seen in the absence of actin stress fibers. During mitosis, cdc2/cyclin B associates with the mitotic apparatus [Bailly et al., 1989; Rattner et al., 1990] and participates in the rearrangement of the MT network [Verde et al., 1992]. Potential targets of cdc2/cyclin B include MT-associated proteins, which on phosphorylation can no longer stabilize MTs [Ookata et al., 1995].

In summary, we altered the function of RhoA through overexpression of $RhoA^{wt}$. Our study clearly illustrates that the expression levels of protein must be carefully considered when interpreting functions of small GTPases after protein overexpression. Previous studies have demonstrated the importance of RhoA in the cell cycle entry. Our study provides evidence that RhoA is also required for cell cycle progression through G_2/M. Although we demonstrated a correlation between the observed cell cycle blocks in the $RhoA^{wt}$ overexpressing cells and the reduction in the actin cytoskeleton, additional studies are warranted to determine the precise role of RhoA and actin cytoskeleton in the cell cycle progression.

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