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Evidence that Farnesyltransferase Inhibitors Suppress Ras Transformation by Interfering with Rho Activity

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Small-molecule inhibitors of the housekeeping enzyme farnesyltransferase (FT) suppress the malignant growth of Ras-transformed cells. Previous work suggested that the activity of these compounds reflected effects on actin stress fiber regulation rather than Ras inhibition. Rho proteins regulate stress fiber formation, and one member of this family, RhoB, is farnesylated in vivo. Therefore, we tested the hypothesis that interference with RhoB was the principal basis by which the peptidomimetic FT inhibitor L-739,749 suppressed Ras transformation. The half-life of RhoB was found to be ~2 h, supporting the possibility that it could be functionally depleted within the 18-h period required by L-739,749 to induce reversion. Cell treatment with L-739,749 disrupted the vesicular localization of RhoB but did not effect the localization of the closely related RhoA protein. Ras-transformed Rat1 cells ectopically expressing N-myristylated forms of RhoB (Myr-rhoB), whose vesicular localization was unaffected by L-739,749, were resistant to drug treatment. The protective effect of Myr-rhoB required the integrity of the RhoB effector domain and was not due to a gain-of-function effect of myristylation on cell growth. In contrast, Rat1 cells transformed by a myristylated Ras construct remained susceptible to growth inhibition by L-739,749. We concluded that Rho is necessary for Ras transformation and that FT inhibitors suppress the transformed phenotype at least in part by direct or indirect interference with Rho, possibly with RhoB itself.

Isoprenylated proteins, which constitute ~0.5% of the proteins in the cell, fall into two classes that are characterized by the presence of either farnesyl (C_{15}) or geranylgeranyl (C_{20}) isoprenoids (for reviews, see references 18 and 32). Isoprenylation is required for the biological function and efficient membrane association of these proteins, possibly because of a role of the isoprenyl groups in protein-protein interactions (33). The signal for isoprenyl modification is located in the C terminus of the polypeptide and dictates not only the specificity of the isoprenoid but also further modifications which occur.

The housekeeping enzyme farnesyltransferase (FT) is one of at least three isoprenyl-protein transferase activities that have been identified (for reviews, see references 5, 10, 35, and 48). FT recognizes on its substrates a C-terminal CAAX motif (where C is cysteine, A is typically an aliphatic amino acid, and X is any amino acid) and catalyzes transfer of the C_{15} isoprenoid from farnesyl PP_i to the CAAX cysteine. The farnesylated product is a substrate for additional modification by enzymes which proteolyze the terminal three amino acids and carboxymethylate the new C terminus. Some farnesylated proteins (e.g., H-Ras) are further modified by the addition of palmitate to an additional cysteine(s) that is proximal to the CAAX motif. In these cases, palmitylation appears to increase the avidity of membrane association (20) but is dispensable for biological activity (26).

Since FT is responsible for farnesylating oncogenic Ras (8), small-molecule inhibitors of FT were sought as possible cancer therapeutics (16, 17). Recently, FT inhibitors have been demonstrated to revert the malignant phenotype of Ras-transformed cells in vitro and in vivo without toxic or anti-proliferative effects on normal cells (15, 24, 28, 28a, 29, 32a). These results present some question as to the biological mechanism of action, since Ras and other farnesylated proteins (e.g., lamin B [14]) are critical for normal cell growth and function. Our previous work revealed that the kinetics of reversion induced by one FT inhibitor, L-739,749, were too rapid to be explained easily by suppression of Ras activity. Instead, the inhibitory effects of L-739,749 correlated with effects on the regulation of actin stress fiber formation (39).

We tested the hypothesis that a putative stress fiber regulator, RhoB, played a role as a direct or indirect target in the mechanism by which L-739,749 reverted Ras-transformed cells. RhoB is a member of the Rho protein family which is implicated in the regulation of various cytoskeletal actin structures (19). Although Rho proteins are typically geranylgeranylated, RhoB exists in both geranylgeranylated and farnesylated forms in vivo (1) and therefore presented a potential target for FT inhibition. RhoB also has a connection to cell growth control, since it had been identified previously as an immediate-early response gene for epidermal growth factor and the v-Src oncogene (23). An important prediction of our hypothesis was that genetic inhibitors of RhoB (dominant inhibitory mutants) should mimic pharmacological inhibitors of FT in their ability to inhibit Ras transformation. We have demonstrated elsewhere that this is the case (42) and report here that the effects of L-739,749 can be ablated by ectopic expression of farnesyl-independent forms of RhoB.

MATERIALS AND METHODS

Plasmid constructs. Standard PCR mutagenesis techniques were used to introduce an N-terminal hemagglutinin (HA) epitope tag into cDNAs encoding rat RhoB (23) or human RhoA (53). For RhoA, the construct was generated from a glutathione *S*-transferase fusion gene (46). N-terminal epitope tags of 10 amino acids do not affect the ability of Rho proteins to induce actin stress fibers (2) or affect cell growth regulation (42). The primers used to generate HA-tagged RhoB have been described elsewhere (42). The primers used to generate HA-tagged RhoA were hem-rhoA5′ (GGAAAGCTTGCTCATGTACCCATAC GATGTGCCAGACTATGCTGCCATCCGG AAG) and rhoA3′ (GAGGG

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ATCCCTGTGCTTGCAGCAAGGTTTC). The HA-specific antiserum 12CA5 has been described elsewhere (36). The myristylation signal in Myr-rhoB^{V14} was derived from the N-terminal 16 residues of the v-Src oncoprotein and was identical to the signal used to generate a myristylated form of H-Ras that was membrane bound and biologically active (13). Myr-rhoB^{V14} was generated in two steps by PCR from HA-RhoB^{V14} (42) by using the primer pair Myr rhoB 5′ (GGAAGATCTACCATGGGGAGTAGCAAGAGCAAGCCTAAGGACCC CAGCCAGCGCCGGATGGCGGCCATCCGCAAGAAG) and rhoB 3′-S KVL (GGAAGATCTTTCATAGCACCTTGCAGCAGTTG). A T37A effector mutation (37) was added to create Myr-rhoB^{V14A37} by using the Myr rhoB^{V14} gene construct as a target for PCR mutagenesis with another pair of complementary oligonucleotides, the sense one of which was CCCGAAGTGTACGT GCCCGCCGTGTTCGAGAACTATG. A disabling point mutation in the myristylation acceptor site (G2A) to make Myr^{G2A}-rhoB^{V14} used the primer pair Myr(GA) (GGAAGATCTACC ATGGCGAGTA GCAAGAGCAAGC) and rhoB 3′-SKVL.

For the COS immunofluorescence and Rat1 cell population experiments, gene constructs were expressed from the cytomegalovirus enhancer-promoter in pcDNAI/neo (Invitrogen), which contains a simian virus 40 origin of replication and a dominant selectable neomycin resistance gene. For the Rat1/ras cell experiments, gene constructs were expressed from the same enhancer-promoter in hygCMV (a gift of D. Defeo-Jones), which contains a dominant selectable *hph* gene allowing selection for hygromycin.

Cell culture. Rat1, Rat1/ras, and COS cells were cultured in Dulbecco modified Eagle medium containing 10% fetal calf serum (Sigma) and 50 U of penicillin-streptomycin (Fisher) per ml. The Ras-transformed cell line Rat1/ras has been described elsewhere (28). The cells were transfected by a modified calcium phosphate precipitation method (9). Rat1 focus assays were performed by transfecting $\sim 5 \times 10^5$ cells with 10 μg of plasmid DNAs and passaging the cells in triplicate (1:6) the day after transfection. Cell foci were scored 14 or 28 days posttransfection by fixing with methanol and staining with crystal violet. To generate cell populations stably expressing various RhoB constructs, >50 colonies that were selected for by culturing transfected cells in medium containing 400 μg of G418 (Gibco/BRL) per ml were pooled and grown in mass culture. The assays for growth in normal or low-serum medium and in soft agar culture have been described elsewhere (11).

Rat1/ras cell lines expressing various Myr-rhoB constructs were generated by selecting transfected cells in 40 μg of hygromycin B (Calbiochem) per ml and selecting colonies for ring cloning and expansion into mass culture. For drug experiments, $\sim 10^5$ cells were treated in monolayer culture or $\sim 10^4$ cells were treated in soft agar culture with 25 μM L-739,749, which is 10- to 25-fold the MIC required to suppress anchorage-independent growth or to induce morphological reversion of Rat1/ras cells, respectively (39). Assays for FT activity in L-739,749-treated cells were performed as described elsewhere (38, 39).

Metabolic labeling. For the pulse-chase experiment, Rat1 and Rat1/ras cells were labeled for 2 h with 100 μCi of [^{35}S]methionine (Amersham) per ml in Dulbecco modified Eagle medium lacking methionine (Gibco/BRL) that was supplemented with 2% fetal calf serum (Sigma). Cells were washed in phosphate-buffered saline and then normal growth medium was added to chase the [^{35}S]methionine label for various times. At the desired intervals, cells were harvested and lysed in Nonidet P-40 lysis buffer containing the protease inhibitors leupeptin, aprotinin, antipain, and phenylmethylsulfonyl fluoride (21). For the mevalonate labeling experiments, Rat1, Rat1/ras, or COS cells were treated 5 h with L-739,749 and labeled for the last 4 h of this period with 50 μCi of [^{3}H]-RS-mevalonolactone (NEN) per ml in normal growth medium. At the end of this interval, cell lysates were prepared as described above.

Immunoprecipitation. Cell lysates were clarified for 10 min in a refrigerated microcentrifuge and quantitated by the Bradford assay. One milligram of protein per sample was precleared with protein G-Sepharose (Pharmacia) in 1 ml of Nonidet P-40 buffer for 30 min at 4°C and then immunoprecipitated for 1 h on a nutator shaker. A rabbit anti-RhoB antiserum (1) was used for the pulse-chase experiment. For the mevalonate labeling experiments, Ras monoclonal antiserum Y13-259, a rabbit anti-Rac1 antiserum (Santa Cruz Biotechnology), and monoclonal anti-HA antiserum 12CA5 (36) were used. Immunoprecipitates were washed four times with Nonidet P-40 buffer, fractionated on sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) gels, and analyzed by fluorography. Exposure times were 1 and 10 to 35 days for ^{35}S- and ^{3}H-labeling experiments, respectively.

Cell immunofluorescence. COS cells were transfected with 10 μg of plasmid DNA and processed for indirect immunofluorescence 24 to 48 h posttransfection as described previously (43), with 2.5 μg of monoclonal anti-HA antiserum 12CA5 (36) or 1 μg of rabbit anti-RhoB antiserum (Santa Cruz Biotechnology) per ml. The secondary antiserum was fluorescein isothiocyanate-conjugated goat anti-mouse or anti-rabbit antiserum used at a 1:1,000 dilution (Cappel). Images from stained cells were gathered on a Leitz confocal microscope apparatus. The figures were generated with Photoshop 2.5.

RT-PCR. Total cytoplasmic RNA was isolated from cell lines as described elsewhere (41). The presence of exogenous Myr-rhoB RNA was verified by using a reverse transcriptase PCR kit (Gibco/BRL) and then by agarose gel electrophoresis of the products. Briefly, 3 μg of RNA and 100 ng of RhoB-specific primer (CAGCTCCGTGCGGACATGCAC) in 20 μl were incubated at 70°C for 5 min and placed on ice, and the mixture was brought to 1× first-strand buffer–10 mM dithiothreitol–1.25 mM deoxynucleoside triphosphates–200 U of Moloney murine leukemia virus RT. The annealed reaction mixture was incubated for 1 h at 42°C, and then PCR was performed with 5 μl of the RT product in 1× *Taq* buffer–1.5 mM $MgCl_2$–0.25 mM deoxynucleoside triphosphates–200 ng each of the RhoB-specific primer and the primer CCCAGCCAGCGCCG GATGGCG, which contained sequences unique to the myristylation signal in the Myr-rhoB constructs. Polymerization reactions were cycled 50 times by using the protocol 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR products were analyzed on 2% NuSieve agarose gels and photographed.

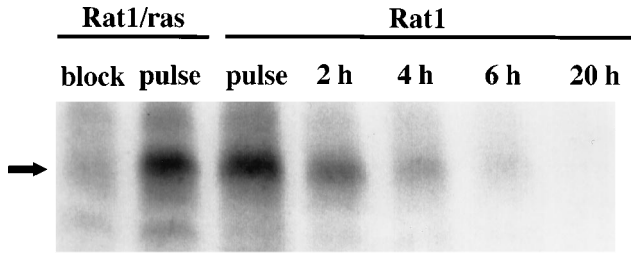

FIG. 1. RhoB has a short half-life. Extracts from cells pulsed for 2 h with 100 μCi of [^{35}S]methionine per ml and then chased with normal medium for the periods indicated were subjected to immunoprecipitation with an antipeptide antiserum specific for RhoB (1). Immunoprecipitates were analyzed by SDS-PAGE and fluorography. block, antiserum blocked by a ~50-fold molar excess of specific peptide before immunoprecipitation.

RESULTS

RhoB has a short half-life. Since fully modified Ras is stable (half-life, 24 h [50]), several days would need to pass before it was functionally depleted from cells treated with L-739,749. However, we observed that L-739,749 induced reversion of Ras-transformed cells within 18 to 24 h of cell treatment, a period in which steady-state Ras levels dropped by only ~50% (39). We reasoned that if L-739,749 acted by altering the activity of a protein which influenced actin regulation, then in the simplest scenario this protein should have a short half-life, so that it could be depleted within the 18- to 24-h period which elapsed before reversion was observed.

The turnover rate of RhoB was examined by a pulse-chase experiment in cells labeled with [^{35}S]methionine. Briefly, cells were pulse-labeled for 2 h and chased in normal medium for various times, and then cell extracts were prepared for immunoprecipitation with an anti-RhoB peptide antiserum (1) and analyzed by SDS-PAGE (Fig. 1). A RhoB polypeptide of the predicted size was immunoprecipitated from both Rat1 and Rat1/ras cells, a Ras-transformed Rat1 cell line (28), and antiserum preblocked with a molar excess of specific peptide failed to precipitate it. In Rat1 cells, RhoB decayed with a half-life of approximately 2 to 3 h. A similar turnover rate was observed in Rat1/ras cells (data not shown), indicating that Ras transformation did not affect RhoB half-life. The rapid turnover rate was consistent with the identification of RhoB as an immediate-early response gene for v-*src* and epidermal growth factor (23), since such genes typically encode short-lived mRNAs and/or proteins. We concluded that RhoB had a short half-life and that it could be functionally depleted within the period required by L-739,749 to revert Ras-transformed cells.

N myristylation of RhoB renders it resistant to changes in cell localization induced by L-739,749. The RhoB C terminus undergoes a series of posttranslational modifications in vivo, including addition of either farnesyl (C_{15}) or geranylgeranyl (C_{20}) isoprenoids to the CAAX cysteine and palmitylation of two cysteines that are immediately proximal (1). Mutation of the CAAX cysteine residue (C193S) destroys C_{15} or C_{20} isoprenyl addition, as well as the subsequent palmitylation steps (1), and eliminates localization to a cytoplasmic vesicle com-

partment (2). On the basis of these results, we postulated that FT inhibition might interfere with the normal localization pattern of RhoB but not with RhoA, which is 88% identical but solely geranylgeranylated, or with RhoB constructs whose membrane association was engineered to be independent of farnesylation.

We used L-739,749, which is a potent and specific peptidomimetic inhibitor of FT (29), to assay the effects of FT inhibition on the cell localization patterns of several constructs expressed transiently in COS cells. Localization was monitored by indirect immunofluorescence. RhoB-S, which contains the CAAX mutation C193S, was used to verify that the pattern of wild-type localization depended on isoprenylation. RhoA, which is 88% identical to RhoB except that it is modified solely by geranylgeranyl isoprenoid (1, 25), was included as a negative control since it would be predicted to be unaffected by L-739,749. We also examined the localization of a farnesyl-independent construct, Myr-rhoB^{V14}, that contained the disabling CAAX mutation C193S and instead a signal for N-myristylation derived from the v-Src oncoprotein. This Myr-rhoB construct was predicted to exhibit a pattern of localization that was unaffected by L-739,749. Transfected cells were treated for 18 h with L-739,749 or methanol vehicle beginning at 30 h posttransfection and were then processed for indirect immunofluorescence.

L-739,749 was observed to disrupt the localization of RhoB but not that of RhoA or Myr-rhoB^{V14} (Fig. 2). In the absence of the drug, RhoB staining was cytoplasmic but largely confined to a vesicular compartment that has been seen previously (2, 42). In contrast, L-739,749-treated cells exhibited a dense punctate staining spread throughout the cytoplasm and the periphery of the nucleus. This pattern appeared to be different than that exhibited by the completely unmodified RhoB-S construct, suggesting the possibility that geranylgeranylated forms of RhoB persisted in drug-treated cells and were responsible for the unique staining pattern seen there (see below). RhoA staining was seen in the cytoplasm and the plasma membrane, as observed previously (2). Myr-rhoB^{V14} staining was confined to a cytoplasmic vesicle compartment similar to wild-type RhoB. However, neither the RhoA nor the Myr-rhoB^{V14} staining patterns were as significantly affected by L-739,749. We concluded (i) that L-739,749 interfered with the normal localization of RhoB but not RhoA and (ii) that N myristylation was sufficient to localize RhoB to cytoplasmic vesicles in a relatively L-739,749-insensitive manner.

L-739,749 has been shown to selectively inhibit FT in vitro (29). We performed two metabolic labeling experiments with [^{3}H]mevalonate (an isoprenoid precursor) to verify that L-739,749 did not affect geranylgeranylation in vivo. In the first experiment, Rat1 and Rat1/ras cells were metabolically labeled for 4 h in the presence or absence of 10 μM L-739,749, and cell extracts were prepared for immunoprecipitation by using antiserum to Ras or to Rac1, a Rho family protein which is modified solely by C_{20} isoprenoid (Fig. 3A). In the second experiment, COS cells were transiently transfected with RhoB or RhoA constructs and 42 h later were labeled for 4 h in the presence or absence of the drug as before (Fig. 3B). As expected, L-739,749 blocked the labeling of Ras, which is modified solely by farnesyl isoprenoid, but had no effect on the labeling of Rac1, RhoA, or RhoB. The inability of L-739,749 to block RhoB labeling was expected since RhoB is modified by both farnesyl and geranylgeranyl isoprenoids in vivo (1) and geranylgeranylation would mask any loss of RhoB farnesylation. This experiment ruled out the trivial possibility that L-739,749 affected the localization of RhoB by inhibiting modification by both C_{15} and C_{20} isoprenoids.

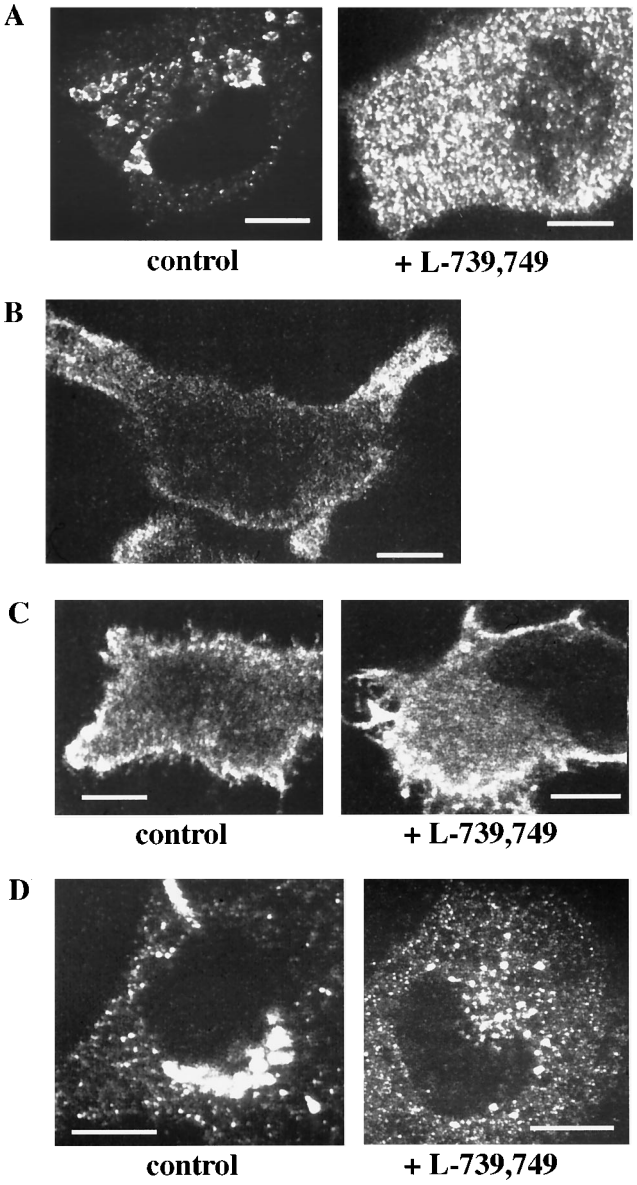

FIG. 2. L-739,749 affects the cell localization of RhoB but has significantly less effect on RhoA or an N-myristylated RhoB construct. RhoB, RhoB-S, and RhoA constructs included an N-terminal HA epitope tag used for immunological detection (see reference 42 and Materials and Methods). COS cells were transiently transfected with the indicated constructs and were treated 30 h later for 18 h with 25 μM L-739,749 or methanol vehicle and then processed for indirect immunofluorescence. The cells shown in panels A to C were stained with anti-HA antiserum, while those shown in panel D were stained with an anti-RhoB peptide antiserum. Under the experimental conditions used, only the transfected construct is seen in panel D. An image containing cytosol and nuclei from at least one cell is shown in each panel; nuclei are seen as darker areas. The scale bar in each figure is ~10 μm. (A) Wild-type RhoB; (B) RhoB-S (no L-739,749 added); (C) RhoA; (D) Myr-rhoB^{V14}.

Ras-transformed cells ectopically expressing myristylated RhoB are resistant to L-739,749-induced growth inhibition and morphological reversion. If RhoB or a related Rho family protein were necessary for Ras to maintain the transformed phenotype, then Ras-transformed cells ectopically expressing farnesyl-independent forms of RhoB would be resistant to reversion by L-739,749. To test this prediction, we generated derivatives of the Rat1/ras cell line which expressed farnesyl-

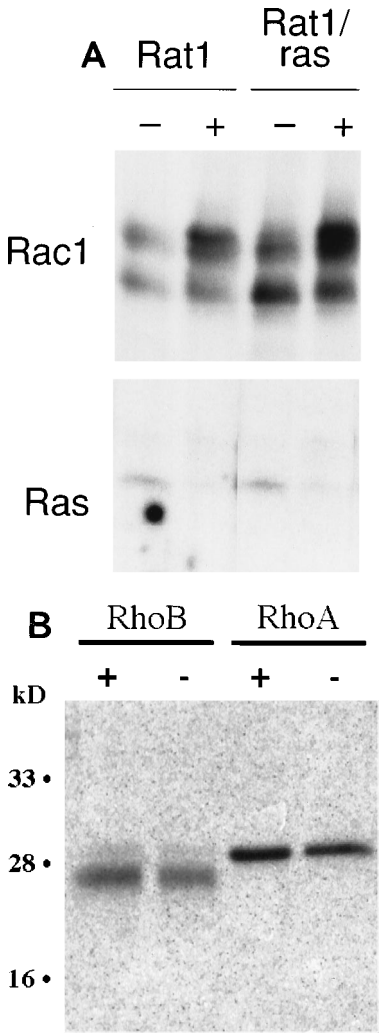

FIG. 3. L-739,749 does not affect geranylgeranylation in vivo. Cells were treated for 5 h with L-739,749 or methanol vehicle and were labeled during the last 4 h of this period with 50 μCi of [^{3}H]RS-mevalonolactone per ml. Cell extracts were prepared for immunoprecipitation and were analyzed by SDS-PAGE and fluorography. (A) Rat1 and Rat1/ras cell lines (10 μM L-739,749 was used); (B) transiently transfected COS cells. The cells were transfected with the RhoB or RhoA constructs as described in the legend to Fig. 2 and were treated with 25 μM L-739,749.

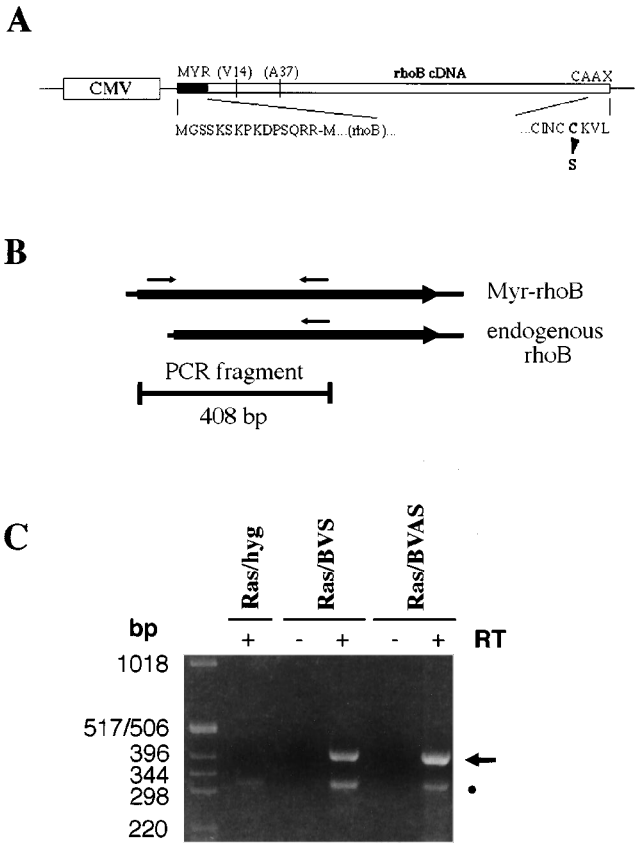

FIG. 4. Myristylated RhoB gene constructs and expression. (A) Gene constructs. CMV, human cytomegalovirus enhancer-promoter region; MYR, 16-amino-acid myristylation signal from v-Src N terminus; ^{186}CKVL→SKVL, mutation which destroys the CAAX cysteine acceptor site for isoprenylation (1); G14V, mutation which compromises the Rho GTPase and activates Rho signaling function (46); T37A, mutation which eliminates the effector function of Rho proteins (37). (B) Predicted RT-PCR product. The top line represents the exogenous Myr-rhoB RNA which gives a predicted 408-bp product; the bottom line is the endogenous message which will not anneal to the MYR-specific primer. Small arrows indicate the relative locations of the primers. (C) RT-PCR results. An ethidium bromide-stained 2% agarose gel containing RT-PCR products is shown (see Materials and Methods). + and −, the products of PCR performed with material from reverse transcription reactions in the presence (+) or absence (−) of RT. Bands that are diagnostic for expression of Myr-rhoB genes are indicated with an arrow, while a dot designates a nonspecific product.

independent Myr-RhoB constructs whose membrane localization depended on N-terminal myristylation instead of C-terminal isoprenylation (Fig. 2).

The set of Myr-rhoB constructs used in these experiments are outlined in Fig. 4A. Each contained the v-Src N-myristylation signal and the CAAX mutation C193S, which destroys isoprenylation and palmitylation in vivo (1). Myr-rhoB^{V14} included an activating mutation to compensate for the possibility of inefficient localization to the same vesicular compartment as wild-type RhoB. Myr-rhoB^{V14A37} included an effector mutation that eliminates stress fiber-inducing activity (37) to demonstrate that Rho activity was necessary for any biological effects of the constructs.

Rat1/ras cell lines stably expressing these constructs were generated, and RT-PCR was performed to analyze gene expression in representative cell lines (Fig. 4B and C). To assay the effects of FT inhibition in these cell lines, we monitored the morphology of cells in monolayer culture at 48 h after drug addition and colony formation of cells in soft agar culture at 12 to 14 days after treatment with L-739,749 or methanol vehicle (control), as previously described (39). The L-739,749 concentrations used for these experiments were 10- to 25-fold those required to suppress soft agar growth and induce morphological reversion, respectively (29, 39). The results of the experiments with these cell lines are shown in Table 1 and Fig. 5.

As expected, the growth and transformed morphology of Ras/hyg control cells containing only empty plasmid vector were suppressed by L-739,749, similarly to the parental Rat1/ras cells (39). In contrast, Ras/BVS cells expressing Myr-rhoB^{V14} retained their transformed morphology and ability to grow in soft agar following drug treatment (Fig. 5). Cells expressing wild-type forms of Myr-rhoB exhibited an intermediate response, with three of five lines exhibiting complete or partial resistance to L-739,749. The stronger effect of Myr-rhoB^{V14} was due not solely to RhoB activation, because L-739,749 still inhibited cell lines expressing normally isoprenylated RhoB^{V14}. Rho activity was required for the Myr-rhoB

TABLE 1. Effects of L-739,749 on Rat1/ras cell lines stably expressing various Myr-rhoB constructs[a]

Construct expressed in Rat1/ras cells (*n*)	Effect of L-739,749		
	Inhibition of FT activity[b]	Inhibition of transformed morphology	Inhibition of soft agar growth
Vector only (2)	++	++	++
Myr-rhoB (wild type) (5)	++	+/−[c]	+/−[c]
Myr-rhoB^{V14} (4)	++	−	−
Myr-rhoB^{V14A37} (3)	ND	++	++
RhoB^{V14} (4)	ND	+	++

[a] Rat1 cells were transfected with the Myr-rhoB constructs indicated, and clonal cell lines were isolated as described in Materials and Methods. Cells were treated with 25 μM L-739,749 or methanol vehicle in assays for FT inhibition, morphological reversion, and soft agar culture colony formation, all as previously described (39). Phenotypes were scored relative to the vector-only control lines. *n*, number of cell lines tested; ND, not done.

[b] FT enzyme assays were performed with extracts from cells treated for 4 h with L-739,749 as described previously (38, 39).

[c] Three of five lines exhibited complete or partial resistance to L-739,749.

protective effect, because the T37A mutation that eliminates Rho effector function abolished drug resistance. The requirement for myristylation was verified by showing that a point mutation at the myristyl acceptor site in the v-Src signal (51) eliminated Myr-rhoB^{V14}-induced drug resistance (data not shown). L-739,749 inhibited FT activity to a similar degree in both sensitive and resistant cell lines (Table 1), proving that Myr-rhoB-induced resistance was not due to inefficient access to or suppression of endogenous enzyme activity. Titration of L-739,749 indicated that the resistance induced by Myr-rhoB^{V14} was not complete but that the response curve was shifted ~100-fold (data not shown). This result suggested either that Myr-rhoB^{V14} substituted a farnesylated RhoB function with less than complete efficiency or that inhibition of another farnesylated protein(s) was involved in the mechanism of L-739,749-induced reversion. We concluded that loss or change of Rho activity, possibly in RhoB itself, played a necessary role in the suppression of Ras transformation by L-739,749.

Myr-RhoB is not oncogenically activated by myristylation. N-terminal myristylation of Ras results in its oncogenic activation (7, 30). Since RhoB is induced by cell growth activators (23) and by itself can exert some growth-stimulating activity in NIH 3T3 cells (42), we were concerned that myristylation of RhoB might act as an oncogenic gain-of-function mutation which indirectly caused drug resistance. To rule out this possibility, the effects of Myr-rhoB^{V14} in transformation and cell growth assays were examined. Focus formation assays were used to test Myr-rhoB^{V14} on its own or to determine whether it was similar to RhoB in its ability to augment Ras activity (42). By themselves, wild-type RhoB, activated RhoB^{V14}, Myr-rhoB^{V14}, or empty vector plasmids all lacked focus-forming activity in Rat1 cells, in contrast to oncogenic Ras plasmid, when monitored up to 4 weeks after transfection (Fig. 6A). For the augmentation assay, Rat1 cells were transfected with 10 μg of oncogenic Ras and 10 μg of RhoB^{V14}, Myr-rhoB^{V14}, or empty vector plasmids. In a manner similar to that seen previously (42), RhoB^{V14} stimulated focus formation 218% relative to the empty vector control. Myr-rhoB^{V14} more weakly augmented Ras-dependent foci, to only 153% of the control, arguing that its protective effect against L-739,749 was not due to an abnormal collaborative effect with Ras.

To further assess the effect of Myr-rhoB^{V14} on cell growth, Rat1 fibroblast populations stably expressing it or the other constructs were tested for changes in morphology, growth in normal or low-serum medium, and anchorage-independent growth capability (Fig. 7; Table 2). Cells transfected with each expression plasmid, which also contained a neomycin resistance gene, were selected in G418-containing medium, and at least 50 G418-resistant colonies were pooled for analysis. Expression of the constructs in each cell population was verified by Northern (RNA) analysis, immunoprecipitation, and/or cell immunofluorescence (data not shown). Each construct exhibited weak growth-stimulatory activity in a manner similar to what was seen previously with NIH 3T3 cells (42). Cells expressing Myr-rhoB^{V14} were observed to be similar to the other Rho proteins in their ability to induce higher saturation densities and weak outgrowth under low-serum and reduced-anchorage conditions. We concluded that N-terminal myristylation did not significantly affect RhoB activity with regard to cell growth control.

Cells transformed by Myr-ras constructs remain susceptible to inhibition by L-739,749. We observed previously that L-739,749 elicited changes in cell phenotype faster than could be explained by suppression of steady-state farnesylated Ras levels, suggesting that reversion was not caused by loss of Ras activity (39). To directly test this idea, we examined the effect of L-739,749 on the anchorage-independent growth of Rat1/Myr-ras cells, a Rat1 cell line generated by transformation with an N-myristylated Ras construct (13). In contrast to Ras/BVS cells, Rat1/Myr-ras cells remained susceptible to growth inhibition by L-739,749 (Fig. 8). This result argued that inhibition of the activity of farnesylated proteins other than Ras was a critical part of the drug mechanism, consistent with a role for RhoB. The inhibitory effect on Rat1/Myr-ras cells was partial relative to that on Rat1/ras cells (data not shown); however, since myristylation activates the oncogenic potential of Ras by itself (7, 30), the synergistic effect of myristylation and Ras mutation might explain the less-potent inhibitory effect. Alternately, the weakly protective effect of farnesyl-independent Ras could indicate a secondary role for Ras as an L-739,749 target. We concluded that loss of Ras function was not a major component of the mechanism by which L-739,749 suppressed Ras transformation.

DISCUSSION

Our results supported the hypothesis that the action of FT inhibitors may be explained by interference with the activity of RhoB or a functionally related protein. RhoB was found to have a short half-life, allowing it to be functionally depleted from drug-treated cells with kinetics paralleling those required for cell reversion. Drug treatment disrupted the cell localization of RhoB, which is consistent with the possibility that it is a target for inhibition in Ras-transformed cells. Finally, myristylated forms of RhoB (but not Ras) had a strong protective effect against drug treatment, arguing that interference with RhoB or a related protein was a critical part of the drug mechanism. These results support other work arguing that Rho is necessary for Ras transformation and suggest differences in the manner in which normal and transformed cells may use Rho functions.

RhoB is a short-lived protein whose cell localization is affected by FT inhibition. The finding that RhoB has a short half-life may help explain the kinetics of L-739,749-induced reversion of Ras-transformed cells (39), which are too rapid to be accounted for simply by a loss of membrane-associated Ras protein. The 2- to 3-h half-life of RhoB indicates that >95% of vesicle-bound protein could be depleted within 18 h to 24 h, the period of cell treatment required before phenotypic rever-

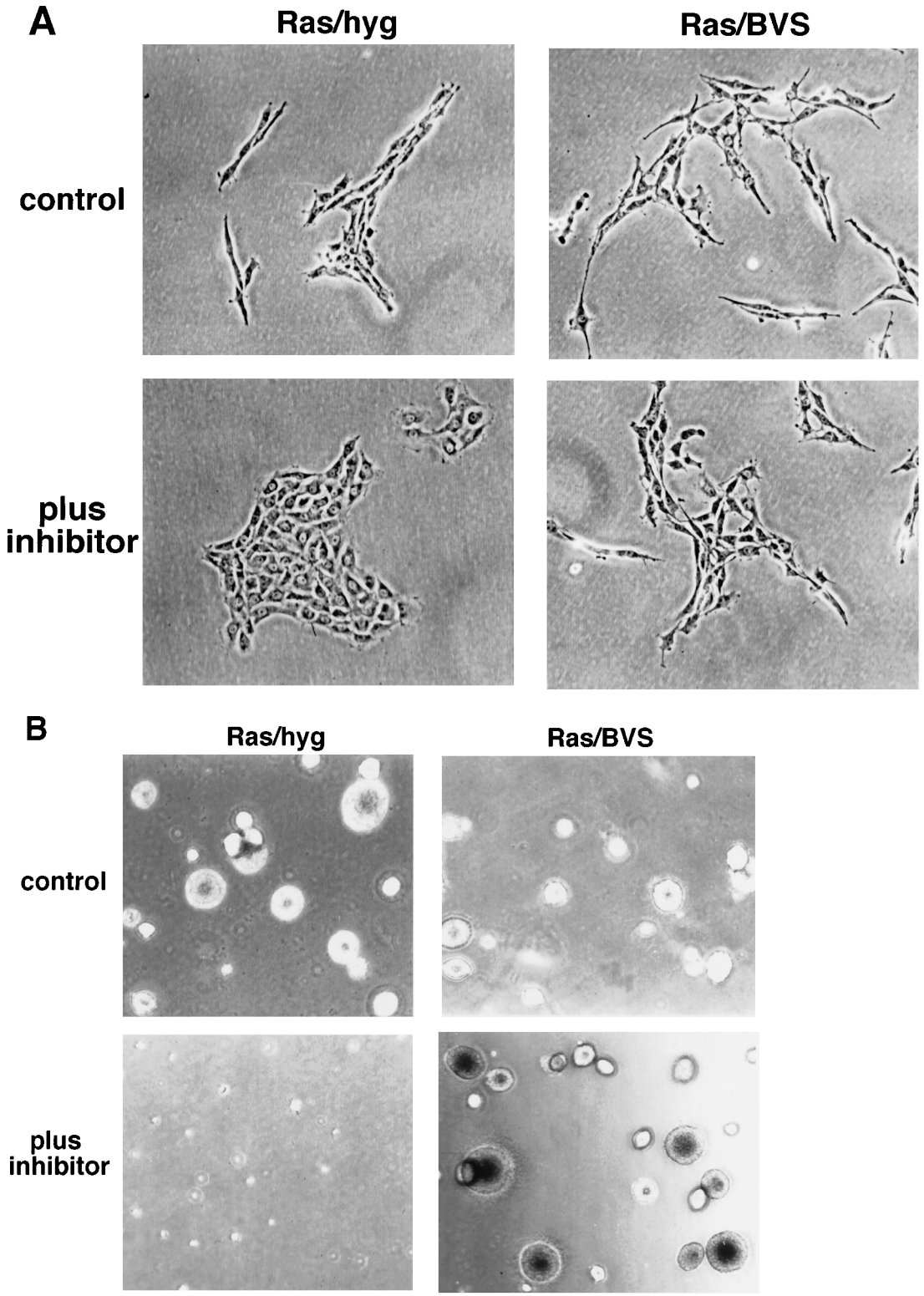

FIG. 5. Rat1/ras cells expressing Myr-RhoB are resistant to treatment with L-739,749. (A) Morphological reversion assay. Cells containing plasmid vector only (Ras/hyg) or expressing Myr-rhoB^{V14} (Ras/BVS) were seeded overnight, treated for 48 h with 25 μM L-739,749 (plus inhibitor) or an equivalent quantity of methanol vehicle (control), and photographed. (B) Soft agar culture growth assay. The same cells were seeded into soft agar culture with 25 μM L-739,749 or methanol vehicle, and the colonies were photographed 12 days later.

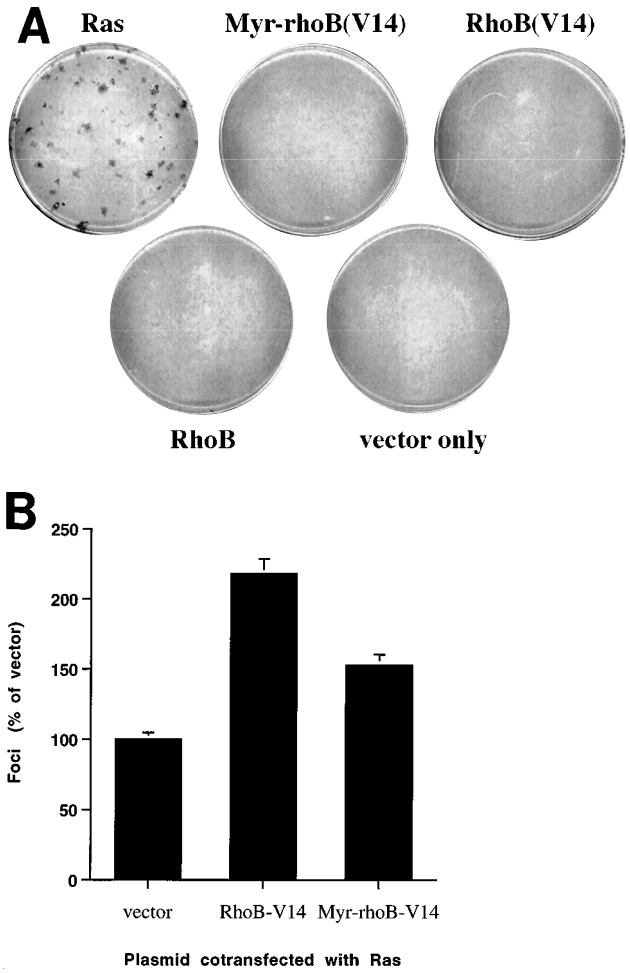

FIG. 6. Myr-rhoB resembles RhoB in transformation assays with or without Ras. (A) Focus formation assay. Cells were transfected with 10 μg of the constructs indicated, and transformed cell foci were scored 4 weeks later (Ras-transfected cells were scored 2 weeks later). (B) Rho stimulation of Ras-dependent transformation. Cells were transfected with 10 μg of oncogenic Ras and 10 μg of the constructs indicated, and transformed foci were scored 2 weeks later. Four trials were performed, three of which were scored in triplicate.

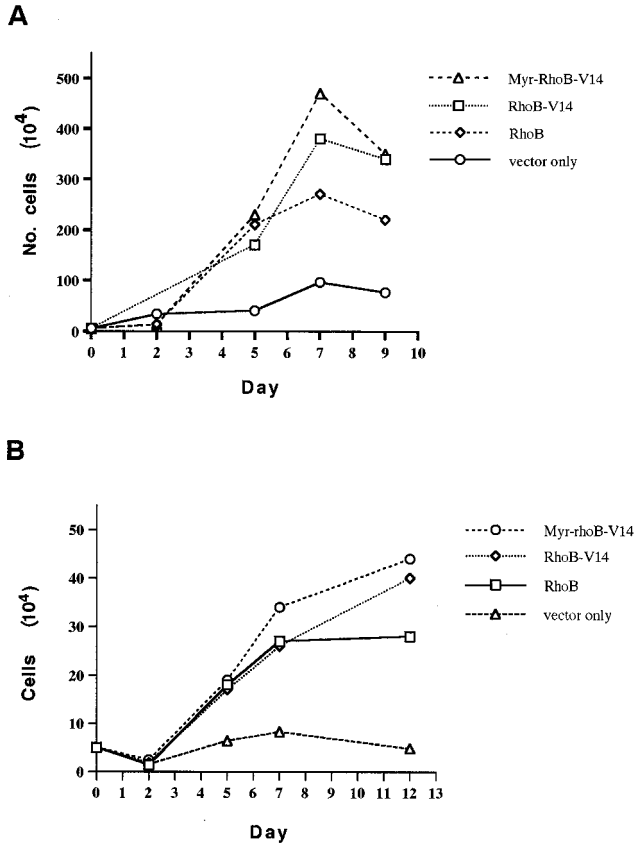

FIG. 7. Similar effects of Myr-rhoB and RhoB on the growth of Rat1 cells. A total of 5×10^4 cells were seeded on day 0, and the cells were counted at the intervals indicated. (A) Growth curve of cells carried in normal medium; (B) growth curve of cells carried in medium containing 0.5% fetal calf serum.

sion begins to be detectable. Since most farnesylated proteins in the cell are more stable (e.g., the half-life of Ras is 24 h [50]), drug treatment could selectively suppress or alter RhoB activity before other farnesylated proteins were functionally depleted from cell membranes. This effect may also explain the relative lack of cytotoxic or cytostatic effects of FT inhibitors despite the requirement for other farnesylated proteins in normal cell growth and function (e.g., lamins [14]). Longer treatments with L-739,749 might be predicted to produce general cytostatic effects, in a manner similar to what was seen when FT was genetically inhibited by antisense or dominant inhibitor approaches (40), since a greater number of farnesyl-dependent processes are affected. Such effects have not been reported to occur in vivo (28a) but can begin to be seen in Rat1/ras cells continuously treated in tissue culture at concentrations that are 50- to 100-fold higher than those required for phenotypic reversion (39).

We demonstrated that FT inhibition caused a change in the cell localization of RhoB but not of the nearly identical protein RhoA, which is consistent with the fact that RhoB exists in both farnesylated and geranylgeranylated forms in vivo while RhoA is only geranylgeranylated (1). However, given the complexity of RhoB isoprenylation, we cannot rule out the possibility that the effects of L-739,749 on localization are indirect and not due to direct inhibition of RhoB farnesylation. The observation that L-739,749 failed to block metabolic labeling by [^{3}H]mevalonate was unsurprising, because RhoB contains a CAAX recognition signal for geranylgeranyl transferase type I (GGT I), is geranylgeranylated by GGT I in vitro, and is modified by both C_{15} and C_{20} isoprenoids in vivo (1, 3, 34).

TABLE 2. Properties of Rat1 cell populations stably expressing Myr-rhoB or other RhoB constructs[a]

Construct	Morphology[b]	Growth in normal medium	Growth in low-serum medium	Soft agar colony formation
Vector only	N	+	−	−
RhoB	N	++	±	±
$RhoB^{V14}$	N	+++	+	±
Myr-$rhoB^{V14}$	N	+++	+	±
v-Ras	T	+++++	ND	++++

[a] Rat1 cell populations stably expressing the indicated constructs were generated and assayed as described in Materials and Methods. Cells were assessed for morphological phenotype, outgrowth in medium containing normal or suboptimal levels of serum growth factors, and colony formation in soft agar culture. Phenotypes were scored in arbitrary units relative to the vector-only and v-Ras control cell lines. ND, not done.

[b] N, normal; T, transformed.

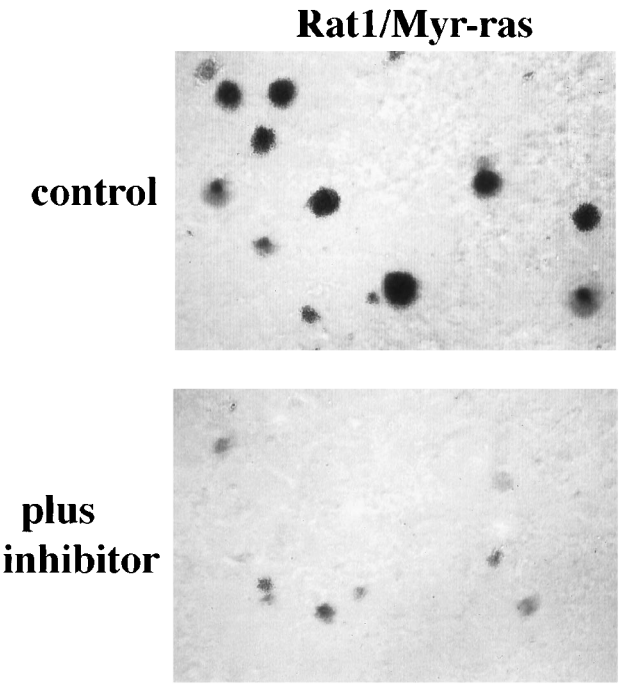

FIG. 8. The anchorage-independent growth of Myr-ras cells is suppressed by L-739,749. Rat1 cells transformed by an N-myristylated Ras (Myr-ras) construct (13) were assayed for growth in soft agar culture in the presence or absence of L-739,749, as in Fig. 5B.

Thus, geranylgeranylation of RhoB by GGT-I in vivo would be expected to mask any loss of farnesylation caused by L-739,749.

Biochemical analysis has indicated that in vitro RhoB is a weak substrate for farnesylation by FT but is a good substrate for farnesylation by GGT I, which can transfer C_{15} isoprenoid when provided as the second substrate (3). The in vivo significance of this result is unclear, but it presents the possibility that RhoB farnesylation could be carried out by GGT I rather than FT and thus may not be a direct target for FT inhibition. However, it is premature to draw this inference. First, it is not known whether GGT I binds C_{15} isoprenoid in vivo. Second, mutation of one of the proximal non-CAAX cysteines on RhoB (C192S) which decreases C_{20} and increases C_{15} addition in vivo (1) actually has the opposite effect on GGT I isoprenylation in vitro (3). Finally, the possibility that RhoB farnesylation is carried out by GGT I in vivo does not in itself rule out RhoB as a target for inhibition by L-739,749, because while this compound does not affect GGT I-dependent geranylgeranylation (29, 39), its effects on GGT I-dependent farnesylation have not been examined. Thus, L-739,749 disrupted the vesicular localization of RhoB directly, by interfering with its farnesylation, or indirectly, by blocking the farnesylation of another protein whose activity affected RhoB localization. In either case, RhoB inhibition or change-of-function would be predicted to be the result of depletion from a normal site of action.

Potential role of RhoB or a related Rho protein(s) in suppression of Ras transformation by FT inhibitors. We have presented evidence that FT inhibitors must inhibit or change RhoB activity to suppress Ras transformation. The results suggest that RhoB is necessary for Ras to maintain the transformed phenotype and corroborate previous demonstrations that dominant inhibitory RhoB mutants inhibit Ras focus formation activity (42). Thus, either pharmacological or genetic inhibition of RhoB leads to suppression of oncogenic Ras activity. The ability of Myr-rhoB^{V14} to confer drug resistance to Ras-transformed cells depended on the integrity of the Rho effector domain and was not due to an oncogenic gain-of-function effect of the myristylation signal. Since RhoB and Myr-rhoB^{V14} appeared to localize to similar but nonidentical cytoplasmic vesicles, we interpreted the potentiating effects of the V14 activating mutation as an augmentation of Rho signaling activity in the wild-type compartment, which might be poorly populated by the myristylated protein. RhoB-RhoA fusions, which are solely geranylgeranylated and localized like RhoA in the cytoplasm and plasma membrane (2), do not appear to confer a protective effect (30a), supporting the likelihood that the protective effect of Myr-rhoB is due to maintenance of a Rho signal in the appropriate vesicle compartment.

Our results corroborated the hypothesis that FT inhibitors suppress Ras transformation by interfering with actin stress fiber regulation. However, they did not prove that RhoB is a direct target for FT inhibition. One could envision other targets, such as (i) a different farnesylated Rho family protein(s) whose function overlaps with that of RhoB or (ii) a farnesylated protein whose activity influences RhoB localization. Nevertheless, we suggest that RhoB's short half-life, change in cell localization in response to L-739,749, and connections to cell growth control make RhoB a more plausible focus than Ras or other known proteins for explaining the effects of FT inhibition, even if it is indirectly affected by drug treatment. Since Rho dominant inhibitory mutants also can suppress Ras transformation (27, 42, 44), the effects of L-739,749 on RhoB and/or related Rho proteins may be sufficient as well as necessary for explaining how FT inhibition leads to reversion of Ras-transformed cells.

We observed that Rat1/Myr-ras cells were still partially sensitive to inhibition by L-739,749, arguing that inhibition of Ras processing is less important than interference with Rho function for reversion. Using different FT inhibitors, others have also observed that Rat1 cells transformed by Myr-ras are partially inhibited by drug treatment (11a). Since by itself myristylation activates the oncogenic capacity of Ras (7, 30), unlike Rho, a clear interpretation of these effects is difficult. One possibility is that synergy between myristylation and the activating mutation present in the Myr-ras construct used makes the drug suppression less potent. Alternatively, the weak protective effect of Myr-Ras could indicate that Ras inhibition played a secondary role in the drug mechanism. However, in this interpretation, we would argue that the role is minor, at best, since the anchorage-independent growth of Myr-ras cells was still quite susceptible to inhibition by L-739,749.

Role of RhoB in cell growth and Ras transformation. Our results support the idea that RhoB plays a role in cell growth control, as suggested by its identification as an epidermal growth factor- and v-*src*-induced immediate-early gene (23). Experiments with C_3 transferase, which inhibits both Rho proteins and cell cycle transit (52), have previously suggested a role in cell growth control. Experiments with dominant inhibitory and activated mutants support this work but have gone on to suggest that there may be differences between normal and transformed cells in the way in which they require and/or respond to Rho activity (27, 42, 44), a finding that has obvious implications for cancer research. RhoB has been studied little but is believed to be functionally similar to RhoA in stress fiber regulation because of its 88% amino acid identity to RhoA (37, 46). Our results suggest that their functions may not overlap entirely, possibly because of a difference in cell localization.

The connection(s) between the effects of Rho proteins on

stress fiber regulation and normal and malignant cell growth is unclear. The molecular mechanisms by which oncogenic Ras affects the actin cytoskeleton (4, 12, 47) are not well-understood but are starting to be characterized (45). One possibility is that Ras's requirement for Rho activity centers on stress fiber organization at focal adhesions, the complex cell surface structures where stress fibers terminate and where many signal transduction molecules regulating cell growth, adhesion, and motility are localized (for reviews, see references 6, 22, and 31). A recent report suggesting that the Sos-binding adapter protein GRB2 can associate with activated focal adhesion kinase supports possible communication between Ras and focal adhesions (49). The relative lack of effect of L-739,749 and other FT inhibitors on normal cell functions, even in animals (28a, 29), suggest that there may be differences in the requirements for Rho and/or focal adhesion-associated functions in transformed cells.

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