v-K-ras leads to preferential farnesylation of p21\textsuperscript{ras} in FRTL-5 cells: Multiple interference with the isoprenoid pathway

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ABSTRACT The isoprenoid pathway in FRTL-5 thyroid cells was found to be deeply altered on transformation with v-K-ras. A dramatic overall reduction of protein prenylation was found in v-K-ras-transformed cells in comparison with the parent FRTL-5 cells, as shown by labeling cells with [\textsuperscript{3}H]mevalonic acid. This phenomenon was accompanied by a relative increase of p21\textsuperscript{ras} farnesylation and by a decrease of the ratio between the amounts of geranylgeraniol and farnesol bound to prenylated proteins. Analysis of protein prenylation in FRTL-5 cells transformed by a temperature-sensitive mutant of the v-K-ras oncogene indicated that these variations represent an early and specific marker of active K-ras. Conversely, FRTL-5 cells transformed with Harvey-ras showed a pattern of [\textsuperscript{3}H]-mevalonate (MVA)-labeled proteins similar to that of nontransformed cells. The K-ras oncogene activation also resulted in an overall decrease of [\textsuperscript{3}H]-MVA incorporation into isopentenyl-tRNA together with an increase of unprocessed [\textsuperscript{3}H]-MVA and no alteration in [\textsuperscript{3}H]-MVA uptake. The effects of v-K-ras on protein prenylation could be mimicked in FRTL-5 cells by lowering the concentration of exogenous [\textsuperscript{3}H]-MVA whereas increasing the [\textsuperscript{3}H]-MVA concentration did not revert the alterations observed in transformed cells. Accordingly, v-K-ras expression was found to: (i) downregulate mevalonate kinase; (ii) induce farnesylpyrophosphate synthase expression; and (iii) augment protein farnesyltransferase but not protein geranylgeranyltransferase-I activity. Among these events, mevalonate kinase downregulation appeared to be related strictly to differential protein prenylation. This study represents an example of how regulation appeared to be related strictly to differential activity. Among these events, mevalonate kinase downregulation may result in the preferential farnesylation of the ras oncogene product p21\textsuperscript{ras}.

EXPERIMENTAL PROCEDURES

Materials. (RS)-[5-\textsuperscript{3}H]-mevalonate ([\textsuperscript{3}H]MVA, 35.0 Ci/ mmol), [1,2-\textsuperscript{3}H]-farnesylpyrophosphate ([\textsuperscript{3}H]FPP, 15 Ci/mmol), and [1,2-\textsuperscript{3}H]-geranylgeranylpyrophosphate, 10 Ci/mmol) were purchased from DuPont/NEC. Biotinylated (Bt)-KTKCVIS and Bt-KKFCAIL kindly were provided by C. M. Allen (Univ. of Florida, Gainesville). Thyrotropin was a highly purified preparation from bovine pituitary extracts (17). Lovastatin was a gift from A. W. Alberts of the Merck, Sharp and Dohme Institute (Rahway, NJ). The rabbit polyclonal antibody to mevalonate kinase kindly was provided by S. Krisans (San Diego State Univ.) (18).

Cells and Culture. FRTL-5 cells were cultured as described (10). KIMol cells were derived from FRTL-5 cells on infection and transformation with a wild-type strain of KiMSV-MolMuLV (13, 14), and Ats cells were derived from FRTL-5 cells transformed with a temperature-sensitive mutant (33°C, 

Abbreviations: MVA, mevalonate; MK, mevalonate kinase; FPP synthase, farnesyl-diphosphate synthase; FTase, protein farnesyltransferase.

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permissive; 39°C, nonpermissive) of Kirsten-murine sarcoma virus (15). H-ras was derived from FRTL-5 cells transformed by Harvey ras oncogene (16). TK-6 and MPTK-6 cells were derived, respectively, from a thyroid carcinoma and lung metastases of this tumor induced in propyl-thiouracil-pretreated Fisher rats by the injection of a retrofitus carrying the v-K-ras oncogene (19). All of these transformed cells kindly were provided by G. Vecchio and A. Fusco (Univ. di Napoli, “Federico II,” Italy). KiMol, H-ras, TK-6, and MPTK-6 cells were grown at 37°C, and Ats cells were grown at 39°C, in Coon’s modified Ham’s F-12 medium, supplemented with 5% calf serum. Where indicated, Ats cells were shifted to 33°C for different periods of time, in different experiments.

**Incorporation of [3H]-Mevalonate into Cellular Proteins.** Proliferating FRTL-5 cells, KiMol cells, and Ats and H-ras cells were incubated with 10 μM lovastatin and 30 μCi/ml [5-3H]-MVA for 7 hr. Density of cell culture ranged between 1.5 and 2.0 × 10^6 cells/ml in 100-mm Petri dishes. Cells then were washed three times with ice-cold PBS, were scraped from the dish, and were lysed in hypotonic buffer. Equal amounts of each protein extract (~100 μg) were analyzed by 12% SDS/PAGE as described (20–22).

**Immunoprecipitation and SDS/PAGE.** [3H]-MVA labeled cells were washed three times with PBS and were lysed in RIPA buffer [20 mM Tris/150 mM NaCl, 1 mM EDTA, 0.5% (vol/vol) Nonidet P-40, 0.5% (wt/vol) Na deoxycholate, 0.1% (vol/vol) Trasylol, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.4]. After 10 min on ice, the lysates were centrifuged at 12,000 × g for 10 min, and supernatants were immunoprecipitated with 5 μg of preimmune rat serum or anti-p21ras mAb (Y13–259, Oncogene Science) followed by incubation with Protein A-Sepharose. Immunoprecipitates were washed three times with RIPA buffer and once with 100 mM Tris-Cl (pH 6.8) and then were dissolved in Laemmli loading buffer with 1 mM DTT before electrophoresis in a 12.5% SDS-polyacrylamide gel. Gels then were permeated with Amplify fluorographic enhancer (Amersham) and were dried and autoradiographed at –90°C.

**Incorporation of [3H]-Mevalonate into Cellular mRNA.** Proliferating FRTL-5 cells and KiMol cells were incubated with 10 μM lovastatin and 30 μCi/ml [3H]-MVA for 7 hr. At the end of the incubation period, cells were processed for total RNA extraction and [3H]-isopentenyl-riboflavin analysis as reported (23).

**HPLC Analysis of Protein-Bound Farnesol and Geranylgeraniol.** [3H]-farnesol and [3H]-geranylgeraniol released by methyl iodide reaction of prenylated proteins were analyzed by HPLC carried out by using a Spherisorb ODS-2 column (Phase Sep, Queen Penny, Clwyd, U.K.) (5 mm × 4.5 mm × 25 cm) eluted with a 40-min linear gradient from 50 to 100% (vol/vol) CH₃CN/25 mM H₃PO₄ in 25 mM H₃PO₄ as described (1). Free [3H]-MVA was analyzed by a slight modification of these elution conditions, i.e., by means of a simple 20-min isocratic step of 50% (vol/vol) CH₃CN in 25 mM H₃PO₄.

**Protein Prenyltransferase Assays.** Fase and protein geranylgeranyltransferase-I activities were assayed by measuring, respectively, the amount of [3H] farnesyl and [3H] geranylgeranyl transferred from [3H]-FPP and [3H]-geranylgeranylophosphosphate to recombinant H-Ras, wild-type and CVL7 type, as described (24). Protein prenyltransferase activities also were assayed by using biotinylated peptides (BiKTCVCS and Bt-KKKFCAIL) as prenyl acceptors by a modification of the method of Farnsworth et al. (25).

**Cell Labeling with [3H]-FPP by Low Density Lipoprotein Carrier.** [3H]-FPP (5 μCi) was dried under N₂ in syliconized glass tubes to which low density lipoprotein (1 mg/ml in PBS, pH 7.4) was added. The mixture was incubated at room temperature under stirring for 1 hr and then was added to culture medium. Cells then were incubated overnight with the modified medium in the presence of 10 μM lovastatin.

**Protein Farnesylation Assay in Vitro.** Cells were incubated with 10 μM lovastatin for 6 hr at 37°C and then were washed with ice-cold PBS and were lysed in 50 mM Tris-HCl (pH 7.4), 10 mM DTT, 10 mM MgCl₂, and 1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 100,000 × g for 45 min, and 5 μCi of [3H]-FPP were added to the supernatant. The mixture was incubated for 1 hr at 37°C, and proteins were precipitated with 9 vol of cold acetone and were analyzed by SDS/PAGE.

**Total RNA Preparation and Northern Blot Analysis.** Total RNA was extracted by the guanidinium thiocyanate- acid phenol procedure. Total RNA (20 μg) from each cell strain was loaded and separated on 1% agarose gel containing 2% formaldehyde and was blotted onto nylon membranes (Hybond-N, Amersham). Prehybridization (1 hr at 65°C), hybridization (16–18 hr at 65°C), and high stringency washes (1 hr at 65°C and 1 hr at 55°C) were carried out as reported (9). An FPP synthase cDNA (CR39), kindly provided by P.A. Edwards (Univ. of California, Los Angeles), was used as radiolabeled probe (26). Normalization was accomplished by using radiolabeled glyceraldehyde-phosphate dehydrogenase cDNA as reference probe.

**RESULTS**

**Protein Prenylation and [3H]-MVA Metabolism in Differentiated, K-ras-, and H-ras-Transformed FRTL-5 Cells.** To improve the incorporation of [3H]-MVA into prenylated proteins, endogenous MVA synthesis was blocked by the addition of 10 μM lovastatin. Under these conditions, FRTL-5 cells incorporated very efficiently [3H]-MVA and displayed a variety of [3H]-labeled proteins with a molecular weight ranging between 14 and 90 (Fig. 1). FRTL-5 cells transformed by Kirsten-Moloney sarcoma virus (KiMol), incubated with lovastatin under similar conditions, displayed an entirely different pattern of [3H]-MVA-labeled proteins, essentially limited to one major protein species with an apparent molecular weight of 21 (Fig. 1). Other [3H]-MVA-labeled proteins were not detected in KiMol cells even on prolonged exposure (40 days) of the gel (data not shown). Specific immunoprecipitation with an anti-p21ras mAb showed that the major [3H]-MVA-labeled protein was the product of the ras oncogene (Fig. 1).

Cells derived from a thyroid carcinoma (TK-6 cells) induced in propyl-thiouracil-pretreated rats by the injection of a retrofitus carrying the v-K-ras oncogene and from lung metastases of this tumor (MPTK-6 cells) displayed a pattern of [3H]-MVA-labeled proteins, essentially limited pattern of [3H]-MVA-labeled proteins, essentially limited to the p21ras protein with an apparent molecular weight of 21 (Fig. 1). Other [3H]-MVA-labeled proteins were not detected in KiMol cells even on prolonged exposure (40 days) of the gel (data not shown). Specific immunoprecipitation with an anti-p21ras mAb showed that the major [3H]-MVA-labeled protein was the product of the ras oncogene (Fig. 1).

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peared much earlier than the latters (Fig. 1B). The decrease of [3H]-MVA-labeled proteins in transformed cells appeared to be evident, although to a lesser extent, even if lovastatin blockade was omitted (data not shown). Experiments with FRTL-5 cells transformed by Harvey-ras (H-ras) also were performed and showed a pattern of prenylated proteins very similar to that of nontransformed cells (Fig. 1A).

The changes in protein prenylation observed in KiMol cells was not caused by reduction of protein synthesis because the levels of proteins other than p21ras, including other prenylated proteins, such as 2',3'-cyclic nucleotide 3'-phosphodiesterase and Rab5, are not varied in KiMol cells, Ats cells at 33°C, and H-ras cells with respect to FRTL-5 cells (data not shown). The expression of p21ras in all cell lines transformed by either v-K-ras or Harvey-ras oncogenes was considerably higher than in FRTL-5 cells (data not shown), in which only a faint, 21-kDa band in SDS/PAGE analyses of proteins immunoprecipitated by an anti-p21mAb was found, in agreement with published data (13–16, 19).

The pattern of prenylated proteins observed in v-ras-transformed cells resembled that of FRTL-5 cells incubated with decreasing concentrations of exogenous [3H]-MVA (Fig. 2). The two patterns became nearly identical when FRTL-5 cells were incubated in the presence of a 5-fold lower [3H]-MVA concentration. On the other hand, when KiMol cells were incubated with increasing concentrations of [3H]-MVA, p21ras appeared more intensely prenylated but still remained the only prenylated protein (Fig. 2). FRTL-5 and KiMol cells were able to incorporate [3H]-MVA at the same rate (Table 1) whereas HPLC analyses revealed a large increase (70%) of unprocessed [3H]-MVA and a significant decrease of isopentenyl-tRNA in KiMol versus normal FRTL-5 cells (Table 1). Moreover, the percentage of farnesol and geranylgeraniol released from prenylated proteins was respectively higher and lower in KiMol cells compared with FRTL-5 cells, thus leading to a reduced geranylgeraniol/farnesol ratio (Table 2). If the concentration of [3H]-MVA used for labeling FRTL-5 cells was reduced by 5-fold, the percentage of farnesol and the geranylgeraniol/farnesol ratio also varied by becoming similar to those observed in KiMol cells incubated with higher [3H]-MVA concentrations (Table 2). Similar results were obtained with Ats cells at 39°C (nontransformed) and at 33°C (transformed) (data not shown). Finally, in agreement with the overall decrease of geranylgeranylation of proteins described above, we found a sensible (2- to 3-fold) increase of the levels of unbound cytosolic Rab5 in KiMol cells (data not shown).

### Table 1. Analysis of [3H]-MVA incorporated into isoprenoid products in FRTL-5 and KiMol cells

<table>
<thead>
<tr>
<th>[3H]-Isoprenoid compounds</th>
<th>FRTL-5</th>
<th>KiMol</th>
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<tbody>
<tr>
<td>Unprocessed [3H]-MVA</td>
<td>21.9 ± 3.1</td>
<td>38.1 ± 4.5</td>
</tr>
<tr>
<td>[3H]-isopentenyl-tRNA</td>
<td>4.50 ± 2.20</td>
<td>1.00 ± 0.20</td>
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Proliferating FRTL-5 and KiMol cells were incubated with 10 μM lovastatin and 30μCi [3H]-MVA for 7 hr. At the end of the incubation period, cells were processed and analyzed as detailed under Experimental Procedures. Activation of K-ras had no influence on the mevalonate uptake because FRTL-5 and KiMol cells show comparable values of total incorporation (20,200 ± 7,200 vs. 21,700 ± 11,100 cpm/μg cell protein). The values indicate the percent of cpm per total incorporated cpm.

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and transformed cells, the expression of MK. Protein levels of MK, as assessed by immunoblot experiments with a polyclonal antibody to MK, were found to be decreased in KiMol and in Ats cells at 33°C when compared, respectively, to FRTL-5 cells and Ats cells at 39°C (Fig. 3). On the other hand, H-ras cells showed MK levels similar to those of FRTL-5 cells (Fig. 3).

**Increased Protein Farnesyltransferase but not GeranylGeranyltransferase-I Activity in K-ras- and H-ras-Transformed FRTL-5 Cells.** We analyzed the activity of FTase and protein geranylgeranyltransferase-I in K-ras- and H-ras-transformed FRTL-5 cells. We observed a 2- to 3-fold increase in FTase activity and no variation in geranylgeranyltransferase I activity in K-ras-transformed cells in comparison with FRTL-5 cells (Fig. 4). H-ras cells behaved like K-ras-transformed cells by exhibiting a < 2-fold increase in FTase activity and, again, no change in geranylgeranyltransferase I activity. We next verified K-ras regulation of FTase activity by incorporating, using low density lipoprotein as physiological carrier, [3H]-farnesyl-PP into cellular proteins and cholesterol.

Under these conditions, we observed a large increase in the incorporation of [3H]-farnesyl-PP, mostly into p21ras (Fig. 5A) as well as a corresponding reduction of incorporation into cholesterol (data not shown) in KiMol vs. FRTL-5 cells. Accordingly, HPLC analysis of prenyl groups attached to the proteins revealed a larger peak of farnesol in KiMol cells compared with FRTL-5 cells (Fig. 5A) whereas the geranylgeraniol/farnesol ratio in prenylated proteins was decreased, and the percent of bound farnesol was increased in KiMol vs. FRTL-5 cells (Table 2), in agreement with the results shown above for cells labeled with [3H]-MVA. Cell-free [3H]-farnesyl-PP labeling experiments carried out in KiMol as well as in H-ras cells also showed an increased incorporation of label in p21ras (Fig. 5B). Ats cells displayed at both temperatures (33 and 39°C) significantly increased FTase activity and [3H]-farnesyl-PP incorporation, mostly into p21ras when compared with FRTL-5 cells (Figs. 4 and 5A).

**DISCUSSION**

We presented data showing that K-ras oncogene activity affects protein prenylation. Transformation of FRTL-5 cells with v-K-ras caused a dramatic change in the pattern of prenylated proteins. An overall decrease of [3H]-MVA-labeled proteins was observed in K-ras transformed cells whereas the ras oncogene product p21ras appeared still farnesylated. Our data strongly suggest that this phenomenon is related directly and specifically to K-ras activity. First, when this oncogene was activated in Ats cells (33°C), a pattern of prenylated proteins closely resembling that of KiMol cells was observed. In addition, cells derived from thyroid carcinoma, induced by expression of v-K-ras, and from lung metastases of this tumor displayed a similar profile of prenylated proteins. Conversely, FRTL-5 cells transformed by H-ras showed a pattern of prenylated proteins very similar to that found in nontransformed cells. There are at least two implications from this finding: (i) The alterations observed on transformation by K-ras provide an early and specific marker for K-ras transforming activity; 6–8 hours of oncogene activation were sufficient to observe an altered prenylation pattern; and (ii) these alterations represent an unprecedented example of how v-K-ras induces metabolic changes in the isoprenoid pathway.

The changes in the protein prenylation pathway observed here in v-K-ras transformed cells were found to be caused by reduction of geranylgeranylation and, only to a lesser extent, farnesylation of proteins, as assessed by HPLC analysis of the prenyl groups attached to proteins from FRTL-5, KiMol, and Ats cells labeled with either [3H]-MVA or [3H]-FPP (Table 2 and data not shown). Indeed, a previous investigation (27), carried out in Chinese hamster ovary cells, correlated (i) the appearance of a protein prenylation pattern similar to that observed here in v-K-ras-transformed cells and (ii) a drastic decrease of the geranylgeraniol/farnesol ratio in total prenylated proteins, with decreasing concentrations of exogenous prenyl acceptors.
autoradiograms were exposed for 20 days. The experiments were repeated with similar results.

Experimental Procedures
detailed in
concentration became identical to those observed in KiMol
proteins from FRTL-5 cells incubated with a low [3H]-MVA
farnesol and the geranylgeraniol
ster ovary cells (Table 2). Moreover, both the percent of
the geranylgeraniol
y
decreased with decreasing [3H]-MVA concentrations in a way
[3H]-MVA. This led to the suggestion that p21
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of farnesylated proteins labeled in whole-cells labeled with [3H]-farnesyl-PP by using low density lipoprotein as a carrier, and HPLC analysis of
prenyl groups attached to proteins. These data are representative of three independent experiments. Molecular weight standards (45 and 21) are
shown on the right. (B) p21ras farnesylated in cell-free experiments carried out with normal, KiMol, and H-ras FRTL-5 cell homogenates. The
autoradiograms were exposed for 20 days. The experiments were repeated with similar results.

[H]-MVA. This led to the suggestion that p21ras farnesylation is less sensitive than prenylation of other proteins to reduced availability of exogenous
[H]-MVA (27). Here, we found that it is possible to reproduce a KiMol cell-like protein prenylation pattern also in nontransformed FRTL-5 cells by lowering the
concentration of exogenous [H]-MVA (Fig. 2). We found that the geranylgeraniol/farnesol ratio in total prenylated proteins decreased with decreasing
[H]-MVA concentrations in a way strikingly similar to that observed previously in Chinese hamster ovary cells (Table 2). Moreover, both the percent of
farnesol and the geranylgeraniol/farnesol ratio in prenylated proteins from FRTL-5 cells incubated with a low [H]-MVA concentration became identical to those observed in KiMol
cells (Table 2). On the other hand, an increase of [H]-MVA concentration did not reverse the changes of protein prenylation observed in KiMol cells (Fig. 2). Therefore, we decided to
assess whether the changes in protein prenylation observed here were caused by some irreversible alteration in the isoprenoid pathway subsequent to v-K-ras activation and leading to (i) conserved or slightly reduced p21ras and protein farnesyl
ylation and (ii) greatly reduced protein geranylgeranylation. On the basis of the data reported here, we suggest that these changes are caused by the following events: (i) Exogenous

![Figure 5](image-url)

**Fig. 5.** Farnesylation in differentiated, K-ras, and H-ras transformed FRTL-5 cells. [3H]-farnesyl-PP labeling in whole-cell and cell-free experiments carried out with FRTL-5, KiMol, Ats (39 and 33°C), and H-ras cells, as described in Experimental Procedures. (A) Electrophoretic profile of farnesylated proteins labeled in whole-cells labeled with [3H]-farnesyl-PP by using low density lipoprotein as a carrier, and HPLC analysis of
prenyl groups attached to proteins. These data are representative of three independent experiments. Molecular weight standards (45 and 21) are
shown on the right. (B) p21ras farnesylated in cell-free experiments carried out with normal, KiMol, and H-ras FRTL-5 cell homogenates. The
autoradiograms were exposed for 20 days. The experiments were repeated with similar results.

![Figure 6](image-url)

**Fig. 6.** FPP synthase expression in FRTL-5, KiMol, Ats (39 and 33°C), and H-ras cells. Northern blot analysis was performed as detailed in Experimental Procedures. Total RNA (20 μg) was used in each lane. (Top) The blot was probed with a FPP synthase cDNA. (Bottom) The blot was stripped and reprobed with a rat glyceraldehyde phosphate dehydrogenase cDNA probe, performed as a control that
similar amounts of RNA were loaded in each lane. Details are given in Experimental procedures. The data presented are representative of
three experiments.
thase and FTase are up-regulated to further increase the incorporation of farnesyl-PP into the overexpressed p21 ras.

From the above observations it follows that the use of Ats and H-ras cells in this study was important, not only inasmuch as it allowed, together with data from other tumoral cells, to establish that the changes in the protein prenylation profile observed in transformed cells were related specifically to v-K-ras activation, but also because it provided a clue as to which of the metabolic changes observed are related to p21 ras synthesis and which to p21 ras activity. In Ats cells, in fact, p21 ras intracellular concentration is increased at both permissive (33°C) and nonpermissive (39°C) temperatures, but the oncogene product is active only at the former. Because MK and FPP synthase expression were modified only at 33°C whereas FTase activity was increased at both temperatures, it is possible that, of the three enzymes studied, only FPP synthase and MK require both the synthesis and the activation of p21 ras for their expression to be modified. Conversely, the presence of high levels of p21 ras, rather than its activity (tumorigenicity), would be sufficient to observe—and, as suggested above, may be the cause of—the increase of FTase activity (28). As for H-ras cells, the differences in the isoprenoid pathway described here between these and KiMol cells are intriguing, although different biological effects of the H-ras and v-K-ras oncogene products have been reported (29, 30).

A possible consequence of the differential decrease of protein farnesylation observed in this study in transformed KiMol cells is that the cell distribution of farnesylated proteins—which depend in part on their prenyl chain for membrane anchoring—and particularly of geranylglyceroylated proteins, also is modified. This, indeed, was found to be the case for Rab5, whose unbound levels were increased significantly in KiMol cells (data not shown). However, high amounts of Rab5 still were associated with membranes, suggesting that other post-translational modifications may occur in v-K-ras-transformed cells to compensate for the loss of prenyl chains and prompting further studies in this direction. It is worthwhile mentioning that this kind of compensatory effects already have been shown to occur for prenylated oncogene products (31).

In conclusion, the findings described herein provide a remarkable example of how v-K-ras oncogene may induce several modifications in the isoprenoid pathway and, in particular, a dramatic decrease in the expression of MK, ultimately leading to preferential farnesylation of p21 ras. To establish whether this phenomenon also results in an improved functional activation of p21 ras was beyond the scope of this study and remains a subject for speculation. It is possible that MK down-regulation leads to the decrease/inactivation of some as yet unidentified mevalonate derivative and/or isoprenylated protein that inhibit p21 ras activation in undifferentiated cells. Recent experiments performed with the rat seminiferous epithelium (31) have shown that changes in the protein prenylation pattern similar to those described here can be observed also during a physiological response, such as the meiotic stages of spermatocyte formation, and are accompanied by comparable increases of FTase activity. Therefore, our findings, by describing the transformation-related alteration of protein prenylation, may represent the starting point for future investigations on this new aspect of oncogene expression during both physiological and pathological responses.

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