MOLECULAR AND CELLULAR BIOLOGY, June 1994, p. 4193–4202
0270-7306/94/$04.00+0

Vol. 14, No. 6

Farnesyltransferase Inhibition Causes Morphological Reversion of *ras*-Transformed Cells by a Complex Mechanism That Involves Regulation of the Actin Cytoskeleton

GEORGE C. PRENDERGAST,[1]† JOSEPH P. DAVIDE,[1] S. JANE DESOLMS,[2] ELIZABETH A. GIULIANI,[2] SAMUEL L. GRAHAM,[2] JACKSON B. GIBBS,[1] ALLEN OLIFF,[1] AND NANCY E. KOHL[1]*

Departments of Cancer Research[1] and Medicinal Chemistry,[2] Merck Research Laboratories, West Point, Pennsylvania 19486

Received 25 January 1994/Returned for modification 20 March 1994/Accepted 28 March 1994

A potent and specific small molecule inhibitor of farnesyl-protein transferase, L-739,749, caused rapid morphological reversion and growth inhibition of *ras*-transformed fibroblasts (Rat1/ras cells). Morphological reversion occurred within 18 h of L-739,749 addition. The reverted phenotype was stable for several days in the absence of inhibitor before the transformed phenotype reappeared. Cell enlargement and actin stress fiber formation accompanied treatment of both Rat1/ras and normal Rat1 cells. Significantly, inhibition of Ras processing did not correlate with the initiation or maintenance of the reverted phenotype. While a single treatment with L-739,749 was sufficient to morphologically revert Rat1/ras cells, repetitive inhibitor treatment was required to significantly reduce cell growth rate. Thus, the effects of L-739,749 on transformed cell morphology and cytoskeletal actin organization could be separated from effects on cell growth, depending on whether exposure to a farnesyl-protein transferase inhibitor was transient or repetitive. In contrast, L-739,749 had no effect on the growth, morphology, or actin organization of v-*raf*-transformed cells. Taken together, the results suggest that the mechanism of morphological reversion is complex and may involve farnesylated proteins that control the organization of cytoskeletal actin.

Activation of the *ras* protooncogene plays a role in the formation of 20 to 30% of all human tumors (4). In normal cells, *ras* encodes a plasma membrane-localized guanine nucleotide-binding protein, Ras, that in its GTP-bound state couples the signals of activated growth factor receptors to downstream mitogenic effectors. In tumor cells, Ras becomes oncogenically activated as a result of mutation, losing its normal GTPase activity and becoming constitutively bound to GTP. There is substantial evidence that oncogenically activated Ras plays an important role in tumorigenesis (for reviews of the biological and biochemical activities of normal and oncogenic Ras proteins, see references 2, 5, and 7). Furthermore, elimination of *ras* function by homologous gene recombination or antisense RNA has demonstrated that expression of activated *ras* is necessary for maintaining the transformed phenotype of human tumor cells (14, 43). These results argue that inhibitors of oncogenic Ras activity may prove useful for anticancer therapy in tumors in which Ras plays a contributing role.

One avenue to impede Ras in vivo may be to inhibit its posttranslational modification. Mutation of the evolutionarily conserved C-terminal CAAX box sequence in Ras (where C is cysteine, A is an aliphatic amino acid, and X is any amino acid) destroys plasma membrane localization and cell transforming activity (reviewed in references 15 and 26). This sequence is the target for recognition by farnesyl-protein transferase (FPTase), a heterodimeric enzyme that transfers the C_{15} isoprenoid group from farnesyl diphosphate to the CAAX box cysteine (for reviews on FPTase and isoprenylation, see references 16 and 31). Farnesyl addition is followed by removal of the remaining three amino acids by an endopeptidase and methylation of the new C terminus by a specific methyltransferase. Although mature Ras undergoes all three modifications, only farnesylation is necessary for its plasma membrane localization and cell transforming potential (25).

On this basis, it has been proposed that oncogenic Ras activity could be blocked by inhibiting the activity of FPTase (see references 15 and 26). However, until recently, the foundation for this proposal was uncertain. First, the biological consequences of FPTase inhibition in animal cells had not been examined. This issue was important because there are other cellular proteins that are farnesylated in addition to Ras (31). Although their number is fairly small, some of these proteins might be expected to be essential for cell growth (e.g., nuclear lamins [11]). A second concern was that, although farnesylation of Ras had been shown to be necessary for its oncogenic activity, it was not known whether inhibition of FPTase would prove sufficient to specifically block Ras in vivo. This issue was considerable because of the overlapping specificity of FPTase and geranylgeranyltransferase type I, a related heterodimeric enzyme that adds C_{20} geranylgeranyl groups to specific CAAX box sequences and that shares a subunit with FPTase (32, 42). Loss of FPTase activity in *Saccharomyces cerevisiae* was found to be nonlethal, despite the fact that *ras* is an essential gene (21). This observation suggested that other prenyltransferases might be able to modify essential proteins in the absence of FPTase. Consistent with this possibility, some normally farnesylated substrates are geranylgeranylated in *S. cerevisiae* strains that lack FPTase (46), and modified forms of oncogenic Ras that are geranylgeranylated have been shown to transform mouse fibroblasts (8). Therefore, it was unclear whether FPTase inhibition would be sufficient to block oncogenic Ras activity.

Recently, however, this hypothesis has been supported by experimental results from several groups. In one study, a

* Corresponding author. Mailing address: Department of Cancer Research, Merck Research Laboratories, P.O. Box 4, WP16-3, West Point, PA 19486. Phone: (215) 652-5646. Fax: (215) 652-7320.

† Present address: The Wistar Institute, Philadelphia, PA 19104.

CAAX analog that suppressed cellular Ras processing inhibited the anchorage-independent growth of *ras*- but not *raf*-transformed fibroblasts (29). This result indicated that *ras*-transformed growth, but not general mitogenic functions, was selectively suppressed by FPTase inhibition. In a second study, benzodiazepine-based CAAX mimetics which suppressed farnesylation of cellular proteins inhibited the anchorage-dependent growth of *ras*-transformed cells, without gross effects on proliferation of normal cells (23). A genetic approach utilizing antisense and dominant negative strategies corroborated the selection against monolayer growth of cells targeted for inhibition of FPTase (37). Finally, another group has shown that peptidomimetic inhibitors of Ras farnesylation blocked the maturation of *Xenopus* oocytes induced by Ras but not progesterone (13). Taken together, these findings implied that FPTase inhibitors could selectively suppress the cellular activities of activated forms of Ras.

In this study, we examined the effects of cell treatment with the CAAX peptidomimetic L-739,749 (30), which is a potent and specific inhibitor of FPTase in vitro and in vivo. L-739,749 caused a rapid phenotypic reversion of *ras*-transformed cells which was characterized by cell enlargement, stimulation of actin stress fiber formation, and inhibition of anchorage-independent and anchorage-dependent cell growth. Our work suggests that the mechanism of morphologic reversion is complex and may involve farnesylated proteins that can control the organization of cytoskeletal actin.

MATERIALS AND METHODS

Cell culture. All cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (Gibco). The Rat1, Rat1/ras, and Rat1/raf cell lines were described previously (29). The Rat1-Mev cell line was produced by transfection of the pMev plasmid (27), followed by selection for the neomycin resistance encoded on pMev by culturing the cells in 400 μg of G418 per ml (Gibco). Single cell clones were expanded into mass culture and verified for MEV RNA expression by Northern (RNA) analysis (9a); one such clone was used for mevalonate labelling experiments.

Experiments to monitor cell morphology and growth were performed by seeding ~5×10^4 cells in a 10-cm plate 18 to 24 h before adding L-739,749 or methanol carrier at a final 1:2,000 dilution in culture medium. After incubation for the times indicated, cells were photographed at ×200 magnification on a Nikon Diaphot inverted microscope with Kodak TX-400 film. For growth curves, 10^4 cells were seeded into culture and treated 3 days later with either a single addition of L-739,749 (single-addition treatment [SAT] protocol) or multiple additions of L-739,749 (continuous treatment [CT] protocol) when cells were fed every 2 to 3 days. At the times indicated, viable cell counts were determined from trypsinized cultures stained with trypan blue (Gibco). Each growth point shown was the mean of three separate cultures carried and treated in parallel.

FPTase assays. Three different assays were employed to assess FPTase activity in vivo. For the Ras processing assay, Rat1/ras cells were treated with L-739,749 during metabolic labelling with [^{35}S]methionine, and Ras protein was analyzed by immunoprecipitation with anti-Ras antiserum Y13-259 as described previously (29).

For the determination of cellular FPTase activity, 2×10^6 to 5×10^6 cells were treated for 24 h with a single addition of L-739,749 or methanol carrier, and cell extracts were prepared as follows. Monolayer cell cultures were washed twice with ice-cold phosphate-buffered saline and harvested by scraping and centrifugation at 4°C. After resuspension in hypotonic lysis buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.4], 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride), cells were subjected to two freeze-thaw cycles in a dry ice-ethanol bath, and the supernatant was collected by centrifugation for 10 min in a microcentrifuge at 4°C. Forty micrograms of cell protein was then analyzed by an in vitro FPTase assay (34).

Prenylation of cellular proteins was analyzed by incubating ~5×10^6 Rat1-Mev cells for 16 h in growth medium containing 50 μCi of [^{3}H]mevalonolactone per ml (Amersham), 15 μM lovastatin, and 0, 5, or 50 μM L-739,749, and then preparing cell lysates as in the Ras processing assay (29). Approximately 100 μg of total protein per sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

Immunofluorescence. Cells were seeded into two-well cover slides (Nunc) 18 to 24 h before a single addition of L-739,749 or an equivalent volume of methanol carrier. Following incubation for various periods, cultures were processed for immunofluorescence as described previously (39). F-actin was visualized by incubating fixed cells for 30 min with fluorescein-phalloidin, with conditions recommended by the vendor (Molecular Probes, Eugene, Oreg.). Processed cover slides were mounted with the fluorescence enhancer VectaStain (Vector Laboratories, Burlingame, Calif.) and viewed at ×200 magnification with a Nikon Diaphot inverted microscope and a mercury light source. Photography was performed with Kodak TX-400 or Tmax-400 film with exposures of 1 to 4 s.

Western blotting (immunoblotting). Extracts were prepared from cells that were treated for various times with 50 μM L-739,749 or methanol solvent control by lysing in Nonidet P-40 lysis buffer (20 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride). Twenty micrograms of cell protein per lane was fractionated on an SDS–12% polyacrylamide gel and transferred to an Immobilon P membrane (Millipore) with a semidry blotting apparatus (Millipore). The blot was probed with the rat monoclonal anti-Ras antibody Y13-259, by standard protocols, and developed with the ECL Western blotting system (Amersham) under conditions recommended by the vendor. Extract from cells treated with 15 μM lovastatin for 24 h was prepared as a control for the migration of unprocessed Ras.

RESULTS

L-739,749 is a potent and specific inhibitor of FPTase in vivo. Improvement in the potency of previous CAAX analog FPTase inhibitors (18, 29) was realized in the compound L-739,749 (30). Three cell assays were used to evaluate the activity of L-739,749 in whole cells. The first was a Ras processing assay similar to that described previously (29). Briefly, NIH-3T3 cells transformed by v-*ras* were treated for 24 h with L-739,749 and metabolically labelled with [^{35}S]methionine during the last 20 h of the incubation. The extent that Ras was processed was monitored by immunoprecipitation and SDS-PAGE (Fig. 1A). Similar to previous observations (29), the two forms of v-H-Ras protein present in untreated cells exhibited a gel mobility shift following treatment of cells with L-739,749. The shifted bands comigrated with bands seen in extracts from cells treated with lovastatin, an inhibitor of cellular isoprenoid biosynthesis (data not shown; see reference 29), confirming that they were diagnostic for unprocessed Ras. In this assay, L-739,749 inhibited Ras processing with a 50% inhibitory concentration between 0.1 and 1 μM (Fig. 1A). Similar results were obtained when Rat1 cells transformed with v-*ras* (Rat1/ras) were used in the assay (data not shown).

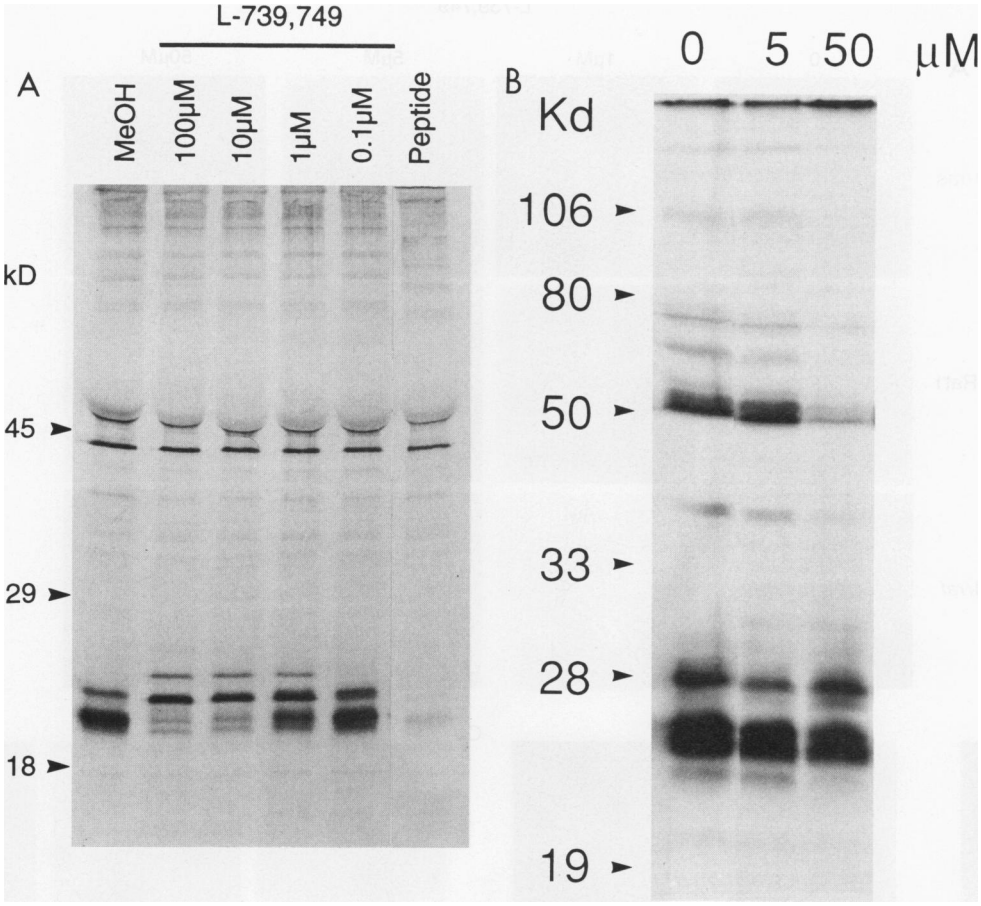

FIG. 1. L-739,749 specifically inhibits FPTase in whole cells. (A) Inhibition of Ras processing. Under conditions described previously (29), extracts from NIH 3T3 cells metabolically labelled with [^{35}S]methionine ± the indicated concentrations of L-739,749 were subjected to immunoprecipitation with anti-Ras Y13-259 antiserum. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Specificity of the Ras bands was demonstrated by blocking the anti-Ras antiserum for 30 min at 4°C with a Ras peptide before immunoprecipitation (lane marked "Peptide"). (B) L-739,749 does not generally inhibit protein prenylation. Total cell extracts (150 μg) from Rat1-Mev cells labelled with 50 μCi of [^{3}H]mevalonolactone per ml ± indicated concentrations of L-739,749 were analyzed by SDS-PAGE and fluorography.

An additional assay was performed (i) to assess the ability of L-739,749 to inhibit the processing of farnesylated cellular proteins in addition to Ras and (ii) to verify the in vitro selectivity of the compound against FPTase relative to the geranylgeranyltransferases in cells. For these experiments, we utilized Rat1-Mev cells that were engineered to express the MEV protein (see Materials and Methods). MEV is a mutant serpentine receptor cloned from a variant CHO cell line that incorporates mevalonate, an isoprenoid precursor, into cellular proteins more efficiently than normal CHO cells (27). For the experiment, Rat1-Mev cells were incubated with tritiated mevalonate in the presence or absence of inhibitor, and then total labelled cell proteins were compared by SDS-PAGE and fluorography. No general inhibition of prenylation was observed (Fig. 1B), similar to previously reported results from use of benzodiazepine-based CAAX mimetic inhibitors (23). For example, the majority of proteins in the 21- to 27-kDa size range were labelled with similar efficiency in extracts from untreated and inhibitor-treated cells. The proteins in this size range represent mainly members of the Ras superfamily, which are predominantly geranylgeranylated (31). By immunoprecipitation from these extracts, it was found that L-739,749 inhibited the tritiated mevalonate labelling of Ras but not the geranylgeranylated small G protein Rac1 (data not shown). We concluded that L-739,749 was specific for FPTase in cells since it did not appreciably affect the prenylation state of proteins that are geranylgeranylated.

A final cell-based assay was performed to verify that L-739,749 inhibited FPTase activity with similar efficiency in all the Rat1 cell lines that were employed in the biological studies described below. This set included Rat1/raf cells (29), a previously described cell line generated by transformation of Rat1 cells by the v-*raf* oncogene. In the experiments described below, Rat1/raf cells were used to control for general transformed cell growth parameters that are independent of Ras because the biological activity of v-*raf* does not require Ras function (45). In this assay for FPTase inhibition, normal Rat1, Rat1/ras, or Rat1/raf cells were incubated for 24 h in the presence of L-739,749, and cell extracts were prepared and assayed for enzyme activity. We observed a similar pattern of inhibition in the extracts from all the cells treated with L-739,749 (data not shown). We concluded that, as expected, FPTase was similarly susceptible to inhibition by L-739,749 in normal and transformed Rat1 cell lines.

Transient exposure of *ras*-transformed fibroblasts to L-739,749 induces rapid phenotypic reversion that is stable for

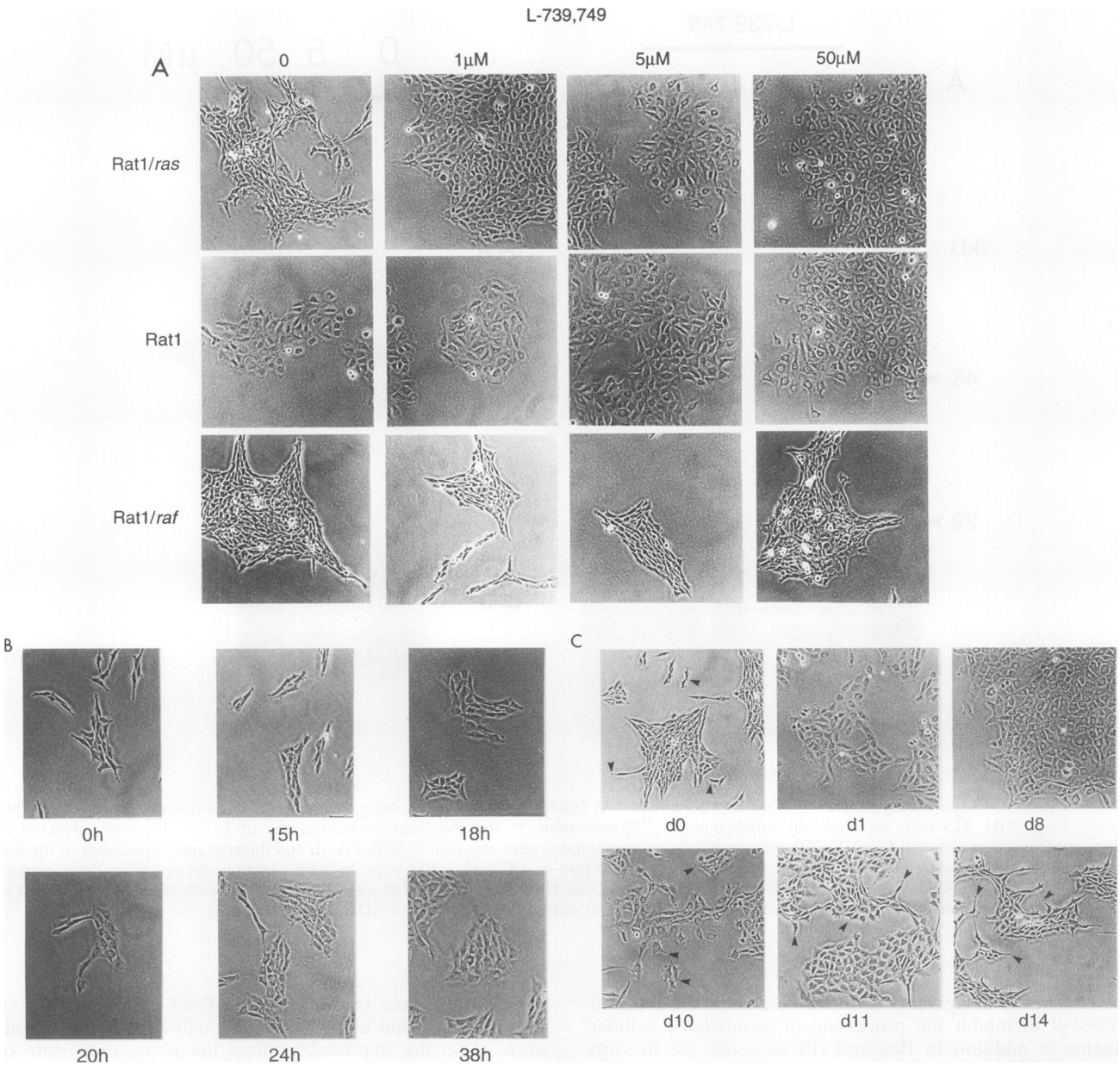

FIG. 2. Transient treatment of Rat1/ras cells with L-739,749 causes a rapid and stable phenotypic reversion of Rat1/ras cells. (A) Morphological reversion. Rat1, Rat1/ras, and Rat1/raf cells were photographed 48 h after incubation in medium containing 0, 1, 5, or 50 μM L-739,749. (B) Short-term kinetics of reversion. Rat1/ras cells were photographed at the indicated times after incubation in medium containing 50 μM L-739,749. (C) Stability of reverted phenotype. Rat1/ras cells were treated on day 0 with a single addition of 50 μM L-739,749 and photographed at the indicated times afterward. During the course of the experiment, cells were passaged normally (as they approached confluence) into medium that was not replenished with L-739,749. Arrowheads indicate cell-substratum attachment structures.

several days in the absence of inhibitor. We have demonstrated elsewhere that L-739,749 selectively inhibited the anchorage-independent growth of Rat1/ras cells in soft agar culture with a MIC of ~2.5 μM (30). These data supported earlier biological studies of FPTase inhibitors (23, 29). To explore the mechanism of this phenomenon, we characterized the effects of the inhibitor in monolayer cell culture. A single addition of L-739,749 caused Rat1/ras cells to phenotypically revert, exhibiting a dramatically flattened and enlarged cell morphology that resembled that of Rat1 cells (Fig. 2A). This effect was noticeable at 1 μM but more apparent and pronounced at higher concentrations. Concentrations of L-739,749 up to 50 μM had no effect on the morphology of Rat1/raf cells, consistent with the lack of an effect of L-739,749 (30) and other CAAX analog inhibitors (29) on the anchorage-independent growth of Rat1/raf cells in soft agar culture. Significantly, in contrast to this result but similar to the effect on Rat1/ras cells, L-739,749 caused a detectable enlargement of normal Rat1 cells (Fig. 2A; see also below). To characterize the features of cell enlargement, we examined Rat1/ras cells since in this cell line the morphological change was more apparent. We found that the phenotypic conversion induced by

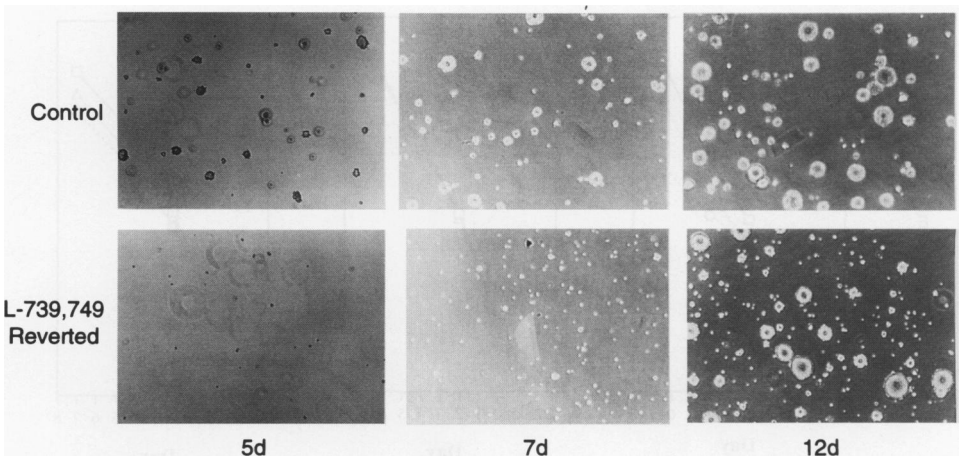

FIG. 3. Reverted Rat1/ras cells stably lack anchorage-independent growth potential. Rat1/ras cells that were treated with methanol or phenotypically reverted by treatment for 2 days with 50 μM L-739,749 were harvested, washed in medium, and seeded into soft agar culture (~10^4 cells) or monolayer culture (~5×10^4 cells) in the absence of inhibitor. The soft agar cultures were photographed at the indicated times.

L-739,749 was rapid, being detectable within 18 h and essentially complete by 24 h. However, cells continued to enlarge beyond this time to at least 38 h (Fig. 2B).

In addition to being induced quickly, the reverted phenotype in Rat1/ras cells was stable. A single addition of L-739,749 without medium replenishment was sufficient to revert Rat1/ras cells for ~8 to 10 days before the transformed phenotype began to reappear (Fig. 2C). During this period, the cells were mitogenically active and could be passaged normally in L-739,749-free medium (see below). The initial sign of re-reversion was the appearance of cell-substratum attachment structures similar to those seen in untreated Rat1/ras cells (arrowheads, Fig. 2C). Rat1/ras cells which had proceeded through one cycle of reversion-rereversion were still susceptible to flattening by L-739,749. The length of the reversion period was not appreciably concentration dependent over a 10-fold range of inhibitor tested, nor was it specific to L-739,749 since other CAAX analog FPTase inhibitors also exhibited this effect (data not shown).

To examine the reversibility of the L-739,749-reverted phenotype and correlate its duration with loss of anchorage-independent growth potential in soft agar, the following experiment was performed. Rat1/ras cells were treated with methanol carrier as a control, or morphologically reverted by incubating them in medium containing 50 μM L-739,749. After 48 h, the cells were washed, trypsinized, and pelleted through ~20 volumes of growth medium to remove any residual inhibitor from the extracellular environment. Cells were then seeded into soft agar or monolayer culture in the absence of additional compound. After 5 days of incubation in soft agar culture, small colonies of the control cells but not the L-739,749-treated cells could be observed (Fig. 3). At 7 days, growth of the L-739,749-treated cells began to be detected. The kinetics of the reappearance of anchorage-independent growth potential at this time, 9 days after L-739,749 addition, correlated well with the re-reversion of Rat1/ras cells seen in the parallel monolayer cultures (Fig. 2C). We concluded that the phenotypic reversion of Rat1/ras cells induced by L-739,749 (i) was rapid, (ii) was correlated with a loss of anchorage-independent growth potential, and (iii) was stable for several days but eventually regressed to a fully transformed phenotype.

CT with L-739,749 is required to substantially reduce the monolayer growth rate of *ras*-transformed cells. We observed that the morphologically reverted Rat1/ras cells were still mitogenically active. Therefore, their growth rate in L-739,749 was examined and compared with that of Rat1 and Rat1/raf control cells. Two treatment regimens were employed. As described in Materials and Methods, cells were treated continuously with methanol carrier (control), once with L-739,749 (SAT), or continuously with L-739,749 by replenishment at each 2-day feeding interval (CT). The growth curves are shown in Fig. 4, and average doubling times calculated from these data are shown in Table 1.

Neither treatment regimen affected the growth of Rat1/raf cells. This observation was consistent with the inability of CAAX analog inhibitors to block the anchorage-independent growth of Rat1/raf cells (29, 30). SAT or CT of Rat1 cells caused a 10 or 22% inhibition, respectively, in their average doubling times. SAT of Rat1/ras caused an analogous inhibition of 17% in doubling time. However, CT of Rat1/ras cells produced a greater inhibition of 77% in cell doubling time. Using other FPTase inhibitors, another group observed a similar suppression of Rat1/ras cell monolayer growth (23), and this result was consistent with the interpretation of animal cell genetic experiments that employed antisense and dominant negative protocols to target FPTase for inhibition (37). Here, the two L-739,749 treatment regimens allowed a dissection of separate biological effects of FPTase inhibition. While morphological reversion and inhibition of anchorage-independent growth required only transient exposure to L-739,749, pronounced suppression of anchorage-dependent cell growth required CT with the inhibitor. We concluded that this latter effect of FPTase inhibition in Rat1/ras cells was separable from cell flattening and loss of anchorage-independent growth potential.

L-739,749 induces cell enlargement and the formation of actin stress fibers in both normal and transformed cells. Oncogenic Ras is known to induce the appearance of cytoskeletal actin structures, termed membrane ruffles, which are associated with cell transformation (3) (for reviews of cellular actin structure, see references 6 and 44; for a review of the regulatory connections between Ras and the actin cytoskeleton, see reference 38). Reverted Rat1/ras cells and other control cell lines were therefore analyzed for changes in the organization of cytoskeletal actin. This objective was accom-

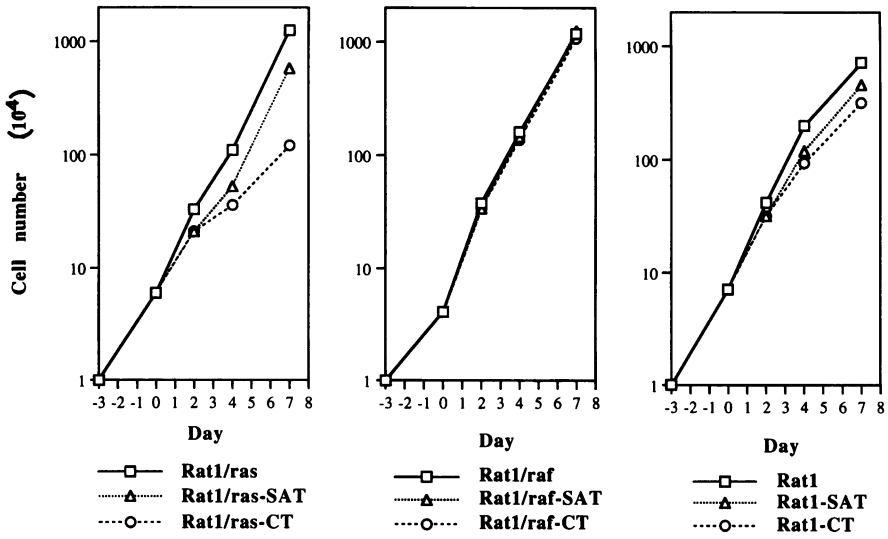

FIG. 4. CT of Rat1/ras cells with L-739,749 is required to reduce their anchorage-dependent growth rate. A total of ~10^4 Rat1/ras, Rat1/raf, or Rat1 cells were seeded at day −3. At day 0, 50 μM L-739,749 or an equivalent dilution of methanol (control) was added to the cultures. One set of cultures received L-739,749 as a SAT while another received CT by readdition of inhibitor at regular 2-day feeding intervals. Mean cell counts from triplicate samples harvested on the days indicated are shown.

plished by staining fixed cells with fluorescein-phalloidin, which avidly binds F-actin, and examining them by fluorescence microscopy (see Materials and Methods).

We observed that treatment of Rat1/ras cells with L-739,749 induced the formation of actin stress fibers concomitantly with cell enlargement (Fig. 5A). Similar to what was seen in other transformed cell types (6), actin was concentrated in membrane ruffles and substratum-attachment sites in the transformed cells while stress fibers predominated in the untransformed Rat1 cells. In Rat1/ras cells, the appearance of actin stress fibers was coordinated kinetically with the reversion phenomenon and correlated with the loss of anchorage-independent growth. Furthermore, membrane ruffles decreased and then disappeared as stress fibers gradually came to predominate (data not shown). In contrast to the dramatic effects observed in Rat1/ras cells, treatment of Rat1/raf cells with L-739,749 had no observable effect on cell size or cytoskeletal actin organization. These observations were consistent with the inability of FPTase inhibitors to block the anchorage-independent growth of Rat1/raf cells (29, 30).

TABLE 1. Effect of L-739,749 on the average doubling time of Rat1, Rat1/ras, and Rat1/raf cell cultures

Cell type	Treatment (day 7)	Avg no. of divisions/week[a]	Avg doubling time (h)[b]	% Change[c]
Rat1/ras	Control	7.6	22.1	NA[d]
	SAT	6.5	25.8	−17
	CT	4.3	39.1	−77
Rat1/raf	Control	8.1	20.7	NA
	SAT	8.2	20.5	+1
	CT	8.0	20.9	−1
Rat1	Control	6.6	25.5	NA
	SAT	6.0	28.0	−10
	CT	5.4	31.1	−22

[a] The average number of cell divisions per week was obtained by dividing by 7 the fold increase in the cell culture population between day 0 and day 7.
[b] Average cell doubling time was determined by dividing 7 by the number of cell divisions per week and multiplying by 24.
[c] Change in the doubling time after exposure to L-739,749 is expressed as a percentage of the average doubling time of untreated cells.
[d] NA, not applicable.

Intriguingly, L-739,749 augmented formation of actin stress fibers and caused detectable cell enlargement in normal Rat1 cells (Fig. 5A). These effects were not specific to L-739,749 but were also observed with other FPTase inhibitors (data not shown). This observation was important because it suggested that Rat1/ras cell reversion might be caused by the effects of FPTase inhibition on regulators of cytoskeletal actin rather than on oncogenic Ras itself. L-739,749 was titrated in Rat1/ras cell cultures to determine whether cell enlargement and stress fiber formation could be separated from phenotypic reversion per se. We observed that both cell enlargement and stress fiber formation were concentration dependent, being augmented by increasing amounts of L-739,749 (Fig. 5B). L-739,749 also caused cell enlargement and an increase in stress fibers in two other transformed cell lines, Rat1/src (Rat1 cells transformed by the v-*src* oncogene) and T24 human bladder carcinoma cells, that contained normal and oncogenic Ras, respectively (data not shown). We concluded that the increase in cell size and actin stress fibers elicited by L-739,749 occurred by a mechanism that was separable from mutated Ras expression.

The reverted cellular phenotype does not correlate with the state of Ras processing. The persistence of the reverted phenotype of Rat1/ras cells after L-739,749 was removed from the culture medium prompted us to examine the state of Ras processing in reverted cells. Western analysis was employed to observe the entire steady-state pool of cellular Ras. Extracts were prepared from cells that were left untreated or treated once with inhibitor and harvested at various times afterward. For the longer time points, cells were passaged normally in medium lacking inhibitor as they approached confluence (conditions similar to those of the experiment whose results are shown in Fig. 2C). The flattened morphology of cells prepared at all the points examined was verified by microscopy. The results from Western analysis of various cell extracts with anti-Ras Y13-259 are shown in Fig. 6.

The two bands seen in untreated cell extracts represented processed forms of phosphorylated (upper band; P-Ras) and

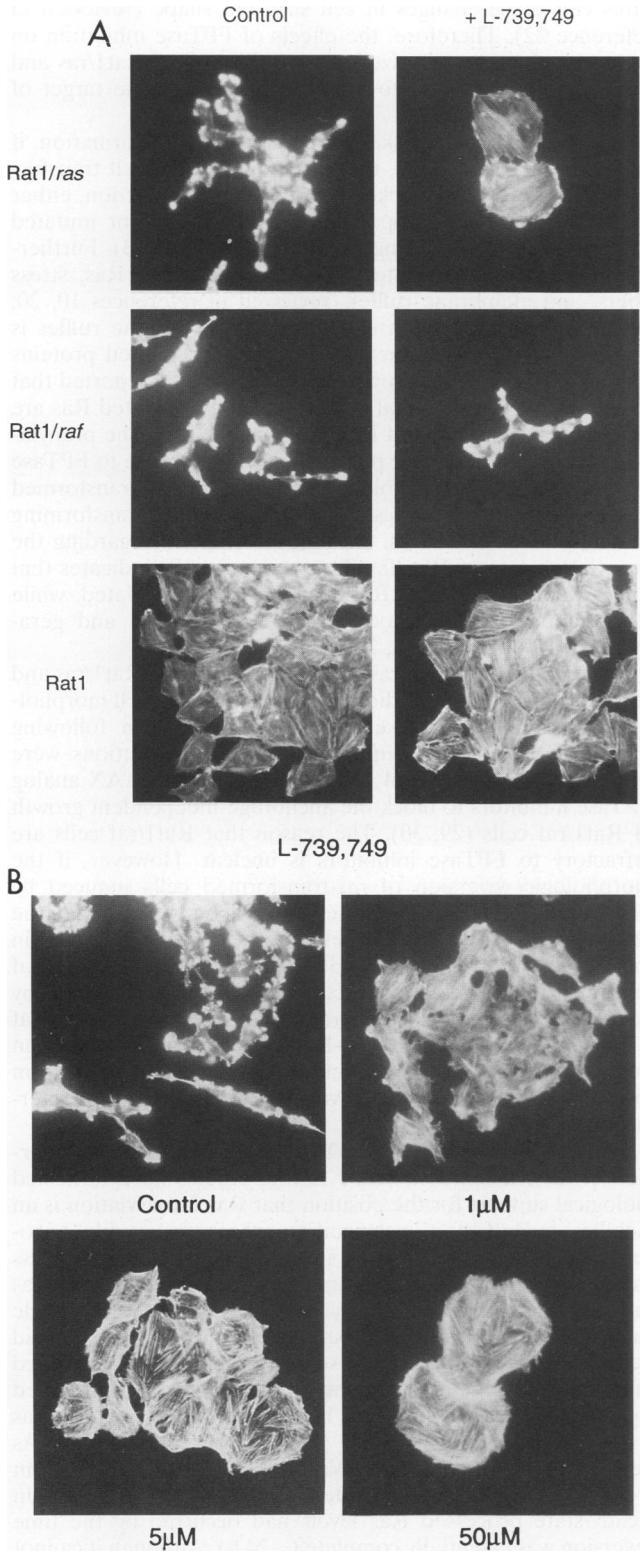

FIG. 5. L-739,749 induces cell enlargement and actin stress fibers in cell lines containing normal or oncogenic Ras. (A) Effects on Rat1, Rat1/ras, and Rat1/raf cells. A total of $\sim 10^3$ cells were plated in tissue culture slides 1 day before treatment with 50 μM L-739,749. Following a 2-day incubation, cells were fixed, stained with fluorescein-phalloidin, and processed for fluorescence microscopy. (B) Concentration dependence of cell enlargement and actin stress fiber formation in Rat1/ras cells. Cells were seeded and treated as above with the indicated concentrations of L-739,749 before being processed for fluorescence microscopy.

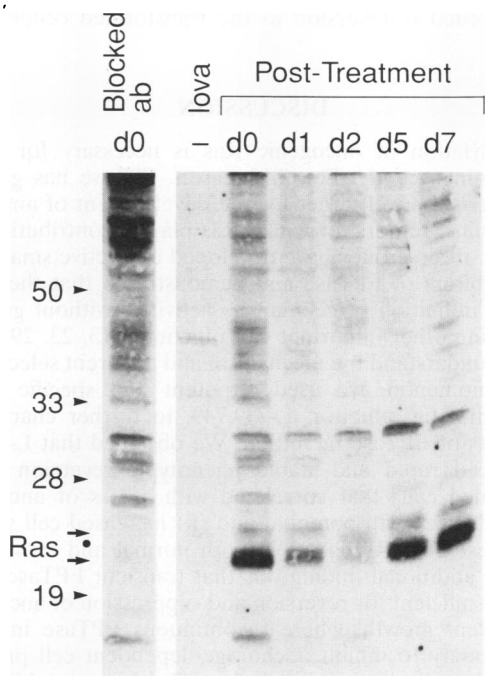

FIG. 6. Steady-state levels of processed Ras correlate poorly with the reverted phenotype. Western analysis with anti-Ras Y13-259 antiserum was performed on 25 μg of protein prepared from Rat1/ras cells incubated for the indicated times after a single addition of 50 μM L-739,749 (see Materials and Methods). A second blot was processed with antiserum that had been blocked with an excess of specific Ras peptide (Blocked ab). The arrows indicate processed forms of unphosphorylated (bottom band) and phosphorylated (top band) v-H-Ras protein. The dot indicates the position of a band observed at day 2 and in the lane containing lovastatin-treated cell extract, representing unprocessed, unphosphorylated Ras (Fig. 2A) (29). Relative molecular size (in kilodaltons) is indicated to the left of the gel.

unphosphorylated (lower band; Ras) v-H-Ras proteins. One day after L-739,749 treatment, there was an approximately twofold decrease in Ras and a slight but detectable decrease in the level of P-Ras. These observations were consistent with the ~20- and ~56-h half-life reported for Ras and P-Ras, respectively (47). Cells at this time were fully reverted even though Ras was expressed at appreciable levels. These data argued that reversion was not initiated solely by a loss of Ras function. After 2 days of L-739,749 treatment, Ras and P-Ras levels were significantly decreased. A novel band appeared at this time with a mobility similar to that seen in extracts from lovastatin-treated cells, representing unprocessed Ras (29). Unprocessed P-Ras was not detected, although it was detectable in metabolically labelled cells (Fig. 2A). These data suggested that the unprocessed forms of Ras were relatively unstable, since they did not accumulate substantially. By 5 days after treatment with L-739,749, processed Ras levels returned to pretreatment levels and were unchanged during the remainder of the assay. At this time, cells were flat and remained so until the experiment was concluded at 7 days after inhibitor addition. We concluded that reversion occurred prior to complete inhibition of Ras processing and that the full reappearance of processed

Ras preceded re-reversion to the transformed cellular morphology.

DISCUSSION

Farnesylation of oncogenic Ras is necessary for its cell transforming activity. For this reason, FPTase has garnered attention as a novel target for the development of anticancer drugs against tumors for which Ras plays a contributing role. Recently, three groups have developed cell-active small molecule inhibitors of FPTase and demonstrated that they cause selective inhibition of cellular *ras* activities without generally suppressing other important cell functions (13, 23, 29). In an effort to understand the mechanism and apparent selectivity of this phenomenon, we used a potent and specific CAAX peptidomimetic inhibitor, L-739,749, to further characterize the effects of FPTase inhibition. We observed that L-739,749 (i) induced rapid and stable phenotypic reversion of *ras*-transformed cells that correlated with a loss of anchorage-independent growth potential and (ii) increased cell size and actin stress fiber formation in both normal and transformed cells. An additional finding was that transient FPTase inhibition was sufficient for reversion and suppression of anchorage-independent growth, whereas continuous FPTase inhibition was necessary to inhibit anchorage-dependent cell proliferation. Although the biochemical mechanism of inhibitor-induced reversion was unclear, the results suggested that the reverted phenotype was not initiated solely by a loss of molecular Ras activity and was not maintained solely because of an inhibition of Ras processing.

Effects of FPTase inhibition by L-739,749 on cell growth and cytoskeletal actin organization. The observation that L-739,749 inhibited the anchorage-independent and anchorage-dependent growth of Rat1/ras cells recapitulated earlier studies of other pharmacological inhibitors of FPTase (23, 29). The results also corroborated genetic experiments performed to assess the effects of FPTase inhibition (21, 37). The genetic approaches that were explored should be considered to be analogous to constitutive pharmacological treatments. We found with Rat1/ras cells that the effects of transient treatment (anchorage-independent growth inhibition, morphological reversion, actin stress fiber induction, cell enlargement) could be separated from the effects of constitutive treatment (anchorage-dependent growth inhibition). This separation suggests that greater selectivity against *ras*-transformed cells may be achieved by transient pharmacological treatment, which was sufficient to block transformation parameters, rather than more stringent repetitive treatment, which caused more pronounced inhibition of anchorage-dependent (i.e., normal) growth in both *ras*-transformed and normal Rat1 cells. The implications of this finding may be important to the design of future experiments required to assess the utility of FPTase inhibitors as antitumor agents.

We found that FPTase inhibition caused an increase in cell size and actin stress fiber formation in normal Rat1, Rat1/ras, Rat1/src, and T24 cells, but not in Rat1/raf cells. We also observed that the level of membrane ruffles, another cellular actin structure, was reduced in Rat1/ras cells by FPTase inhibition. Stress fibers are rigid elements that terminate at focal adhesions, which are located at plasma membrane sites where cells attach to substratum. Focal adhesions contain many factors that are important for cell structure and signal transduction, including integrins, actin-tipping proteins, oncogene products, and kinases (for reviews of cellular actin structures and focal adhesion function, see references 6, 44, and 48). Changes in actin stress fibers and other actin structures can cause changes in cell size and shape (reviewed in reference 22). Therefore, the effects of FPTase inhibition on actin organization, cell size, and morphology of Rat1/ras and Rat1 cells may be due to modification of a single target of inhibitor treatment.

A causal role for cytoskeletal actin in *ras* transformation, if any, is unclear. However, there is evidence that cell transformation by *ras* can be blocked by altering actin function, either by overexpression of tropomyosin 1 cDNA (35) or mutated forms of the actin-severing protein gelsolin (12, 33). Furthermore, there exist regulatory connections among Ras, stress fibers, and membrane ruffles (reviewed in references 10, 20, and 38). Formation of stress fibers and membrane ruffles is regulated by the Rho-Rac proteins, a set of related proteins that are part of the Ras superfamily. It has been reported that the changes in cytoskeletal actin induced by activated Ras are mediated by the Rho and Rac proteins (40, 41). The prenylation state of the Rho-Rac proteins may be germane to FPTase inhibitor-induced morphologic reversion of *ras*-transformed cells, if Rho-Rac functions are required for the transforming activity of oncogenic Ras. Current information regarding the prenylated state of Rho family proteins in cells indicates that Rac1, Rac2, RhoA, and RhoC are geranylgeranylated, while RhoB appears to be modified by both farnesyl and geranylgeranyl isoprenoids (1, 28).

We found that, in contrast to the effects seen in Rat1/ras and Rat1 cells, Rat1/raf cells did not show changes in cell morphology, cell size, or cytoskeletal actin organization following treatment with FPTase inhibitors. These observations were consistent with inability of L-739,749 and other CAAX analog FPTase inhibitors to block the anchorage-independent growth of Rat1/raf cells (29, 30). The reason that Raf1/raf cells are refractory to FPTase inhibitors is unclear. However, if the morphologic reversion of *ras*-transformed cells induced by FPTase inhibitors is due to an effect on actin cytoskeleton, the resistance of *raf*-transformed cells may be due to a difference in the regulatory pathways used by oncogenic Ras and Raf proteins to regulate actin structure(s). One potential route by which Ras may control actin structure independently from Raf is through the so-called Rho-Rac pathway, which has been implicated as a signal transduction route that is distinct from the MAPK kinase pathway involving Raf (reviewed in reference 38).

What is the key step(s) in FPTase inhibitor-induced reversion of *ras*-transformed cells? Earlier studies have furnished biological support for the position that Ras farnesylation is an Achilles' heel of the transformation process induced by oncogenic *ras*. Our analysis of the morphologic reversion process caused by FPTase inhibitors indicates that inhibition of Ras function due to a loss of farnesylated Ras may not be the single causative step in reversion. First, the onset of reversion is rapid ($\leq$18 h). If reversion were due solely to the loss of farnesylated oncogenic Ras, the reverted phenotype would not be expected to appear for several days after FPTase inhibition, because Ras is a relatively stable protein (half-life of ~20 h [47]). As demonstrated above (Fig. 6), Western analysis of extracts from L-739,749-treated cells indicated that a ~50% reduction in steady-state processed Ras levels had occurred by the time reversion was essentially complete (~24 h). Although it cannot be rigorously ruled out, it seems unlikely that a twofold reduction in oncogenic Ras levels would be sufficient to cause reversion. Second, morphologic reversion occurred at a point when the steady-state accumulation of unprocessed v-H-Ras in Rat1/ras cells was undetectable. This observation argued against a model in which soluble unprocessed Ras could act as a dominant inhibitor, and was consistent with previous work

showing that soluble valine 12 Ras mutants (like v-H-Ras) lack significant inhibitory activity even when overexpressed (24). Thus, loss of oncogenic Ras function(s) due to a loss of farnesylated Ras protein may not be the sole causative event for reversion induced by FPTase inhibitors.

There are other results that strengthen this interpretation. Analysis of a variant Rat1/ras-derived cell line that is resistant to L-739,749-induced reversion (36) cast doubt on a necessary role for soluble Ras in L-739,749-induced reversion. The resistant cell line, termed 749^r-1, did not morphologically revert following treatment with FPTase inhibitors, despite the fact that its FPTase activity was inhibited by L-739,749 and its processed Ras levels decreased transiently similar to that observed in Rat1/ras cells. These data further uncoupled L-739,749-induced reversion of *ras*-transformed cells from inhibition of Ras processing. One cannot rule out the possibility that inhibition of Ras farnesylation is necessary but by itself is not sufficient for morphological reversion. However, when taken together, the data reported here and elsewhere (36) suggest that FPTase inhibitor-induced morphologic reversion of *ras*-transformed cells is neither initiated by a loss of Ras function nor maintained by inhibition of Ras farnesylation.

If it is not Ras alone, then what might be the other critical farnesylated protein target(s) of FPTase inhibition in *ras*-transformed cells? A key step in reversion might involve regulators of actin-based cell structure since FPTase inhibitors had direct effects on cell size and stress fiber formation in normal cells. In *ras*-transformed cells, these effects were separable from reversion per se, since the efficiency of cell size and stress fiber induction but not the reversion phenomenon were affected by varying inhibitor concentration. One model for explaining the mechanism by which reversion was initiated proposes the loss of function of a short-lived, farnesylated protein whose cellular activity depends on farnesylation. In its simplest form, this model is not sufficient to explain the mechanism by which reversion is maintained, and therefore must be considered incomplete. Other, more complicated variations of this model for initiation of reversion would invoke a change of function in a farnesylated protein(s) when it is not processed correctly. For example, a nonfarnesylated form of the target protein(s) might stably accumulate and cause a dominant inhibitory effect that leads to reversion. Such an effect has been observed with soluble forms of Ras encoded by the activated c-H-$ras^{Leu\text{-}61}$ allele (17). Alternately, following FPTase inhibition in cells, one might envision geranylgeranylation of a normally farnesylated protein that results in a change-of-function activity.

What is the basis for the stability of the reverted state? After being induced, the reverted cell state had two surprising and intriguing features. One was its persistence for several days in the absence of inhibitor in the cell growth medium. Both morphological reversion and inhibition of anchorage-independent growth of Rat1/ras cells required only a single exposure to L-739,749; replenishment of the drug in cell culture was unnecessary. This feature was unusual since one might have predicted that inhibitor would be required to maintain as well as to initiate phenotypic reversion. In our experiments, it seems unlikely that L-739,749 persists inside the cell for the 7- to 10-day course of the growth and morphology assays. Cells were washed and passaged several times during the course of the monolayer re-reversion experiments. Any intracellular pool of inhibitor would be significantly diluted after several cell divisions. In any case, Western analysis showed that in reverted cells v-H-Ras was farnesylated with pretreatment efficiency within 5 days of L-739,749 addition, even though cells remained flat for at least two additional days. This observation indicated that FPTase must be active at this time since it could utilize Ras as a substrate.

Another unanticipated feature of the reverted state was that, by itself, the reappearance of processed, oncogenic Ras in the reverted cells was apparently insufficient to cause retransformation. Together with its prolonged stability, this feature suggested that the physiology of the morphologically reverted state was different from that of normal cells, despite some phenotypic resemblance. These attributes prompt a comparison with cellular differentiation in vitro. Similar to the L-739,749-induced phenomenon, after differentiation has been induced, the differentiated state remains stable after removal of the inducing agent if commitment has been reached. Additionally, the reverted cell state induced by FPTase inhibition may resemble that seen in tumor regression studies of the nontoxic plant-specific monoterpene limonene (see reference 19 and references therein), which is reported to block cellular protein prenylation (9). Reminiscent of the effects of cell treatment with FPTase inhibitors, tumors that were caused to regress by dietary limonene exhibited a histopathology that resembled a differentiation-like process with prolonged stability, rather than apoptosis, immune infiltration, or other observed regression phenomena (19). We speculate that the normal fibroblast phenotype that is induced in *ras*-transformed cells by FPTase inhibition may be in fact a more complicated physiological state than can be explained by simple antagonism of *ras*-induced biological properties. Future studies will be required to assess this possibility.

ACKNOWLEDGMENTS

We thank our colleagues S. Barnett, J. Condra, P. Huang, K. Koblan, C. Omer, and D. Pompliano for discussions and criticism. The technical assistance of D. Shah is gratefully acknowledged. G.C.P. thanks C. Woods for technical advice on immunofluorescence.

REFERENCES

1. **Adamson, P., C. J. Marshall, A. Hall, and P. A. Tilbrook.** 1992. Post-translational modification of $p21^{rho}$ proteins. J. Biol. Chem. **267**:20033–20038.
2. **Barbacid, M.** 1987. *ras* genes. Annu. Rev. Biochem. **56**:779–827.
3. **Bar-Sagi, D., and J. R. Feramisco.** 1986. Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by *ras* proteins. Science **233**:1061–1066.
4. **Bos, J. L.** 1990. ras gene mutations and human cancer, p. 273–287. *In* J. Cossman (ed.), Molecular genetics in cancer diagnosis. Elsevier Scientific Publishing Co., Amsterdam.
5. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature (London) **348**:125–132.
6. **Bretscher, A.** 1991. Microfilament structure and function in the cortical cytoskeleton. Annu. Rev. Cell Biol. **7**:337–374.
7. **Chardin, P.** 1991. Small GTP-binding proteins of the Ras family: a conserved functional mechanism? Cancer Cells **3**:117–126.
8. **Cox, A. D., M. M. Hisaka, J. E. Buss, and C. J. Der.** 1992. Specific isoprenoid modification is required for function of normal, but not oncogeneic, Ras function. Mol. Cell. Biol. **12**:2606–2615.
9. **Crowell, P. L., R. R. Chang, Z. Ren, E. C. Elson, and M. N. Gould.** 1991. Selective inhibition of isoprenylation of 21-26-kDa proteins by the anticarcinogen d-limonene and its metabolites. J. Biol. Chem. **266**:17679–17685.

9a. **Davide, J., and G. C. Prendergast.** Unpublished data.

10. **Downward, J.** 1992. Rac and Rho in tune. Nature (London) **359**:273–275.

11. **Farnsworth, C. C., S. L. Wolda, M. H. Gelb, and J. A. Glomset.** 1989. Human lamin B contains a farnesylated cysteine residue. J. Biol. Chem. **264:**20422–20429.
12. **Fujita, H., H. Suzuki, N. Kuzumaki, L. Müllauer, Y. Ogiso, A. Oda, K. Ebisawa, T. Sakurai, Y. Nonomura, and S. Kijimoto-Ochiai.** 1990. A specific protein, p92, detected in flat revertants derived from NIH/3T3 transformed by human activated c-Ha-*ras* oncogene. Exp. Cell Res. **186:**115–121.
13. **Garcia, A. M., C. Rowell, K. Ackermann, J. J. Kowalczyk, and M. D. Lewis.** 1993. Peptidomimetic inhibitors of Ras farnesylation and function in whole cells. J. Biol. Chem. **268:**18415–18418.
14. **Georges, R. N., T. Mukhopadhyay, Y. Zhang, N. Yen, and J. A. Roth.** 1993. Prevention of orthotopic human lung cancer growth by intratracheal instillation of a retroviral antisense K-ras construct. Cancer Res. **53:**1743–1746.
15. **Gibbs, J. B.** 1991. Ras C-terminal processing enzymes—new drug targets? Cell **65:**1–4.
16. **Gibbs, J. B.** 1993. Lipid modifications of proteins in the Ras superfamily, p. 335–344. *In* L. Birnbaumer and B. Dickey (ed.), GTPases in biology. Springer-Verlag, New York.
17. **Gibbs, J. B., M. D. Schaber, T. L. Schofield, E. M. Scolnick, and I. S. Sigal.** 1989. *Xenopus* oocyte germinal-vesicle breakdown induced by [Val^{12}]Ras is inhibited by a cytosol-localized Ras mutant. Proc. Natl. Acad. Sci. USA **86:**6630–6634.
18. **Graham, S. L., S. J. deSolms, E. A. Giuliani, N. E. Kohl, S. D. Mosser, A. I. Oliff, D. L. Pompliano, E. Rands, M. J. Breslin, A. A. Deana, V. M. Garsky, T. H. Scholz, J. B. Gibbs, and R. L. Smith.** 1994. Pseudopeptide inhibitors of Ras farnesyl-protein transferase. J. Med. Chem. **37:**725–732.
19. **Haag, J. D., M. J. Lindstrom, and M. N. Gould.** 1992. Limonene-induced regression of mammary carcinomas. Cancer Res. **52:** 4021–4026.
20. **Hall, A.** 1992. Ras-related GTPases and the cytoskeleton. Mol. Biol. Cell. **3:**475–479.
21. **He, B., P. Chen, S.-Y. Chen, K. L. Vancura, S. Michaelis, and S. Powers.** 1991. *RAM2*, an essential gene of yeast, and *RAM1* encode the two polypeptide components of the farnesyltransferase that prenylates a-factor and Ras proteins. Proc. Natl. Acad. Sci. USA **88:**11373–11377.
22. **Ingber, D.** 1993. Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. J. Cell Sci. **104:**613–627.
23. **James, G. L., J. L. Goldstein, M. S. Brown, T. E. Rawson, T. C. Somers, R. S. McDowell, C. W. Crowley, B. K. Lucas, A. D. Levinson, and J. C. Marsters.** 1993. Benzodiazepine peptidomimetics: potent inhibitors of Ras farnesylation in animal cells. Science **260:**1937–1942.
24. **Johnston, C., J. Morris, and A. Hall.** 1991. The role of Ras in signal transduction. Biochem. Soc. Trans. **19:**296–299.
25. **Kato, K., A. D. Cox, M. M. Hisaka, S. M. Graham, J. E. Buss, and C. J. Der.** 1992. Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. Proc. Natl. Acad. Sci. USA **89:**6403–6407.
26. **Khosravi-Far, R., A. D. Cox, K. Kato, and C. Der.** 1992. Protein prenylation: key to Ras function and cancer intervention? Cell Growth Differ. **3:**461–469.
27. **Kim, C. M., J. L. Goldstein, and M. S. Brown.** 1992. cDNA cloning of MEV, a mutant protein that facilitates cellular uptake of mevalonate, and identification of the point mutation responsible for its gain of function. J. Biol. Chem. **267:**23113–23121.
28. **Kinsella, B. T., R. A. Erdman, and W. A. Maltese.** 1991. Carboxyl-terminal isoprenylation of *ras*-related GTP-binding proteins encoded by *rac*1, *rac*2 and *ral*A. J. Biol. Chem. **266:**9786–9794.
29. **Kohl, N. E., S. D. Mosser, S. J. deSolms, E. A. Giuliani, D. L. Pompliano, S. L. Graham, R. L. Smith, E. M. Scolnick, A. Oliff, and J. B. Gibbs.** 1993. Selective inhibition of *ras*-dependent transformation by a farnesyltransferase inhibitor. Science **260:** 1934–1937.
30. **Kohl, N. E., F. R. Wilson, S. D. Mosser, E. Giuliani, S. J. deSolms, M. W. Conner, N. J. Anthony, W. J. Holtz, R. P. Gomez, T.-J. Lee, R. L. Smith, S. L. Graham, G. D. Hartman, J. B. Gibbs, and A. Oliff.** Farnesyltransferase inhibitors block the growth of *ras*-dependent tumors in nude mice. Submitted for publication.
31. **Maltese, W. A.** 1990. Posttranslational modification of proteins by isoprenoids in mammalian cells. FASEB J. **4:**3319–3327.
32. **Moores, S. L., M. D. Schaber, S. D. Mosser, E. Rands, M. B. O'Hara, V. M. Garsky, M. S. Marshall, D. L. Pompliano, and J. B. Gibbs.** 1991. Sequence dependence of protein isoprenylation. J. Biol. Chem. **266:**14603–14610.
33. **Müllauer, L., H. Fujita, A. Ishizaki, and N. Kuzumaki.** 1993. Tumor-suppressive function of mutated gelsolin in *ras*-transformed cells. Oncogene **8:**2531–2536.
34. **Pompliano, D. L., E. Rands, M. D. Schaber, S. D. Mosser, N. J. Anthony, and J. B. Gibbs.** 1992. Steady-state kinetic mechanism of Ras farnesyl:protein transferase. Biochemistry **31:**3800–3807.
35. **Prasad, G. L., R. A. Fuldner, and H. L. Cooper.** 1993. Expression of transduced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the *ras* oncogene. Proc. Natl. Acad. Sci. USA **90:**7039–7043.
36. **Prendergast, G. C., J. P. Davide, J. B. Gibbs, and N. E. Kohl.** Resistance of a variant *ras*-transformed cell line to phenotypic reversion by farnesyltransferase inhibitors. Submitted for publication.
37. **Prendergast, G. C., J. P. Davide, A. Kral, R. D. Diehl, C. A. Omer, J. B. Gibbs, and N. E. Kohl.** 1993. Negative growth selection against rodent fibroblasts targeted for genetic inhibition of farnesyl transferase. Cell Growth Differ. **4:**1–7.
38. **Prendergast, G. C., and J. B. Gibbs.** 1993. Pathways of Ras function: connections to the actin cytoskeleton. Adv. Cancer Res. **62:**19–63.
39. **Prendergast, G. C., and E. B. Ziff.** 1991. Mbh1: a novel gelsolin/severin-related protein which binds actin *in vitro* and exhibits nuclear localization *in vivo*. EMBO J. **10:**757–766.
40. **Ridley, A. J., and A. Hall.** 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell **70:**389–399.
41. **Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann, and A. Hall.** 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell **70:**401–410.
42. **Seabra, M. C., Y. Reiss, P. J. Casey, M. S. Brown, and J. L. Goldstein.** 1991. Protein farnesyltransferase and geranylgeranyltransferase share a common α subunit. Cell **65:**429–434.
43. **Shirasawa, S., M. Furuse, N. Yokoyama, and T. Sasazuki.** 1993. Altered growth of human colon cancer cell lines disrupted at activated Ki-Ras. Science **260:**85–88.
44. **Small, J. V.** 1988. The actin cytoskeleton. Electron Microsc. Rev. **1:**55–174.
45. **Smith, M. R., S. J. DeGudicibus, and D. W. Stacey.** 1986. Requirement for c-*ras* proteins during viral oncogene transformation. Nature (London) **320:**540–543.
46. **Trueblood, C. E., Y. Ohya, and J. Rine.** 1993. Genetic evidence for in vivo cross-specificity of the CaaX-box protein prenyltransferases farnesyltransferase and geranylgeranyltransferase-I in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**4260–4275.
47. **Ulsh, L. S., and T. Y. Shih.** 1984. Metabolic turnover of human c-*ras*^{H} p21 protein of EJ bladder carcinoma and its normal cellular and viral homologs. Mol. Cell. Biol. **4:**1647–1652.
48. **Zachary, I., and E. Rozengurt.** 1992. Focal adhesion kinase (p125^{FAK}): a point of convergence in the action of neuropeptides, integrins, and oncogenes. Cell **71:**891–894.