Dislodgment and Accelerated Degradation of Ras[†]

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ABSTRACT: Membrane anchorage of Ras oncoproteins, required for transforming activity, depends on their carboxy-terminal farnesylcysteine. We previously showed that *S-trans,trans*-farnesylthiosalicylic acid (FTS), a synthetic farnesylcysteine mimetic, inhibits growth of ErbB2- and Ras-transformed cells, but not of v-Raf-transformed cells, suggesting that FTS interferes specifically with Ras functions. Here we demonstrate that FTS dislodges Ras from membranes of H-Ras-transformed (EJ) cells, facilitating its degradation and decreasing total cellular Ras. The dislodged Ras that was transiently present in the cytosol was degraded relatively rapidly, causing a decrease of up to 80% in total cellular Ras. The half-life of Ras was 10 ± 4 h in FTS-treated EJ cells and 27 ± 4 h in controls. The dislodgment of membrane Ras and decrease in total cellular Ras were dose-dependent: 50% of the effects occurred at 10−15 μM, comparable to concentrations (7−10 μM) required for 50% growth inhibition in EJ cells. Higher concentrations of FTS (25−50 μM) were required to dislodge Ras from Rat-1 cell membranes expressing normal Ras, suggesting some selectivity of FTS toward oncogenic Ras. Membrane localization of the prenylated G$\beta\gamma$ of heterotrimeric G proteins was not affected by FTS in EJ cells. An FTS-related compound, *N*-acetyl-*S*-farnesyl-L-cysteine, which does not inhibit EJ cell growth, did not affect Ras. FTS did not inhibit growth of Rat-1 cells transformed by N-myristylated H-Ras and did not reduce the total amount of this Ras isoform. The results suggest that FTS affects docking of Ras in the cell membrane in a rather specific manner, rendering the protein susceptible to proteolytic degradation.

Association of Ras proteins with the inner surface of the plasma membrane is required for Ras signaling and transforming activity (*1*−*3*). This association is promoted by sequential posttranslational modifications at the carboxy-terminal CAAX motif (C = cysteine, A = aliphatic amino acid, X = Met/Ser) of Ras where the cysteine residue is farnesylated, the AAX is removed, and the *S*-farnesylcysteine is carboxyl methylated (*4*−*10*). The last two modifications are not essential for Ras transforming activity, though farnesylated but unmethylated forms of Ras have lower transforming potencies than their fully processed counterparts (*5*) and associate less avidly with the plasma membrane (*7*).

A rather specific role for the farnesylcysteine of Ras and for additional sequences at the Ras carboxy terminus in targeting and binding of Ras to plasma membranes has been proposed in several studies (*1*, *7*, *11*, *12*). It was shown that both are required for these functions (*7*). The type of prenyl group in Ras appears to be important as well. For example, the cellular distributions of geranylgeranyl forms of K-Ras4B (*7*) or of H-Ras (*1*) were shown to differ from those of the naturally farnesylated forms of these Ras proteins, suggesting the possibility of a specific targeting and binding role for the farnesylcysteine in Ras. Although N-myristylated forms of Ras (*11*) as well as activated geranylgeranylated Ras (*7*, *12*) do exhibit transforming activities, their membrane anchorage and activities may differ from those of farnesylated Ras-proteins (*1*, *12*). For example, activation of the transforming activity of normal Ras that is myristylated but not farnesylated has been demonstrated, and dominant-negative effects on the growth of NIH3T3 cells exerted by geranylgeranylated normal Ras were also observed (*11*, *12*). The precise mode of interaction of Ras proteins with the plasma membrane is, however, not known, nor is it known whether the farnesylcysteine of Ras interacts with distinctive sites in the membrane.

We recently proposed the use of a new pharmacological tool to address questions related to Ras membrane anchorage (*13*, *14*). In those studies we showed that farnesyl derivatives of rigid carboxylic acids can inhibit the growth of H-Ras-transformed (EJ) cells in a rather specific manner. First we showed that there were distinctive structure−activity relationships among these analogues and that the most potent inhibitor of EJ cell growth was *S-trans,trans*-farnesylthiosalicylic acid (FTS).[1] Next we found that FTS did not inhibit Ras farnesylation in vitro, and that at concentrations at which it inhibited EJ cell growth it did not inhibit Ras methylation. Finally we showed that FTS inhibits the growth

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of ErbB2-transformed cells, but not of v-Raf-transformed cells. Taken together, these results suggested that FTS interferes with the functions of Ras. Here we show that FTS dislodges Ras from intact EJ cell membranes and facilitates its degradation.

EXPERIMENTAL PROCEDURES

FTS and *N*-acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine (AFC) were prepared by a general procedure and purified and analyzed as described in detail elsewhere (*14*). The pcDNA3-myr-v-h-ras(V^{12}) plasmid, obtained from D. Bar-Sagi, contains a 15-amino acid N-myristylation signal sequence and a substitution of Ser for Cys^{186} (*15*). Rat-1 cells were transfected with this plasmid or with the pcDNA3 vector by the Lipofectamine (Gibco BRL) method. Cultures were maintained in DMEM/10% fetal calf serum (FCS) containing 800 μg/mL G418 to establish stable cell lines expressing the myristylated Ras (myr-Ras). An individual G418-resistant clone was then isolated and maintained in medium containing 400 μg/mL G418. The effects of FTS on myr-Ras cell growth were estimated by direct counting. Cells were plated at a density of 2500 cells per well in 24-well plates in the presence of 0.1% DMSO or FTS and counted on day 5.

Ras was assayed by enhanced chemiluminescence (ECL) assays following immunoprecipitation and immunoblotting. H-Ras-transformed Rat1 cells (EJ cells) were plated at a density of 4×10^6 cells/10-cm dish. After 3 h, the cells were treated for 15 h (dose response experiments) or for 2, 4, 6, 12, or 24 h (time course experiments) with FTS (0−50 μM for dose response, 25 μM for time course) or with 0.1% DMSO (control). The cells were then detached from the dishes, washed in phosphate-buffered saline (PBS), and counted. All subsequent procedures were carried out at 4 °C. In agreement with previous results (*13*), FTS had no effect on the cell number during the first 24 h of treatment. Cell pellets were homogenized in 750 μL of homogenization buffer (20 mM Tris-HCl, pH 7.6, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, 5 units/mL aprotinin, and 10 mM $MgCl_2$) as detailed previously (*13*), and the total amount of protein was determined. FTS had no effect on this total amount. Total cell membrane (P_{100}) and total cytosol (S_{100}) fractions were obtained by centrifugation at 100000*g* (30 min). Following resuspension of the P_{100} fraction in 750 μL of homogenization buffer, both S_{100} and P_{100} were placed in 75 μL of ×10 immunoprecipitation buffer (100 mM Tris-HCl, pH 7.5, 1.5 M NaCl, 10% Triton X-100, 5% sodium deoxycholate, 1% SDS). Samples were frozen for 1 h at −70 °C and then thawed. Insoluble material was removed by centrifugation for 10 min at 10000*g*, and the clear supernatants were used for immunoprecipitation. Samples containing 500 μg of protein of the P_{100} fraction and 4 equiv of S_{100} proteins were incubated for 12 h with 2 μg of Y13−259 anti-Ras antibodies coupled to agarose beads (Oncogene Research Products). The beads were then precipitated, washed 4 times with 1 mL of immunoprecipitation buffer and twice with 1 mL of 20 mM Tris-HCl, pH 7.6, and resuspended in 20 μL of SDS-sample buffer. Proteins were separated by 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (mini-gels) and blotted onto nitrocellulose paper. The paper was blocked with 10%

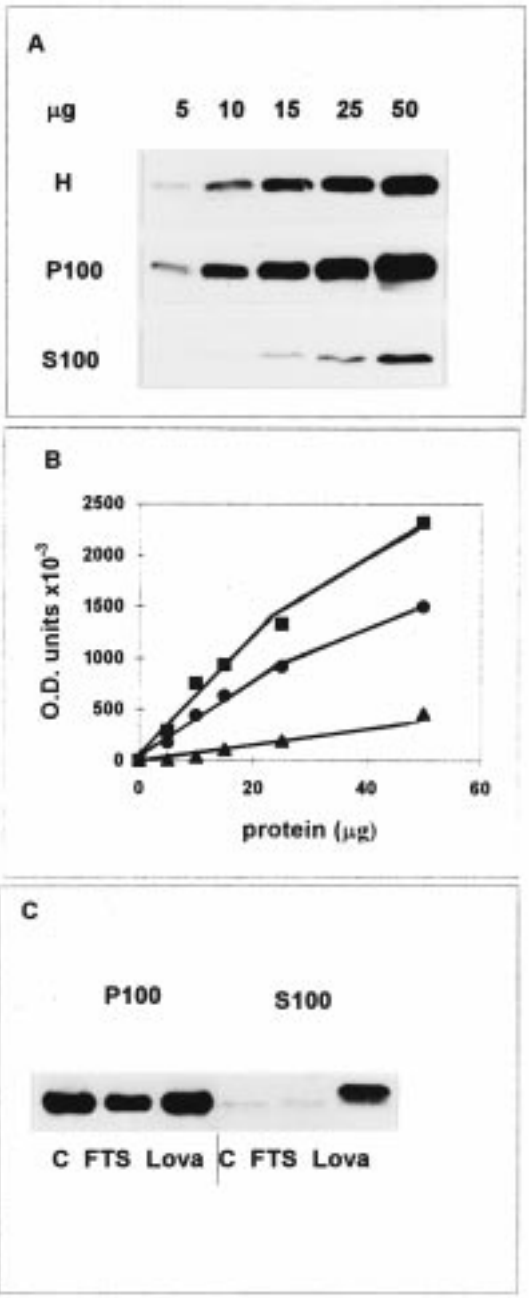

FIGURE 1: Comparison of the effects of FTS and lovastatin on Ras in EJ cells. EJ cells were plated at a density of 4×10^6 cells/10-cm dish. One day after plating, 0.1% DMSO (control, C) or 25 μM FTS or 25 μM lovastatin (Lova) was added for 24 h. Ras proteins were then determined in whole cell homogenates (H) or in the particulate (P_{100}) and in the cytosolic (S_{100}) fractions of the cells by immunoblotting and ECL assay with pan Ras Ab, as described under Experimental Procedures. (A) Amount of Ras in control (DMSO-treated) whole cell homogenates, P_{100} fractions, and S_{100} fractions, expressed as a function of the amount of protein. (B) Densitometric analysis of the Ras bands shown in (A): P100 (■), H (●), and S100 (▲). (C) Amount of Ras in the P_{100} and S_{100} fractions of control, FTS-treated, and lovastatin-treated cells (15 μg of protein from each fraction was used).

[1] Abbreviations: FTS, *S*-*trans*,*trans*-farnesylthiosalicylic acid; AFC, *N*-acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine; ERK, extracellular regulated protein kinase; ECL, enhanced chemiluminescence; FCS, fetal calf serum; HRP, horseradish peroxidase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

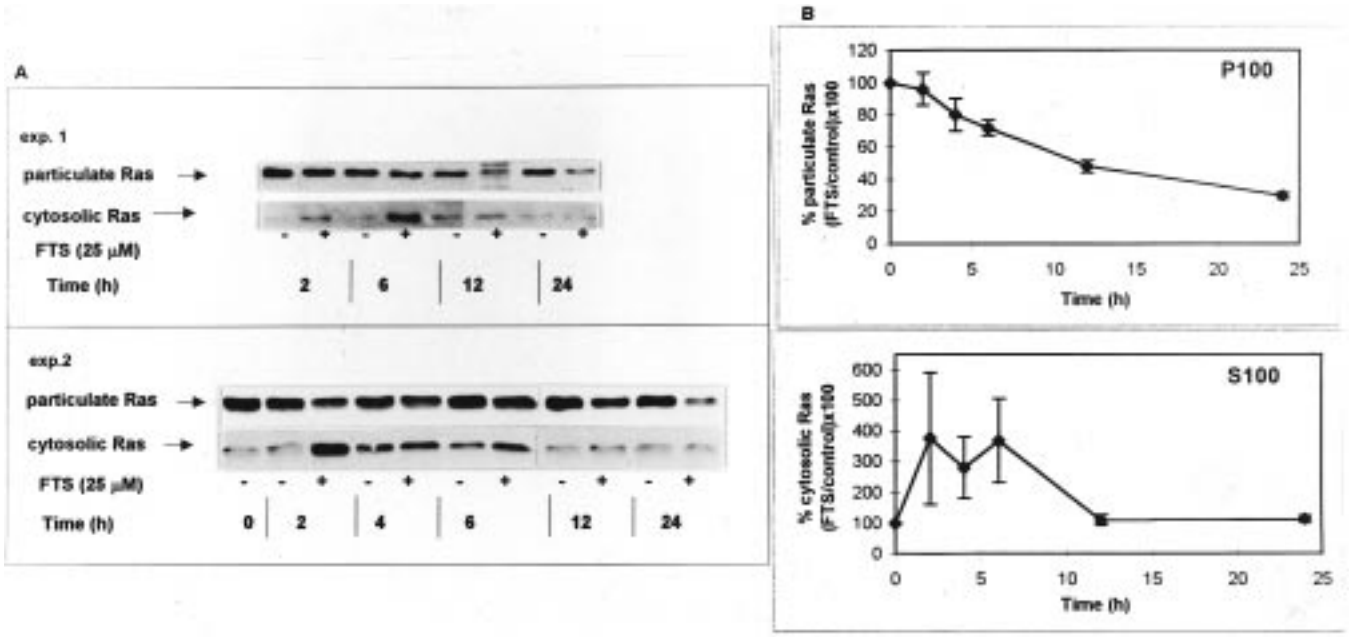

FIGURE 2: Time-dependent dislodgment of Ras in EJ cells by FTS. (A) EJ cells were plated at a density of 4×10^6 cells/10-cm dish. After 3 h, FTS (25 μM) or DMSO (0.1%) was added for the indicated periods of time. Ras proteins were then determined in the particulate fraction (P_{100}) and in the cytosolic fraction (S_{100}). Results of two experiments are shown. In experiment 1, Ras proteins were first immunoprecipitated and then assayed by immunoblotting and ECL as described under Experimental Procedures. In experiment 2, Ras proteins were determined by direct immunoblotting and ECL assay, as in Figure 1. (B) Densitometric analysis of data from experiments performed as described in (A), presented as the ratio of values recorded in FTS-treated cells to values recorded in control cells (mean ± SD values from five separate experiments).

skim milk in Tris-buffered saline (pH 7.6) and then incubated for 2 h with 1:1000 dilution of rabbit anti-Ras serum (*13*) in Tris-buffered saline containing 10 mg/mL bovine serum albumin and 0.05% Tween-20. Immunoblots were incubated for 1 h with 1:5000 dilution of goat anti-rabbit IgG−horseradish peroxidase (HRP) conjugate (Sigma) and subjected to ECL assays.

In several experiments aimed at estimating the amount of Ras in EJ, Rat1, or myr-Ras cells, ECL assays following direct immunoblotting were used. The cells were homogenized as described above. Samples of the total cell homogenate (15 μg of protein unless otherwise indicated) or of P_{100} and S_{100} fractions (15 and 30 μg of protein unless otherwise indicated) were loaded onto the gel. For immunoblotting in these experiments, we used the pan-Ras antibody (Oncogene Research Products) at a dilution of 1:2000 and anti-mouse IgG−HRP conjugate (Sigma) at a dilution of 1:7500. The blots were subjected to ECL assays. Gβ was determined in P_{100} and S_{100} fractions of EJ cells grown in the absence or in the presence of FTS under the same conditions as those used for Ras detection. Membrane proteins (25 μg samples) were separated by SDS−PAGE (12.5%) and blotted onto nitrocellulose paper. Immunoblotting was performed with 1:1000 dilution of rabbit anti-Gβ antibodies (a gift from P. Gierschik) and 1:5000 dilution of goat anti-rabbit IgG−HRP conjugate (Sigma).

The effects of FTS (or AFC) on membrane-bound Ras were examined also in a cell-free system. Total EJ cell membranes were prepared by homogenizing 6×10^7 cells in 3 mL of ice-cold 50 mM Tris-HCl, pH 7.6, containing 1 mM EGTA, 3 mM EDTA, 5 μg/mL pepstatin, and 5 units/mL aprotinin as detailed previously (*13*). The cell homogenate was centrifuged (100000*g*, 30 min), and the membrane pellet thus obtained was resuspended in 3 mL of ice-cold homogenization buffer to yield a preparation of 3−4 mg of protein/mL which was kept at −70 °C. Release of Ras from the membranes was assayed at 37 °C, using 15 μg of membrane proteins that were incubated in 100 μL of buffer A (10 mM Tris-HCl, pH 8.5, containing 0.32 M sucrose, 1 mM EDTA, 5 μg/mL pepstatin, 5 units/mL aprotinin, 5 μg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, and 1 mM $MgCl_2$), containing 2% DMSO and FTS at the indicated concentration or 2% DMSO only (control). Following 60 min incubation (or as indicated), the reaction mixture was diluted by the addition of 900 μL of buffer B (10 mM Tris-HCl, pH 8.5, containing only the antiproteases and 10 μg of BSA). The samples were kept for 5 min at room temperature and then centrifuged (100000*g*, 30 min). The membrane pellets were then subjected to the direct immunoblotting assay as detailed above. To examine whether Ras was present in the diluted 100000*g* supernatant, it was immunoconcentrated from this solution with 2 μg of Y13−259 Ab coupled to agarose beads as detailed above.

The rate of Ras degradation was determined by pulse−chase experiments. EJ cells were plated at a density of 3×10^6 cells/10-cm dish in DMEM/10% FCS. One day after plating, the cells were starved of serum and methionine and pulsed with 100 μCi/mL [^{35}S]cysteine/methionine (1000 Ci/mmol, DuPont NEN) for 12 h. The cells were then chased by removal of the medium and addition of fresh medium containing methionine and 25 μM FTS or 0.1% DMSO (control). At zero time or after the indicated periods of time, the cells were detached, washed as described, and homogenized in 500 μL of homogenization buffer. For determination of ^{35}S-labeled Ras in whole cell homogenates, ×10 immunoprecipitation buffer (50 μL) was then added. This step was preceded by preparation of P_{100} and S_{100} fractions

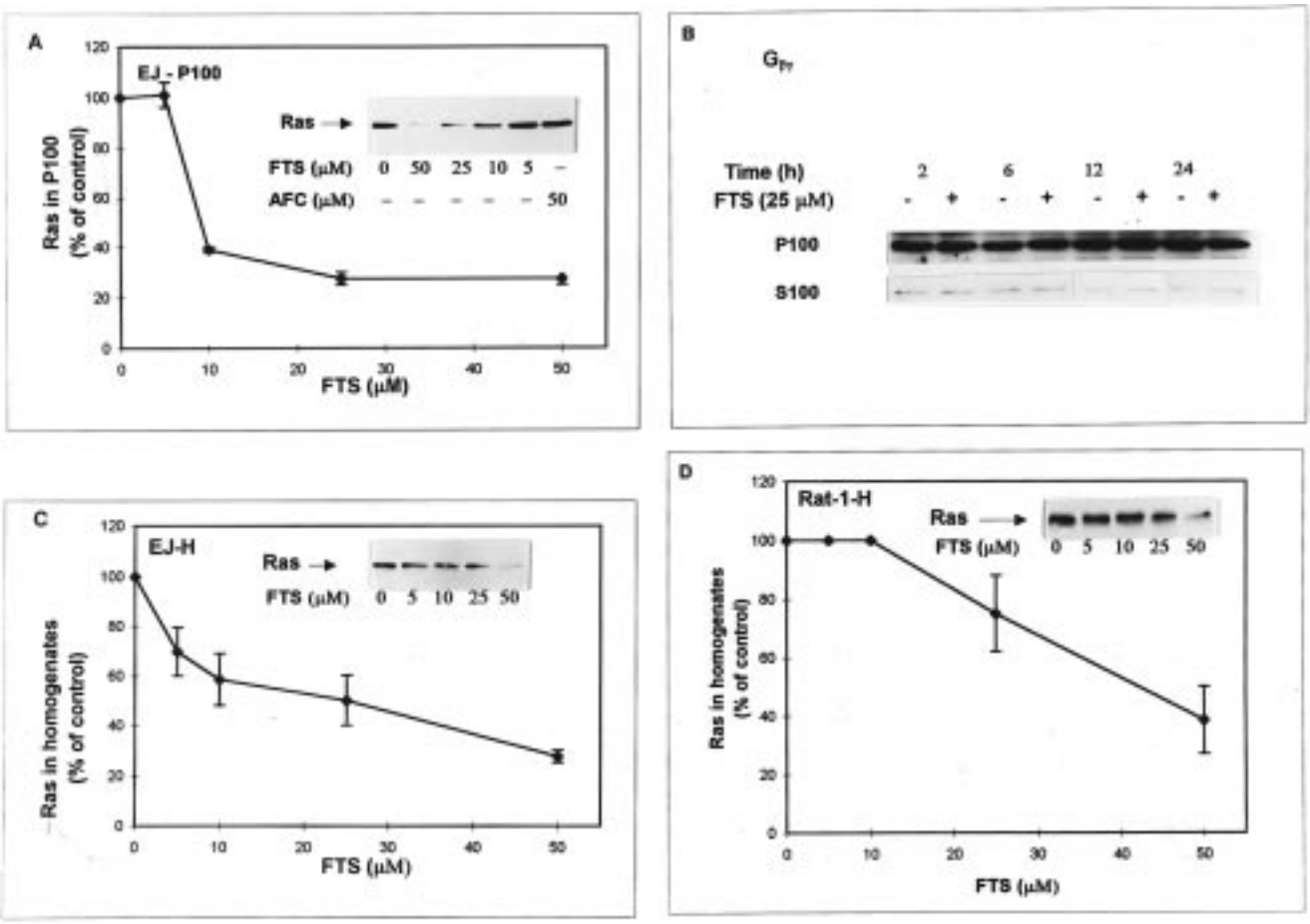

FIGURE 3: FTS induces a specific and dose-dependent decrease in the amount of Ras in EJ and Rat1 cells. EJ or Rat1 cells were grown for 15 h (or as indicated) in the presence of 0.1% DMSO (control) or in the presence of the indicated concentrations of FTS or AFC. Ras proteins were then determined in the P_{100} fraction of the cells (A) by immunoprecipitation/immunoblotting/ECL assay as detailed in Figure 2, or in whole-cell homogenates (panels C and D) by immunoblotting/ECL assay as described for Figure 1. The effects of FTS (25 μM) on Gβ in P_{100} and in S_{100} of EJ cells (B) were examined by means of the immunoblotting/ECL assay, as described under Experimental Procedures, using P_{100} and S_{100} samples from experiment 2 in Figure 2. The immunoblot data (shown in insets of panels A, C, and D), along with the data of two additional experiments, were analyzed densitometrically, and the mean values (±SD), expressed as percentages of control, were plotted as a function of FTS concentration. Densitometric analysis of the Gβ bands in P_{100} or in S_{100} revealed no differences between control and FTS-treated cells (lowest values were 90% and highest were 115% of controls).

in experiments where ^{35}S-labeled Ras was determined in these subcellular fractions. The samples were frozen for 1 h at −70 °C and then thawed. Insoluble material was removed by centrifugation at 10000*g* for 10 min, and the supernatants (600 μg of protein) were precleared by incubation for 1 h with 2 μg of native rat IgG and 40 μL of 10% protein G−agarose in a total volume of 500 μL of immunoprecipitation buffer. The precleared samples were immunoprecipitated as described above. Proteins were separated by 12.5% SDS−PAGE (mini-gels), stained, destained, and dried. The dried gels were exposed to X-ray film for 12 h.

In all experiments, the amount of protein loaded onto the gels and the exposure times of immunoblots to ECL (15−60 s) or of [^{35}S]cysteine-labeled gels to the X-ray films were calibrated. This enabled subsequent densitometric quantitation at the linear ranges of each assay. The actual background value in each lane was subtracted from the value recorded in the Ras protein band.

RESULTS

Using direct immunoblotting and ECL assays with pan Ras Ab, p21 Ras proteins were detected in EJ whole cell homogenates, in the particulate cell fraction (100000*g* pellet, P_{100}) and in the cytosolic cell fraction (S_{100}) (Figure 1A). The pan Ras Ab, as well as Y13−259 Ab and the rabbit anti-Ras Ab used in our experiments, recognizes all Ras isoforms. The intensity of the p21 Ras band increased as a function of the amount of protein (Figure 1A), enabling the densitometric estimations of the apparent amount of Ras in each fraction (Figure 1B). Most of Ras (>95%) was found in the P_{100} fraction, and cytosolic Ras accounted for only 1.5−3% of the Ras found in the whole cell homogenates.

The effects of FTS on Ras localization in EJ cells were examined using drug concentrations of 5−50 μM, previously shown to inhibit cell growth without cytotoxicity (*13*, *14*). Figure 1C shows that FTS (25 μM, 24 h) caused a significant decrease in the amount of Ras in the P_{100} fraction. Notably, FTS treatment did not result in the accumulation of cytosolic Ras protein with a slow gel mobility (Figure 1C). Accumulation of cytosolic Ras was observed in cells treated with lovastatin (25 μM, 24 h), an inhibitor of hydroxymethylglutaryl-coenzyme A reductase. Lovastatin inhibits the formation of mevalonic acid, the isoprenoid precursor, thereby preventing farnesylation of Ras. The unprocessed Ras that accumulates in the cytosol has a relatively slow gel mobility (*15*, Figure 1C).

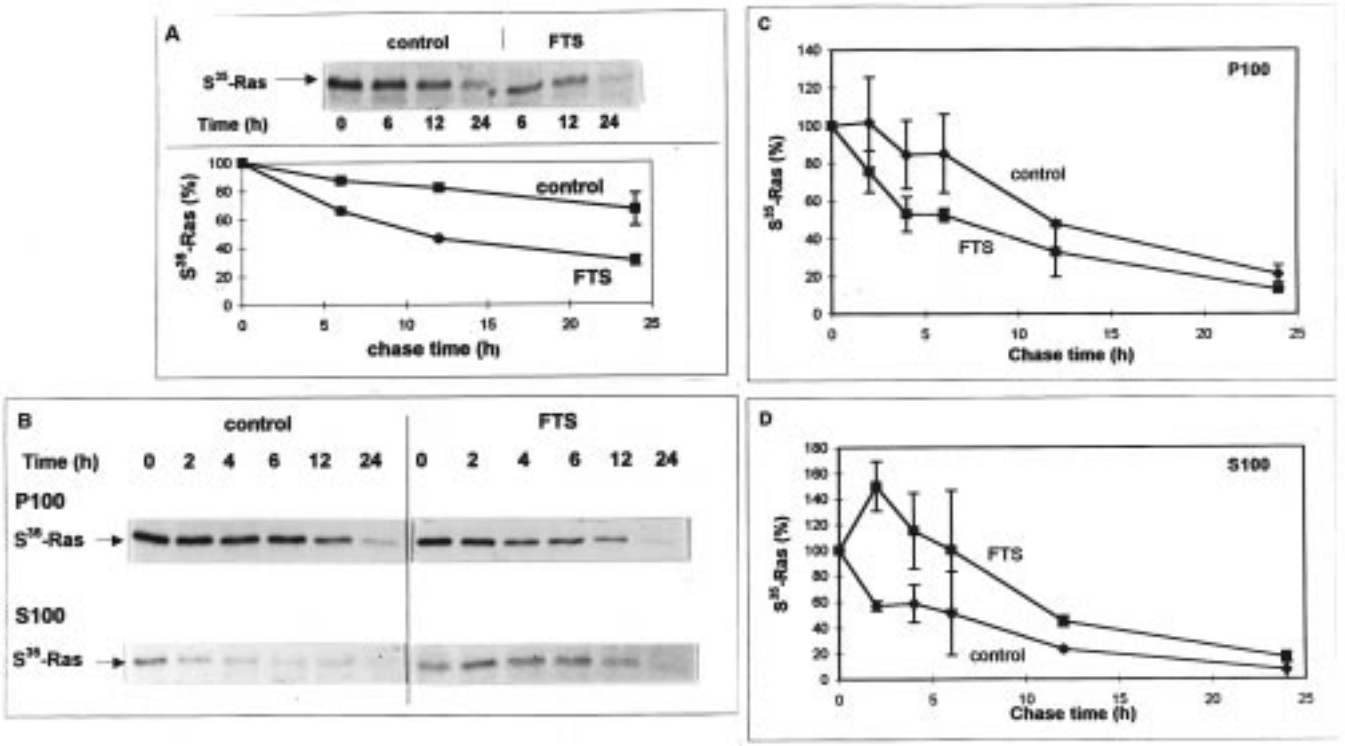

FIGURE 4: FTS facilitates Ras degradation in EJ cells. EJ cells (3×10^6 cells/10-cm dish) were pulsed with [^{35}S]cysteine/methionine (100 μCi/mL) in serum-free and methionine-free medium for 12 h and then chased in serum-free medium, in the absence or in the presence of 25 μM FTS, for 0−24 h. Ras proteins were immunoprecipitated from total cell homogenates or from P_{100} and S_{100} fractions, as described under Experimental Procedures. The immunoprecipitated proteins were separated by SDS−PAGE (12.5%), and gels were exposed to X-ray film for 48 h. (A) Results of a typical experiment in which ^{35}S-Ras determined in whole-cell homogenates (inset) and densitometric analysis of data from four separate experiments are presented as the ratios of values obtained in FTS-treated cells to values obtained in control cells (mean ± SD). (B) Determination of ^{35}S-Ras in P_{100} and in S_{100} fractions of the cells. In this experiment, cycloheximide (3 μg/mL) was present during the first hour of the chase. (C) Densitometric analysis of the ^{35}S-Ras bands in P_{100} (mean values from two separate experiments). (D) Densitometric analysis of the ^{35}S-Ras bands in S_{100} (mean values from two separate experiments).

Figure 2 shows typical results of two separate experiments in which the effect of FTS is plotted as a function of time. The immunoprecipitation/immunoblot/ECL method was used in the first experiment and the direct immunoblot/ECL assay in the second. As shown in Figure 2A, both methods detected a similar time-dependent decrease in particulate Ras in the EJ cells treated with FTS (25 μM). The decrease was already apparent after 4−6 h, was augmented at 12 h, and appeared to reach a maximum value at 24 h. Densitometric analysis of data obtained in five separate experiments indicated that after 24 h FTS caused an 80% decrease in the amount of particulate Ras relative to controls (Figure 2B). The amount of Ras was also determined in the cytosolic cell fraction (S_{100}). Relative to controls, a significant increase in cytosolic Ras occurred 2−6 h after exposure to FTS (Figure 2A). These results, together with the decrease in particulate Ras, suggest that FTS dislodges Ras from the plasma membrane. The dislodged protein did not however appear to accumulate in the cytosol, as the observed increase in cytosolic Ras was transient (Figure 2A,B). This phenomenon was consistently observed; the only variation observed from experiment to experiment was in the rate of disappearance of cytosolic Ras. The mean values obtained by densitometric analysis of data from five separate experiments (presented as the ratio of cytosolic Ras in FTS-treated cells to that in control cells) suggest that cytosolic Ras peaks between 2 and 6 h after drug application and then declines to control levels between 6 and 10 h. The reason for these variations is not known. Whatever the explanation, the data in Figure 2A,B show that FTS causes a continuous decrease in particulate Ras followed by a transient relative increase and then a decrease in cytosolic Ras. Taken together, these results suggest that Ras is dislodged from anchorage sites in the plasma membrane by FTS and then degraded.

The effect of FTS in dislodging Ras from EJ cell membranes was dose dependent (Figure 3A). As shown in Figure 3A, the EC_{50} was about 10 μM, comparable to the estimated EC_{50} for inhibition of EJ cell growth by FTS (*13*, *14*). If Ras is degraded after being dislodged, total cellular Ras should decline after FTS treatment. EJ cells were incubated for 12 h with various concentrations of FTS, and then homogenized and assayed for Ras. Results of a typical experiment (shown in Figure 3C) indicate that FTS indeed caused a dose-dependent decrease in the total amount of Ras in EJ cells. The EC_{50} for FTS was 15 μM (Figure 3C), similar to that obtained for the effects of FTS on the particulate Ras (Figure 3A). We also examined the effect of FTS on total Ras in Rat1 cells, which have normal as opposed to oncogenic Ras. Ras in these cells was significantly decreased (Figure 3D), but the dose−response curve for the effect of FTS on total Ras in Rat1 cells was shifted to the right relative to that in EJ cells (Figure 3C,D). Moreover, FTS (50 μM) caused a decrease in particulate Ras in Rat1 cells, just as it did in EJ cells (not shown). We found, however, that dislodgment of normal Ras in Rat1 cells required 50 μM FTS, whereas the oncogenic Ras found in EJ cells was dislodged at lower FTS concentrations (Figure

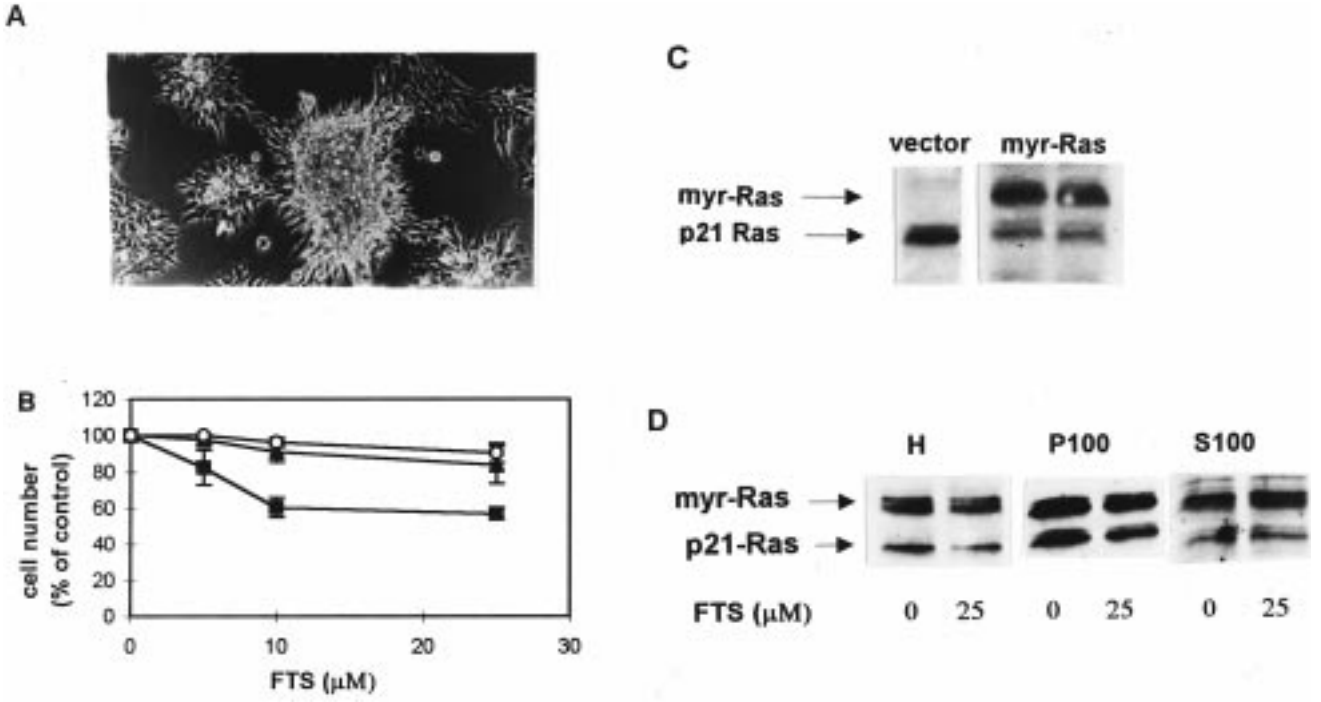

FIGURE 5: FTS does not affect myristylated Ras in myr-Ras cells. (A) Typical photomicrograph of myr-Ras cells in culture (day 5). (B) Dose-dependent inhibition of EJ (■) but not of myr-Ras (▲) or of vector-transfected (○) cells by FTS. Cells were grown for 5 days in the absence or in the presence of FTS, and then counted as described under Experimental Procedures. (C) Immunoblots of vector-transfected cell homogenates and of myr-Ras-transfected cell homogenates. Ras proteins were detected in whole cell homogenates as described under Experimental Procedures. The myristylated v-H-Ras exhibits slower gel mobility than that of the endogenous p21 Ras proteins (*15*). (D) FTS does not induce a decrease in the total amount of myristylated Ras or in particulate myristylated Ras in myr-Ras cells. Cells were grown and treated with 25 μM FTS as described for Figure 1. Results of two separate experiments are shown. In the first experiment, Ras was determined in whole-cell homogenates, as described for Figure 1. In the second experiment, Ras was determined in P_{100} and in S_{100} fractions, as described for Figure 1.

3A). Thus, FTS may exhibit some selectivity toward activated forms of Ras.

The possibility that FTS renders Ras proteins more susceptible to degradation was examined in pulse−chase experiments. EJ cells were labeled with [^{35}S]cysteine/methionine for 12 h and then chased for various periods of time with unlabeled cysteine/methionine. FTS was added at the beginning of the chase period. Ras proteins were then immunoprecipitated from the cell lysates, separated by SDS−PAGE, and assayed by autoradiography. Results of a typical experiment (Figure 4A) indicate that in control EJ cells Ras degradation was relatively slow, with an estimated half-life of 27 ± 3 h ($n = 4$) (Figure 4B). These results are consistent with earlier studies (*16*, *17*). The rate of Ras degradation in FTS-treated cells was far higher (Figure 4A); the estimated half-life of Ras in the presence of 25 μM FTS was 10 ± 4 h ($n = 4$).

In a second set of pulse−chase experiments, the rates of disappearance of ^{35}S-labeled Ras from the particulate and cytosolic fractions of the cells were determined. The pulse−chase experiments were performed as described above except that cycloheximide was added for the first hour of the chase period. Labeled Ras was then immunoprecipitated from the P_{100} and S_{100} fractions. As shown in Figure 4B, most of the labeled Ras was found in the P_{100} fraction. Its pattern of decay in this fraction resembled that of labeled Ras in whole cell homogenates: the half-life of the particulate Ras was 12 h in control fractions and 4−6 h in FTS-treated fractions (Figure 4C). Labeled cytosolic Ras in control cells decayed rapidly, within less than 4 h (Figure 4B,D). In contrast, in FTS-treated cells, the disappearance of labeled cytosolic Ras was delayed: during the first 2−4 h of chase, a small increase in labeled Ras was observed, and only then a decrease (Figure 4B,D). These results strengthen the notion that FTS dislodges Ras from the cell membranes and the dislodged protein is then degraded in the cytosol. They do not, however, rule out the possibility that Ras is also degraded in the membrane.

We next examined the effects of AFC on membrane Ras in EJ cells. This farnesyl derivative is ineffective in inhibiting the growth of Ras-transformed cells (*18*), although it does enter cells readily (*18*). As shown in Figure 3A, AFC did not affect the membrane localization of Ras in EJ cells.

We also examined the distribution subunits of the trimeric G-proteins to which a large number of receptors couple. Specifically, we looked at the Gβ subunit, which is tightly associated with the prenylated Gγ subunit of the trimer (*19*, *20*). As shown in Figure 3B, FTS did not reduce the amount of Gβ associated with the EJ cell membrane. It also did not induce an increase in cytosolic Gβ (Figure 3B). It thus seems that FTS dislodges Ras specifically, a property that is not shared by the related compound, AFC. Our results do not exclude the possibility that FTS may have additional effects besides dislodgment of Ras from the membrane. For example, FTS may affect the membrane localization of farnesylated Gγ subunits (e.g., Gγ_1, Gγ_9, Gγ_{11}) and/or the function of other prenylated proteins.

We also examined the effects of FTS on Rat-1 cells stably expressing myr-Ras. This v-H-Ras isoform has an N-myristylation signal, but because of the substitution of Ser

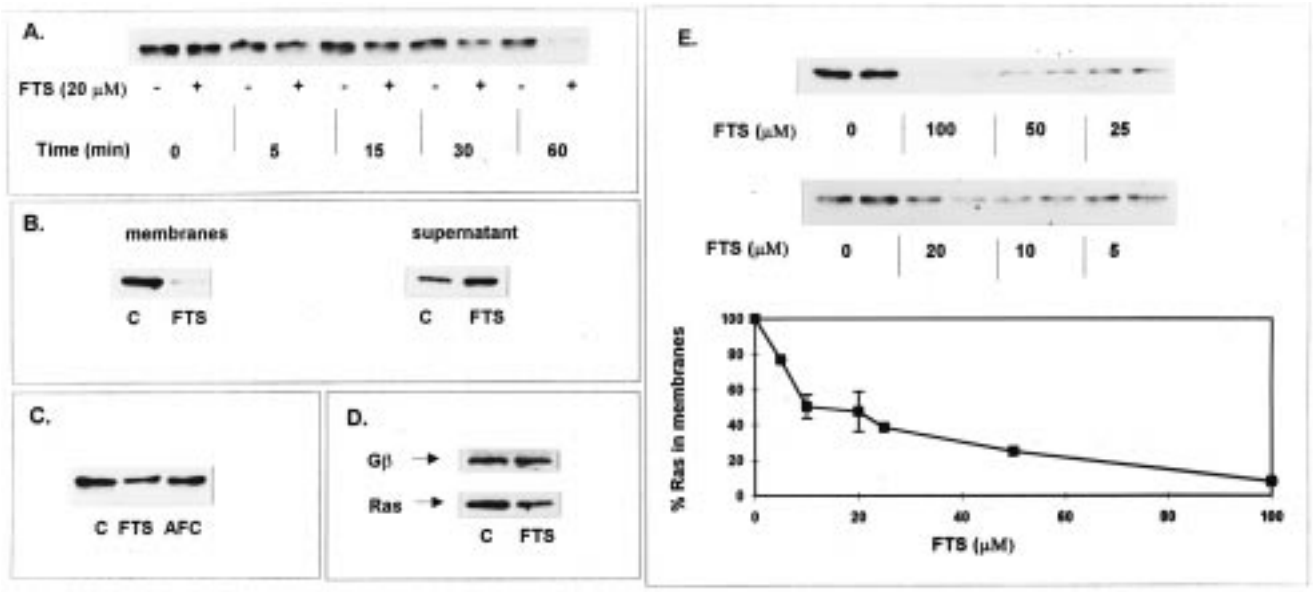

FIGURE 6: Dislodgment of Ras from EJ cell membranes in a cell-free system. EJ cell membranes (15 μg of protein) were incubated in 100 μL of buffer A at 37 °C in the absence or in the presence of FTS for the indicated periods of time. Membranes were then diluted 10-fold by the addition of buffer B, collected by 100000*g* spin, and subjected to immunoblotting/ECL assay as detailed under Experimental Procedures. (A) Time-dependent decrease in the amount of membrane-bound Ras determined in the absence or in the presence of 20 μM FTS. (B) Recovery of the dislodged Ras in the 100000*g* supernatant. Membranes were incubated 60 min in the absence or in the presence of 20 μM FTS. Ras proteins in the membranes were then determined as detailed above. The supernatants were collected, and Ras proteins were immunoprecipitated with Y13−359 Ab coupled to agarose beads. The immunoprecipitated Ras was then determined by the immunoblotting/ECL assay. (C) Dislodgment of Ras from EJ cell membranes by 20 μM FTS but not by 20 μM AFC. Membranes were incubated for 60 min in the absence or in the presence of each compound, and membrane-Ras was then determined as detailed above. (D) Dislodgment of Ras but not of Gβ from EJ cells membranes by 20 μM FTS. Membranes were incubated for 60 min in the absence or in the presence of FTS, and the reaction was terminated as detailed above. Ras proteins and Gβ were then determined in the membranes by immunoblotting/ECL assays where blots of the same samples were exposed either to Pan Ras Ab or to Gβ Ab. (E) Dose-dependent dislodgment of Ras from EJ cell membranes by FTS. Membranes were incubated 60 min in the absence or in the presence of the indicated concentrations of FTS, and Ras proteins were then determined in the membranes as detailed above. Immunoblots of two representative experiments are shown. The immunoblot data shown along with data of two additional experiments ($n = 6$) were analyzed densitometrically, and the mean values (±SD), expressed as percentages of control, were plotted as a function of FTS concentration. Values of SD equal or smaller than the size of symbols are not seen.

for Cys^{186} (*15*) it is not farnesylated. In agreement with earlier studies (*15*, *21*), the cells expressing myr-Ras formed foci typical of malignant transformation (Figure 5A). Growth of these cells, or of the vector-transfected cells, was not inhibited by FTS, although under the same conditions growth of EJ cells was inhibited (Figure 5B). As shown in Figure 5C, the myr-Ras cells express significant amounts of the 24-kDa myristylated v-H-Ras protein (*15*, *21*), an isoform not detected in the vector-transfected Rat-1 cells. No decrease was observed in the total amount of myristylated Ras in cells exposed to FTS or in the amount of myristylated Ras in their membranes (Figure 5D). FTS also did not induce an increase in cytosolic myristylated Ras (Figure 5D). Thus, FTS appears to affect the farnesylated H-Ras expressed by the EJ cells (Figure 3D), but not the myristylated H-Ras expressed by the myr-Ras cells (Figure 5D).

In a separate set of experiments, we examined the effects of FTS on membrane-bound Ras of EJ cells in a cell-free system. The membranes were incubated for various periods of time in the absence or in the presence of 20 μM FTS, and then precipitated and subjected to Ras-immunoblotting assays. As shown in Figure 6A, FTS (20 μM) caused a time-dependent decrease in membrane-bound Ras which appeared to reach a maximum value at 1 h. A significant amount of Ras was also detected in the supernatant, although not all of the dislodged Ras was recovered (Figure 6B). As shown in Figure 6C, AFC (20 μM) did not induce a significant decrease in membrane-bound Ras. We also found that FTS (20 μM) did not reduce the amount of Gβ associated with the membranes (Figure 6D). The FTS-induced decrease in membrane-bound Ras was dose-dependent (Figure 6E). The estimated EC_{50} was 10 μM, comparable to the estimated EC_{50} for the dislodgment of Ras by FTS in intact EJ cells.

DISCUSSION

The results of this study show that FTS causes a time- and dose-dependent decrease in membrane-bound Ras. This is accompanied by a transient increase in cytosolic Ras, accelerated degradation of Ras, and a decrease in the total amount of cellular Ras. To the best of our knowledge, previous studies of pharmacological (*8*, *10*, *21*−*23*) and molecular (*5*−*7*, *11*, *12*) reagents have not shown such effects on Ras in intact cells. Our results support the hypothesis that FTS dislodges Ras from its membrane anchorage sites and that the dislodged Ras is susceptible to proteolytic degradation. The observation that p21 Ras is lost from cell membranes and gained by the cytosol is consistent with the previously described lack of effect of FTS on Ras farnesylation and Ras methylation (*13*). Moreover, inhibition of Ras farnesylation would have caused a slowly migrating form of Ras to appear in the cytosol (*8*, *10*, *23*; see also Figure 1), but no such form of Ras was observed here (Figure 1). It therefore appears that the Ras dislodged from the FTS-treated cell membranes was fully processed. In support of

this conclusion, we also found that FTS dislodges Ras from EJ cell membranes in a cell-free system, where the observed effects were time- and dose-dependent (Figure 6). It seems that FTS dislodges Ras from specific anchorage sites in the membrane. This claim is supported by three experiments showing (a) that a similar molecule (AFC) has no effect on Ras, (b) that FTS has no effect on members of another class of lipid-modified proteins (i.e., Gβ), and (c) that FTS has no effect on the growth of myr-Ras-transformed Rat1 cells or on the amounts of myristylated Ras in whole cell homogenates or in membrane fractions of these cells.

The observed specificity suggests that FTS dislodges Ras from specific anchorage sites in the membrane. We believe that Ras must first associate with and then dissociate from its putative anchorage site, and that FTS must compete for this site. Once Ras dissociates from its site or is displaced in the presence of a large amount of FTS, it cannot reassociate, nor can newly formed molecules of Ras associate with their docking sites. In support of the existence of a specific Ras docking site are the observations that Ras binds specifically to the plasma membrane and that distribution of the geranylgeranyl form of Ras differs from that of farnesyl forms (*1*, *7*, *11*, *12*). Evidence for specific anchorage sites for H-Ras comes from the observed localization of the mature protein in caveolae (*24*, *25*), and from the demonstrated association of Ras-GDP with caveolin (*25*). However, the lack of caveolae in Ras-transformed cells (*26*) suggests that oncogenic Ras associates with a distinct membrane domain. The relatively low concentrations of FTS (<25 μM) required to dislodge Ras from cell membranes, and the lack of any FTS effect on G-protein subunits, are also consistent with the existence of a specific anchorage site, i.e., a distinct protein receptor or a rather unique lipid environment.

Our results suggest that FTS acts as a true Ras antagonist and that FTS-induced inhibition of cell growth in Ras-transformed cells (*13*) is associated primarily with the inhibition of Ras-dependent signaling pathways. The similar concentration ranges at which FTS inhibits EJ cell growth (*13*) and dislodges Ras from EJ cell membranes are consistent with this suggestion. We also now know that the Raf1-ERK cascade, which is associated with cell growth and transformation (*27*−*29*), is inhibited by FTS (*30*, *31*). The relatively high concentrations of FTS required to affect Ras in untransformed Rat1 cells (Figure 2B,D) and inhibit their ERK activity (*30*, *31*) appear to correlate with the failure of FTS to inhibit growth of these cells. These findings may be in line with the described specific requirement of a farnesyl group for the functions of normal, but not of constitutively active Ras (*12*). In other words, perhaps normal forms of Ras, which in our experiments appear to associate with the cell membrane more avidly than at least the oncogenic H-Ras, gain a specific C-terminus-dependent conformation appropriate for tight binding with a putative receptor site such as caveolin (*24*). The activated forms of Ras may have less stringent conformational requirements for this binding function, and may therefore associate less tightly with the putative receptor site. Whether or not Ras proteins dock in the plasma membrane, and how tightly they bind, may depend also on the host cell and not only on the lipid modification or the conformation of the particular Ras protein. Future examination of the effects of FTS on a variety of Ras isoforms (expressed in different cell lines) may substantiate one or more of these possibilities. The unusual specificity and potency of FTS in dislodging and accelerating the rate of Ras degradation suggest that this and related compounds may be valuable pharmacological tools for studies of the nature of Ras anchorage sites and the mechanisms of Ras degradation.

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