K- and N-Ras Are Geranylgeranylated in Cells Treated with Farnesyl Protein Transferase Inhibitors*

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The association of mutant forms of Ras protein with a variety of human cancers has stimulated intense interest in therapies based on inhibiting oncogenic Ras signaling. Attachment of Ras proteins to the plasma membrane is required for effective Ras signaling and is initiated by the enzyme farnesyl protein transferase. We found that in the presence of potent farnesyl protein transferase inhibitors, Ras proteins in the human colon carcinoma cell line DLD-1 were alternatively prenylated by geranylgeranyl transferase-1. When H-Ras, N-Ras, K-Ras4A, and K-Ras4B were expressed individually in COS cells, H-Ras prenylation and membrane association were found to be uniquely sensitive to farnesyl transferase inhibitors; N- and K-Ras proteins incorporated the geranylgeranyl isoprene group and remained associated with the membrane fraction. The alternative prenylation of N- and K-Ras has significant implications for our understanding of the mechanism of action of farnesyl protein transferase inhibitors as anti-cancer chemotherapeutics.

Newly synthesized Ras proteins are partitioned to the cytoplasmic face of the plasma membrane by a series of post-translational modifications. The first step, catalyzed by the enzyme farnesyl protein transferase, is the addition of the 15-carbon isoprenyl group farnesyl to the sulfhydryl group of cysteine in the Ras carboxyl-terminal CAAX box (where C is cysteine, A is aliphatic, and X is typically Met or Ser) (1–3). Farnesylation is followed by proteolytic removal of the AAX amino acids and methylation of the carboxyl group of the farnesylated cysteine (4). Ras proteins at the plasma membrane cycle between an active GTP-bound state and an inactive GDP-bound state. Mutations that stabilize the active GTP-bound state have been identified in over 30% of human tumors, with particularly high incidences in pancreatic (∼90%) and colon (∼50%) cancers. Four oncogenic Ras proteins have been described, H-Ras, N-Ras, K-Ras4A, and K-Ras4B. The majority of mutations associated with human cancer have been found in the K-Ras gene. The two K-Ras proteins are products of a single alternatively spliced transcript, with K-Ras4B the predominant isoform (>80%) (5, 6).

Ras proteins that have been genetically modified so that they lack the isoprenylated cysteine do not associate with the plasma membrane and cannot transform fibroblasts (7). These genetic experiments provided the basis for the development of farnesyl transferase inhibitors (FTIs) as anti-cancer agents. A number of reports have demonstrated that pharmacological inhibition of farnesyl protein transferase by CAAX analogs reduces anchorage-independent growth of Ras-transformed cells in soft agar (8) and slows growth of Ras-transformed cells in nude mice (9, 10). The FTIs appear relatively non-toxic in that they do not interfere with normal cell proliferation (11). This result was somewhat surprising because Ras function was shown to be necessary for normal growth factor signaling and cell proliferation (12). A mechanism through which cells may proliferate in the presence of FTIs was suggested by the observation that, in vitro, the K-Ras4B protein, but not H-Ras, can act as a substrate for geranylgeranyl transferase-1 (13). Geranylgeranyl transferase-1 adds the 20 carbon geranylgeranyl isoprenyl unit to the cysteine residue of the CAAX motif (14). Geranylgeranyl transferase-1 substrate proteins generally have a carboxyl-terminal leucine (15). James et al. (16) recently reported that a chimeric Ras protein consisting of the first 164 amino acids of H-RasV12 followed by the carboxyl-terminal 24 amino acids of K-Ras4B incorporates [9H]mevalonate in Rat-1 cells even in the presence of the peptidomimetic FTIs. The prenyl group attached to H/K-Ras4BV12 in this study was reported to be farnesyl, both in the absence and presence of the FTI. We report here that Ras proteins in the human cancer cell line DLD-1 become geranylgeranylated in the presence of farnesyl transferase inhibitors. Plasmids expressing H-, N-, and the two K-Ras isoforms were transfected into COS cells to determine the effects of FTI on the prenylation and membrane association of each isoform.

EXPERIMENTAL PROCEDURES

Chemicals and Tissue Culture Reagents—[9H]mevalonolactone (40 Ci/mmol) in 100% ethanol at 1 mCi/ml was from DuPont NEN (NET-1075). It was dried under nitrogen to increase the concentration to 100 mCi/ml. G418 and trypsin-EDTA were from Life Technologies, Inc.; Amplify fluorographic reagent was from Amersham Life Science; Pefabloc SC was from Boehringer Mannheim. All other chemicals were from Sigma.

Generation of DLDpMev Cell Line—The pMev construct, which contains the coding sequence for the mevalonate transporter under control of a cytomegalovirus immediate early gene promoter (17) was co-transfected with a neo-expressing plasmid (pCI-neo, Promega Corp.) into DLD-1 cells (American Type Culture Collection, Rockville, MD) using Lipofectamine as described by the manufacturer (Life Technologies, Inc.). Control cells were transfected with pCI-neo alone. Neo-expressing colonies were selected in DMEM, 10% fetal calf serum containing 800 μM G418 and maintained in 400 μM G418. Treatment of the parental line, control neo-resistant lines, and pMev transfectants with mevastatin (20 μM) resulted in complete cell killing within several days. In the presence of exogenous mevalonate (100 μM), several pMev-transfected clones were resistant to 20 μM mevastatin. In contrast, pCI-neo...
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transfectants and the parental line were equally sensitive to mevastatin in the presence or absence of exogenous mevalonate.

**Labeling of Prenylated Proteins in DLDp-Mev Cells**—DLDp-Mev cells were incubated overnight (18 h) at 37 °C, 5% CO₂ with SCH 44342 or SCH 56582 at 0, 1.25, 2.5, 5.0, 10, and 20 µM, or with the farnesyl pyrophosphate antagonist, α-hydroxyfarnesyl-phosphonic acid (Biomol Research Laboratories, Inc., Plymouth Meeting, PA), at 0, 0.08, 0.2, 0.7, 2.2, and 6.6 µM. The following morning, the cells were treated with fresh compound or vehicle control, mevastatin (20 µM, to block endogenous mevalonate synthesis), and [³H]mevalonolactone (40 Ci/mmol) at 100 µCi/µl. After 18 h of incubation, the cells were rinsed twice with phosphate-buffered saline and lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, 50 µM leupeptin, 1 mM Pefabloc SC, 2 µg/ml aprotinin, 2 µg/ml soybean trypsin inhibitor, 1 µg/ml pepstatin, 2 mM benzamide, 2 mM EDTA).

**Ras Immunoprecipitation**—Ras proteins were immunoprecipitated from approximately 20 million cpm of lysate in a 1-ml volume of RIPA buffer. Antibodies were the pan-Ras antibody, Y13–259 (SC-35 for N-Ras coding sequence, a gift from A. Wolfman (The Cleveland Clinic, Cleveland, OH), was cloned into the pCI-neo vector (Promega Corp.). Mutant (G12N) N-Ras was PCR amplified from HTB-177 cells and cloned into pCI-neo. Mutant (G12V) K-Ras4B cDNA was amplified from the human colon cancer line SW620 and cloned into the mammalian expression vector pCI-neo. The following morning, the cells were treated with [³H]mevalonolactone (40 Ci/mmol) at 100 µCi/µl and 20 µM mevastatin. The cells were lysed in RIPA buffer and Ras proteins were immunoprecipitated with Y13–259-agarose conjugate (Santa Cruz Biotechnology, Inc.).

**Preynol Group Analysis**—Structural characterization of prenyl groups attached to immunoprecipitated proteins was performed as described (18). Briefly, protein was precipitated in acetone, washed twice with acetone to remove free lipids, and digested with trypsin overnight at 37 °C in 100 mM Tris-Cl, pH 7.5, 5% acetonitrile, and 0.04% w/v trypsin-EDTA. Cleavage of prenyl groups was initiated by addition of 0.1 volumes of methyl iodide in 3% formic acid. Released prenyl groups were extracted into chloroform:methanol in the presence of 7.5 µM SCH 56582, 20 µM mevastatin (Sigma), or vehicle. Following transfection, cells were suspended in 0.9 ml of ice-cold hypotonic buffer (25 mM Tris-Cl, pH 8.0, 1 mM EDTA, 5 µM leupeptin, 1 mM Pefabloc SC, 50 µg/ml aprotinin, 5 µg/ml soybean trypsin inhibitor, 4 mM benzamidine) and sonicated for 5 s. The cell debris was pelleted at 1500 × g for 4 min at 4 °C and discarded, and the supernatant was transferred to Beckman polyallomer tubes and spun at 46,000 rpm (100,000 × g) for 45 min. The pellet was washed once with ice-cold hypotonic buffer with protease inhibitors, re-pelleted at 46,000 rpm for 15 min, and then resuspended in 200 µl of ice-cold hypotonic buffer with protease inhibitors. The supernatant was concentrated in Microcon 10 concentrators (Amicon). Particulate and soluble samples (approximately 15–20% of total) were loaded on an 18 × 18 cm 14% polyacrylamide gel, run at 30 mA for approximately 4 h, and then transferred to Hybond-ECL nitrocellulose (Amersham Corp.). The filter was blocked for 30 min in 5% dried skim milk in TBST (20 mM Tris-Cl, pH 7.6, 137 mM sodium chloride) and incubated with primary antibody (SC-030 for K-Ras, SC-029 for H-Ras, SC-31 for N-Ras (Santa Cruz)) at 1 µg/ml and rotated at 4 °C for 4 h. The gel was fixed in 25% methanol, 7% acetic acid for 15 min, immersed for 15 min in Amplify fluorographic reagent, dried, and exposed (Hyperfilm-MP, Amersham Life Science) for 1–30 days.

**Ras Expression Vectors**—Mutant (G12V) H-Ras cDNA was PCR amplified from human bladder carcinoma cell line T24 cDNA and cloned into the mammalian expression vector pSV-Sport (Life Technologies, Inc.). Mutant (G12V) K-Ras4B cDNA was PCR amplified from the human colon carcinoma cell line SW620 and cloned into the mammalian expression vector pCI-neo (Promega Corp.). Mutant (Q61H) K-Ras4A was PCR amplified from HTB-177 cells and cloned into pCI-neo. Mutant (G12N) N-Ras coding sequence, a gift from A. Wolfman (The Cleveland Clinic, Cleveland, OH), was cloned into the pCI-neo vector. All PCR products were sequenced and were transforming in NIH 3T3 cells.

**Labeling of Ras Proteins in COS Cells**—COS-7 cells were co-transfected (Lipojectamine, Life Technologies, Inc.) with 15 µg each of pMEV and H-, N-, K-Ras4A, or K-Ras4B expression vector and incubated for 48 h in control DMEM medium or DMEM with 7.5 µM SCH-56582. For the final 18 h, cells were treated with [³H]mevalonolactone (40 Ci/mmol) at 100 µCi/µl and 20 µM mevastatin. The cells were lysed in RIPA buffer, and Ras proteins were immunoprecipitated with Y13–259-agarose conjugate (Santa Cruz Biotechnology, Inc.).
RESULTS AND DISCUSSION

To examine the effects of farnesyl transferase inhibitors SCH 44342 and SCH 56582 (19) on the prenylation of endogenous Ras proteins in a human tumor-derived cell line, we introduced the mevalonate transporter (17) into DLD-1 cells. (DLD-1 cells are derived from a colon carcinoma and contain one normal K-Ras allele and one activated mutant K-Ras allele. Genetic disruption of the mutant allele causes altered morphology, loss of capacity to grow in soft agar, and reduced tumorigenesis in nude mice (20)). Expression of the mevalonate transporter in the clonal DLD-1 cell line DLDpMev allows the cells to efficiently utilize exogenous \(^{3}H\)mevalonate in the synthesis of farnesyl and geranylgeranyl pyrophosphate. Proteins labeled in DLDpMev cells following incubation in 100 \(\mu\)Ci/ml \(^{3}H\)mevalonate, 20 \(\mu\)M mevastatin, and various concentrations of farnesyl transferase inhibitor were visualized by polyacrylamide gel separation followed by autoradiography (Fig. 1).

Treatment with increasing amounts of SCH 56582 clearly inhibited mevalonate incorporation into several proteins present in the total cell lysate between 46 and 66 kDa (Fig. 1, lanes 1–6). These proteins are believed to be farnesylated (21). Mevalonate incorporation into proteins that comprise a broad band between 21 and 28 kDa was not inhibited. These proteins consist of a number of members of the small G protein family, which are primarily geranylgeranylated. These results are compatible with the \textit{in vitro} selectivity of this compound for farnesyl protein transferase over geranylgeranyl transferase-1.

Ras proteins were immunoprecipitated from the cell lysate with the pan-Ras antibody Y13–259. Surprisingly, incorporation of the mevalonate label into immunoprecipitated Ras proteins was not substantially inhibited by increasing concentrations of SCH 56582 (Fig. 1, lanes 7–12). Western blot analysis of DLDpMev lysates revealed approximately equal signals using antibodies specific for N-Ras and K-Ras proteins; the H-Ras signal was very weak relative to the N- and K-Ras signals (data not shown). Since Y13–259 precipitates all Ras isoforms, the proteins immunoprecipitated in Fig. 1 are most likely a mixture of K- and N-Ras.

We examined the nature of the prenyl group attached to the immunoprecipitated Ras proteins (Fig. 2) using methyl iodide cleavage and HPLC analysis (18). Ras-associated prenyl groups derived from untreated DLDpMev cells (Fig. 2A) migrated as farnesol and geranylgeraniol standards. Farnesol elutes between 16 and 18 min, and geranylgeraniol elutes between 31 and 33 min. B, the predominant alcohol derived from Ras proteins immunoprecipitated from untreated DLDpMev cells is farnesol and the rearrangement isomer nerolidol (farnesol elutes first). C, the predominant alcohol derived from Ras proteins in cells treated with farnesyl transferase inhibitor (20 \(\mu\)M SCH 44342, a compound similar to SCH 56582 but lacking the bromine at position 3 on the tricyclic ring (19)) is geranylgeraniol and its rearrangement isomer geranylinalool (geranylgeraniol elutes first). D, dose response for alternative prenylation of Ras proteins in DLD pMev. Ras proteins, predominantly farnesylated in the absence of FTI, become increasingly geranylgeranylated as the concentration of the farnesyl protein transferase inhibitor SCH 56582 is increased.

FIG. 2. Analysis of the prenyl groups attached to Ras proteins in DLDpMev cells treated with farnesyl protein transferase inhibitor. Prenyl groups on immunoprecipitated Ras proteins were determined by methyl iodide cleavage and HPLC analysis. A, \(^{3}H\)farnesol and \(^{3}H\)geranylgeraniol standards. Farnesol elutes between 16 and 18 min, and geranylgeraniol elutes between 31 and 33 min. B, the predominant alcohol derived from Ras proteins immunoprecipitated from untreated DLDpMev cells is farnesol and the rearrangement isomer nerolidol (farnesol elutes first). C, the predominant alcohol derived from Ras proteins in cells treated with farnesyl transferase inhibitor (20 \(\mu\)M SCH 44342, a compound similar to SCH 56582 but lacking the bromine at position 3 on the tricyclic ring (19)) is geranylgeraniol and its rearrangement isomer geranylinalool (geranylgeraniol elutes first). D, dose response for alternative prenylation of Ras proteins in DLD pMev. Ras proteins, predominantly farnesylated in the absence of FTI, become increasingly geranylgeranylated as the concentration of the farnesyl protein transferase inhibitor SCH 56582 is increased.

FIG. 3. Prenylation of Ras proteins in COS cells. H-Ras prenylation is blocked by the farnesyl transferase inhibitor SCH 56582 (7.5 \(\mu\)M), whereas N-Ras, K-Ras4B, K-Ras4A, and H-Ras-CVLL (a geranylgeranylated version of H-Ras) are resistant to the inhibitor and continue to incorporate mevalonate. Endogenous Ras proteins are barely visible in vector control (pCI-neo) lanes. COS-7 cells were co-transfected with pMev and H-, N-, K-Ras4B, K-Ras4A, or H-Ras-CVLL expression vectors (or pCI-neo, as a negative control), and prenylated proteins were labeled by incubating in 100 \(\mu\)Ci/ml \(^{3}H\)mevalonate and 10 \(\mu\)M mevastatin. Cells were treated with 7.5 \(\mu\)M SCH 56582 (+) or Me2SO control (-) for 48 h after transfection. Immunoprecipitated Ras proteins were run on a 15% polyacrylamide gel, which was then fixed, soaked in fluorographic solution, and dried. Unprocessed H-Ras protein can be detected in COS cell lysates, following transfection and treatment with SCH 56582, by Western blotting (see Fig. 5).
FIG. 4

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Treated with 10 μM SCH 44342, the large majority of Ras-derived prenyl groups migrate with the geranylgeranyl standard (Fig. 2C). A full dose-response curve for SCH 56582 is shown in Fig. 2D. Ras proteins in DLDpMev cells are nearly completely farnesylated in the absence of FTI but become progressively geranylgeranylated in the presence of increasing amounts of SCH 56582.

To determine what effect treatment with FTI would have on processing of each of the Ras proteins, we co-transfected COS cells with the mevalonate transporter construct pMEV (17) and human, activated Ras genes (H-Ras Val-12, N-Ras Asn-12, K-Ras4A Val-12, K-Ras4B Val-12, or the geranylgeranylated transferase-1 substrate H-Ras Val-12-CVLL). Prenylated proteins were labeled by treatment with 10 μM mevastatin and 100 μCi/ml [3H]mevalonate, and Ras proteins were immunoprecipitated with the pan-Ras antibody Y13–259 (Fig. 3). K-Ras4A, K-Ras4B, N-Ras, and H-Ras(CVLL) continue to incorporate [3H]mevalonate in the presence of 7.5 μM SCH 56582. H-Ras labeling, on the other hand, is completely inhibited (H-Ras protein is expressed at similar levels in untreated cells and cells treated with SCH 56582 and mevastatin (see Fig. 5), confirming that the loss of labeled H-Ras in Fig. 3 is due to inhibition of prenylation and not to loss of H-Ras protein). Labeling patterns observed in the whole cell lysates of COS cells were similar to those observed in the whole cell lysates of DLDpMev cells (data not shown).

Isoprene analysis was performed on Ras immunoprecipitates from COS cells transfected with Ras expression vectors (Fig. 4). COS-7 cells were co-transfected with pMev and individual Ras expression vectors, and prenylated proteins were labeled and isolated as described above. Prenyl analysis of immunoprecipitated Ras proteins was done as described for Fig. 2. In the absence of FTI, N-Ras, K-Ras4A, and H-Ras contained predominantly (>90%) farnesyl isoprene. This is similar to our results in DLD-1 cells, where Ras proteins were predominantly (>90%) farnesylated in the absence of drug treatment. In the absence of FTI, K-Ras4B was found to contain a higher proportion (approximately 20% of the total) of geranylgeranyl groups, compared with N-, H-, and K-Ras4A. This result was unexpected since previous studies have not detected incorporation of geranylgeranyl groups into K-Ras proteins in untreated cells (for example, see Ref. 18) and may be a consequence of the high level of expression of K-Ras4B in COS cells. In COS cells treated with 10 μM SCH 56582, immunoprecipitates of both N-Ras and K-Ras proteins contained predominantly (>90%) geranyl-geranyl isoprenes. H-Ras prenylation is blocked by the inhibitor leading to a loss of label from the immunoprecipitated H-Ras protein. These results extend our observation of alternative prenylation in DLD-1 cells to another cell type, COS-7 cells, and allow analysis of each form of Ras individually.

Our ability to detect geranylgeranyl incorporation into K-Ras expressed in COS cells is in contrast to the results of James et al. (16). They detected farnesyl incorporation into K-Ras4B3125 expressed in Rat-1 cells grown in the presence or absence of BZA-5B, a benzodiazepine-based FTI. The differences in our results may be due to the different cell lines or Ras substrates employed. Alternatively, the difference may be due to significantly higher intracellular concentration of mevalonate in our studies due to co-expression of the mevalonate transporter. Rilling et al. (22) reported previously that protein prenylation in Chinese hamster ovary cells can vary as a function of the extracellular mevalonate concentration.

The effect of SCH 56582 on processing and localization of the individual Ras proteins was also analyzed by Western blots (Fig. 5). COS cells transfected with Ras expression vectors in the presence of 7.5 μM SCH 56582, 20 μM mevastatin (as a positive control for inhibition of processing), or Me2SO (vehicle control) were fractionated into a particulate fraction (100,000 × g pellet) enriched in cellular membranes, and a particulate fraction (100,000 × g supernatant) enriched in soluble protein.

**Fig. 5. Western blots of Ras proteins expressed in COS cells.** COS-7 cell lysates were fractionated following transfection with Ras expression vectors into a membrane-enriched particulate fraction (100,000 × g pellet) and a soluble protein fraction (supernatant 100,000 × g). In untreated controls, H-Ras, K-Ras4B, K-Ras4A, N-Ras, and H-Ras-CVLL (geranylgeranylated version of H-Ras) proteins are processed and associate with the particulate fraction. In lysates from cells treated with mevastatin (10 μM), synthesis of farnesyl and geranylgeranyl pyrophosphate is blocked, and all of the Ras proteins accumulate as unprocessed, soluble precursors. Treatment with SCH 56582 (7.5 μM) displaces H-Ras from the particulate to the soluble fraction, whereas K-Ras4B, K-Ras4A, N-Ras, and H-Ras-CVLL remain associated with the particulate fraction. Note faster mobility of processed Ras proteins (associated with particulate fraction) compared with unprocessed proteins (in the soluble fraction).

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**Fig. 4. Isoprene analysis of individual Ras proteins.** In the absence of farnesyl transferase inhibitor (untreated), H-, N- and K-Ras proteins predominantly yield a farnesol group. In the presence of farnesyl transferase inhibitor SCH 56582 (7.5 μM), N-, K-Ras4A, and K-Ras4B become alternatively prenylated and contain the 20-carbon geranylgeranyl group. H-Ras prenylation, in contrast, is blocked by SCH 56582. COS-7 cells were co-transfected with Ras expression vectors and with pMev as described for Fig. 3. Ras proteins were immunoprecipitated with Y13–259 pan-Ras antibody, and the prenyl group attached to each Ras was analyzed as described under “Experimental Procedures.” The farnesol standard elutes at approximately 19 min, whereas the geranylgeraniol standard elutes at approximately 32 min.
soluble fraction (supernatant after 100,000 × g). Inhibition of Ras processing can be detected by the slower migration of unprocessed Ras proteins in 15% polyacrylamide gels (23) and by the loss of Ras protein from the membrane fraction. In the absence of SCH 56582 or of mevastatin, each of the Ras proteins associated exclusively with the particulate fraction. Treatment with mevastatin causes loss of all forms of Ras from the particulate fraction and the appearance of their unprocessed, slower-migrating forms in the soluble fraction. In cells treated with SCH 56582, H-Ras is lost from the particulate fraction and appears in the soluble fraction. Both isoforms of K-Ras, in contrast, are prenylated, as determined by their relative (faster) migration, and remain exclusively associated with the particulate fraction. The results with N-Ras in the presence of the farnesyl transferase inhibitor are more complex; most of the N-Ras protein expressed in COS cells is processed in the presence of SCH 56582 and remains associated with the particulate fraction. Some (~10% of the total) N-Ras protein, however, consistently appears as unprocessed precursor in the soluble fraction (we have noted this partial inhibition of N-Ras prenylation in several experiments, both with antibody detection and with [35S]methionine labeling). These results using Western analysis confirm the results using mevalonate labeling, i.e. N-Ras, K-Ras4A, and K-Ras4B are prenylated in the presence of FTI. They reveal further that alternatively prenylated versions of N- and K-Ras remain associated with the membrane fraction in treated cells.

The switch in prenylation that occurs in N- and K-Ras proteins in the presence of FTI is probably a consequence of the greater catalytic efficiency of farnesyl protein transferase, compared with geranylgeranyl transferase-1, for these substrates. Thus, in the absence of FTI, N- and K-Ras precursor proteins are preferentially farnesylated by farnesyl protein transferase. When farnesyl protein transferase is inhibited, N- and K-Ras become available as substrates for geranylgeranyl transferase-1. H-Ras, on the other hand, is not a suitable substrate for geranylgeranyl transferase-1 and so remains unprenylated. The unique sensitivity of H-Ras processing to inhibition suggests that cells whose transformed phenotype depends on H-Ras activity may be particularly responsive to FTI treatment. In agreement with this, we have found that Rat-2 cells transformed with mutant H-Ras are approximately 10–20-fold more sensitive to FTI in the soft agar assay than cells transformed with mutant K-Ras (data not shown); James et al. (16) have made similar observations. Normal Ras proteins also play a role in oncogenesis, serving as mediators of transforming signals from mutant tyrosine kinase receptors for example, and one might expect cells in which normal H-Ras plays a significant role in mediating upstream oncogenic signals to be relatively sensitive to FTI treatment. The accumulation of unprocessed H-Ras in the cytosol of cells treated with FTI (see Fig. 5) may also play a part in the mechanism of action of these compounds, perhaps by perturbing normal signaling pathways (for example, see Ref. 24).

The alternative processing of N- and K-Ras proteins in the presence of FTI suggests that they may continue to interact with effector proteins under these conditions. In support of this, we have found in co-transfection studies that, whereas H-Ras stimulation of Ras-responsive promoters can be blocked by farnesyl transferase inhibitors, K- and N-Ras stimulation of transcription is only marginally reduced. Nonetheless, anchorage-independent growth of K-Ras transformed mouse fibroblasts, and of human tumor cells containing K- and N-Ras mutations, can be inhibited by FTI treatment (9). The mechanism by which FTIs inhibit anchorage-independent growth in such cells is unclear. The incorporation of geranylgeranyl rather than farnesyl into N- and K-Ras proteins may cause subtle changes in Ras signaling, perhaps by altering subcellular localization or protein-protein interaction, that lead to reduced ability to grow in an anchorage-independent fashion. Several bifurcating pathways are stimulated by activation of Ras proteins, for example membrane ruffling and mitogenesis (25), and it is possible that alternative prenylation affects these pathways differently. Alternatively, the mechanism of action of FTIs may involve inhibiting the farnesylation of a target protein other than Ras.

The mechanism of action of FTIs is clearly more complex than originally envisioned. The overall result of treatment with FTI may reflect the combined effects of inhibiting the prenylation of some proteins, such as H-Ras and the proteins >30 kDa in the DLDpMev lysate (Fig. 1), and altering the pattern of prenylation of others, such as N- and K-Ras. Other cellular proteins that are normally farnesyl protein transferase substrates may also become geranylgeranylated when farnesyl protein transferase activity is inhibited.

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