Protein farnesyltransferase inhibitors (FTIs) inhibit Ras transformation and Ras-dependent tumor cell growth, but the biological mechanisms underlying these activities is unclear. In previous work, we presented support for the hypothesis that the anti-transforming effects of FTIs depend upon alterations in the function of RhoB, a member of the Rho family of proteins that regulate cytoskeletal actin, cell adhesion, and cell growth. A significant question that needed to be addressed was whether FTIs could directly alter the prenylation as well as the function of RhoB in cells. This issue is complex because farnesylated and geranylgeranylated forms of RhoB (RhoB-F and RhoB-GG) both exist in cells. Here, we show that RhoB farnesylation in vitro can be catalyzed by protein farnesyltransferase and that the peptidomimetic FTI L-739,749 inhibits the farnesylation of RhoB both in vitro and in intact cells. In drug-treated cells, the level of RhoB-GG increased in parallel with the decrease in RhoB-F. In addition to altering RhoB prenylation, L-739,749 suppressed RhoB-dependent cell growth. Taken together, the results suggest that the inhibitory effects of FTIs on RhoB function can be mediated by a relative loss of RhoB-F, a gain of RhoB-GG, or both. Our findings strengthen the causal link between RhoB inhibition and the anti-transforming effects of FTIs and indicate that differently prenylated forms of RhoB may have unique functions.

The Rho proteins share about 30% homology with Ras and are crucial regulators of the actin cytoskeleton, cell adhesion, and motility (1). Rho family members play a critical role in Ras transformation (2–4), making them potential targets for anti-cancer therapy (5, 6). Like Ras, Rho proteins are post-translationally modified by either farnesyl (C15) or geranylgeranyl (C20) isoprenoids at their C-terminal CAAX box sequence, where C is cysteine. A is generally an aliphatic amino acid, and X is usually methionine, serine, glutamine, or leucine (7). This prenylation is important for proper membrane localization and protein-protein interactions (8). Two enzymes are involved in the isoprenylation of Ras and Rho proteins, protein farnesyltransferase (FTase)1 and protein geranylgeranyltransferase I (GGTase-I), which transfer farnesyl and geranylgeranyl isoprenoid groups, respectively (7).

FTase inhibitors (FTIs) were developed as potential anti-cancer therapeutics to exploit the farnesylation requirement of Ras for its oncogenic activity (9). In support of this potential, FTIs have been shown to revert Ras-transformed cells to a normal phenotype and to cause dramatic tumor regression in animal models without significant toxicity (10, 11). However, inhibition of Ras farnesylation does not seem to fully explain their anti-transforming activity (5, 10, 12). We have previously presented evidence that inhibition of the Rho family member RhoB is a critical event in FTI-induced reversion of Ras-transformed cells (5). First, unlike most Rho proteins, which are solely geranylgeranylated in vivo, RhoB can be either farnesylated or geranylgeranylated in cells (13), making it a reasonable candidate as an FTI target. Second, RhoB is up-regulated by growth factors and is weakly oncogenic (3, 14). Third, RhoB has a short half-life and therefore can be functionally depleted within the 18–24 h required by FTIs to induce phenotypic reversion (5, 10). Fourth, a dominant inhibitory form of RhoB mimics FTIs in its ability to inhibit Ras transformation (3). Finally, a myristylated form of RhoB that functions independently of prenylation renders Ras-transformed cell resistant to FTI-induced reversion (5).

Although the hypothesis was supported by these biological experiments, recent in vitro biochemical experiments raised the possibility that RhoB might not be a physiological substrate for FTase (15). If so, the effects of FTIs on RhoB activity would reflect an indirect rather than a direct effect. In an attempt to clarify this issue, we analyzed the ability of FTIs to inhibit RhoB farnesylation in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Materials—1-[3H]Farnesylphosphate (1[3H]FPP; 15 Ci/mmol), 1-[3H]-geranylgeranyldiphosphate (1[3H]GGPP; 15 Ci/mmol), and [3H]mevalonolactone (60 Ci/mmol) and unlabeled FPP and GGPP were obtained from American Radiolabeled Chemicals (St. Louis, MO).

Plasmid Constructs—The HA-RhoB, HA-RhoB124, and Myr-RhoB124 plasmids were described previously (3). Standard polymerase chain reaction mutagenesis techniques were used to generate a C193S mutant designated HA-RhoB193S, and all constructs were cloned into pcDNA3 (Invitrogen). RhoB without an HA-epitope tag was cloned into pQE30 and pQE50 (Qiagen) for bacterial expression. Bacterial expression plasmids for H-Ras was a gift from Channing Der (University of North Carolina-Chapel Hill).

Cell Culture—All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (HyClone). Rat 1 growth curves were performed in 0.25% fetal calf serum as described (10).

1 The abbreviations used are: FTase, protein farnesyltransferase; GGTase-I, protein geranylgeranyltransferase type-I; FPP, farnesyl diphosphate; GGPP, geranylgeranyldiphosphate; PAGE, polyacrylamide gel electrophoresis; HA, hemaglutinin; FTI, farnesyltransferase inhibitor; HPLC, high pressure liquid chromatography.

† Supported by a research grant from Merck Research Laboratories.
‡ To whom correspondence should be addressed: 3601 Spruce St., Philadelphia, PA 19104. Tel: 215-898-3792; Fax: 215-898-2205; E-mail: prendergast@wistar.upenn.edu.

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.
either HA-RhoB or HA-RhoBS were immunoprecipitated with an anti-HA antibody and analyzed by SDS-PAGE and fluorography. Equal expression between samples was verified by Western analysis. B, isoprenoid analysis by HPLC. The isoprenoids attached to RhoB in the immunoprecipitates described in A were determined following methyl iodide cleavage by reverse-phase HPLC. \(^{3}H\)-Labeled isoprenoids were extracted after methyl iodide treatment of each immunoprecipitate were analyzed for SDS-PAGE analysis and fluorography or for isoprenoid analysis by HPLC. The isoprenoids attached to RhoB in the immunoprecipitates described in A were determined following methyl iodide cleavage by reverse-phase HPLC. \(^{3}H\)-Labeled isoprenoids were extracted after methyl iodide treatment of each immunoprecipitate were resolved by C18 reverse-phase HPLC as follows: ● RhoB control; ○, RhoB + FTI L-739,749; ▲, RhoB C193S mutant; ●, RhoB C193S mutant + FTI L-739,749. Isoprenoids were identified by their co-elution with authentic, unlabeled C15, and C20 isoprenoid standards, as indicated above each peak.

**Immunoprecipitation**—COS cells were co-transfected with 15 \(\mu\)g of RhoB and 5 \(\mu\)g of pMEV (a mammalian expression vector containing the cDNA for a mevalonate transporter) and labeled for 9 h with \([^{3}H]\)mevalonate, in the presence or the absence of 15 \(\mu\)M L-739,749. Aliquots of cell lysate were reserved for Western analysis, and the remainder was used for immunoprecipitation with the anti-HA monoclonal antibody 12CA5 using standard methods. Equivalent aliquots of each immunoprecipitate were analyzed for SDS-PAGE analysis and fluorography or for isoprenoid analysis.

**Western Analysis**—Total cellular protein (4 \(\mu\)g/lane) was fractionated via SDS-PAGE, blotted to nitrocellulose, and analyzed by standard methods. Anti-HA antibody, 12CA5, was used at 2.5 dilution. A chemiluminescence reagent (Pierce) was used for detection following the protocol recommended by the vendor (Pierce).

**Isooprenoid Analysis**—Isoprenoids attached to the anti-HA immunoprecipitated proteins were determined by HPLC analysis of the methyl iodide cleavage product essentially as described previously (16, 17).

**Prenylation Assays**—Recombinant FTase and GGTase-I were produced in Sf9 insect cells by infection with recombinant baculoviruses and purified as described (18, 19). Recombinant six-histidine (His6) fusion of RhoB was produced in *Escherichia coli* and purified using Ni\(^{2+}\) affinity chromatography as described previously (20). Untagged versions of RhoB and H-Ras were expressed in *E. coli* and purified as described previously (21). Prenylation reactions were conducted at 37 °C and contained the following components in a final volume of 50 \(\mu\)l: 50 mM Tris-Cl, pH 7.7, 5 mM MgCl\(_2\), 5 mM ZnCl\(_2\), 2 mM dithiothreitol, 0.02% lumbrol, 1 \(\mu\)M \([^{3}H]\)FPP or \([^{3}H]\)GGPP (typically at 8 Ci/mmol), Ras or RhoB protein at concentrations in figure legends, and 50 ng of either FTase or GGTase-I. The reactions were processed as described previously (22).

**RESULTS**

**Inhibition of RhoB Farnesylation but Not Geranylgeranylation by Treatment of Cells with the Peptidomimetic FTI, L-739,749**—To determine whether FTIs inhibit the farnesylation of RhoB in intact cells, we examined the isoprenylation status of RhoB, introduced transiently into COS cells, in the presence and the absence of the peptidomimetic FTI, L-739,749 (10). COS cells were transfected with HA-tagged versions of RhoB and RhoBS; the latter is a nonprenylatable C193S mutant that lacks an acceptor cysteine residue (13). The day after transfection, cells were metabolically labeled with \([^{3}H]\)mevalonate, an isoprenoid precursor, in the presence or the absence of 15 \(\mu\)M L-739,749, which blocks FTase, but not GGTase-I. The reactions were processed as described previously (21).
the absence of L-739,749, whereas, as expected, RhoB was not labeled under either condition (Fig. 1A). Based on these results, we concluded that the total amount of isoprenoid transferred to RhoB in vivo was not significantly affected by L-739,749.

To determine if L-739,749 treatment influenced RhoB farnesylation, the anti-HA immunoprecipitates used above were processed by cleavage of the isoprenoids from immunoprecipitated protein followed by reverse-phase HPLC to analyze isoprenoid moieties released (Fig. 1B). This analysis revealed that L-739,749 completely inhibited farnesylation of RhoB, whereas geranylgeranylation of RhoB increased correspondingly. Taking into account that C-20 isoprenoid would incorporate radioactivity from four mevalonate molecules versus three for the C-15 isoprenoid, quantitation of the radioactivity in the farnesol and geranylgeraniol peaks revealed that the total amount of prenylated RhoB was conserved after treatment of cells with L-739,749. This observation explained why the extent of [3H]mevalonate labeling in immunoprecipitates from drug-treated and untreated cells was similar. We concluded that RhoB is solely geranylgeranylated in cells treated with L-739,749.

RhoB Can Be Farnesylated by Both FTase and GGTase-I in Vitro—The finding that farnesylation of RhoB was inhibited by treatment of cells with the FTase-selective inhibitor L-739,749 prompted us to re-examine the ability of this G protein to be recognized by the two CAA \textit{X} prenyltransferases. In vitro prenylation assays were performed using purified recombinant FTase and GGTase-I and selected substrates for these enzymes (Fig. 2). The results of this analysis, which are summarized in Table I, revealed that in contrast to a previous report (15), farnesylation of RhoB could be catalyzed by both FTase and GGTase-I with approximately the same efficiency ($V/K$ of 2.0 for each). In addition and also in contrast to the previous study,

Table I

| Enzyme | Protein | Isoprenoid | $K_m$ | $V_{max}$ | $V/K$ | IC$_{50}$ |
|--------|--------|-----------|------|-----------|-------|----------|
| FTase  | H-Ras  | FPP       | 0.3  | 7.0       | 23    | 0.06     |
| FTase  | Rho B  | FPP       | 3.0  | 6.0       | 2.0   | 0.06     |
| GGTase | Rho B  | FPP       | 1.0  | 2.0       | 2.0   | >20      |
| GGTase | Rho B  | GGPP      | 0.1  | 3.0       | 30    | >20      |

The results of these experiments with L-739,749 showed that farnesylation of RhoB was inhibited by L-739,749 but not by GGTase-I. Protein substrates, either H-Ras or RhoB, were incubated as described under “Experimental Procedures” in the presence of the indicated concentrations of the FTI L-739,749, with the following enzyme (50 ng) and isoprenoid (1 $\mu$M) combination: ● H-Ras + FPP + FTase; ○ RhoB + FPP + FTase; ● RhoB + GGPP + GGTase; ○ RhoB + FPP + GGPP. IC$_{50}$ values for each inhibition curve are listed in Table I.

![Graph](image1)

**Fig. 3.** L-739,749 selectively inhibits \textit{in vitro} farnesylation of RhoB by FTase but not by GGTase-I. Protein substrates, either H-Ras or RhoB, were incubated as described under “Experimental Procedures” in the presence of the indicated concentrations of the FTI L-739,749, with the following enzyme (50 ng) and isoprenoid (1 $\mu$M) combination: ● H-Ras + FPP + FTase; ○ RhoB + FPP + FTase; ● RhoB + GGPP + GGTase; ○ RhoB + FPP + GGPP. IC$_{50}$ values for each inhibition curve are listed in Table I.

![Graph](image2)

**Fig. 4.** L-739,749 inhibits RhoB-dependent cell growth. A, B, and C, growth curves in the presence or the absence of 5 $\mu$M L-739,749. Cell populations stably transfected with RhoB$^{V14}$ (A), vector (B), or Myr-rhoB$^{V14}$ (C), were cultured in 0.25% fetal calf serum with or without L-739,749. Cell counts were taken at the indicated time points. The results are representative of four separate trials. □ control; ○ , with FTI. D, cell counts. The results represent the average and standard deviation of three trials.

RhoB was a significantly better substrate for geranylgeranylation by GGTase-I ($V/K$ of 30) than for farnesylation by the same enzyme ($V/K$ of 2.0, as noted above). The basis for the discrepancy with previously reported results is unclear, but it is not due to differences in substrates used, because both His-tagged RhoB and untagged RhoB behaved similarly in the assays (data not shown).
L-739,749 Inhibits in Vitro Farnesylation of RhoB by FTase but Not by GGTase-I—The finding that FTase was able to catalyze farnesylation of RhoB suggested a mechanism for the ability of the FTase-selective inhibitor L-739,749 to block this modification of RhoB in cells. However, it remained possible that the compound, although a poor inhibitor of geranylgeranylation by GGTase-I, might effectively inhibit the ability of this enzyme to transfer farnesyl group onto substrate proteins. To assess this possibility, L-739,749 was tested for its ability to inhibit farnesylation of RhoB by both GGTase-I and FTase in an in vitro assay. The results of this analysis, shown in Fig. 3, indicate that, as expected for this FTase-selective inhibitor, L-739,749 was a poor inhibitor of reactions catalyzed by GGTase-I. The IC₅₀ values for inhibition of both RhoB farnesylation and geranylgeranylation by GGTase-I was >20 μM, whereas farnesylation of the protein by FTase was inhibited with an IC₅₀ of 0.06 μM, a similar IC₅₀ as that determined for inhibition of FTase action on H-Ras. These findings, together with the finding that L-739,749 blocks RhoB farnesylation in intact cells, provides compelling evidence that RhoB farnesylation in vivo is mediated by FTase.

Growth Stimulatory Function of RhoB Is Attenuated by L-739,749 Treatment—Previously we have shown that stable expression of RhoB in Rat1 cells stimulates their proliferation and allows them to grow to a greater saturation density (5). To test whether inhibition of RhoB farnesylation compromises its growth-stimulating function, we investigated the effects of L-739,749 on the growth characteristics of Rat1 cells stably expressing RhoB constructs. Pooled cell populations stably transfected with either constitutively activated RhoB (RhoBV₁₄), a myristylated form of activated RhoB (Myr-RhoBV₁₄), or vector have been described previously (5). The Myr-RhoBV₁₄ construct has been shown to function similar to RhoBV₁₄; however, its function is independent of farnesylation.

As observed previously, the RhoBV₁₄ and Myr-rhoBV₁₄ cell populations grew faster and to a greater saturation density than the control cell population in the absence of L-739,749 (Fig. 4). Growth curves determined in the presence of 5 μM L-739,749 revealed that the growth rate and saturation density of RhoBV₁₄-expressing cells decreased significantly, whereas that of cells containing vector or Myr-rhoBV₁₄ was comparatively less affected (Fig. 4). Cell counts for vector and Myr-rhoBV₁₄ cells were reduced by ~12% in the presence of L-739,749, consistent with the slight suppression of cell growth of normal Rat1 cells seen previously (10). In contrast, cell counts for RhoBV₁₄ cells treated with L-739,749 were decreased ~35%. This result implied that either farnesylated RhoB had a specific growth stimulatory activity that geranylgeranylated RhoB could not replace or that increased levels of geranylgeranylated RhoB was growth inhibitory. We concluded that L-739,749 attenuated the growth stimulatory function of RhoB as well as selectively inhibiting its farnesylation.

DISCUSSION

Among prenylated proteins, RhoB provides an exception to the simple rules for prenylation because its CAAX box (CKVL) suggests that it should be geranylgeranylated but not farnesylated. Although previous in vitro work suggested that GGTase-I transfers both isoprenoid groups, our data argue that the GGTase-I farnesylation of RhoB is not physiologically relevant. Thus, the apparent inability of GGTase-I to farnesylate RhoB when the latter is expressed in an intact cell is also consistent with a recent report showing that CAAX prenyltransferases exhibit a very strong preference for utilization of their “correct” isoprenoid if presented both prenyldiphosphates (23). Despite the fact that RhoB is not a particularly good substrate for FTase in vitro, it appears that this enzyme is able to mediate farnesylation of RhoB in vivo.

This report significantly strengthens our hypothesis that RhoB inhibition forms the basis for the anti-transforming effects of FTIs. (5, 24). Initial biochemical data suggested that RhoB might not be farnesylated by FTase, so the effect of FTIs could have been indirect. We reconciled these data by showing that RhoB farnesylation and growth-stimulatory function are indeed directly blocked by FTI treatment.

An interesting implication of our results is that farnesylated RhoB and geranylgeranylated RhoB may not be functionally interchangeable. Consistent with the results presented in this report, we have previously shown that FTI treatment alters the cellular localization of RhoB (5), suggesting that the differently prenylated forms of the protein may act at different subcellular locations. Our data suggest that farnesylation, but not geranylgeranylation, of RhoB is critical for Ras transformation. Although simple models for understanding how FTIs reverse cell transformation and cause tumor regression propose that the drugs act by loss of function of a farnesylated protein, our studies on RhoB open the possibility that FTIs could act instead by a gain of function effect, by preferentially inducing the accumulation of geranylgeranylated RhoB. Further investigation is needed to determine (i) whether the ratio or level of differently prenylated RhoB species can affect cell growth and transformation and (ii) whether loss of farnesylated RhoB, gain of geranylgeranylated RhoB, or both represent the key trigger for FTI-induced reversal.

Acknowledgments—We thank John Moomaw and Carolyn Diesing for purification of the protein prenyltransferase and the Ras proteins. We also thank Channing Der (University of North Carolina-Chapel Hill) for expression plasmids for H-Ras.

REFERENCES

1. Hall, A. (1994) Annu. Rev. Cell Biol. 10, 31–54
2. Khsoravi-Far, R., Solski, P. A., Kinech, M. S., Burridge, K., and Der, C. J. (1995) Mol. Cell. Biol. 15, 6443–6453
3. Prendergast, G. C., Khsoravi-Far, R., Solski, P. A., Kurzawa, H., Lebowitz, P. F., and Der, C. J. (1995) Oncogene 10, 2289–2296
4. Qiu, R.-G., Chen, J., Kirk, D., McCormick, F., and Simons, M. (1995) Nature 374, 457–459
5. Lebowitz, P. F., Davide, J. P., and Prendergast, G. C. (1995) Mol. Cell. Biol. 15, 6613–6622
6. Symons, M. (1995) Curr. Opin. Biotechnol. 6, 668–674
7. Casey, P. J., and Seabra, M. (1996) J. Biol. Chem. 271, 5289–5292
8. Marshall, C. J. (1993) Science 259, 1865–1866
9. Gibbs, J., Oliff, A., and Kohl, N. E. (1994) Cell 77, 175–178
10. Prendergast, G. C., Davide, J. P., deSams, S. J., Giuliani, E., Graham, S., Gibbs, J. B., Oliff, A., and Kohl, N. E. (1994) Mol. Cell. Biol. 14, 4183–4192
11. Kohl, N. E., Omer, C. A., Conner, M. W., Anthony, N. J., Davide, J. P., deSams, S. J., Giuliani, E. A., Gomez, R. P., Graham, S. L., Hamilton, K., et al. (1995) Nat. Med. 1, 792–797
12. Sepp-Lorenzino, L., Ma, Z., Rands, E., Kohl, N. E., Gibbs, J. B., Oliff, A., and Rosen, N. (1995) Cancer Res. 55, 5302–5309
13. Adamson, P., Marshall, C. J., Hall, A., and Tillbrook, P. A. (1992) J. Biol. Chem. 267, 20033–20038
14. Jahnner, D., and Hunter, T. (1991) Mol. Biol. Cell. 1, 3682–3690
15. Armstrong, S. A., Hannah, V. C., Goldstein, J. L., and Brown, M. S. (1995) J. Biol. Chem. 270, 7864–7868
16. Casey, P. J., Sokal, P. A., Der, C. J., and Buss, J. E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8323–8327
17. Farnsworth, C. C., Casey, P. J., Howald, W. N., Glomset, J. A., and Gelb, M. H. (1999) Methods: A Companion to Methods in Enzymology 1, 231–240
18. Chen, W. J., Moowam, J. F., Overton, L., Kost, T. A., and Casey, P. J. (1993) J. Biol. Chem. 268, 9675–9680
19. Zhang, F. L., Moowam, J. F., and Casey, P. J. (1994) J. Biol. Chem. 269, 23465–23470
20. James, G. L., Goldstein, J. L., and Brown, M. S. (1995) J. Biol. Chem. 270, 6221–6226
21. Casey, P. J., Thiessen, J. A., and Moowam, J. F. (1993) Proc. Natl. Acad. Sci. U. S. A. 86, 8631–8635
22. Thiessen, J. A., and Casey, P. J. (1996) Anal. Biochem. 243, 80–85
23. Yokoyama, K., Zimmerman, K., Scholten, J., and Gelb, M. H. (1997) J. Biol. Chem. 272, 3944–3952
24. Lebowitz, P., Sakamuro, and Prendergast, G. C. (1997) Cancer Res. 57, 708–713