Selective Inhibition of Ras-dependent Cell Growth by Farnesylthiosalisylic Acid*

(Received for publication, January 3, 1995, and in revised form, June 5, 1995)

M. Maromt, R. Haklait, G. Ben-Baruch§, D. Marciano, Y. Egazi†, and Y. Kloog‡

From the Department of Biochemistry, The George S. Wise Faculty of Life Sciences, the Department of Obstetrics & Gynecology, Sheba Medical Center, Tel Hashomer and the Sackler School of Medicine, Tel Aviv University, Tel Aviv 699787, and the Israel Institute for Biological Research, POB 19, Ness Ziona 70450, Israel

S-trans,farnesylthiosalisylic acid (FTS) is a novel farnesylated rigid carboxylic acid derivative. In cell-free systems, it acts as a potent competitive inhibitor ($K_i = 2.6 \mu M$) of the enzyme prenylated protein methylytransferase (PPMTase), which methylates the carboxyl-terminal S-prenylycysteine in a large number of prenylated proteins including Ras. In such systems, FTS inhibits Ras methylation but not Ras farnesylation. Inhibition of the PPMTase by FTS in homogenates or membranes of a variety of tissues and cell lines is inferred from a block in the methylation of exogenously added substrates such as N-acetyl-S-trans,farnesyld-cysteine and of endogenous substrates including small GTP-binding proteins. FTS can also inhibit methylation of these proteins in intact cells (e.g., in Rat-1 fibroblasts, Ras-transformed Rat-1, and B16 melanoma cells). Unlike in cell-free systems, however, relatively high concentrations of FTS (50–100 $\mu M$) are required for partial blocking (10–40%) of protein methylation in the intact cells. Thus, FTS is a weak inhibitor of methylation in intact cells. Because methylation is the last step in the processing of Ras and related proteins, FTS is not likely to affect steps that precede it, e.g., protein prenylation. This may explain why the growth and gross morphology of a variety of cultured cell types (including Chinese hamster ovary, NIH3T3, Rat1, B16 melanoma, and PC12) is not affected by up to 25 $\mu M$ FTS and is consistent with the observed lack of FTS-induced cytotoxicity. Nevertheless, FTS reduces the levels of Ras in cell membranes and can inhibit Ras-dependent cell growth in vitro, independent of methylation. It inhibits the growth of human Ha-ras-transformed cells (E cells) and reverses their transformed morphology in a dose-dependent manner (0.1–10 $\mu M$). The drug does not interfere with the growth of cells transformed by v-Raf or T-antigen but inhibits the growth of ErbB2-transformed cells and blocks the mitogenic effects of epidermal and basic fibroblast growth factors, thus implying its selectivity toward Ras growth signaling, possibly via modulation of Ras-Raf communication. Taken together, the results raise the possibility that FTS may specifically interfere with the interaction of Ras with a farnesylcysteine recognition domain in the cell membrane. This drug, and perhaps other farnesylated rigid carboxylic acid analogs, may be used for in vitro characterization of the PPMTase and for the identification of a putative Ras farnesylcysteine recognition domain in cell membranes.

The free carboxyl group of the terminal farnesylcysteine or geranylgeranylcysteine in a large number of prenylated proteins is methylated by the prenylated protein methyltransferase (PPMTase), a unique enzyme among methyltransferases (1). This enzyme associates tightly with the particulate fractions in eukaryotic cells (2–5) and has an absolute requirement that its substrates contain the free carboxyl group and S-farnesyl- or S-geranylgeranyl moieties (4–6). The sequence of the yeast PPMTase gene (STE14) indeed predicts a hydrophobic membrane-associated protein (7, 8). Many of its natural substrates are key regulatory elements in receptor signal transduction pathways (9–15), for example Ras and the heterotrimeric G-proteins. Methylation of these proteins would increase the hydrophobicity of their carboxyl terminus, already rendered hydrophobic by prenylation (16–19). Because methylation is the last and only possible reversible step in the processing of such prenylated proteins, it is likely that the PPMTase modulates their interactions with membranes and other proteins. It is also reasonable to assume that the methyl-acceptor recognition site in the PPMTase shares some similarities with prenyltransferase recognition domains found in target sites for prenylated proteins. Such sites are not necessarily located within the lipid bilayer (20).

The view that protein prenylation would mediate protein-protein interaction (20), as well as the possibility of separate targets for the methylated and unmethylated forms of prenylated proteins, has recently been discussed (5). Very little is known about the nature of prenyltransferase recognition domains. They are likely to exist, however, in at least three other proteins besides the PPMTase: the GDP-dissociation inhibitory protein, which binds only to the fully processed rab3A (21), and the yeast a-factor transporter (8) and receptor (8, 22), which may require the fully processed a-factor. Also, the existence of farnesyltransferase recognition domains for Ras proteins may be predicted from the absolute requirement of the farnesylcysteine for Ras oncprotein membrane anchorage and transforming activity (10, 19, 23).

An approach that has provided valuable information on the prenyltransferase recognition domain of the PPMTase is the use of synthetic farnesyl derivatives. To be recognized by this domain, a PPMTase substrate need not be a peptide; N-acetyl-S-trans,farnesyl-L-cysteine (AFC) is an excellent substrate for the enzyme (5, 6). Other N-acetylated farnesylcysteine analogs can also act as substrates provided that they do not have bulky moieties (24). Whereas such moieties can prevent association of

* This work was supported by The Israel Cancer Research Foundation and by the SAFHAO Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom all correspondence should be addressed. Tel.: 972-3-6406699; Fax: 972-3-6407643.

1 The abbreviations used are: PPMTase, prenylated protein methyltransferase; FTS, S-trans,farnesylthiosalisylic acid; AFC, N-acetyl-S-trans,farnesyl-L-cysteine; AdoMet, S-adenosyl-L-methionine; MTT, (3-(4,5-dimethylthiosol-2-yl))-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; CHE cells, Chinese hamster embryonic cells.
farnesyl derivatives with the methyl-acceptor recognition domain (24), the total absence of an amino group is tolerated, as shown by the fact that S-farnesylthioacid mimics the diphosphonate moiety and serve as a PPMTase substrate (6, 24, 25). Moreover, S-farnesylthioacetate acid is recognized by the PPMTase, though it does not affect their ability to inhibit the PPMTase.

On the basis of such observations, we have now prepared a novel rigid S-farnesyl carboxylic acid derivative, which, like S-farnesylthioacetate acid, is not an amino acid. We show here that it acts as a pure competitive inhibitor of the PPMTase in cell-free systems. Most interestingly, the results presented here together with those presented in a related study (26) suggest that rigid farnesylated carboxylic acid derivatives can inhibit Ras-dependent cell growth in a mechanism unrelated to their ability to inhibit the PPMTase.

**EXPERIMENTAL PROCEDURES**

**Materials**—AFC and FTS were prepared by a general procedure (6) and purified and analyzed as described in detail elsewhere (26). Purity of compounds, as assessed by TLC, 1H NMR, and mass spectra analysis was >95%. S-[methyl-3H]-adenosyl-l-methionine ([methyl-3H]AdoMet, 85 Ci/mmol) was from ARC, and S-[methyl-3H]-methionine (85 Ci/mmole) and [3H]thymidine (6.7 Ci/mmol) were from DuPont NEN. Other chemicals were from Sigma and Merck. Gel electrophoresis supplies were from Pharmacia Biotech Inc. Tissue culture supplies were from BectonDickinson (Isreal).

**PPMTase Assays**—Synaptosomal membranes of rat brain cerebellum (25) or total membranes (23, 25) of cultured cell lines (100,000 viable cells) were used for methyltransferase assays in the cell-free systems. Methyltransferase assays were performed at 37°C in 50 mM Tris-HCl buffer, pH 7.4, using 100 μg of protein, 25 μM [methyl-3H]AdoMet (300,000 cpmmg), and 50 μM AFC (prepared as a stock solution in Me2SO) in a total volume of 50 μl. Me2SO concentration in all assays was 5%. Various AFC concentrations were used in several experiments and yielded the following results: 10 min after incubation with 500 μl of chloroform:methanol (1:1) with subsequent addition of 250 μl of H2O, mixing, and phase separation. A 125-μl portion of the chloroform phase was dried at 40°C, and 200 μl of 1 N NaOH, 1% SDS solution was added. The [3H]methanol thus formed was counted by the vapor phase equilibrium method, as previously described (2, 25, 26). The data were expressed in terms of 3H-labeled methyl groups (dpm) determined in gel slices of 20–30-kDa gel migration zones. A reconstituted system for cell-free Ras processing was obtained by a two-step procedure. In the first step (30), bacterially expressed human Ha-Ras (25 μg) and partially purified rat brain farnesyltransferase (2 μg) were incubated (total volume 10 μl) for 1 h at 37°C with 15 pmol [3H]farnesylpyrophosphate (15 Ci/mmole, ARC), 50 mM Tris, 50 μM ZnCl2, 5 mM MgCl2, 20 mM KCl, 1 mM dithiotreitol, 4% Me2SO (control), or 50 μM FTS in 4% Me2SO. Proteins were separated by SDS-PAGE, and [3H]-farnesylated Ras was determined by direct counting of 2-mm gel sections in a Lipoluma Lumasolve scintillation mixture. Neither Ras alone nor farnesyltransferase alone yielded a signal above background. In the second step, samples incubated as described above were treated with 15 μg of rat brain microsomal membranes (5 μg of protein), which served as a source of protease and methyltransferase, and 10 μl (110 pmol) of S-[methyl-3H]-adenosyl-l-methionine (85 Ci/mmmole). Me2SO and FTS concentrations in the test samples were adjusted to 4% and 50 μM, respectively, and the samples were then incubated for 30 min at 37°C. Proteins were separated by SDS-PAGE, and [3H]-labeled methylated primary antibodies were determined by the base hydrolysis vapor phase diffusion assay (2, 26, 27). Assay background counts (no Ras added) were 10% of signals. Control experiments showed that by itself the [3H]-farnesylated Ras, which was formed in the first step, did not yield base-labile difluorescent counts. This enabled us to perform the double tritium labeling procedure described above.

**Cell Culture Procedures**—Appropriate specified conditions were used for culturing of the various cell types employed: Chinese hamster embryonic (CHE) fibroblasts and T-antigen transformed CHE (31), Chinese hamster ovary fibroblasts and B16 melanoma (31), pheochromocytoma PC12 (27), NIH3T3 and v-Raf-transformed NIH3T3 (32), Erib-B2-transformed NIH3T3 (33), Rat1 and human Ha-Ras-transformed Rat1 (E) (28), human endo-derial carcinoma HEC1A (34), bovine capillary endothelial cells (35), mouse cerebellar granule cells (36), and rat brain astrocytes (37). All cultured cells were grown at 37°C humidified 96%, 4% CO2 in media containing 10% serum. Except for the biochemical experiments, which were carried out as described above, cells were grown in 24-well plates. Plating density was as follows (cells/well): PC12, 105; PC12, 106; CHE, endothelial cells, astrocytes, and B13, 6 x 104; Rat1; E, v-Raf-transformed NIH3T3, 2 x 104. 2 h after plating, the cells received either solvent or FTS freshly prepared from a stock solution to yield the final indicated concentrations in 0.1% Me2SO. Media were replaced every 4 days with fresh medium containing the solvent or the drug. Separate experiments indicated that the solvent itself had no effect on cell growth. On the indicated days, the cells were detached from plates by trypsin/EDTA and counted under the light microscope. All assays were performed in quadruplicate. In parallel experiments, cells were stained either with trypan blue or with 3-(4,5-dimethylthio- syl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (38), and the stained cells were examined under the light microscope. In some MTT-stained cultures, the cells were collected in 0.2 ml of 100% Me2SO and the extent of staining was determined spectrophotometrically (A650, A510) using an enzyme-linked immunosorbent assay reader.

**Immunoprecipitation and Immunoblotting**—EJ cells were plated at a density of 2 x 105 cells/75-cm2 flask, grown for 2 days as detailed above, and then incubated for 12 h with 25 μM FTS or with 0.1% Me2SO (control). The cells were then detached from the flasks and washed in phosphate-buffered saline. All of the subsequent procedures were carried out at 4°C. The cell pellets were homogenized in 2 ml of 20 mM Tris-HCl, pH 7.4, containing 5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM EDTA, 1 mM benzamidin, 1 mM phenylmethylsulfonyl fluoride, and 5 units/ml aprotinin. Total cell membranes (P100 and cytosol (S100) were obtained by a 100,000 x g centrifugation step (1h). Following resuspension of the P100 in 2 ml of homogenization buffer, both S100 and P100 received 220 μl of 10x immunoprecipitation buffer (100 mM Tris-HCl, pH 7.5, 1.5 mM NaCl, 10% Triton X-100, 500 μg/ml sodium deoxycholate, 1% SDS). Following 10 min on ice, the insoluble material was removed by a 10-min 10,000 x g spin, and the clear supernatant was saved at −70°C. Samples containing a total of 103–104 P100 protein were preincubated with 1 μg of naive rat IgG and protein G-agarose in a total volume of 500 μl of immunoprecipitation buffer containing the anti-proteases mentioned above. The preincubated samples were incubated for 12 h with 2 μg of Y13-259 antibodies coupled to agarose beads (Oncoogene Science Ab-1A). The beads were then precipitated and washed four times with 1 ml of immunoprecipitation buffer and resuspended in 1 ml of 3% SDS sample buffer. These samples were then incubated for 1 h with 1 μg of 37°C, and then resuspended in 20 μl of SDS sample buffer. Proteins were then separated on 15% SDS-polyacrylamide mini gels and blotted onto a...
nitrilotriacetate paper. The paper was blocked with 10% skim milk in Tris-buffered saline and then incubated for 2 h with 1:250 dilution of a rabbit anti-Ras serum in Tris-buffered saline containing 10 mg/ml bovine serum albumin and 0.05% Tween 20 (Ras antiserum was prepared by immunizing rabbits with recombinant human Ha-Ras). Immunoblots were then incubated for 1 h with 1:5000 dilution of goat anti-rabbit IgG-horseradish peroxidase conjugate (Sigma) and exposed to ECL. Results similar to those described here were also obtained when blots were incubated with Y13–259 antibodies and then with rabbit anti-rat IgG-horseradish peroxidase conjugate (Sigma).

RESULTS

In an attempt to determine whether FTS (Fig. 1a, inset) can interact with PPMTase, we used a rat cerebellum-derived preparation of synaptosomal membranes, which are highly enriched in the enzyme (25). With AFC as a substrate, Vmax in this preparation is 60 pmol/min/mg protein. Thus, together with a sensitive methylation assay (25, 26), it should be possible to detect substrate or inhibitor activity. Incubation of synaptosomal membranes with 25 μM [methyl-3H]AdoMet and 200 μM FTS under conditions that promote efficient methylation of either AFC or S-farnesylthiopropionic acid (25, 26) yielded no methylated products in the standard methylation assay. If FTS were a substrate, the formation of an H3-methylated FTS should be detected in this assay, in which total lipophilic compounds are extracted by chloroform/methanol. We conclude that FTS is not a substrate for rat cerebellum PPMTase. In contrast, FTS inhibited the methylation of AFC in a dose-dependent (Fig. 1a) and competitive manner (Fig. 1b). The estimated K value for FTS in this preparation is 2.6 ± 1.5 μM (n = 4), comparable to the values recorded for S-farnesylthioacetic acid in the same preparation (25) or in rod outer segments (6). As shown in Fig. 1c, in the synaptosomal membrane preparation FTS could also inhibit methylation of endogenous 21–26-kDa proteins, which represent mostly GTP-binding proteins (26). Thus, in this cell-free system, FTS can compete with natural substrates for the prenylcyte recognition domain in the PPMTase. To examine whether FTS can also inhibit the PPMTase in other cell-free systems, we repeated the experiments with homogenates of a variety of tissues and cell types. These include mouse brain, kidney, pancreas, heart and testis, human endometrium and endometrial carcinomas, Rat1 and NIH3T3 fibroblasts, pheochromocytoma PC12 cells, B16 melanoma cells, human Ha-Ras-transformed rat1 (EJ) cells, and human endometrial carcinoma HEC1A cells. In all cases, FTS caused a dose-dependent inhibition of AFC methylation. Typical results obtained with homogenates of EJ cells are shown in Fig. 1a. We conclude that FTS is likely to inhibit PPMTase in any cell-free system, even though its inhibitory potency may vary among cell types and tissues (see Fig. 1a). Such variations were also observed with the PPMTase inhibitor S-farnesylthioacetic acid (24).

Further studies were performed with intact cells. We first examined whether FTS can inhibit protein carboxyl methylation in the cells. Cells were metabolically labeled with [methyl-3H]methionine in the absence (●) and in the presence (○) of 10 μM FTS (see “Experimental Procedures”). Cell homogenates were separated by SDS-PAGE, and carboxyl methylesters were determined in gel slices (25, 26).

Fig. 2. Inhibition of methylation in intact cells by FTS. Methylation was determined following metabolic labeling for 2 h with [methyl-3H]methionine in the absence (●) and in the presence (○) of 10 μM FTS (see “Experimental Procedures”). Cell homogenates were separated by SDS-PAGE, and carboxyl methyl esters were determined in gel slices corresponding to gel migration of 20–30 kDa. a, Rat1 cells; b, EJ cells; and c, B16 cells.

The ability of FTS to inhibit carboxyl methylation of Ras in intact cells was examined in [methyl-3H]methionine-labeled human Ha-ras-transformed Rat1 (EJ) cells. As shown in Fig. 3a, FTS (100 μM) inhibited Ras methylation by 40%. Labeling of EJ cells exposed to 50 μM FTS for 3 days gave similar results.
except that Ras methylation was inhibited by only 20–30%. Thus, FTS appears to be a weak inhibitor of carboxyl methylation in intact cells. It therefore probably does not affect processing steps that precede methylation of prenylated proteins, such as farnesylation (16, 19, 20, 23). Indeed, FTS (50 μM) did not inhibit farnesylation of Ha-Ras in a reconstituted cell-free system. Partially purified farnesyltransferase (2 μg) and Ha-Ras (2.5 μg) were incubated with 15 pmol of [14C]farnesylpyrophosphate for 1 h at 37 °C in the absence (●) or in the presence (○) of 50 μM FTS (see “Experimental Procedures”).

In view of the fact that several prenylated proteins apparently play important roles in cell growth and in cytoskeleton–membrane interactions (16–20), it was reasonable to examine whether FTS can affect the growth and/or morphology of cultured cells. Accordingly, a variety of cell types (Tables I and II) were grown under normal serum and medium conditions for 5 days in the presence of the solvent (0.1% Me2SO) or of 25 μM FTS in the solvent (see “Experimental Procedures”). Cell morphology was examined under the light microscope, with cell counting and trypan blue exclusion staining to detect dead cells and MTT staining to detect live cells. A typical example of an experiment in which Rat1 cells were stained with MTT is shown in Fig. 4, a–c, and the estimated numbers of cells in the various cell types tested are summarized in Tables I and II. The MTT data indicated that under the specified conditions, FTS was not toxic and did not affect the growth of Rat1 (Fig. 4, a–c) or of the other tested cell types, namely NIH3T3, CHE, Chinese hamster ovary, PC12, and B16 cells, bovine capillary endothelial cells, mouse cerebellum granular cells, and rat brain astrocytes. This was confirmed by direct cell counting (Tables I and II). No gross effects of FTS on cell morphology were detected in these cell types, and trypan blue staining indicated that cell death in the presence of 25 μM FTS (5–10%) was similar to that observed in controls.

We therefore conclude that FTS is not cytotoxic and would probably have no effect in vitro on the unstimulated growth and morphology of a variety of non-transformed cells or of some types of transformed cells such as the pheochromocytoma PC12 and melanoma B16 cells. In contrast, FTS had a profound effect on the growth of Rat1 cells transformed by the human Ha-ras oncogene (Ej). At concentrations lower than required for inhi-
bition of Ras methylation, FTS caused a dose-dependent (0.1–10 μM) and time-dependent (3–10 days) inhibition of cell growth in EJ cells (Fig. 5). Cultures grown for 5 days in the presence of 5 μM FTS had fewer and smaller foci than control cultures, and most cells had the flat morphology reminiscent of non-transformed Rat1 cells (Fig. 5b).

The change in EJ cell morphology developed rapidly and was apparent already 24 h to 48 h after drug treatment (Fig. 6). Reversibility of the effect of FTS on EJ cell growth was evident in experiments in which the cells were exposed to 25 μM FTS for 48 h, then washed and replated in a drug-free medium. The number of cells in the replated cultures (determined 5 days after replating) presented 90 ± 7% (n = 4) of controls. These results are also consistent with lack of FTS-induced cytotoxicity in EJ cells.

Fig. 4, d and e, shows MTT-stained EJ cells that were grown in the absence and in the presence of 10 μM FTS. Despite the reduction in cell number, most of the EJ cells were stained by MTT. Trypan blue staining confirmed that FTS did not cause death of the EJ cells. Thus, the observed reduction in the spectrophotometrically determined MTT staining of the FTS-treated EJ cultures (Fig. 4f) probably reflects the reduced number of cells and not cell death. In separate experiments, it could be demonstrated that [3H]thymidine incorporation was reduced by 55% when EJ cells were grown for 24 h in the presence of 25 μM FTS.

While FTS (up to 50 μM) had no effect on the growth or morphology of non-transformed Rat1 cells (Figs. 4 and 5), it did block their mitogenic response to basic fibroblast and epidermal growth factors (Table II). We conclude that FTS can affect growth signaling either when Ras is activated transiently through a tyrosine-kinase growth factor-receptor pathway (39) or when it is activated constitutively. This conclusion was supported by experiments showing that the growth of NIH3T3 cells transformed by the Ras-activating tyrosine-kinase ErbB2 oncogene (40), and of human endometrial carcinoma HEC1A cells that express activated K-Ras (34), was inhibited by FTS (Table II). In contrast, growth of cells transformed by the v-Raf oncogene, whose normal cellular form c-Raf1 is recruited by Ras (41,42) to the plasma membrane where it is activated (43), or of cells transformed by the nuclear T-antigen oncogene (31), was not affected by up to 25 μM FTS (Table II).

Taken together, the results suggest that FTS may directly affect the membrane anchorage of Ras and thereby interfere with Ras-Raf communication. This implies that FTS alters the distribution of cellular Ras. To test this possibility, EJ cells were exposed to 25 μM FTS for 12 h, and the amounts of Ras present in the particulate (P100) and in the cytosolic (S100) fractions of the cells were determined by immunoprecipitation combined with immunoblotting (see “Experimental Procedures”). In agreement with earlier studies (19), Ras was found to be localized predominantly to the P100 fraction of the untreated Ha-ras-transformed cells (Fig. 7). In contrast, most of the Ras in the FTS-treated cells was localized to the S100 fraction (Fig. 7).

**DISCUSSION**

The results of this study indicate that the farnesylated rigid carboxylic acid derivative FTS is a potent competitive inhibitor of the PPMTase (K_i = 2.6 μM) in cell-free systems. In such systems, FTS can inhibit methylation of added synthetic substrates, such as AFC and added Ras, and methylation of endogenous proteins.

Unlike in cell-free systems, in intact cells FTS is a relatively
weak inhibitor of methylation. It is possible that FTS crosses the plasma membrane with low efficacy, perhaps due to its carboxyl moiety. Alternatively, the PPMTase in intact cells, unlike the enzyme in broken cells, may be partially protected from interactions with exogenously added farnesyl derivatives.

Whatever the cause, it is clear that FTS may be a useful tool for studies of PPMTase in cell-free systems but not for studies of methylation of prenylated proteins in intact cells. To achieve significant inhibition of methylation of such proteins in the cells, concentrations higher than 100 μM would be required. This, however, could be an advantage for studying interactions
at relatively low FTS concentrations with farnesylcysteine recognition domains other than one known in the PPMTase.

Such recognition domains for farnesylcysteine are likely to be critical for the activity of Ras oncoproteins, since their carboxyl-terminal farnesylcysteine, but not in its carboxymethylated form (44), is essential for membrane anchorage and transforming activity (10, 19, 23). Similarities between farnesylcysteine domains in Ras targets and in the PPMTase may therefore allow farnesylcysteine mimetics independently to inhibit both carboxymethylation and Ras-dependent cell growth. This is demonstrated here with FTS, which inhibited the growth of Ha-ras-transformed Rat1 cells and reversed their transformed morphology at concentrations (0.1–10 μM) lower than those required to inhibit protein methylation. FTS is therefore one example of a farnesyl derivative that may actually bind to a distinctive farnesylcysteine recognition domain with a higher affinity than its affinity for the PPMTase. This possibility is strengthened by the demonstrated effect of FTS on Ras localization (Fig. 7) and by recent results obtained in a related study with human platelets (24). In that case, it was demonstrated that the PPMTase substrate, AFC, and farnesylcysteine derivatives that do not interact with the enzyme can all inhibit platelet aggregation (24).

The possibility that aside from inhibiting Ras membrane association, FTS may also affect the interaction of proteins other than Ras with their own targets cannot be ruled out. In such a case, parameters other than Ras signaling would be affected by FTS. Additional experiments are required to test this possibility and to examine whether, for example, the effects of FTS on cell morphology and on cell growth are separable, as in the case of the farnesyltransferase inhibitor L-739,749 (45). The latter was found to induce morphological reversion of ras-transformed Rat1 cells and to inhibit their anchorage-independent growth by a mechanism that is unrelated to inhibition of Ras processing yet involves regulation of the actin cytoskeleton (45). Inhibition of the anchorage-dependent growth by L-739,749 appears, however, to correlate with the inhibition of Ras processing (45). Because the effects of FTS on EJ cell morphology and on Ras localization are both relatively fast (apparent within 24 h or less) and because the inhibition of DNA synthesis by FTS is also detected after 24 h of treatment, we cannot tell whether or not the effects of FTS on cell morphology and on cell growth are separable.

The lack of FTS toxicity at concentrations that effectively inhibit Ras-dependent cell growth may be attributable to its low efficacy in inhibiting the processing of prenylated proteins in intact cells. In addition to Ras, many of the proteins involved in regulatory mechanisms in mammalian cells are prenylated. In those that undergo both prenylation and methylation, prenylation occurs first (16–20, 23, 44). Therefore, despite its ability to inhibit methyltransferase in vitro, FTS probably does not affect processing steps that precede methylation in intact cells, and as shown here, even Ras methylation is only weakly affected. Our results then suggest that the consequence of FTS action is a diminution in the levels of Ras in the membranes with a concomitant accumulation of fully processed Ras in the cytosol.

In this respect, the action of FTS is in sharp contrast to the actions of farnesyltransferase inhibitors (29, 46, 47), which inevitably prevent all of the covalent modifications in the Ras CAA box (C = cysteine, A = aliphatic and, X = any amino acid). It is unlikely that FTS, and perhaps other farnesyl derivatives of rigid carboxylic acids (26), interact specifically with Ras farnesylcysteine anchorage sites. High-affinity binding of FTS to such sites would probably affect proper anchoring of Ras in the plasma membrane and thereby disrupt the Ras-dependent activation of c-Raf1. Indeed, our results suggest that FTS affects membrane-anchorage of Ras and inhibits the growth signaling of tyrosine kinases (epidermal and basic fibroblast growth factor receptors and Erb-B2), which use Ras-Raf1 to recruit the mitogen-activated protein kinase cascade (39, 40, 43). This and the lack of an effect on cells transformed by the constitutively activated v-Raf strongly suggest that FTS indeed interferes with tyrosine kinase-activated Ras-Raf communication. In this sense, the consequences of FTS actions would be similar to those of CAAX farnesyltransferase inhibitors. Indeed, neither farnesyltransferase inhibitors (29, 46) nor FTS affected the growth of untransformed fibroblasts grown in the absence of tyrosine kinase-receptor ligands. We report here that the unstimulated growth of cell types other than fibroblasts is also not affected by FTS. Together, our results suggest that FTS can selectively affect Ras-dependent cell growth but not Ras-independent cell division.

It is important to note, however, that the CAAX farnesyltransferase inhibitor BZA-5B does not block the epidermal growth factor-induced activation of the mitogen-activated protein kinase cascade in Rat1 cells (47). This would imply that BZA-5B, unlike FTS, does not block the growth factor’s mitogenic effect. Two alternative explanations have been proposed for the observed resistance of Rat1 cells to BZA-5B (47). The one assumes that this drug does not affect farnesylation of endogenous K- or N-Ras and the other that the cells may use alternative, Ras-independent signaling pathways. FTS is not expected to be selective toward one or other types of Ras, as it only mimics the common structure of their farnesylcysteine. Therefore, usage of Ras-independent pathways is probably the more likely explanation for the resistance of unstimulated Rat1 cells to FTS. Because of the different actions of FTS and CAAX farnesyltransferase inhibitors, it will be interesting to examine whether these two types of inhibitors of Ras oncoprotein-dependent cell growth can act synergistically. Our results suggest potential uses for FTS and its analogs (26) in the characterization of farnesylcysteine recognition domains and in the design of treatments of Ras-dependent cancers.

Acknowledgments—We thank Y. Yarden for the EJ and RB-22 cells, S. Lavi for CO60 cells, S. Gutkind for v-Raf-transformed cells, and S. Smith for editorial assistance.

REFERENCES
1. Clarke, S. (1992) Annu. Rev. Biochem. 61, 355–386
2. Haklai, R., and Kloog, Y. (1990) Biochem. Pharmacol. 40, 1365–1372
3. Yamane, H. K., and Fung, B. K. K. (1989) J. Biochem. (Tokyo) 261, 20100–20105
4. Stenson, R. C., and Clarke, S. (1990) J. Biol. Chem. 265, 16248–16254
5. Volker, C., Miller, R. A., McCleary, W. R., Rao, A., Poenie, M., Backer, J. M., and Stock, J. B. (1991) J. Biol. Chem. 266, 21515–21522
6. Tan, E. W., Perez-Sala, D., Canada, F. J., and Rando, R. R. (1991) J. Biol. Chem. 266, 10719–10722
7. Ashby, M., Errada, P. R., Boyartchuk, V. L., and Rine, J. (1993) Yeast 9, 807–913
8. Sapperson, S., Berkower, C., and Michaelis, S. (1994) Mol. Cell. Biol. 14, 1348–1449
9. Clarke, S., Vogel, J. P., Deschenes, R. J., and Stock, J. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 4643–4647
10. Gutierrez, L., Magee, A. I., Marshall, C. J., and Hancock, J. F. (1989) EMBO J. 8, 15572–15576
11. Backlund, J. S., Simonis, W. F., and Spiegel, A. M. (1990) J. Biol. Chem. 265, 15572–15576
12. Fukada, Y., Takano, T., Ohguro, H., Yoshizawa, T., Akino, T., and Shimonishi, Y. (1990) Nature 346, 658–660
13. Fung, B. K. K., Yamane, H. K., Ota, I. M., and Clarke, S. (1990) FEBS Lett. 260, 313–317
14. Maltese, W. A., Sheridan, K., Repko, E. M., and Erdman, R. A. (1990) J. Biol. Chem. 265, 2148–2155
15. Phillips, M. B., Pillinge, M. H., Staud, R., Volker, C., Rosenfeld, M. E., Weissmann, G., and Stock, J. B. (1993) Science 259, 977–980
16. Gimsaert, J. A., Gelb, M. H., and Famsworth, C. C. (1990) Trends Biochem. Sci. 15, 139–142
17. Maltese, W. A. (1990) FASEB J. 4, 3319–3328
18. Brown, M. S., and Golden, J. L. (1993) Nature 365, 14–15
19. Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989) Cell 57, 1167–1177
An Inhibitor of Ras-dependent Cell Growth

20. Marshall, C. J. (1993) Science 259, 1865–1866
21. Araki, S., Kato, M., Sasaki, T., Hata, Y., and Takai, Y. (1991) Mol. Cell. Biol. 11, 1438–1447
22. Andergott, R. J., Betz, R., Carr, S. A., Crabb, J. W., and Duntze, W. (1988) J. Biol. Chem. 263, 18236–18240
23. Casey, P. J., Solski, P. A., Der, C. J., and Buss, J. E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8323–8327
24. Ma, Y.-T., Shi, Y.-Q., Lim, Y. H., McGrait, S. H., Ware, A., and Rando, R. R. (1994) Biochemistry 33, 5414–5420
25. Ben-Baruch, G., Paz, A., Mariano, D., Egzai, Y., Haklai, R., and Kloor, Y. (1993) Biochem. Biophys. Res. Commun. 195, 282–288
26. Mariano, D., Ben-Baruch, G., Marom, M., Egzai, Y., Haklai, R., and Kloor, Y. (1995) J. Med. Chem. 38, 1267–1272
27. Haklai, R., Lerner, S., and Kloor, Y. (1991) Neuropeptides 24, 11–25
28. Land, H., Parada, L. F., and Weinberg, R. A. (1983) Nature 304, 596–602
29. James, G. L., Goldstein, J. L., Brown, M. S., Rawson, T. E., Somers, T. C., McDowell, R. S., Crowley, C. W., Lucas, B. K., Levinson, A. D., and Marsters, J. C. (1993) Science 260, 1937–1942
30. Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J., and Brown, M. S. (1990) Cell 62, 81–88
31. Lavi, S. (1983) Proc. Natl. Acad. Sci. U. S. A. 78, 6144–6148
32. Xu, N., Bradley, L., Ambdukar, I., and Gutkind, J. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6741–6745
33. Peles, E., Ben-Ley, R., Or, E., Ulrich, A., and Yarden, Y. (1991) EMBO J. 10, 2077–2086
34. Enomoto, T., Enoke, M., Pernantoni, A. O., Terakawa, N., Tanizawa, O., and Rice, J. M. (1990) Cancer Res. 50, 6139–6145
35. Audus, K. L., and Borchardt, R. T. (1987) Ann. N. Y. Acad. Sci. 507, 9–18
36. D’Mello, S. R., Galli, C., Cottit, T., and Calissano, P. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10989–10993
37. McCarthy, K. D., and de Vellis, J. (1980) J. Cell Biol. 85, 890–902
38. Mosmann, T. (1983) J. Immunol. Methods 65, 55–63
39. Schlessinger, J., and Ulrich, A. (1992) Neuron 9, 383–391
40. Ben-Ley, R., Paterson, H. F., Marshall, C. J., and Yarden, Y. (1994) EMBO J. 13, 3302–3311
41. Leevers, S. J., Paterson, H. F., and Marshall, C. J. (1994) Nature 369, 411–414
42. Stokoe, D., MacDonald, S. G., Cadwallader, K., Symons, M., and Hancock, J. F. (1994) Science 264, 1463–1467
43. Zhang, X., Setteman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993) Nature 364, 308–313
44. Kato, K., Cox, A. D., Hisaka, M. M., Graham, S. M., Buss, J. E., and Der, C. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6403–6407
45. Prendergast, G. C., Davide, J. P., deSdms, S. D., Giuliani, E. A., Graham, S. L., Gibbs, J. B., Oliff, A., and Kohl, N. E. (1994) Mol. Cell. Biol. 14, 4193–4202
46. Kohl, N. E., Mosser, S. D., deSdms, S. J., Giuliani, E. A., Pompliano, D. L., Graham, S. L., Smith, R. L., Scdinick, E. M., Oliff, A., and Gibbs, J. B. (1993) Science 260, 1934–1937
47. James, G. L., Brown, M. S., Cobb, M. H., and Goldstein, J. L. (1994) J. Biol. Chem. 269, 27705–27714