RhoA Prenylation Is Required for Promotion of Cell Growth and Transformation and Cytoskeleton Organization but Not for Induction of Serum Response Element Transcription*

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The importance of post-translational geranylgeranylation of the GTPase RhoA for its ability to induce cellular proliferation and malignant transformation is not well understood. In this manuscript we demonstrate that geranylgeranylation is required for the proper cellular localization of V14RhoA and for its ability to induce actin stress fiber and focal adhesion formation. Furthermore, V14RhoA geranylgeranylation was also required for suppressing p21WAF transcription, promoting cell cycle progression and cellular proliferation. The ability of V14RhoA to induce focus formation and enhance plating efficiency and oncogenic Ras anchorage-dependent growth was also dependent on its geranylgeranylation. The only biological activity of V14RhoA that was not dependent on its prenylation was its ability to induce serum response element transcriptional activity. Furthermore, we demonstrate that a farnesylated form of V14RhoA was also able to bind RhoGDI-1, was able to induce cytoskeleton organization, proliferation, and transformation, and was just as potent as geranylgeranylated V14RhoA at suppressing p21WAF transcriptional activity. These results demonstrate that RhoA geranylgeranylation is required for its biological activity and that the nature of the lipid modification is not critical.

Small G proteins of the Ras superfamily are regulatory proteins whose activity is controlled by a GDP/GTP cycle. Several members of the Ras superfamily are regulators of signaling pathways that control cell growth, differentiation, and oncogenic transformation as well as actin cytoskeletal organization (1). The Rho protein branch of this superfamily includes at least eight distinct Rho families (RhoA, B, C, D, and G, Rac1 and 2, TC10, Cdc42, and Rnd1, 2, and 3) (2) that are regulated by Rho-GTPase activating proteins and a large family of guanine nucleotide exchange factors of the Dbl family proteins. Moreover Rho guanine nucleotide dissociation inhibitors (RhoGDI(s)) stabilize the inactive GDP-bound form of the Rho proteins. Rho proteins notably regulate signal transduction from cell surface receptors to intracellular molecules and are involved in a variety of cellular processes including cell morphology (3), motility (4), cytokinesis (5, 6), cell proliferation (7, 8), and tumor progression (9–11).

Ras and Rho proteins are post-translationally modified by the isoprenoid lipids, farnesyl, and geranylgeranyl (12). Two prenyltransferases, farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I), catalyze the covalent attachment of the farnesyl and geranylgeranyl groups, respectively, to the carboxyl-terminal cysteine of proteins ending in a CAAX motif (C is a cysteine, A usually alphatic amino acid, and X any amino acid). FTase prefers CAAX sequences where X is a serine, methionine, cysteine, alanine, or glutamine, as in Ras or in nuclear lamins (13–15). When X is a leucine or isoleucine the protein, as in the Rho/Rac family of proteins, is geranylgeranylated by GGTase I (16, 17). Protein prenylation is important in targeting proteins to cellular membranes but also in protein-protein interactions (18–20). This process appeared to be critical for the oncprotein Ras functions as observed with the dependence of its transforming activity on prenylation (21, 22). Hence, over the past decade the functional role of protein prenylation has been intensively studied for Ras, while a few studies took interest in the other proteins. It has been shown that geranylgeranylation of RhoA is required for its correct subcellular localization (23) and for interaction with its GDP/GTP cycle regulators, guanine nucleotide dissociation inhibitor and guanine nucleotide exchange factor (24, 25). Furthermore, RhoA prenylation is needed for phospholipase D activation (26, 27) and potentiation of AP-1 transcription (28). However, although it was demonstrated that the prenylation of the highly homologous Rho protein, RhoB, is required for its cell transforming function but not its ability to activate serum response element-dependent transcription (29), whether RhoA geranylgeranylation is required for suppression of p21WAF, induction of proliferation, and cytoskeleton organization was still not established. It was only shown that inhibition of protein geranylgeranylation by the GGTase I inhibitor, GGTI-298, resulted in G0/G1 cell cycle arrest, induction of p21WAF transcrip-

1 The abbreviations used are: RhoGDI(s), Rho guanine nucleotide dissociation inhibitor(s); FTase, farnesyltransferase; GGTase I, geranylgeranyltransferase I; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; SRE, serum response element; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide.

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tion, programmed cell death, and disorganization of actin cytoskeleton (30–32). Similar effects observed with the Clostridium butulinum C3 exoenzyme, a specific inhibitor of Rho A, B, and C proteins (32–34), as well as with a dominant negative mutants of RhoA (7, 32) have suggested a role of Rho proteins in these processes.

Specific isoprenoids may facilitate distinct consequences for protein functions. Hence, normal Ras function is critically dependent on modification by a farnesy1 group, and a geranylgeranylated normal Ras protein appeared to be a potent inhibitor of cellular proliferation (35). In contrast either a C15 or C20 isoprenoid can promote membrane interaction of the oncoprotein Ras necessary for triggering the cell transformation (35–37). On the other hand, it was demonstrated that a specific prenylation of the γ subunits of G protein is needed for the membrane localization, interaction with the Gβ and their effectors (38–42). Thus an evaluation of the role of specific isoprenoid modification in the function of other prenylated proteins such as Rho GTPase would be needful for a better understanding of the functional modification of a protein.

In this manuscript, we determined whether RhoA prenylation is required for its activity on cytoskeleton organization, proliferation, transcription, and transformation. Furthermore, we also investigated whether the nature of the prenyl group (i.e. farnesyl versus geranylgeranyl) influences the biological activities of RhoA. To this end, we generated RhoA mutants by deleting the CAAX sequence to produce an unprenylated protein as well as by mutating the CAAX box to produce farnesylated RhoA.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Standard polymerase chain reaction mutagenesis techniques were used to generate plasmids coding for RhoA with the wild type (RhoA-CLVL), farnesylated, (RhoA-CVLS), or deleted (RhoA-Δ) CAAX sequence. Polymerase chain reaction amplification of pEXmyc-tagWT RhoA and pEXmyc-tagV14RhoA (a generous gift of Dr. A. Hall, London) were done with the forward primer (CCAGACGTGGGCGTCATGAGCAGAAGGCTGATCCTC) and the reverse primer (RhoA-CLVL, GGAATTCCGGATCCCTACAAGACAAGGCAACCAGATGTTTACAGGTCCACGCT; and RhoA-Δ, GGAATTCCGGATCCCTACAACCAGATTTTTTCTCCTCAACCTAG), respectively. The amplified fragments were next washed twice with interaction buffer and resuspended directly in Laemmli buffer. RhoA content was analyzed by SDS–polyacrylamide gel electrophoresis followed by Western blot with anti-c-Myc (Calbiochem) antibodies. RhoA and vinculin were detected with anti-c-Myc (Calbiochem) or anti-vinculin antibodies (Sigma Immuno Chemical), respectively, by fixing with AFA and staining with crystal violet.

Fluorescence—Cells were seeded on glass coverslips in six-well plates (Nunc) at a density of 8 × 10^4 cells/well in DMEM containing 10% FCS. 48 h later cells were serum-starved for 48 h. Then cells were fixed in 3% paraformaldehyde and permeabilized to 0.1% Triton X-100 in phosphate-buffered saline. Actin fibers were detected by incubation with tetramethylrhodamine isothiocyanate-labeled phalloidin (Molecular Probes). RhoA and vinculin were detected with either anti-c-Myc (Calbiochem) or anti-vinculin antibodies (Sigma Immuno Chemical), respectively, and fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (Sigma Immuno Chemical). Cells were viewed on a Zeiss Axiopt microscope, and pictures were taken with a Princeton camera.

Cell Growth Determination—NIH-3T3 cells were seeded at 2000 cells/well in a 96-well plate six times on day 0 in DMEM containing 10% or 2.5% FCS, and the amount of cells was evaluated on day 0 (6 h post-plating) and at the intervals indicated in the figure legends by a MTT test as described previously (45).

Fluorescence—Cells were seeded on glass coverslips in six-well plates (Nunc) at a density of 8 × 10^4 cells/well in DMEM containing 10% or 2.5% FCS, and the amount of cells was evaluated on day 0 (6 h post-plating) and at the intervals indicated in the figure legends by a MTT test as described previously (45).

Plating Efficiency Determination—For plating efficiency assays, NIH-3T3 cells were seeded at 400 cells/60-mm culture dishes in DMEM 10% or 2.5% FCS. Cell foci were scored 15 days after confluence after fixing with AFA (ethanol/formol/acetic acid, 75/20/5) and staining with crystal violet.

Prenylation Status Analysis—NIH-3T3 cell lines expressing the V14RhoA constructs were seeded at 25000 cells in a 25-cm2 flask. Cell foci were scored 15 days after confluence after fixing with AFA (ethanol/formol/acetic acid, 75/20/5) and staining with crystal violet.

Plating Efficiency Determination—For plating efficiency assays, NIH-3T3 cells were seeded at 400 cells/60-mm culture dishes in DMEM containing 10% or 2.5% FCS. Cell foci were scored 15 days after confluence after fixing with AFA (ethanol/formol/acetic acid, 75/20/5) and staining with crystal violet.
vested 30 h later and lysed in 200 μl of lysis buffer (Promega). For SRE activity analysis, 24 h after transfection cells were washed twice with phosphate-buffered saline and incubated in DMEM supplemented with 0.5% FCS during the next 24 h, before harvesting and lysis. Cell extracts were used for β-galactosidase (CLONTECH) and luciferase (Promega) assays.

RESULTS

Prenylation Status and Subcellular Localization of RhoA Mutants in NIH-3T3 and COS-7 Cell Transfectants—To evaluate the role of prenyl group and of its nature (C15 farnesyl or C20 geranylgeranyl) in RhoA functions we generated RhoA mutants by altering the CAA\textsubscript{X} sequence to render RhoA a substrate either of geranylgeranyltransferase I or farnesyltransferase or not prenylated. Using standard polymerase chain reaction mutagenesis, the CLVL sequence of RhoA (RhoA-CLVL) was either replaced by the CAA\textsubscript{X} box of the farnesylated Ha-Ras protein (RhoA-CVLS) or deleted (RhoA-Δ). The plasmids encoding these RhoA mutants were transfected in the murine NIH-3T3 fibroblasts. Expression of RhoA was controlled by Western blot in 10 different clones picked for each construction. Representative clones with similar expression level to each other were selected (Fig. 1).

We determined the prenylation status of each V14RhoA mutant by using FTI-277 and GGTI-298, specific inhibitors of FTase and GGTase I, respectively. NIH-3T3 cells expressing either V14RhoA-CLVL or V14RhoA-CVLS were treated with FTI-277 or GGTI-298, and the lysates were analyzed for inhibition of prenylation of Ha-Ras (exclusively farnesylated control), Rap1A (exclusively geranylgeranylated control), RhoA-CLVL, and RhoA-CVLS, by Western blotting as described under “Experimental Procedures.” As expected, treatment with FTI-277 resulted in inhibition of farnesylation of Ha-Ras but had no effects on the geranylgeranylation of Rap1A (Fig. 1). Similarly, GGTI-298 inhibited the geranylgeranylation of Rap1A without any effects on the farnesylation of Ha-Ras. Fig. 1 also shows that FTI-277 inhibited the prenylation of RhoA-CVLS but not RhoA-CLVL, whereas GGTI-298 inhibited the prenylation of RhoA-CLVL but not RhoA-CVLS. These results suggest that RhoA-CVLS is exclusively farnesylated, whereas RhoA-CLVL is exclusively geranylgeranylated.

The prenylation status of a protein being important for its proper subcellular localization (23), we checked the localization of the RhoA mutants by immunofluorescence. V14RhoA-CLVL cells displayed a diffuse staining throughout the cytoplasmic compartment (Fig. 2A) with an increased staining in the perinuclear area of some cells. RhoA-CLVL cells showed a similar pattern of fluorescence (Fig. 2A). In contrast RhoA-Δ staining was not confined to a specific subcellular compartment but was rather spread throughout the whole cell (Fig. 2A). This pattern was also observed when all of RhoA mutants were treated with lovastatin, a potent inhibitor of prenylation (data not shown), confirming that prenylation is essential for proper protein subcellular localization.

We next analyzed the subcellular localization of RhoA mutants by Western blot after separation of cell homogenates in cytosol and membrane proteins by Triton X114 partitioning as described under “Experimental Procedures.” As shown in Fig. 2B unprenylated V14RhoA was essentially associated with the cytosolic fraction, whereas prenylated RhoA, either farnesylated or geranylgeranylated, was distributed in both fractions. About 40% of prenylated V14RhoA expressed in NIH-3T3 cells was observed in membrane fraction. When analyzed in COS-7 cells after transient expression, prenylated V14RhoA display a similar subcellular distribution, whereas wild type RhoA was essentially found in cytosol fraction (data not shown), illustrating the shift toward the membrane of RhoA GTPase after activation but only when prenylated. These results together confirmed that prenylation is essential for proper protein subcellular localization and also indicated that farnesyl can sub-

Fig. 1. Expression and prenylation of RhoA mutants. RhoA transfected cells NIH-3T3 cells were treated either with 10 μM GGTI-298 or with 10 μM FTI-277 for 48 h. The cells were lysed and analyzed for RhoA, Rap1A, and Ha-Ras expression and processing by Western blotting. Data are representative of four independent experiments.

Fig. 2. Subcellular distribution of RhoA mutants localization. A, NIH-3T3 cells grown on coverslips were analyzed for RhoA localization by immunofluorescence as described under “Experimental Procedures.” B, after separation of cell homogenates into membrane (Mb) and cytosolic (SN) fractions by Triton X-114 partition, RhoA Myc-tagged content was analyzed by Western blot with monoclonal anti-Myc antibodies.
stitute for geranylgeranyl without any dramatic effect on RhoA subcellular localization.

**Interaction of RhoA with its Guanine Dissociation Inhibitor, RhoGDI-1**—Although it was well established that isoprenoid modification of RhoA is required for interaction with RhoGDI (24, 47), it is still not known whether prenylation of RhoA has to be specific. To assess the role of isoprenoid the *in vitro* interaction of RhoA mutants with GST-RhoGDI-1 was examined. Because it was described that RhoGDI-1 binds poorly RhoA-GTP (48, 49), we used COS-7 cells transiently with wild type RhoA bearing or deleted of the different CAAX boxes. We found as expected that nonprenylated RhoA was unable to bind GST-RhoGDI-1 (Fig. 3). In contrast geranylgeranylated as well as farnesylated RhoA strongly interacted with GST-RhoGDI-1. As illustrated by the quantitation of the signal of precipitated RhoA normalized by the signal of total RhoA on Western blot, GST-RhoGDI-1 appeared to bind equal levels of both farnesylated and geranylgeranylated RhoA (Fig. 3).

**Role of RhoA Prenylation on Cytoskeleton Organization**—The Rho protein family whose members include RhoA has been shown to influence a number of cellular processes including actin stress fiber organization, cell adhesion, cell proliferation, and transformation. We next determined whether prenylation and moreover the nature of the added prenyl group play a role on RhoA implication on actin stress fibers and focal adhesions.

We observed that whereas NIH-3T3 fibroblasts transfected with the empty vector (mock) or V14RhoA-Δ have conserved a typical fibroblast morphology, V14RhoA-CLVL and V14RhoA-CLVS expressing cells were smaller and displayed a more epithelial-like morphology (Fig. 4A). Similar changes of cell size were observed in Swiss 3T3 cells microinjected with active RhoA (3) as well as in murine tumor cells (50).

To examine the effect of the V14RhoA mutants on stress fibers and focal adhesions, the cells were serum-starved for 48 h before analysis. Indeed removal of growth factors and LPA (a well known stimulator of RhoA functions) from culture medium led to the reduction of actin stress fiber content in mock and V14RhoA-Δ cells (Fig. 4B). In contrast, under these conditions V14RhoA-CLVL and V14RhoA-CLVS cells displayed well preserved stress fibers (Fig. 4B). In parallel experiments, vinculin immunostaining showed that in V14RhoA-CLVL and V14RhoA-CLVS but not V14RhoA-Δ expressing cells a retention of numerous focal adhesions in the absence of serum (Fig. 4B). These results would indicate that prenylation is required for RhoA implication in stress fibers and focal adhesions and that geranylgeranylation could be substituted for by farnesylation.

**Role of Prenylation of RhoA on Cell Growth Properties**—We next assessed the role ofay of RhoA on cell growth. Mock-transfected NIH-3T3 cells and those expressing V14RhoA-CLVL, V14RhoA-CLVS, or V14RhoA-Δ maintained in DMEM supplemented with 10% FCS displayed similar growth rate (Fig. 5A). However, in lower serum concentration (2.5%), V14RhoA-CLVL and -CLVS cells showed a significantly

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**FIG. 3.** *In vitro* interaction of GST-RhoGDI with prenylated RhoA. 100 μg of cell lysates of COS-7 cells transiently transfected with pCMV-WT RhoA were incubated with purified GST-RhoGDI-1 fusion protein as described under "Experimental Procedures." The proteins bound on beads were then denatured in sample buffer and separated by SDS-polyacrylamide gel electrophoresis, followed by Western blot (GST-RhoGDI-1 bound RhoA). 20 μg of cell lysates were analyzed for expression of RhoA (total RhoA) by Western blot with anti-Myc antibodies.

**FIG. 4.** Prenylated V14RhoA mutants induce a dramatic morphological modification and an increase in actin stress fiber content and focal adhesion formation. A, morphology of RhoA transfected NIH-3T3 cells maintained in growth medium. B, 2 days after being seeded, cells were serum-starved for 48 h and actin fibers, and focal adhesions were visualized with tetramethylrhodamine isothiocyanate-phalloidin and mouse anti-vinculin followed by fluorescein isothiocyanate-conjugated anti-mouse antibody, respectively.
higher growth rate than mock and V14RhoA-D cells (Fig. 5B), indicating that prenylated V14RhoA expression reduced the serum requirement of the NIH-3T3 cells as described by Perona et al. (9).

We next determined whether the ability of RhoA to transform cells depends on its geranylgeranylation and whether farnesylated RhoA remains transforming. To this end, we have compared various V14RhoA mutants in focus formation, plating efficiency, and anchorage-independent growth assays. For the focus formation assay, NIH-3T3 cells stably expressing V14RhoA mutants were seeded at 25,000 cells/25-cm² flask, and cell foci were scored 12 days after confluence by fixing with AFA and staining with crystal violet. Moreover, only prenylated V14RhoA were able to develop clones after plating in drastic conditions (400 cells in 60-mm culture dishes) (Fig. 6B). But we observed in each of these experiments that a fewer number of clones grew from cells expressing farnesylated RhoA than geranylgeranylated RhoA. When testing on anchorage-independent growth by soft agar cultures, V14RhoA-CLVL and V14RhoA-CVLS as well as V14RhoA-D expression in NIH-3T3 cells did not induce growth (data not shown). However, prenylated V14RhoA but not V14RhoA-D expression increased significantly the anchorage-independent growth of oncogenic Ha-RasL61 transformed NIH-3T3 cells (Fig. 7).

Role of RhoA Prenylation on Transcriptional Activity—RhoA was described to stimulate the SRE activity (51) as well as to repress p21WAF transcription (32). Our recent results suggested indirectly that geranylgeranylation of RhoA was required for its activity on p21WAF transcription (32). To confirm this hypothesis we compared the respective ability of V14RhoA-CLVL, -CVLS, and -D to regulate luciferase transcription under the control of p21WAF promoter. We initially cotransfected NIH-3T3 wild type cells with p21WAF promoter and RhoA expression plasmids. Aliquots of cell homogenates were assayed 48 h after transfection for β-galactosidase and luciferase activities. As we have shown previously RhoA-CLVL induced an important (4-fold) repression of p21WAF promoter activity (Fig. 8A). Furthermore, the farnesylated RhoA-CVLS also displayed a similar effect on p21WAF activity, but the unprenylated form of RhoA, RhoA-D, was not able to regulate the activity of the promoter (Fig. 8A). Thus, the prenylation of RhoA is critical to its ability to repress the p21WAF transcriptional activity. Furthermore, farnesylated RhoA was just as potent as geranylgeranylated RhoA at repressing p21WAF transcription.

To determine whether or not prenylation was required for the role of RhoA on SRE-mediated transcription, we have performed similar experiments co-transfecting NIH-3T3 wild type cells with luciferase under the control of SRE and RhoA expression plasmids. Cells were incubated 24 h post-transfection with DMEM supplemented with 0.5% FCS for the next 18 h. Then as a control of SRE activity FCS was added for 4 h. As expected, addition of FCS resulted in a 4–5-fold stimulation of luciferase activity, whereas the expression of constitutively
Each experiment was done in triplicate. Photographed. Data are representative of three independent experiments.

Described under "Experimental Procedures," and the clones were phophor in 0.3% agar containing medium. The formation of clones was scored as dent growth of Ha-Ras transformed NIH-3T3 cells.

A

CVLS, L61Ras/V14RhoA-
or RhoA and L61Ha-Ras (L61Ras/pCMV), L61Ha-Ras (L61Ras/pCMV), transfected by mock vectors (pZip/pCMV), L61Ha-Ras (L61Ras/pCMV), or RhoA and L61Ha-Ras (L61Ras/V14RhoA-CLVL; L61Ras/V14RhoA-CVLS, L61Ras/V14RhoA-Δ). B, cells were plated onto a 0.6% agar layer in 0.3% agar containing medium. The formation of clones was scored as described under "Experimental Procedures," and the clones were photographed. Data are representative of three independent experiments. Each experiment was done in triplicate.

Active geranylgeranylated RhoA induced a 3-fold increase of luciferase activity (Fig. 8B). Then to directly compare the capacity of the prenylated and unprenylated RhoA to regulate SRE transcriptional activity, the luciferase induction was normalized by RhoA protein expression determined in parallel by Western blot. As shown in Fig. 8C, the geranylgeranylated RhoA-CLVL and the farnesylated RhoA-CVLS displayed a similar effect on SRE activity. Surprisingly RhoA-Δ showed also a stimulatory activity on SRE dependent transcription; however, this unprenylated form of RhoA was less efficacious than the geranylgeranylated and the farnesylated forms.

DISCUSSION

Although a number of studies showed that functions of Ras proteins depend on its farnesylation little is known about the role of prenylation for geranylgeranylated proteins. Using CAAX box mutants of RhoA, we demonstrated that prenylation of RhoA is essential for most but not all of its cellular functions. Furthermore, replacing the geranylgeranylated group by a farnesyl had little effect on the biological activities of RhoA.

The carboxyl-terminal amino acid sequence of the protein, the CAAX box, appears to contain all the critical determinants for interaction with a specific prenyl-transferase (15). The only substitution of the last three carboxyl-terminal amino acids of prenylated proteins such as Ras (15), γ subunit of G protein (40) or of RhoB as we recently described (52), which could be either geranylgeranylated or farnesylated, is sufficient to mod-ify the nature of their prenylation. Thus, to create RhoA prenylation mutants we used a similar strategy deleting the CAAX box or substituting the RhoA CLVL sequence by CVLS, the CAAX box of Ha-Ras, a protein exclusively farnesylated. This RhoA-CVLS mutant appeared to be a substrate for the FTase because FTI-277, a highly specific inhibitor of the enzyme, but not GGTI-298, blocked its prenylation.

Prenylated RhoA was detected in the cytoplasmic compartment in NIH-3T3 cells stably transfected with RhoA, as previously observed in Rat-2 cells or in Madin-Darby canine kidney cells (23, 53). Adamson et al. (23) showed that only the cysteine within the CAAX box is important for the correct intracellular localization of RhoA protein. Indeed its substitution for serine lead to a marked reduction of the normal cytoplasmic fluorescence in Madin-Darby canine kidney cells in favor of a strong nuclear localization. We obtained similar results in NIH-3T3 transfected with RhoA deleted of its CAAX box. Whereas the prenylated RhoA localized preferentially in the cytosol, the RhoA mutant accumulated in whole cells with a predominant nuclear localization as observed either with the unprenylated form of Ras (54) or RhoB (11, 23). A similar subcellular redistribution of unprenylated protein to the nucleus was also reported for protein tyrosine phosphatases PRL-1, -2, and -3 in NIH-3T3 cells (55). Numerous cells displayed a marked staining of RhoA in the perinuclear area that might suggest that RhoA is associated with some vesicles. Indeed after separation of membrane and cytosolic proteins, a large proportion of unprenylated V14RhoA appeared to be associated with membrane fractions. However, RhoA was not fully associated with plasma membrane as it was observed in NE-115 neuronal cells (56) or endothelial cells (57). The pattern of perinuclear fluorescence suggested that RhoA might accumulate in the endoplasmic reticulum or endosomal vesicles as described previously (58). Nevertheless, it is noteworthy that regardless of the prenyl group, a C20 geranylgeranyl or a C15 farnesyl, linked to the RhoA protein, NIH-3T3 transfected cells displayed similar RhoA immunofluorescence patterns, suggesting that the shorter farnesyl group is sufficient for directing the intracellular destination of RhoA.

It was well established that regulators of small G protein activity interact only with the post-translationally modified protein (4, 24, 25, 59). For example RhodGDI inhibits the dissociation of the GDP bound form of RhoA only if the protein is prenylated (24). Similar prenyl-dependent protein interactions should be required for the association of the RhoA-GTP bound form with its effectors and thus for its biological roles such as actin cytoskeleton organization and cell proliferation and transformation control. Nevertheless the role of isoprenylation in the control of stress fibers formation was still contradictory. Although it was shown that HMG-CoA reductase inhibitors induce cell rounding and breakdown of the actin cytoskeleton in cultured cells (60–62), suggesting a role of prenylated protein in stress fiber control, Kranenburg et al. (56) showed that isoprenylation is not required for stress fiber formation in N1E-115 neuronal cells. Herein we showed with a CAAX box mutant that prenylated RhoA ensured stress fiber and focal adhesion maintenance in NIH-3T3 cells, whereas unprenylated form of RhoA did not. Furthermore we demonstrated that the role of RhoA on NIH-3T3 cell proliferation and transformation was also dependent on the prenyl group. Hence, the unprenylated form of RhoA was not able to promote cell proliferation or anchorage-dependent cell growth, whereas farnesylated and geranylgeranylated RhoA acted similarly on cell cytoskeleton and proliferation.

RhoA protein has been suggested to regulate cell cycle progression by modulating the protein stability of cell cycle regu-

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Prenylation Is Required for RhoA Biological Activities

FIG. 8. The prenylation of RhoA is required for suppressing on p21WAF transcription but not for inducing SRE-mediated transcrip- tion. A, NIH-3T3 wild type cells were cotransfected with luciferase under the control of p21WAF promoter, V14RhoA mutants, and β-galactosidase plasmids. 48 h later cell homogenates were prepared, and then β-galactosidase activity and luciferase activity were assayed. B and C, NIH-3T3 wild type cells were cotransfected with luciferase under the control of SRE, V14RhoA mutants, and β-galactosidase plas- mids. 24 h post transfection cells were incubated in 0.5% FCS containing medium for additional 18 h. Where indicated 10% FCS was added 4 h before the end of the incubation. In C the induction factor of luciferase activity was normalized against RhoA mutant expression analyzed by Western blotting. In each experiment the luciferase activity was normalized for transfection efficiency against β-galacto- sidase activity and expressed as relative luciferase. Bars represent standard deviation. The data are representative of three independent experiments.
ence of a prenyl group, is not affected by its nature, i.e., farnesyl or geranylgeranyl, as illustrated by the equal amounts of farnesylated and geranylgeranylated RhoA precipitated by GST-RhoGDI-1 (Fig. 3). Moreover, it was shown that the binding affinity of RhoGDI-1 for the farnesylated and geranylgeranylated moiety is not really important (42).

We described previously a potent and selective GGTase I inhibitor GGTI-298 that blocks human tumors in G0/G1, induces apoptosis, and inhibits the tumor growth in nude mice xenografts (30, 46, 67). More recently we showed that GGTI-298 induces apoptosis, and inhibits the tumor growth in nude mice RhoGDI-1 (Fig. 3). Moreover, it was shown that the binding of a prenylated and geranylgeranylated RhoA precipitated by GST- 

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