Post-translational Modification of Low Molecular Mass GTP-binding Proteins by Isoprenoid*

William A. Maltese†, Kathleen M. Sheridan, Ellen M. Repko, and Robert A. Erdman
From the Weis Center for Research, Geisinger Clinic, Danville, Pennsylvania 17822

Several proteins in mammalian cells are modified post-translationally by the isoprenoid, farnesol. Incubation of cultured cells with [3H]mevalonate, an isoprenoid precursor, results in the labeling of multiple polypeptides, the most prominent of which migrate in the range of 21–26 kDa on sodium dodecyl sulfate-polyacrylamide gels. In Rat-1 fibroblasts transformed by H-ras, one of the farnesylated proteins was identified as p21™ by two-dimensional immunoblotting. However, this protein accounted for only a small proportion of the [3H]mevalonate-derived radioactivity incorporated into 21–26-kDa proteins. Murine erythroleukemia cells, which did not express immunodetectable quantities of p21™, contained several 21–26-kDa farnesylated proteins distributed in both the cytosolic and particulate fractions. At least eight of these proteins were capable of binding [α-32P]GTP on nitrocellulose membranes. Pulse-chase studies showed that the isoprenoid modification did not necessarily result in the translocation of the cytosolic proteins to the cell membrane. A prominent group of carboxyl-methylated proteins in murine erythroleukemia cells overlapped with the 21–26-kDa farnesylated proteins on one-dimensional sodium dodecyl sulfate gels. Methylation of this group of proteins was selectively abolished when cells were treated with lovastatin, an inhibitor of isoprenoid synthesis. Addition of exogenous mevalonate to the lovastatin-treated cells fully restored carboxyl methylation. These studies suggest that the 21–26-kDa farnesylated proteins in mammalian cells are members of a recently discovered family of low molecular mass GTP-binding proteins which, although ras-related, appear to be distinct structurally and possibly functionally from the products of the ras genes. The observed isoprenoid-dependent carboxyl methylation of a group of 21–26-kDa proteins suggests that the low molecular mass GTP-binding proteins may undergo a series of post-translational C-terminal cysteine modifications (i.e. farnesylation, carboxyl methylation) analogous to those recently elucidated for p21™.

Mevalonic acid (MVA)† occupies a central position as a precursor in the biosynthetic pathway for cellular isoprenoid compounds such as cholesterol, dolichol, and ubiquinone (for review, see Ref. 1). It has been known for some time that depriving cells of MVA leads to arrest of cell proliferation (2–7) and, in some cases, increased expression of differentiated properties (6, 7). This occurs even when the cellular requirement for cholesterol is satisfied by exogenous lipoproteins in the culture medium, suggesting that nonsterol isoprenoid derivatives of MVA play a permissive or regulatory role in cell replication (3–7). In recent years, interest in this area has been stimulated by reports that several proteins in mammalian cells are modified covalently by isoprenoid derivatives of MVA (8–15). This novel post-translational modification has been discovered in a variety of cell types by tracing the incorporation of radiolabeled MVA into proteins separated on SDS-polyacrylamide gels.

In all of the mammalian cell lines studied to date, most of the radioactivity derived from 3H- or 14C-labeled MVA is incorporated into a cluster of proteins with molecular masses between 21 and 26 kDa. Smaller amounts of radioactivity are detected in proteins with molecular masses of 17, 45, 53, and 66–70 kDa (8, 9, 12–15). The majority of the modified proteins remain to be identified, but progress has been made in two specific cases. Isoprenylated proteins in the range of 66–70 kDa have been localized to the nuclear matrix (12, 15), and two of these proteins have been shown to react with antibodies against lamins A and B (16, 17). One of the low molecular mass farnesylated proteins has been clearly identified as p21™. Hancock et al. (18) demonstrated that the H-, K-, and N-ras™ proteins undergo isoprenoid modification at Cys16 in what appears to be the first step in a series of post-translational C-terminal processing events, i.e. isoprenylation, proteolytic removal of 3 amino acids distal to the isoprenylation site, carboxyl methylation of the exposed isoprenylated C-terminal cysteine, and palmitoylation of a cysteine residue upstream from the isoprenylated cysteine (18–20). Consistent with the isoprenoid modification of p21™, Schafer et al. (21) demonstrated that expression of the activity of an oncogenic H-c-ras protein in Xenopus oocytes could be blocked by microinjection of an inhibitor of MVA synthesis.

The exact chemical structure of the isoprenoid moiety involved in the modification of mammalian cell proteins remains to be defined. However, our recent chromatographic characterization of the isoprenoid released by sulfonium salt cleavage of low molecular mass proteins from murine erythroleukemia (MEL) cells suggests that the modifying group is farnesol, linked to cysteine via a thioether bond (22). Parallel studies focusing specifically on the isoprenoids released from immunoprecipitated p21™ (23) and the nuclear lamins (24) have provided independent evidence for a farnesyl modification of these proteins. Thus, throughout this report, the covalent modification of cellular proteins by isoprenoid derivatives of MVA will be referred to as farnesylation.

We found the recent demonstration of an isoprenoid modification of p21™ to be of particular interest in light of an

---

* This work was supported by United States Public Health Service Grant ROI CA 34569. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be sent.

‡ The abbreviations used are: MVA, mevalonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MEL, murine erythroleukemia; EGTA, [ethylenebis(oxyethylenenitrilo)] tetracetic acid.
early study in which we observed no obvious differences in the [3H]MVA labeling of 21-26 kDa proteins in H ras-transformed fibroblasts compared with nontransformed fibroblasts (12). In the present report, we demonstrate that although p21™ is indeed modified by isoprenoid in ras-transformed cells, most of the 21-26 kDa MVA-labeled proteins in mammalian cells are immunologically and electrophoretically distinct from p21™. We also provide the first direct evidence that several of these non-ras farnesylated proteins are capable of binding GTP and that methylation of proteins with the same molecular mass as the farnesylated GTP-binding proteins is blocked by an inhibitor of isoprenoid synthesis. Based on these observations, we suggest that the unidentified 21-26 kDa farnesylated proteins are members of the large family of low molecular mass GTP-binding proteins (25, 26) that may undergo C-terminal modifications similar to those described for p21™.

EXPERIMENTAL PROCEDURES

Materials—Lovastatin (mevinolin), a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (27), was a gift from Alfred Alberts of the Merck, Sharp and Dohme Research Laboratories. The lactone form of lovastatin was converted to the sodium salt as described by Kita et al. (28). (R,S)-[H-3H]Mevalonolactone was prepared by reduction of mevalonic acid precursor with sodium borohydride (Amersham Corp.) as described by Keller (29). Diabetes trinitroethylene thiocyanate (DNS) was the trinitium-labeled product was checked by thin-layer chromatography as described previously (14). The specific radioactivity of the [3H]MVA used for all of the studies in this report was 2.25 Ci/mmol. Guanidino 5′-[32P]triphosphate (approximately 3000 Ci/mmol) and L-[methyl-3H]methionine (70 Ci/mmol) were obtained from Amersham Corp. Anti-p21™ (pan) mouse monoclonal antibody (ras-11) was obtained from Du Pont New England Nuclear. The Y13 250 rat monoclonal anti-p21™ antibody was purchased from Oncogene Sciences. Tissue culture medium and fetal calf serum (GIBCO/Bethesda Research Laboratories), tissue culture flasks (Corning), X-Omat AR film (Kodak), nitrocellulose (Schleicher & Schuell), and Amplify fluorographic enhancer (Amersham Corp.) were obtained from the designated sources. Electrophoresis reagents, horseradish peroxidase-conjugated goat anti-mouse IgG (l/3000 dilution) and 4-chloro-l-napthol reagent (Bio-Rad) according to the instructions supplied by the manufacturer.

Binding of GTP to Proteins—Proteins separated by one- or two-dimensional gel electrophoresis were electroblotted onto nitrocellulose membranes as described by Towbin et al. (34) using a Bio-Rad Transblot apparatus and a transfer buffer containing 20% methanol, 192 mM glycine, and 20 mM Tris, pH 8.3. Assay of GTP binding to proteins on the blot was performed by a modification of the procedure of Papetina and Reep (35). Blots were preincubated for 90 min at 37 °C in 50 mM Tris-HCl, pH 7.5, containing 5 mM MgCl2, 1 mM EGTA, and 0.2 mM ATP. Incubation with 50 μCi of [3H]GTP was then carried out for 2 h at room temperature in the same buffer with the addition of 0.3% Tween 20. Blots were washed three times with buffer, air dried, covered with Saran Wrap, and exposed to X-Omat AR film at ~80 °C.

Assay for Protein Methylation—MEL cells were suspended at 5 × 10⁵ cells/ml in 4 ml of methionine-free Dulbecco’s medium containing 10% fetal calf serum and incubated for 3 h with 25 μM lovastatin, 25 μM lovastatin + 200 μM MVA or no addition. [methyl-3H]Methionine (20 μCi/ml medium) was then added, and the incubation was continued for 16 h. Cells were collected by centrifugation, washed three times with ice-cold phosphate-buffered saline, and homogenized in 5 volumes of buffer containing 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 0.1 μM pepstatin. The Triton X-100-soluble protein fraction, which contained all of the farnesylated proteins except the nuclear lamins, was subjected to one-dimensional SDS-PAGE as described above. After staining with Coomassie Blue, the protein bands were transferred to nitrocellulose using a Bio-Rad Transblot apparatus and a transfer buffer containing 20% methanol, 192 mM glycine, and 20 mM Tris. DNA, serum proteins, and phospholipids were removed from the membrane by washing with TBS + 0.5% Tween 20 and one wash with TBS, the blots were incubated for 2 h at room temperature with anti-p21™ antibodies (1/50 dilution of ras-11 or 1/100 dilution of Y13-259 in blocking solution). The blots were washed and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (1/3000 dilution). Detection of the bound horseradish peroxidase-IgG on washed blots was accomplished with 4-chloro-l-napthol reagent (Bio-Rad) according to the instructions supplied by the manufacturer.

RESULTS

Contribution of p21™ to the Profile of [3H]MVA-labeled Proteins in Transformed Fibroblasts and MEL Cells—In the studies depicted in Fig. 1, the subcellular distribution of [3H] MVA-labeled proteins is compared with the distribution of p21™ in two cell lines: one a rapidly growing murine erythroleukemia line shown previously to incorporate relatively large amounts of [3H]MVA into proteins (12), and the other an H-ras-transformed fibroblast cell line (Rat-6) (30). In both cell lines, most of the radioactivity was incorporated into a cluster of proteins with molecular masses between 21 and 26 kDa. However, there were notable differences in the subcellular distributions of these proteins, i.e. in the MEL cells the modified proteins were about equally distributed between the particulate fraction and the soluble fraction, whereas in the
It was shown that the immunologically detectable labeling of the p21ras protein was confined to the particulate fraction (Fig. 1, B and C). Rat-6 cells showed immunologically detectable p21^ras, and this was separated by two-dimensional electrophoresis. Western blots of the [3H]MVA-labeled proteins were subjected to fluorography, then immunoblotted with the antibody. As shown in Fig. 2, the p21^ras-immunoreactive protein accounted for a relatively small portion of the [3H]MVA-labeled proteins in the 21-26-kDa region, and it was only one of many farnesylated proteins, most of which were not recognized by the antibody and had slightly higher molecular masses than the p6 protein.

**GTP Binding to 21-26-kDa Farnesylated Proteins**—A number of GTP-binding proteins with molecular masses in the 21-26-kDa range are not recognized by the standard pan-ras antibodies (37-40). To determine whether any of the nonimmunoreactive farnesylated proteins in the MEL cells might be members of this family of proteins, we took advantage of the fact that the low molecular mass GTP-binding proteins retain their capacity to bind guanine nucleotides after being transferred from SDS gels to nitrocellulose membranes (35, 38). As shown in Fig. 3A, MEL cells contained several proteins that bound [32P]GTP in the presence of excess ATP. The two-dimensional profile of these proteins was similar to the profile of proteins labeled with [3H]MVA, prompting us to perform the overlap experiments shown in Fig. 3, B-E. Soluble and particulate MEL cell proteins labeled with [3H]MVA were separated on two-dimensional gels and blotted onto nitrocellulose. After fluorographic determination of the positions of the farnesylated proteins (Fig. 3, B and D), the Amplify enhancing reagent was washed out, and the blots were probed with [32P]GTP in the presence of excess unlabeled ATP. The [32P]-labeled blots were covered with Saran Wrap during reexposure to preclude detection of tritium. Preliminary studies comparing fluorographed with freshly run blots indicated that fluorography of the blots prior to probing with GTP caused a reduction in the GTP-binding to some of the proteins and a complete loss of binding to others. This necessitated longer exposures to visualize the bound GTP and raised the possibility that the number of GTP-binding proteins might be underestimated. Nevertheless, these disadvantages were outweighed by the ability to match [3H]MVA-labeled proteins and [32P]GTP-binding proteins precisely on the same blot. Using this approach, it appeared that at least eight of the farnesylated proteins in the MEL cell-soluble fraction and two of the proteins in the particulate fraction were capable of binding GTP (Fig. 3, C and E). The two prominent farnesylated GTP-binding proteins in the particulate fraction (Fig. 3E) closely matched two of the farnesylated GTP-binding proteins in the soluble fraction (Fig. 3C) with respect to their electrophoretic mobility, suggesting that these two proteins might be the same or closely related proteins.
Isoprenoid Modification of GTP-binding Proteins

**Fig. 2.** Two-dimensional electrophoresis and immunoblotting of low molecular mass \( [3H] \) MVA-labeled protein from H-ras-transformed Rat-6 fibroblasts. Rat-6 cells were plated in a 75-cm\(^2\) flask at a density of 13,000 cells/cm\(^2\) and grown for 24 h. Medium containing 200 \( \mu \)Ci/ml \( [3H] \) MVA and 25 \( \mu \)M lovastatin was then added, and the incubation was continued for 16 h. The particulate protein (150 \( \mu \)g) was separated by two-dimensional electrophoresis and transferred to nitrocellulose (see “Experimental Procedures”). Panel A shows the fluorograph of the blot after a 10-day exposure, with the arrow indicating the \( [3H] \) MVA-labeled protein overlapping with the \( \sim 21\text{K} \) protein detected by immunoblotting with the ras-11 (pan) antibody (panel B). The radiolabeled (panel A) or prestained (panel B) molecular mass standards shown on the right edge of the gel were run in the second dimension. The acidic end of the isoelectric focusing (IEF) gel was oriented toward the left.

**Distribution of Farnesylated Proteins between Soluble and Particulate Fractions in MEL Cells**—Farnesylated \( \sim 21\text{K} \) was detected only in the particulate fraction of transformed Rat-6 cells (see Fig. 1), presumably because the mature palmitoylated form of the protein is localized in the cell membrane (18, 19, 41). In contrast, the farnesylated 21–26-kDa proteins in MEL cells were equally distributed between the soluble and particulate fractions. To test the possibility that the soluble \( [3H] \) MVA-labeled proteins were intermediates in a series of processing steps which would ultimately lead to their insertion into the membrane, we performed a pulse-chase study depicted in Fig. 4. Cells were incubated with \( [3H] \) MVA in the presence of lovastatin to allow maximum labeling of farnesylated proteins. The labeled MVA was then removed from the medium, and the cells were incubated for 24 h without lovastatin, allowing the endogenous biosynthetic pathway to generate MVA for continued cell growth. When the electrophoretic profiles of the labeled proteins in the soluble and particulate fractions were examined at intervals after removing \( [3H] \) MVA from the medium, there was no evidence that the farnesylated proteins present in the cytosol at the end of the pulse-labeling period were translocated to the membrane compartment during the chase period. Instead, there was little change in the distribution of radiolabeled proteins between the soluble and particulate fractions. By 24 h we observed a uniform diminution of radioactivity in the proteins in both subcellular compartments as cell growth proceeded in the absence of labeled MVA. In view of our previous finding that the farnesylated proteins in MEL cells are relatively stable (14), we attribute the decrease in radioactivity to dilution of the labeled farnesylated proteins as cell growth proceeds.

**Fig. 3.** GTP binding to low molecular mass proteins labeled with \( [3H] \) MVA in MEL cells. Each panel shows a section of a nitrocellulose blot of MEL cell proteins separated by two-dimensional electrophoresis. Only the region of the gel between 15 and 30 kDa is shown, with the acidic end of the isoelectric focusing (IEF) gel oriented to the left. Panel A shows 100 \( \mu \)g of soluble protein from unlabeled MEL cells, probed with \( [\alpha-\text{\textsuperscript{32}}P] \) GTP immediately after transfer to nitrocellulose. The blot was exposed for 12 h. Panels B–E show the results of an overlap experiment in which \( [3H] \) MVA-labeled proteins were matched with \( [\alpha-\text{\textsuperscript{32}}P] \) GTP-binding proteins in the soluble and particulate fractions prepared from MEL cells. MEL cells were incubated with \( [3H] \) MVA as described in the legend to Fig. 1, and the soluble and particulate fractions were prepared as described under “Experimental Procedures.” Panel B shows the \( \text{H} \) profile of 150 \( \mu \)g of soluble protein after a 10-day fluorographic exposure of the nitrocellulose blot. Panel C shows the same blot after washout of the fluorography reagent and incubation with \( [\alpha-\text{\textsuperscript{32}}P] \) GTP. Bound GTP was detected after a 4-day exposure of the blots. Panels D and E show the same experiment performed with 150 \( \mu \)g of protein from the particulate fraction of the \( [3H] \) MVA-labeled MEL cells. The arrows indicate proteins that were labeled with \( [3H] \) MVA and also bound \( [\alpha-\text{\textsuperscript{32}}P] \) GTP. Similar results were obtained in a separate experiment in which the combined Triton X-100-soluble proteins from MEL cells were run on two-dimensional gels.
Isoprenoid Modification of GTP-binding Proteins

**FIG. 4.** Electrophoretic profiles of farnesylated proteins in MEL cells determined at intervals after removal of \[^{3}H\]MVA from the culture medium. A 50-ml suspension culture of MEL cells was initiated at a density of 200,000 cells/ml and grown for 24 h. The cells were concentrated to 5 x 10⁶ cells/ml, and farnesylated proteins were labeled by incubating the cells with \[^{3}H\]MVA (200 μCi/ml medium) and 25 μM lovastatin for 18 h. At the end of the labeling period, the cells were collected by centrifugation, washed with fresh medium, and suspended in 200 ml of medium without labeled MVA or lovastatin. 50-ml aliquots of cell suspension were collected at 0, 2, and 6 h after removal of labeled MVA from the medium. 18 ml of cell suspension was removed at 24 h. Soluble (S) and particulate (P) fractions were prepared from the cells as described under “Experimental Procedures,” and the total protein in each fraction was subjected to SDS-PAGE and fluorography. The actual amounts of protein loaded on each lane were as follows: 0 h (P), 230 μg; 0 h (S), 100 μg; 2 h (P), 200 μg; 2 h (S), 120 μg; 6 h (P), 260 μg; 6 h (S), 130 μg; 24 h (P), 320 μg; 24 h (S), 230 μg. All lanes shown in the figure were exposed for 4 days.

*Isoprenoid-dependent Methylation of 21-26-kDa Proteins—* As mentioned earlier, newly synthesized p21\(^{ras}\) (pro-p21) is rapidly farnesylated at Cys\(^{185}\), triggering proteolytic removal of three C-terminal amino acids and carboxyl methylation of the exposed C-terminal cysteine (18, 19). With this sequence of events in mind, we considered the possibility that the non-ras GTP-binding proteins also might undergo carboxyl methylation in conjunction with their farnesylation. The gel slice profiles in Fig. 5 show that a prominent group of methylated proteins overlapped with the 21-26-kDa \[^{3}H\]MVA-labeled proteins in MEL cells (compare panels A and B). This in itself was not particularly informative, since the methylation assay detects O-methyl derivatives of aspartate and glutamate (36) as well as the newly discovered cysteine carboxyl methylation (20). However, upon addition of lovastatin, an inhibitor of MVA synthesis which blocks the formation of cellular isoprenoids, the methylation of the 21-26-kDa proteins was selectively diminished, whereas methylation of other proteins in the gels was not noticeably affected (Fig. 5C). To confirm that the decreased methylation of these proteins was due specifically to MVA deprivation, we added exogenous MVA to the cultures along with lovastatin and were able to restore methylation of the 21-26-kDa proteins (Fig. 5D). Because the proteins were separated by one-dimensional SDS-PAGE, it is not possible to state definitively that the methylated proteins in the 21-26-kDa region were the same proteins that were labeled with \[^{3}H\]MVA. However, the sensitivity of the methylation of this specific group of proteins to lovastatin strongly supports the notion that their methylation was dependent on prior or concurrent isoprenoid modification.

**DISCUSSION**

Since Schmidt et al. (8) first described the covalent modification of 3T3 cell proteins by unidentified isoprenoids derived from MVA, reports from several laboratories have established that this novel type of post-translational modification is ubiquitous in mammalian cells (9-15). Although it has been known for some time that yeast peptide-mating factors undergo isoprenoid modification and post-translational processing at their C termini (i.e. farnesylation of cysteine, proteolytic removal of 3 distal amino acids, and carboxyl methylation of the farnesylated cysteine) (44-48), this fact initially received little attention because of the apparent lack of structural or functional relationships between these peptides and the isoprenylated proteins in mammalian cells. However, recent reports that p21\(^{ras}\) undergoes isoprenoid modification at Cys\(^{185}\) (18, 23), C-terminal proteolytic processing (18, 19), and carboxyl methylation (18, 20) have suggested that a homologous C-terminal amino acid motif in the yeast-mating peptides and p21\(^{ras}\) (i.e. Cys-A-A-X where A is an amino acid with an aliphatic side chain and X is any amino acid), may represent a consensus sequence for farnesylation. Two additional observations tend to support this hypothesis. The first is the finding that the modifying isoprenoid released from mammalian proteins by thioether cleavage does indeed behave as farnesol or its rearrangement products in several chromatography systems (22-24), and the second is the observation that the nuclear lamins, which undergo isoprenoid modification (16, 17, 24) and carboxyl methylation (49, 50), apparently share the Cys-A-A-X C-terminal motif (51-53).

The present study shows that MEL cells, which do not synthesize immunodetectable p21\(^{ras}\), incorporate \[^{3}H\]MVA...
Triton X-100-soluble proteins (100 μg/lane) were subjected to SDS- or [methyl-3H]methionine (see "Experimental Procedures"), and the on carboxyl methylation of low molecular mass proteins in MEL cells. Cells were labeled with [3H]MVA (see legend for Fig. 1) and the methylation assays were performed on duplicate lanes of the gel, indicated by protein standards.

Lovastatin, with exogenous MVA (200 μM) added to the medium during preincubation and labeling. For each condition, the methylation was performed on duplicate lanes of the gel, indicated by the solid and dashed lines in panels B–D. The molecular mass markers shown at the top of the figure are based on the mobility of prestained protein standards.

**Fig. 5.** Effects of an inhibitor of MVA synthesis (lovastatin) on carboxyl methylation of low molecular mass proteins in MEL cells. Cells were labeled with [3H]MVA (see legend for Fig. 1) or [methyl-3H]methionine (see "Experimental Procedures"), and the Triton X-100-soluble proteins (100 μg/lane) were subjected to SDS-PAGE. Panel A shows the typical profile of proteins labeled with [3H]MVA, obtained by counting 2-mm gel slices. Panels B–D show the profiles of carboxyl methylated proteins, determined by assaying [3H]methanediol released from gel slices containing proteins labeled with [methyl-3H]methionine (see "Experimental Procedures"). Panel B, control (no addition to medium). Panel C, MVA synthesis blocked by addition of 25 μM lovastatin during preincubation (1 h) and labeling (16 h). Panel D, MVA synthesis blocked by the addition of 25 μM lovastatin, with exogenous MVA (200 μM) added to the medium during preincubation and labeling. For each condition, the methylation assays were performed on duplicate lanes of the gel, indicated by the solid and dashed lines in panels B–D. The molecular mass markers shown at the top of the figure are based on the mobility of prestained protein standards.

Isoprenoid Modification of GTP-binding Proteins

2153

into 21–26-kDa proteins to an extent comparable to that seen in H-ras-transformed fibroblasts. In both the MEL cells and the ras-transformed fibroblasts, most of the radioactivity derived from [3H]MVA was incorporated into nonimmunoreactive proteins with slightly higher molecular masses (23–26 kDa) than the immunoreactive p21<sup>ras</sup>. Several of these farnesylated proteins in the MEL cells were able to bind GTP when transferred to nitrocellulose membranes, a feature that is characteristic of a growing number of non-ras guanine nucleotide-binding proteins identified recently in mammalian cells. Although the physiological functions of these proteins have yet to be defined, they generally exhibit some degree of homology with p21<sup>ras</sup> and have hence been termed “ras-related” (25, 26). Using the information available in the literature, it is possible to separate these proteins into three general groups based on their C-terminal amino acid sequences and their sizes. The first group consists of 21-kDa proteins such as rho (40, 54, 55), smg-p21 (56), ral (57), and rap1 (58), which have a relatively high degree of homology to the H-, K-, and N-ras proteins and share the C-terminal Cys-A-A-X motif. The second group consists of 23–24-kDa proteins such as ypt1 (59), rab1, and rab2 (60), which are more closely related to the yeast YPT1 and SEC4 proteins than to p21<sup>ras</sup>, and have C-terminal sequences ending with 2 consecutive cysteine residues. The third group includes the smg25 proteins (61) and the rab3 and rab4 proteins (60, 62), which have molecular masses of approximately 25 kDa and a C-terminal motif of Cys-X-Cys. In addition to the proteins for which sequence data are available, a number of poorly characterized 24–26-kDa guanine nucleotide-binding proteins have been isolated from diverse tissues such as human placenta and platelets (63), bovine brain (64), human leukemia cells (65), and 3T3 fibroblast membranes (39). Based on their molecular masses, most of the farnesylated GTP-binding proteins in the MEL cells correspond more closely to proteins such as ypt1, rab, and smg25 than to the ras-like 21-kDa proteins rho, ral, and smg21. Thus, our findings raise the possibility that classes of GTP binding proteins with C-terminal sequences ending in Cys-X-Cys, instead of Cys-A-A-X, may also undergo post-translational modification by isoprenoid.

Studies of mammalian and yeast ras proteins have suggested that they are methylated by a novel carboxyl methyltransferase activity that is specific for C-terminal cysteine (20, 21). The observation that a Cys→Ser<sup>196</sup> point mutation prevents carboxyl methylation of p21<sup>ras</sup> (18) is consistent with a model in which methylation of Cys<sup>196</sup> is contingent upon its isoprenoid modification and the subsequent proteolytic removal of 3 distal amino acids. Although it is not known whether other low molecular mass GTP-binding proteins undergo C-terminal cysteine carboxyl methylation, two recent observations suggest that this may occur. First is the demonstration of this type of methylation in a group of 23–29-kDa proteins from retinal rod outer segment membranes (43), and second is the finding that in vitro methylation of non-ras 20–23-kDa macrophage membrane proteins is stimulated by GTP (66). In the present report we provide the first direct evidence that carboxyl methylation of 21–26-kDa proteins in cultured mammalian cells is selectively abolished by an inhibitor of isoprenoid synthesis and restored by provision of the isoprenoid precursor, MVA. Thus, it is reasonable to speculate that the non-ras low molecular mass GTP-binding proteins undergo C-terminal modification similar to those described for p21<sup>ras</sup> and that farnesylation of cysteine residues at or near the C-terminal is a prerequisite for their methylation.

The pulse-chase experiment described in this report (Fig. 4) indicates that the patterns of [3H]MVA-labeled proteins in
the soluble and particulate fractions from MEL cells do not change with time. There are several possible interpretations of these observations. For example, the proteins found in the soluble and particulate fractions may be structurally unique so that in some cases farnesylation confers sufficient hydrophobicity for stable membrane localization, whereas in other cases it does not. Alternatively, the soluble and particulate proteins may be closely related or identical translation products that are in a state of dynamic equilibrium between the two subcellular compartments, possibly determined by an additional post-translational modification such as fatty acylation. Although further studies are needed to distinguish between these models, the present studies would seem to rule out the concept that farnesylation necessarily targets all low molecular mass GTP-binding proteins to the cell membrane.

The biochemical basis for the well-documented arrest of cell cycling which occurs when cells are deprived of MVA remains to be firmly established. The fact that growth arrest cannot be reversed by providing exogenous sterols (3–7) has led to a search for nonsterol isoprenoids that may be required for cell replication. Initially it was thought that isopentenyladenine, a precursor for isopentenyl tRNA, might be a key isoprenoid involved in growth regulation (6), but subsequent studies failed to support this hypothesis (9, 12, 68, 69). The intracellular supply of ubiquinone (coenzyme Q), an isoprenoid carrier of reducing equivalents in the mitochondrial electron transport chain (70), also does not appear to be critical, since cells can tolerate a 50% reduction in the mitochondrial ubiquinone pool with little effect on respiratory function (71). Moreover, supplementing cells with ubiquinone does not reverse the arrest of growth induced by inhibiting MVA synthesis (12), despite the fact that exogenous ubiquinone is readily taken up by mitochondria in cultured cells (72). Similar studies have shown that supplementing cells with dolichol cannot reverse the arrest of cell cycling in MVA-depleted cells (12, 73). However, because of uncertainties regarding the extent to which free dolichol may be converted to the phosphate ester, the depletion of intracellular dolichyl phosphate and consequent perturbation of N-linked protein glycosylation still must be considered as a viable mechanism that could lead to growth inhibition.

The discovery that multiple proteins in mammalian cells undergo covalent post-translational modification by MVA-derived farnesyl groups has placed the MVA requirement for cell cycling in an entirely new perspective. Soon after the potential connection between protein isoprenylation and cell growth was strengthened by our recent finding that this modification is tightly coupled to MVA synthesis and isoprenoid supply, i.e. if MVA synthesis is blocked, the pool of isoprenoid (farnesyl) groups available for protein modification is immediately depleted, leading to a rapid accumulation of nonmodified proteins (14). The nuclear lamin proteins are thought to play important roles in the organization of the nuclear envelope (74), and it is therefore conceivable that blocking their isoprenoid modification (16, 17) could disrupt their normal interactions with components of the nuclear membrane and scaffold. At the same time, prevention of the farnesylation of ras proteins or other GTP-binding proteins could impinge on cellular responses to growth factors, signal transduction, or intracellular transport functions in which these proteins have been postulated to play a role (25, 26, 75–77). Thus, decreased MVA synthesis may ultimately affect cell cycling by interfering with a multitude of protein-mediated events that are presently difficult to differentiate from one another. With this in mind, it would seem that the most productive approach to understanding the complex interrelationships between isoprenoid synthesis and cell growth must involve the delineation of the structures and functions of the individual farnesylated proteins and the determination of how the modification of these proteins affects their normal activity. The findings reported herein represent a starting point for such studies insofar as they demonstrate that post-translational modification by isoprenoid has broad relevance for a class of low molecular mass GTP-binding proteins distinct from p21ras.

REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1980) J. Lipid Res. 21, 505–517
2. Konecko, I., Hazama-Shimada, Y., and Endo, A. (1978) Eur. J. Biochem. 87, 313–321
3. Quesney-Huneves, V., Wiley, M. H., and Siperstein, M. D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5056–5060
4. Hallett, A. J. R., Glomset, J. A., and Ross, R. (1980) J. Biol. Chem. 255, 5134–5140
5. Fairbanks, K. F., Witte, L. D., and Goodman, D. S. (1984) J. Biol. Chem. 259, 1546–1551
6. Maltese, W. A., and Sheridan, K. M. (1985) J. Cell. Physiol. 125, 540–556
7. Langan, T. J., and Voipe, J. J. (1987) J. Neurochem. 49, 513–521
8. Schmidt, R. A., Schneider, C. J., and Glomset, J. (1984) J. Biol. Chem. 259, 10175–10180
9. Sinensky, M., and Logel, J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3257–3261
10. Bruegger, E., and Rilling, H. C. (1986) Biochem. Biophys. Res. Commun. 139, 209–214
11. Faust, J., and Krieger, M. (1987) J. Biol. Chem. 262, 1996–2004
12. Maltese, W. A., and Sheridan, K. M. (1987) J. Cell. Physiol. 133, 471–491
13. Maltese, W. A., and Sheridan, K. M. (1988) J. Biol. Chem. 263, 10104–10110
14. Schmidt, R. A., and Maltese, W. A. (1989) J. Biol. Chem. 264, 9945–9952
15. Sepp-Lorenzino, L., Azrolan, N., and Coleman, P. S. (1989) FEBS Lett. 245, 110–116
16. Wolda, S. L., and Glomset, J. A. (1988) J. Biol. Chem. 263, 5997–6000
17. Beck, L. A., Hosick, T. J., and Sinensky, M. (1988) J. Cell. Biol. 107, 1507–1516
18. Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989) Cell 57, 1167–1177
19. Gutierrez, L., Magee, A. I., Marshall, C. J., and Hancock, J. F. (1989) EMBO J. 8, 1093–1098
20. Clarke, S., Vogel, J. P., Deschenes, R. J., and Stock, J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4643–4647
21. Schafar, W. R., Kim, R., Sterne, R. Thorner, J., Kim, S. H., and Rine, J. (1989) Science 245, 379–385
22. Maltese, W. A., and Erdman, R. A. (1989) J. Biol. Chem. 264, 18172–18176
23. Casey, P. J., Solski, P. A., Der, C. J., and Buss, J. E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8323–8327
24. Farnsworth, C. C., Wolda, S. L., Gelb, M. H., and Glomset, J. A. (1989) J. Biol. Chem. 264, 20422–20429
25. Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827
26. Darnell, E., and Nevedal, A. R. (1988) PASJ 0, 2151–2159
27. Alberts, A. W., Chen, J., Kurok, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapeley, E., Albers-Schonberg, G., Hensens, O., Hirschfield, J., Hoogsteen, K., Liesch, J., and...
Springer, J. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3957–3961
28. Kita, T., Brown, M. S., and Goldstein, J. L. (1980) J. Clin. Invest. 66, 1094–1100
29. Keller, R. K. (1986) J. Biol. Chem. 261, 12053–12059
30. Hsiao, W.-L., Wu, T., and Weinstein, I. B. (1986) Mol. Cell. Biol. 6, 7943–1950
31. Laemmli, U. K. (1970) Nature 227, 680–685
32. Garrels, J. J. (1979) J. Biol. Chem. 254, 7961–7977
33. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
34. Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
35. Lapetina, E. G., and Reep, B. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2261–2265
36. Stock, J. B., Clarke, S., and Koshland, D. E. (1984) Methods Enzymol. 106, 310–321
37. Kahn, R. A., Goddard, C., and Newkirk, M. (1988) J. Biol. Chem. 263, 8282–8287
38. Deschenes, R. J., Stimmel, J. B., Clark, S., Stahl, P. D., and Broach, J. R. (1989) J. Biol. Chem. 264, 11865–11873
39. Ota, I. M., and Clarke, S. (1989) J. Biol. Chem. 264, 12394–12401
40. Avraham, H., and Weinberg, R. A. (1989) Mol. Cell. Biol. 9, 2058–2066
41. Willumsen, B. M., Christensen, A., Hubbert, N. L., Papageorge, A. G., and Lowry, D. R. (1984) Nature 310, 583–586
42. Deschenes, R. J., Stimmel, J. B., Clarke, S., Stock, J., and Broach, J. R. (1989) J. Biol. Chem. 264, 11865–11873
43. Ota, I. M., and Clarke, S. (1989) J. Biol. Chem. 264, 12879–12884
44. Kamiya, Y., Sakurai, A., Tamura, S., Takahashi, N., Tsuchiya, E., Abe, K., and Fukui, S. (1979) Agric. Biol. Chem. 43, 363–369
45. Sakagami, Y., Yoshida, M., Isogai, A., and Suzuki, A. (1981) Science 212, 1529–1537
46. Ishibashi, Y., Sakagami, Y., Isogai, A., and Suzuki, A. (1984) Biochemistry 23, 1399–1404
47. Anderegg, R. J., Betz, R., Carr, S. A., Crabb, J. W., and Duntze, W. (1988) J. Biol. Chem. 263, 18236–18240
48. Akada, R., Minomi, K., Kai, J., Yamashita, I., Mivakawa, T., and Fukui, S. (1989) Mol. Cell. Biol. 9, 3491–3498
49. Chelsky, D., Olson, J. F., and Koshland, D. E., Jr. (1987) J. Biol. Chem. 262, 4303–4309
50. Chelsky, D., Sabotka, C., and O’Neill, C. L. (1989) J. Biol. Chem. 264, 7637–7643
51. Fisher, D. Z., Chaudhary, N., and Blobel, G. (1990) Proc. Natl. Acad. Sci. U. S. A. 83, 6450–6454
52. Krohne, G., Wolin, S. L., McKeon, F. D., Franke, W. W., and Kirschner, M. W. (1987) EMBO J. 6, 3801–3808
53. Kitten, G. T., Vorburger, K., and Nigg, E. A. (1989) J. Cell Biol. 109, 132 (abstr.)
54. Mariani, P., and Axel, R. (1985) Cell 41, 31–40
55. Yamamoto, K., Kondo, J., Hishida, T., Teranishi, Y., and Takai, Y. (1988) J. Biol. Chem. 263, 9226–9232
56. Kawata, M., Matsui, Y., Kondo, J., Hishida, T., Teranishi, Y., and Takai, Y. (1988) J. Biol. Chem. 263, 18965–18971
57. Chardin, P., and Tavitian, A. (1986) EMBO J. 5, 2203–2208
58. Pizon, V., Chardin, P., Lerosey, I., Olofsson, B., and Tavitian, A. (1988) Oncogene 5, 201–204
59. Hauksson, H., Disela, C., Wagner, P., and Gallwitz, D. (1987) EMBO J. 6, 4049–4055
60. Touchot, N., Chardin, P., and Tavitian, A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8210–8214
61. Matsui, Y., Kikuchi, A., Kondo, J., Hishida, T., Teranishi, Y., and Takai, Y. (1988) J. Biol. Chem. 263, 11071–11074
62. Zabraoui, A., Touchot, N., Chardin, P., and Tavitian, A. (1989) J. Biol. Chem. 264, 12394–12401
63. Evans, T., Brown, M. L., Fraser, E. D., and Northup, J. K. (1986) J. Biol. Chem. 261, 7052–7059
64. Waldo, G. L., Evans, T., Fraser, E. D., Northup, J. K., Martin, M. W., and Harden, T. K. (1987) Biochem. J. 246, 431–439
65. Uning, R. J., Polakis, P. G., and Snyderman, R. (1987) J. Biol. Chem. 262, 15575–15579
66. Backlund, P. S., Jr., and Akssan, R. M. (1986) J. Biol. Chem. 263, 15864–15867
67. Questney-Honeeus, V., Wiley, M. H., and Siperstein, M. D. (1980) J. Biol. Chem. 255, 1943–1950
68. Perkins, S. L., Ledin, S. F., and Stubbs, J. D. (1982) Biochim. Biophys. Acta 137, 81–89
69. Adair, W. L., Jr., and Brennan, S. L. (1986) Biochem. Biophys. Res. Commun. 137, 208–214
70. Leuz, G., DeSantis, A., and Bertoli, E. (1985) in Coenzyme Q: Biochemistry, Bioenergetics and Clinical Application of Ubiqui-

tone (Lenaz, G., ed) pp. 165–199, John Wiley & Sons, New York
71. Maltese, W. A., and Aprilre, J. R. (1985) J. Biol. Chem. 260, 11524–11529
72. Maltese, W. A., Aprilre, J. R., and Green, R. A. (1987) Biochem. J. 246, 441–447
73. Doyle, J. W., and Kandutsch, A. A. (1988) J. Cell. Physiol. 137, 133–140
74. Gerace, L., and Burke, B. (1988) Annu. Rev. Cell Biol. 4, 335–374
75. Segev, N., Mulholland, J., and Botstein, D. (1989) Cell 52, 915–924
76. Mayorga, L. S., Diaz, R., and Stahl, P. D. (1989) Science 244, 1475–1477
77. Balch, W. E. (1989) J. Biol. Chem. 264, 16965–16968
Post-translational modification of low molecular mass GTP-binding proteins by isoprenoid.

W A Maltese, K M Sheridan, E M Repko and R A Erdman

*J. Biol. Chem.* 1990, 265:2148-2155.

Access the most updated version of this article at [http://www.jbc.org/content/265/4/2148](http://www.jbc.org/content/265/4/2148)

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/265/4/2148.full.html#ref-list-1](http://www.jbc.org/content/265/4/2148.full.html#ref-list-1)