Isoprenylation is Necessary for the Full Invasive Potential of RhoA Overexpression in Human Melanoma Cells

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Rho GTPases are signaling molecules known to control cell motility. Several recent studies have suggested a role for Rho proteins in mediating tumor metastasis independent of their effects on cell proliferation. As Rho proteins require post-translational modification with a geranylgeranyl moiety for full activity, we tested the effect of blocking geranylation on localization, downstream signaling, and stimulation of invasion. Expression of a constitutively active Rho construct in A375 melanoma cells dramatically stimulated invasion through Matrigel membranes; however, a constitutively active RhoA mutated so that it cannot be geranylated, failed to stimulate invasion. Moreover, expression of epitope or GFP tagged modifications of this nongeranylatable constitutively active Rho demonstrated that geranylation is necessary for correct cellular localization of Rho. Geranylation was also found to be necessary for full downstream activation of serum response factor mediated transcription. Pharmacologic inhibition of Rho geranylation produced similar inhibition of Rho localization, signaling, and invasion. Our results suggest that inhibition of Rho geranylation may be an attractive pharmacologic target for inhibiting melanoma metastasis. Key words: metastasis/statins/isoprenylation/RhoA. J Invest Dermatol 119:1172–1176, 2002

The lethality of melanoma is due to its high metastatic potential rather than local tumor invasion. As therapy provides little benefit after metastasis has occurred (Rigel et al, 1996) anti-metastatic chemoprevention strategies may reduce mortality in melanoma prone individuals by delaying or preventing metastasis of the primary neoplasm. Evidence from epidemiologic studies and animal models (Jani et al, 1993) suggests that the statin class of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors inhibit melanoma progression and metastasis. Statins are known to inhibit the post-translational lipidation of Rho GTPases, a family of proteins that have recently been implicated in causing melanoma metastasis (Clark et al, 2000). In order to investigate the potential role of Rho signaling in mediating the anti-metastatic effects of statins, we have examined the significance of blocking Rho lipidation in modulating melanoma cell invasion.

The 3-hydroxy-3-methylglutaryl coenzyme A reductase cholesterol biosynthetic pathway has numerous effects on cellular metabolism. In addition to producing cholesterol, mevalonate metabolism also supplies lipids required for protein isoprenylation. The mevalonate pathway produces farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which in turn post-translationally modify the carboxy termini of many signaling molecules, such as the Ras superfamily of small GTPases (Laufs and Liao, 2000). Farnesylation of Ras proteins, which has been extensively studied, facilitates their appropriate localization to cellular membranes, where interaction with other signaling molecules allows these GTPases to participate in mitogen signal transduction (Bar-Sagi and Hall, 2000). In contrast to Ras, most Rho GTPases control cell motility and adhesion rather than mitogenesis and are geranylgeranylated rather than farnesylated (Adamson et al, 1992).

Indirect lines of evidence suggest that, analogous to farnesylation of Ras, geranylgeranylation of Rho is essential for full activation of their downstream effectors. Rho function can be inhibited by statins, which deplete the cell of the geranylgeranyl pyrophosphate intermediate (Kranenburg et al, 1997) or by geranylgeranyl transferase inhibitors (GGTI), which block the enzyme responsible for attachment of the geranylgeranyl moiety to the carboxy terminus of Rho (Lesh et al, 2001). These drugs likely affect Rho signaling by abrogating its ability to interact with upstream and downstream components of various signal transduction cascades.

Both statins and GGTI have been shown to prevent growth of tumor cells in vivo (Sun et al, 1998; Kusama et al, 2002). These results suggest that pharmacologically inhibiting Rho geranylgeranylation is responsible for the prevention of metastasis observed; however, these drugs theoretically target all post-translationally geranylgeranylated proteins in addition to Rho, and formal demonstration that prevention of Rho geranylation is specifically responsible for the effects of these inhibitors on tumor metastasis has been lacking to date.

In this study, we evaluated the effect of Rho isoprenylation on its ability to promote invasiveness. As the isoprenylation status and downstream effectors of RhoA have been the best studied of the Rho family, we concentrated our experimental efforts on this protein. We used both pharmacologic and genetic approaches to block geranylation of RhoA. We then examined the cellular distribution of RhoA after treatment with statins or mutation of a residue critical for the geranylgeranyl post-translational modification of RhoA. We then compared the effects of both statin treatment and RhoA mutation of geranylgeranyl acceptor residues on the in vivo invasiveness of human melanoma cells. Our results highlight the importance of Rho isoprenylation in
melanoma invasiveness and suggest that pharmacologic inhibition of this post-translational modification is an attractive approach for the prevention of melanoma metastasis.

MATERIALS AND METHODS

Cell lines and chemicals  Madin–Darby canine kidney (MDCK) cells were purchased from ATCC (Manassas, VA). 293T cells were a gift from Dr Charles L. Sawyer (UCLA School of Medicine). A375 cells expressing the murine cpecotropic receptor were a gift from Dr R. Hyne (Dana-Farber, Somerville, MA), and have been described previously (Clark et al, 2000). All cells were maintained in Dulbecco’s minimal Eagle’s medium with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen, Carlsbad, CA) in a 7.5% CO2 incubator.

Atorvastatin (Pfizer, Groton, CT, USA) 20 mg tablets were dissolved in methanol, deionized water or cell culture medium. Ten centiliters of cell culture medium were used at a concentration of 1 µM unless otherwise indicated. The geranylgeranyl transferase inhibitor GGTI-298 or farnesyl transferase inhibitor (FTI)-277 (both from Calbiochem, San Diego, CA) were dissolved in dimethyl sulfoxide and used at 10 µm.

Plasmids, transfections, and retroviruses  V5 epitope tagged V5-EGFP-RhoAwt and V5-EGFP-RhoAdt were engineered by polymerase chain reaction (PCR) by Dr. Claude Bruneau (University of Texas, Dallas, TX) and Dr Alan Hall (University College, London), and is deficient in GTPase activity, rendering it constitutively active. The forward primer AAGCG-GCCGCTTTCACAAGACAAGGCGCCCAGATTTTT) (V5-G14V) or (AAGCG-GCCGCTTTCACAAGACAAGGCCAGAGTTTTT) (V5-G4V) were used to generate an in-frame fusion product. The qG protein was cloned into the plasmid GGAGCAGCAAGCCGACCCAGTGGC and ligated into the pSi2 vector (Stratagene). Twenty-four hours later, cells were transfected with the indicated expression construct using the calcium phosphate method. Twenty-four hours later drugs were added as described above, and incubated for 24h. At this time, cells were washed twice with cold PBS and collected in physiologic buffer 10 mM Tris pH 7.4, 140 mM NaCl, 5 mM ethylendiamine tetraacetic acid, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 µg of aprotin in per ml, 100 µg of leupeptin per ml, and 2 µg of pepstatin per ml (500 µl per 100 mm confluent dish). The cells were homogenized in physiologic buffer by repeated passage through a 21-gauge hypodermic needle, and the lysate was centrifuged with a microcentrifuge for 10 min at 500 x g at 4°C to clear insoluble debris. Membranes and cytosolic material was obtained by ultracentrifugation at 34,000 (20,000 g) r.p.m. for 30 min at 4°C. The supernatant was designated the cytosolic fraction, and the pellets were resuspended in physiologic buffer containing 0.1% sodium deoxycholate (100 µl per 30 mm confluent dish) and designated the membrane fraction. V5-EGFP-RhoA was analyzed by electrophoretic separation of cytosolic and membranous protein pools on sodium dodecyl sulfate–12% polyacrylamide gels followed by western blotting analyses with an anti-V5 monoclonal antibody as described above. Film was scanned by a phosphorimager (Molecular Dynamics, Sunnyvale, CA). The total area under the curve in the cytosolic plus the membranous fraction was used as the denominator, and each fraction’s relative protein amount was calculated as a percentage of this total.

RESULTS

Cell fractionation  V5-EGFP-RhoAwt/dt protein levels were assessed in cytosolic and membranous pools prepared by fractionation of cellular lysates as described previously (Shimizu et al, 1997). Briefly, cells were transfected with the indicated expression construct using the calcium phosphate method. Twenty-four hours later drugs were added as described above, and incubated for 24h. At this time, cells were washed twice with cold PBS and collected in physiologic buffer 10 mM Tris pH 7.4, 140 mM NaCl, 5 mM ethylendiamine tetraacetic acid, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 µg of aprotin in per ml, 100 µg of leupeptin per ml, and 2 µg of pepstatin per ml (500 µl per 100 mm confluent dish). The cells were homogenized in physiologic buffer by repeated passage through a 21-gauge hypodermic needle, and the lysate was centrifuged with a microcentrifuge for 10 min at 500 x g at 4°C to clear insoluble debris. Membranes and cytosolic material was obtained by ultracentrifugation at 34,000 (20,000 g) r.p.m. for 30 min at 4°C. The supernatant was designated the cytosolic fraction, and the pellets were resuspended in physiologic buffer containing 0.1% sodium deoxycholate (100 µl per 30 mm confluent dish) and designated the membrane fraction. V5-EGFP-RhoA was analyzed by electrophoretic separation of cytosolic and membranous protein pools on sodium dodecyl sulfate–12% polyacrylamide gels followed by western blotting analyses with an anti-V5 monoclonal antibody as described above. Film was scanned by a phosphorimager (Molecular Dynamics, Sunnyvale, CA). The total area under the curve in the cytosolic plus the membranous fraction was used as the denominator, and each fraction’s relative protein amount was calculated as a percentage of this total.

Serum response factor (SRF) luciferase assays  293T cells at 50% confluence in 24 well plates were cotransfected with 0.1 µg of the reporter plasmid CMV-SRF-luciferase (Stratagene) and 1 µg of the pGL-SV40 promoter luciferase reporter pGL3-Basic (Promega). LacZ, and 0.1 µg of pMSCV-GFV-RhoAwt or pMSCV-GFV-RhoAdt or pMSCV GFP (totaling 0.3 µg DNA) by the calcium phosphate coprecipitation method (Stratagene). Twenty-four hours later, cells were lysed in 200 µl of cell lysis buffer (Promega). Luciferase values were obtained by analyzing 10 µl of lysate according to the standard protocol provided in the Luciferase Assay Kit (Promega), in a Lumat LB 9501 luminometer for 20 s. β-galactosidase activity was determined by the OPN€G method. Relative luciferase activity is represented as firefly luciferase value/β-galactosidase value.

Invasion assays  A375 cells were plated in rehydrated Matrigel (Becton Dickinson) chambers at a density of 5 x 10^5 per chamber with 0% serum in the top well and 5% serum in the bottom chamber with Atorvastatin added to both the top and bottom chambers as indicated or an equal volume of ethanol was added as a control. After 48 h, invaded cells were counted and the number of invaded cells was expressed in graphic form, with standard deviation from the mean of the three trials expressed as error bars. Each statin dosage was examined in triplicate wells, and all experiments were duplicated. Invasion assays with G4V Rhorwt, G4VRhoAdt, and GFP expressing A375 cell lines were conducted identically, with the exception of Atorvastatin. In parallel experiments, the effect of Atorvastatin on A375 cell viability was ascertained by use of an ethidium calcium/homodimer cytotoxicity assay (Molecular Probes, Eugene, OR) exactly as recommended by the manufacturer.
system. As seen in Fig 1, atorvastatin potently inhibited the invasion of A375 cells at a concentration that did not cause appreciable cell death.

**Isoprenylation status and subcellular localization of RhoA**

We hypothesized that geranylgeranylation was essential for the correct subcellular distribution of RhoA. To evaluate the role of the geranylgeranylation modification of RhoA in cells, we generated V5 epitope tagged GI4V (constitutively active) RhoA mutants. V5-GI4V RhoAwt contains the wild-type CAAX box (CLVL), whereas V5-GI4V RhoAdt has the C-terminal cystine mutated to an arginine (RLVL), and is thus unable to accept an isoprenoid modification.

We next tested if our V5 tagged constructs localized properly. These constructs were transiently expressed in 293T cells treated with vehicle (Con), FTI or GGTI. The amount of tagged protein in membrane and soluble fractions was quantified by western blotting, scanned, digitally quantified, and presented in graphic form. Figure 2(A) demonstrates that activated GI4V RhoAwt is predominantly in the cell membrane but is released into the cytoplasm in GGTI or statin treated cells, and is unaffected by a specific FTI (FTI-277). As expected isoprenylation-deficient Rho is recovered exclusively in the soluble cell fraction.

To visualize geranylgeranylated RhoA in living cells, we generated GFP fusions of the aforementioned GI4V (constitutively active) RhoA mutants. GFP-GI4V RhoAwt contains the wild-type CAAX box (CLVL), whereas GFP-GI4V RhoAdt has the C-terminal cystine mutated to an arginine (RLVL), and is thus unable to accept an isoprenoid modification. These constructs were expressed in MDCK cells chosen for their semicolumnar morphology allowing clear membrane visualization. As seen in Fig 2(C), a substantial portion of GFP-GI4V RhoAwt is localized in the membrane compartment. Figure 2(D,E) demonstrates the exclusion of

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**Figure 1.** Statins inhibit A375 invasion. A375 melanoma cells were incubated with the indicated concentrations of atorvastatin for 48 hours and assayed for invasion in Matrigel chambers. Error bars are ± one SD.

**Figure 2.** Cellular Localization of RhoA mutants. In Fig 2A, 293 T cells were transiently transfected with the indicated expression constructs and 24 hours later treated with EtOH (Con), Farnesyltransferase inhibitor (FTI), geranylgeranyltransferase inhibitor (GGTI) or Atorvastatin (Statin) for 24 hours. At this point cells were fractionated into soluble and membranous pools as described in Materials and Methods. Soluble (Sol) and membranous (Mem) fractions were immunoblotted with an anti V5 antibody. In Fig 2B blots were scanned and quantified as described in Materials and Methods. In Fig 2C, D, and E, pEGFP-GI4V/rhoAwt (C and D) or pEGFP-GI4Vdt (E) constructs were transfected into MDCK cells as described. Atorvastatin was added to D at 4 μM at the time of transfection. Images of live cells were obtained as described in Materials and Methods.
RhoA from the plasma membrane with either statin treatment of GFP-G14V RhoAwt (Fig 2D) or mutation of the terminal cysteine (Fig 2E). Of note, either statin treatment or ablation of the isoprenoid acceptor residue excluded activated RhoA from both the plasma membrane and the interior Golgi and endoplasmic reticular membranes. In contrast, the prenylatable GTP bound RhoA is enriched in these compartments as evidenced by the positively (GFP-G14V RhoAwt) or negatively (GFP-G14V RhoAwt plus statin or GFP-G14V RhoAdt) imaged membranous organelles.

Isoprenylation is necessary for full transcriptional response to Rho signaling As most cellular signaling is initiated at the membrane, we hypothesized that RhoA isoprenylation is necessary for full activation of its downstream effectors. To test this premise, we examined the ability of the aforementioned constructs to activate a known Rho-responsive transcriptional promoter, SRF. As seen in Fig 3, the G14V RhoAwt construct with the wild-type CAAX box strongly enhanced transcription of a SRF driven luciferase reporter construct, whereas G14V RhoAdt did so only weakly, indicating the necessity of an intact prenylation acceptor cysteine for complete activation of downstream Rho effectors.

Isoprenylation is necessary for Rho-Augmented invasive potential Given the signaling deficit observed in ungeraylatable forms of RhoA we hypothesized that an ungeraylatable RhoA would be unable to promote invasion to the extent geranylgemerylated RhoA does in A375 cells. To this end, we generated recombinant retroviruses containing V5 epitope tagged G14V (constitutively active) RhoA mutants with (V5-G14V RhoAwt) or without (V5-G14V RhoAdt) the C-terminal isoprenoid acceptor cysteine, along with an IRES GFP sequence. Viruses were pseudotyped with the MMLV envelope and used to infect dividing A375-P cells expressing GFP-G14V RhoAwt (plus statin or GFP-G14V RhoAdt) imaged membranous organelles.

DISCUSSION

A number of studies have highlighted the importance of Rho GTases in cell motility, migration, and cancer metastasis. It is well known that these proteins are post-translationally modified by geranylgeranyl moieties; however, the function this modification plays in cells remains unclear. Although a number of studies have shown that the oncogenic properties of the closely related ras proteins depend on their farnesylation state, little is known about the function of Rho prenylation. Here, we show that a constitutively active form of RhoA profoundly augments the invasion of a human melanoma cell line in vitro. Furthermore, by using carboxyterminal mutants of RhoA, we demonstrate that prenylation of RhoA is essential to its localization to cell membranes, activation of downstream effectors, and promotion of cell invasion.

The carboxyl-terminal amino acid sequence of small GTases, the CAAX box, appears to contain all the critical determinants for the specificity of their interaction with prenyl-transferases. Adamson et al (1992) showed that only the cysteine within the CAAX box is important for the correct intracellular localization of RhoA protein. Thus, to create a prenylation deficient constitutively GTP bound RhoA construct, we mutated the cysteine essential for prenyl conjugation in the CAAX box of G14V RhoA. Prenylatable G14V RhoA with the wild-type CAAX box (G14V RhoAdt) localized to both the membrane and cytoplasmic compartments of 293 and A375 cells expressing this protein. Of note, RhoA was not exclusively associated with plasma membranes as was observed in NE-115 neuronal cells (Kranenburg et al, 1997) or endothelial cells (Lesh et al, 2001). This is likely due to a combination of factors, including cell type specific handling of RhoA as well as relative expression levels achieved in each experiment. As reported previously (Allal et al, 2000), GGTTI and statins abrogated this membrane association, whereas FTI did not alter localization. These findings confirm that the recombinant RhoA used in this study is geranylgemylated. Furthermore, the CAAX to RAAX mutation (G14V RhoAdt) completely abolished membrane association. We obtained similar results in live cell microscopy experiments with MDCK cells transfected with GFP tagged versions of the wild-type and CAAX-RAAX mutated versions of G14V RhoA. The perinuclear pattern of fluorescence suggested that activated RhoA might accumulate in intracellular membranes as well as the plasma membrane as described previously (Strasheim et al, 2000). These results raise the possibility that these endocellular organelles may well represent a nidus of Rho signaling. The experiments here described, however, did not directly address this
possibility. Importantly, we also noted that treatment with a sta-
tin was sufficient to redistribute membrane-associated RhoA to the cytoplasm thereby assuming a cellular distribution indistinctly from the G4V-RhoAdt RAAX mutant. Taken together, our findings underscore the essential nature of the CAAX isoprenyl modification of RhoA for membrane localization.

The previous observation of Clark et al. (2000) that overexpression of RhoC augmented metastasis of a human melanoma cell line both in vivo and in vitro, suggested to us that Rho’s metastasis promoting activity might be dependent on its isoprenylation status. As RhoA has been found to be purely geranylgeranylated and no such information exists for RhoC, we chose to focus our efforts on RhoA isoprenylation. To this end, we generated cell lines expressing GFP RhoAwt and G4V RhoAdt from the identical parental strain of A375 human melanoma cells as in Clark et al., and assayed ability to transverse a Matrigel invasion chamber. A375 cells expressing G4V RhoAwt were dramatically more invasive than GFP expressing controls. As predicted, expression of G4V RhoAdt failed to augment the invasive ability of A375 cells. These experiments demonstrate that G4V RhoA overexpression confers an invasive phenotype to human melanoma cells and that its potential to do so is dependent on geranylgeranylation modification.

These studies extend the growing body of literature suggesting a role for the Rho family of proteins in the invasion and metastasis of tumor cells. Overexpression of RhoA and RhoC have been shown to correlate with a poorer prognosis in colorectal (Yoshioka et al., 1999) and breast (Fritz et al., 1999) carcinoma. Furthermore, a large randomized clinical trial recently found that statins may prevent mortality due to malignancy (Pedersen et al., 2000). Taken together these studies suggest that the isoprenylation step of Rho activation plays a crucial part in the in vivo promotion of metastasis.

The work described herein represents, to our knowledge, the first formal demonstration that isoprenylation deficient Rho proteins are incapable of promoting invasion of human cancer cells. Rho overexpression in human cancers likely initiates remodeling of the actin cytoskeleton through its downstream effectors ROCK and mDia. This reorganization in turn alters the tumor cells affinity for neighboring cells through integrin and cadherin based adhesions. We show herein that abrogation of the isoprenylation step of RhoA affects its localization to cellular membranes. This disruption of Rho localization may modulate its interaction with upstream and downstream signaling components, thereby attenuating Rho’s ability to promote invasion. Although further in vivo work is required, our data strongly suggest that therapeutic strategies targeting Rho isoprenylation have the potential to inhibit tumor cell metastasis.

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REFERENCES

Sun J, Qian Y, Hamilton AD, Sehbi SM: Both farnesyltransferase and geranylgeranyltransferase I inhibitors are required for inhibition of oncogenic K-Ras prenylation but each alone is sufficient to suppress tumor growth in nude mouse xenografts. Oncogene 16:1467–1473, 1998