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v-K-*ras* leads to preferential farnesylation of p21^{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 [^{3}H]mevalonic acid. This phenomenon was accompanied by a relative increase of p21^{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 [^{3}H]-mevalonate (MVA)-labeled proteins similar to that of nontransformed cells. The K-*ras* oncogene activation also resulted in an overall decrease of [^{3}H]-MVA incorporation into isopentenyl-tRNA together with an increase of unprocessed [^{3}H]-MVA and no alteration in [^{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 [^{3}H]-MVA whereas increasing the [^{3}H]-MVA concentration did not revert the alterations observed in transformed cells. Accordingly, v-K-*ras* expression was found to: (*i*) down-regulate mevalonate kinase; (*ii*) induce farnesyl-pyrophosphate synthase expression; and (*iii*) augment protein farnesyltransferase but not protein geranylgeranyl-transferase-I activity. Among these events, mevalonate kinase down-regulation appeared to be related strictly to differential protein prenylation. This study represents an example of how expression of the v-K-*ras* oncogene, through multiple interferences with the isoprenoid metabolic pathway, may result in the preferential farnesylation of the *ras* oncogene product p21^{ras}.**

Recent observations established a direct connection between the mevalonate pathway and transformation by *ras*. It was demonstrated that farnesol, a mevalonate-derived metabolite, is required for the modification that leads to the activation of the *ras* oncogene product p21^{ras} (1–5). The linkage of a farnesyl group to p21^{ras}, catalyzed by the protein farnesyltransferase (FTase), initiates a set of additional post-translational modifications that promote the anchorage of the Ras protein to the cell membrane, thus leading to the functional activation of this oncogene-encoded protein. An increased expression of normal or mutated *ras* has been detected in 40% of human cancers (6), including anaplastic and follicular thyroid carcinoma (7). It has been shown that v-K-*ras* oncogene down-regulates the expression of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme of isoprenoid biosynthesis, in FRTL-5 thyroid cells through the involvement of a novel cyclic AHP-responsive element site identified in its promoter (8, 9). However, a possible effect of *ras* activity on protein prenylation and in particular on p21^{ras} farnesylation has not been investigated yet. To date, no evidence exists for the occurrence of alterations in protein prenylation after *ras*-induced cell transformation. The aim of this study was to analyze the prenylated proteins in normal and v-K-*ras*-transformed cells to assess whether such a correlation exists. We addressed this issue in FRTL-5 thyroid cells, a cell strain whose proliferation and differentiated functions, like iodide uptake and thyroglobulin synthesis, depend on thyrotropin (10–12) and, in the same cells, are transformed by (*i*) the Kirtsen–Moloney sarcoma virus (KiMol cells) (13, 14), (*ii*) a temperature-sensitive mutant of v-K-*ras* (Ats cells) (15), and (*iii*) the Harvey-*ras* oncogene (H-*ras* cells) (16). FRTL-5 cells loose their differentiated functions on v-K-*ras* activation and represent a good model for investigations of possible correlations between K-*ras* activity and the isoprenoid pathway (9).

Evidence for the occurrence of simultaneous regulation by K-*ras* oncogene on several enzymes of the isoprenoid biosynthesis and metabolism, i.e., mevalonate kinase (MK), farnesyl-PP synthase (FPP synthase), and FTase, concomitantly with a profoundly altered pattern of protein prenylation, will be presented. These effects may be considered as an example of cellular adaptation in response to K-*ras*-induced transformation and may result in the preferential farnesylation of the *ras* oncogene product p21^{ras}.

EXPERIMENTAL PROCEDURES

Materials. (RS)-[5-^{3}H]-mevalonate ([^{3}H]MVA, 35.0 Ci/mmol), [1-^{3}H]-farnesylpyrophosphate ([^{3}H]FPP, 15 Ci/mmol), and [1-^{3}H]-geranylgeranylpyrophosphate, 10 Ci/mmol) were purchased from DuPont/NEN. Biotinylated (Bt)-KTKCVIS and Bt-KKFFCAIL 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,

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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 metastates of this tumor induced in propyl-thyouracyl-pretreated Fisher rats by the injection of a retrovirus 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 [^{3}H]-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-^{3}H]-MVA for 7 hr. Density of cell culture ranged between 1.5 and 2.0 $\times$ 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 ($\approx$100 μg) were analyzed by 12% SDS/PAGE as described (20–22).

Immunoprecipitation and SDS/PAGE. [^{3}H]-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 $\times$ g for 10 min, and supernatants were immunoprecipitated with 5 μg of preimmune rat serum or anti-p21^{ras} 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 −80°C.

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

HPLC Analysis of Protein-Bound Farnesol and Geranylgeraniol. [^{3}H]-farnesol and [^{3}H]-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 $\times$ 4.5 mm $\times$ 25 cm) eluted with a 40-min linear gradient from 50 to 100% (vol/vol) CH_3CN/25 mM H_3PO_4 in 25 mM H_3PO_4 as described (1). Free [^{3}H]-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) CH_3CN in 25 mM H_3PO_4.

Protein Prenyltransferase Assays. FTase and protein geranyl-geranyltransferase-I activities were assayed by measuring, respectively, the amount of [^{3}H] farnesyl and [^{3}H] geranylgeranyl transferred from [^{3}H]-FPP and [^{3}H]- geranylgeranylpyrophosphate to recombinant H-Ras, wild-type and CVLL type, as described (24). Protein prenyltransferase activities also were assayed by using biotinylated peptides (Bt-KTKCVIS and Bt-KKFFCAIL) as prenyl acceptors by a modification of the method of Farnsworth *et al.* (25).

Cell Labeling with [^{3}H]-FPP by Low Density Lipoprotein Carrier. [^{3}H]-FPP (5 μCi) was dried under N_2 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 $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 100,000 $\times$ g for 45 min, and 5 μCi of [^{3}H]-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 [^{3}H]-MVA Metabolism in Differentiated, K-*ras*-, and H-*ras*-Transformed FRTL-5 Cells. To improve the incorporation of [^{3}H]-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 [^{3}H]-MVA and displayed a variety of [^{3}H]-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 [^{3}H]-MVA-labeled proteins, essentially limited to one major protein species with an apparent molecular weight of 21 (Fig. 1). Other [^{3}H]-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-p21^{ras} mAb showed that the major [^{3}H]-MVA-labeled protein was the product of the *ras* oncogene (Fig. 1).

Cells derived from a thyroid carcinoma (TK-6 cells) induced in propyl-thyouracyl-pretreated rats by the injection of a retrovirus carrying the v-K-*ras* oncogene and from lung metastases of this tumor (MPTK-6 cells) displayed a pattern of [^{3}H]-MVA-labeled proteins identical to that of KiMol cells (Fig. 1). Moreover, FRTL-5 cells transformed with a Kirsten-murine sarcoma virus variant carrying a temperature sensitive v-K-*ras* allele (Ats cells), exhibited, at the nonpermissive temperature for p21^{ras} activity (39°C), a pattern similar to FRTL-5 cells (Fig. 1). On the other hand, when Ats cells were shifted over 7 days to the permissive temperature (33°C) for the "transformed phenotype," they gradually lost [^{3}H]-MVA-labeled proteins until the 7th day, when they showed p21^{ras} as the only prenylated protein (Fig. 1). This phenomenon required just 6–8 hr from the temperature shift to be evident and was not caused by a heat-shock mechanism because FRTL-5 cells did not change their prenylation pattern at either 33 or 39°C (data not shown). Densitometric analysis of the major prenylated proteins in Ats cells in experiments was performed at different times after p21^{ras} activation, so as to characterize the kinetics of the K-*ras*-induced changes in the protein prenylation profile, and revealed a different time-dependent disappearance of prenylation for the 48- and 21- to 25-kDa proteins. In fact, the former protein, previously identified as 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNP) (22), disap-

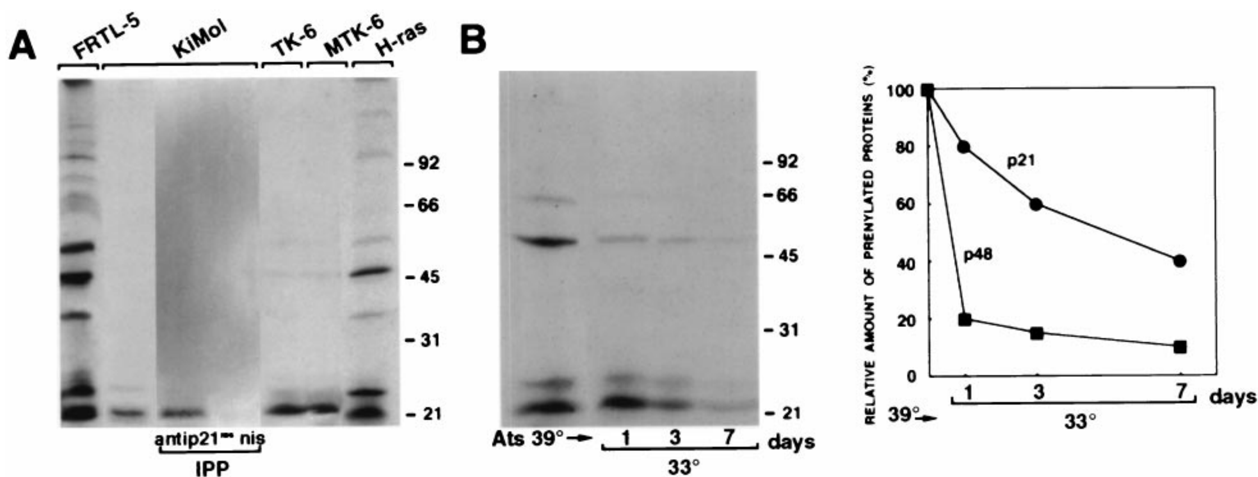

FIG. 1. Alterations in prenylation pattern are a specific marker of active K-*ras*. (*A*) The pattern of [^{3}H]-MVA-labeled proteins in FRTL-5 and KiMol cells, in cells derived from a thyroid carcinoma (TK-6) and lung metastates (MPTK-6) by the injection of v-K-*ras*, and in H-*ras*-transformed FRTL-5 cells. KiMol-labeled proteins were immunoprecipitated with an anti-p21^{ras} mAb and nonspecific immunoserum. (*B*) Electrophoretic profile (left) and densitometric analysis (right) of the major proteins (p48 and p21) in FRTL-5 cells transformed with the temperature-sensitive mutant v-K-*ras* (Ats) at 39°C and in the same cells shifted to 33°C for 1, 3, and 7 days. Cells were incubated with [^{3}H]-MVA (30 Ci/mmol) for 7 hr in the presence of 10 μM lovastatin and were processed as described in *Experimental Procedures*. Protein extracts were analyzed by 12% SDS/PAGE and fluorography. Molecular weight standards are shown on the right. These data are representative of four independent experiments. The autoradiogram was exposed for 20 days.

peared much earlier than the latters (Fig. 1*B*). The decrease of [^{3}H]-MVA-labeled proteins in transformed cells appeared to be evident, although to a lower 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. 1*A*).

The changes in protein prenylation observed in KiMol cells was not caused by reduction of protein synthesis because the levels of proteins other than p21^{ras}, 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 p21^{ras} 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-p21^{ras} mAb was found, in agreement with published data (13–16, 19).

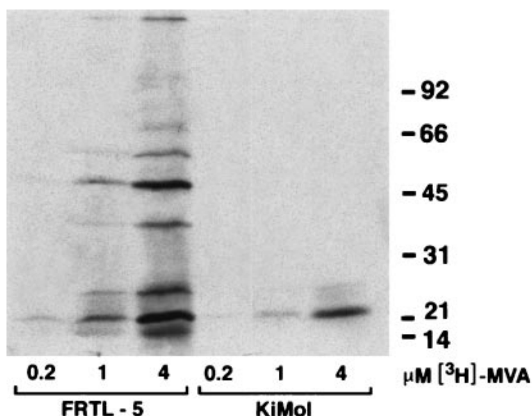

FIG. 2. Alterations in prenylation pattern of FRTL-5 and KiMol cells depending on different concentrations of exogenous [^{3}H]-MVA (0.2, 1, and 4 μM) added to cells. Cells were incubated and processed as described in the legend to Fig. 1. Molecular weight markers are shown on the right. The data presented are representative of three experiments yielding very similar results. The autoradiogram was exposed for 30 days.

The pattern of prenylated proteins observed in v-K-*ras*-transformed cells resembled that of FRTL-5 cells incubated with decreasing concentrations of exogenous [^{3}H]-MVA (Fig. 2). The two patterns became nearly identical when FRTL-5 cells were incubated in the presence of a 5-fold lower [^{3}H]-MVA concentration. On the other hand, when KiMol cells were incubated with increasing concentrations of [^{3}H]-MVA, p21^{ras} appeared more intensely prenylated but still remained the only prenylated protein (Fig. 2). FRTL-5 and KiMol cells were able to incorporate [^{3}H]-MVA at the same rate (Table 1) whereas HPLC analyses revealed a large increase (70%) of unprocessed [^{3}H]-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 [^{3}H]-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 [^{3}H]-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).

Down-Regulation of Mevalonate Kinase in K-*ras*- but not H-*ras*-Transformed FRTL-5 Cells. We investigated, in normal

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

[^{3}H]-Isoprenoid compounds	FRTL-5	KiMol
Unprocessed [^{3}H]-MVA	21.9 ± 5.1	38.1 ± 4.5
[^{3}H]-isopentenyl-tRNA	4.50 ± 2.20	1.00 ± 0.20

Proliferating FRTL-5 cells and KiMol cells were incubated with 10 μM lovastatin and 30μCi [^{3}H]-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.

Table 2. Differential prenylation in FRTL-5 and KiMol cells

Cells	[^{3}H]Isoprenoid, μM	Farnesol (%)	Geranylgeraniol/Farnesol
Mevalonate			
FRTL-5 (CHO)*	0.2	71 (83)	0.41 (0.2)
FRTL-5 (CHO)*	1	53 (50)	0.92 (1.0)
KiMol	1	69	0.40
Farnesyl-PP			
FRTL-5	1	48	1.0
KiMol	1	73	0.37

HPLC analysis of ^{3}H-MVA/Farnesyl-derived isoprenoids released from the prenylated proteins. The results are the means of three experiments.
*Data for CHO cells are from Rilling *et al.* (27).

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 Geranyl-Geranyltransferase-I Activity in K-*ras*- and H-*ras*-Transformed FRTL-5 Cells. We analyzed the activity of FTase and protein geranyl-geranyltransferase-I in differentiated and transformed FRTL-5 cells. We observed a 2- to 3-fold increase in FPTase activity and no variation in geranyl-geranyltransferase 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 geranyl-geranyltransferase I activity. We next verified K-*ras* regulation of FTase activity by incorporating, using low density lipoprotein as physiological carriers, [^{3}H]-farnesyl-PP into cellular proteins and cholesterol. Under these conditions, we observed a large increase in the incorporation of [^{3}H]-farnesyl-PP, mostly into p21^{ras} (Fig. 5*A*) 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. 5*A*) 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 [^{3}H]-MVA. Cell-free [^{3}H]-farnesyl-PP labeling experiments carried out in KiMol as well as in H-*ras* cells also showed an increased incorporation of label in p21^{ras} (Fig. 5*B*). Ats cells displayed at both temperatures (33 and 39°C) significantly increased FTase activity and [^{3}H]-farnesyl-PP incorporation, mostly into p21^{ras} when compared with FRTL-5 cells (Figs. 4 and 5*A*).

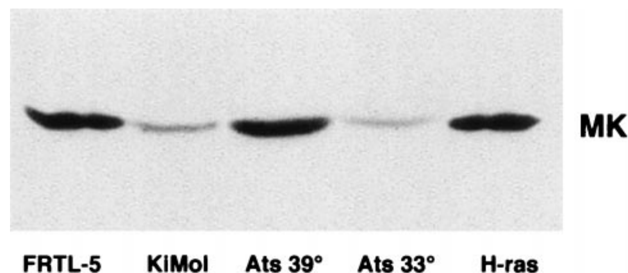

FIG. 3. Mevalonate kinase expression in FRTL-5, KiMol, Ats (39° and 33°C), and H-*ras* cells. Immunoblot analysis was performed as described in *Experimental procedures*. All lanes are from 12% SDS/PAGE and contained 90 μg of protein, transferred to cellulose nitrate and stained with antibody to MK. The data presented are representative of four experiments.

Increased Farnesyl-PP Synthase Expression in K-*ras*- and H-*ras*-Transformed FRTL5 Cells. We examined the expression of FPP synthase by analyzing its mRNA levels in differentiated and transformed FRTL-5 cells. Northern blot analysis showed that, in Ats cells incubated at 33°C and in KiMol cells, the levels of FPP synthase mRNA were 5-fold higher than those found in Ats cells grown at 39°C and in FRTL-5 cells, respectively (Fig. 6). In H-*ras* cells, an up-regulation of FPP synthase mRNA levels also was observed (Fig. 6).

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 [^{3}H]-MVA-labeled proteins was observed in K-*ras* transformed cells whereas the *ras*-oncogene product p21^{ras} 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 [^{3}H]-MVA or [^{3}H]-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

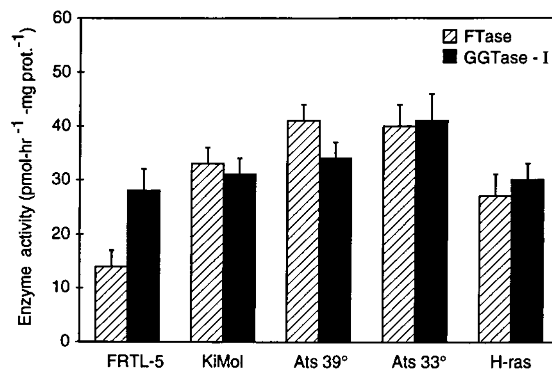

FIG. 4. FTase and protein geranyl-geranyltransferase-I activity in FRTL-5, KiMol, Ats (39 and 33°C), and H-*ras* cells. Enzyme activities were assayed by using 30 μg of cytosolic protein, by measuring, respectively, the amount of [^{3}H] farnesyl and [^{3}H] geranylgeranyl transferred from [^{3}H]-FPP and [^{3}H]-geranyl geranylpyrophosphate to recombinant H-Ras, wild-type and CVLL type cells, as described *Experimental Procedures*. Bars represent three separate experiments. Similar results also were obtained by using biotinylated peptides (Bt-KTKCVIS and Bt-KKFFCAIL) as prenyl acceptors.

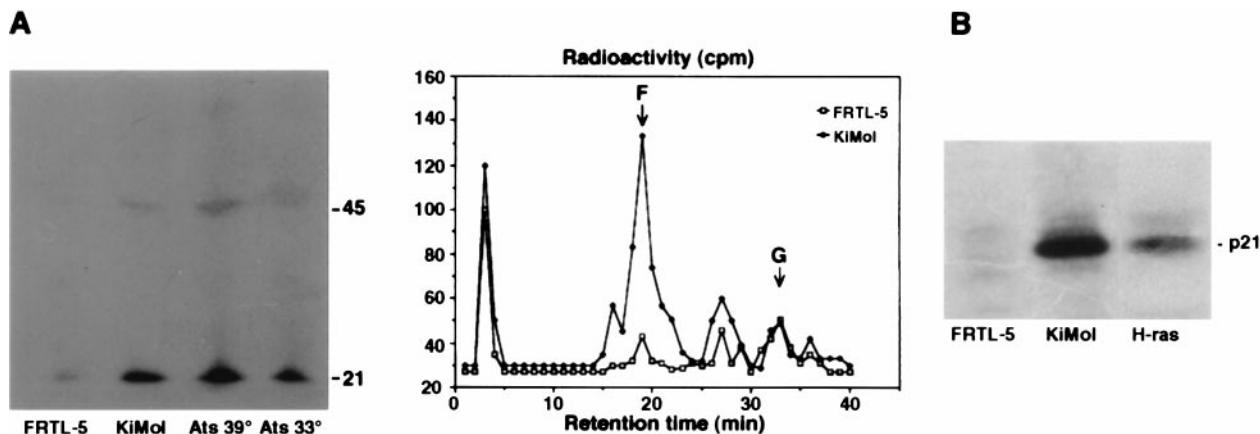

FIG. 5. Farnesylation in differentiated, K-*ras*, and H-*ras* transformed FRTL-5 cells. [^{3}H]-farnesyl-PP labeling in whole-cell and cell-free experiments carried out with FRTL-5, KiMol, Ats (39 and 33°C), and H-*ras* cells, as described in *Experimental Procedures*. (*A*) Electrophoretic profile of farnesylated proteins labeled in whole-cells labeled with [^{3}H]-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*) p21^{ras} 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.

[^{3}H]-MVA. This led to the suggestion that p21^{ras} farnesylation is less sensitive than prenylation of other proteins to reduced availability of exogenous [^{3}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 [^{3}H]-MVA (Fig. 2). We found that the geranylgeraniol/farnesol ratio in total prenylated proteins decreased with decreasing [^{3}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 [^{3}H]-MVA concentration became identical to those observed in KiMol cells (Table 2). On the other hand, an increase of [^{3}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 p21^{ras} and protein farnesylation 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 [^{3}H]-MVA, internalized at the same rate by normal and transformed FRTL-5 cells, is metabolized to a lower extent in the latter cells because of v-K-*ras*-induced down-regulation of MK, thus creating favorable conditions for selective prenylation of farnesylated proteins over geranylgeranylated proteins, in general, and of p21^{ras}, in particular (see above); (*ii*) concomitantly, to counterbalance the decreased availability of dimethylallyl-PP, the expression of FPP synthase is up-regulated, thereby shunting the available dimethylallyl-PP to the synthesis of farnesyl-PP; and (*iii*) finally, the preferential prenylation of p21^{ras} is enhanced further by increasing the activity of the enzyme responsible for its farnesylation, i.e., FTase. It is possible that this latter event is in part a direct consequence of increased expression of p21^{ras}, i.e., one of the FTase substrates, as observed in rabbit reticulocyte lysates (28). Overexpression of p21^{ras} may explain why this protein, under conditions of limited amounts of farnesyl-PP and increased FTase activity, is selected over other farnesylated proteins (e.g., 2′, 3′-cyclic nucleotide 3′-phosphodiesterase) for preferential farnesylation. Overexpression of p21^{ras}, however, it is not necessary nor is sufficient as a prerequisite to direct cells toward selective farnesylation because the latter can be obtained, albeit to a lesser extent, also in nontransformed FRTL-5 cells (by lowering [^{3}H]-MVA concentration) but not in H-ras cells nor in Ats cells at 39°C, where the synthesis of p21^{ras} also is increased. Also, up-regulation of either FPP synthase and/or FTase alone would not be sufficient to cause the change in protein prenylation observed in transformed cells. In fact, H-*ras* transformed cells and Ats cells at 39°C, where increased expression/activity, respectively, of FPP synthase and FTase or of FTase only were observed, did not exhibit preferential farnesylation of p21^{ras}. Indeed, this phenomenon is only observed in those transformed cells that exhibit impaired MK expression. Therefore, among the metabolic alterations that we observed, MK down-regulation appears to be related strictly with the alteration of protein prenylation. In summary, two types of v-K-*ras*-induced metabolic alterations contribute to direct transformed cells toward preferential farnesylation of p21^{ras}. First, the biosynthesis of all of the precursors necessary for protein prenylation must be down-regulated, so as to lead to a differential decrease of the overall protein prenylation and p21^{ras} farnesylation. *In vivo*, this is probably achieved by impairing the expression of MK as well as that of 3-hydroxy-3-methylglutaryl-CoA reductase, as described (9). Subsequently, expression/activity of FPP syn-

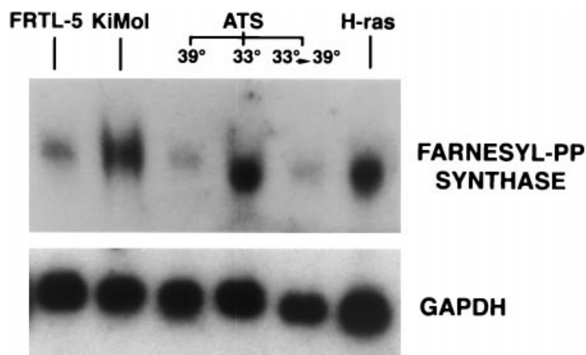

FIG. 6. FPP synthase expression in FRTL-5, KiMol, Ats (39 and 33°C), and H-*ras* cells. Northern blot analysis was performed as detailed in *Experimental Procedures*. Total RNA (20 μg) was used in each lane. (*Top*) The blot was probed with a FPP synthase cDNA. (*Bottom*) The blot was stripped and reprobed 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 p21ras 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 geranylgeranylated 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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