Isoprenoid Modification of G25K (Gp), a Low Molecular Mass GTP-binding Protein Distinct from p21\textsuperscript{ras}

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Cultured murine erythroleukemia (MEL) cells synthesize a number of low molecular mass GTP-binding proteins that undergo post-translational modification by isoprenoids. We used two-dimensional electrophoresis and immunoblotting to show that a 23−24-kDa protein labeled by the isoprenoid precursor \[^3H\]mevalonate was specifically recognized by an antibody to G25K (Gp), a low molecular mass GTP-binding protein originally purified from placental, platelet, and brain membranes. Several isoelcetric variants of G25K were detected in MEL cells, and all were radiolabeled with \[^3H\]mevalonate. The G25K-immunoreactive protein did not cross-react with pan-ras antibody. Although mature p21\textsuperscript{ras} is known to be localized in the cell membrane, most of the isoprenylated G25K was found in the 100,000 \times g supernatant fraction when cells were lysed in buffer without detergent. Blocking isoprenoid synthesis by incubation of MEL cells with lovastatin resulted in a decrease in the concentration of G25K in the particulate fraction and a corresponding increase in immunodetectable protein in the soluble fraction. Lovastatin treatment also produced shifts in the electrophoretic mobilities of the G25K isoforms on two-dimensional gels. These observations are consistent with the idea that isoprenylation plays a permissive role in the association of G25K with the cell membrane. Several isoelectric variants of G25K were isolated from cultured MEL cells and fibroblasts containing several low molecular mass (20−30 kDa) GTP-binding proteins in addition to H-, K-, and N-p21\textsuperscript{ras}, e.g. rho (25−27), ypt1/rab1 (28, 29), rab 2,3,4 (29, 30), smg21 (rap1) (31, 32), rul (33, 34), and rac 1,2 (34, 35). Although these proteins are structurally distinct from each other and from p21\textsuperscript{ras}, their sequences all show some degree of homology with that of p21\textsuperscript{ras}, particularly in the regions contributing to the GTP-binding site. We reported recently that cultured MEL cells and fibroblasts contain several low molecular mass GTP-binding proteins that are not recognized by antibodies to p21\textsuperscript{ras} but nevertheless undergo isoprenylation (36). Although some of these may be novel gene products, it seemed likely that others might be previously identified GTP-binding proteins. Among the low molecular mass GTP-binding proteins described in the literature, one particular protein named G25K (formerly Gp) attracted our interest because of its seemingly ubiquitous distribution in normal tissues and a variety of cultured cell lines (37−39).

Mammalian cells contain many low molecular mass (20−30 kDa) GTP-binding proteins in addition to H-, K-, and N-p21\textsuperscript{ras}. Mevalonic acid (MVA) serves as a precursor for the biosynthesis of a variety of isoprenoid products including cholesterol, dolichols, the side chain of ubiquinone (coenzyme Q), and the farnesyl moiety of heme a (1−3). In 1984, Schmidt et al. (4) demonstrated that cultured mammalian cells were able to incorporate isoprenoid products derived from MVA into a discrete set of proteins, suggesting the existence of a new type of post-translational modification. Subsequent studies have established that isoprenylation of proteins occurs in a wide variety of cell types (5−9) and that the modified proteins fall into several categories with respect to their electrophoretic mobilities on SDS gels and their subcellular localizations (7, 9). Most recently, progress has been made in characterizing the modifying isoprenoid structures as being either farnesyl or diterpene groups, linked to the proteins via cysteine thioether bonds (10−13).

With only a few exceptions, the identities of the isoprenylated proteins remain to be determined. Two of the isoprenylated proteins that are localized in the nuclear matrix and which migrate between 66 and 72 kDa have been identified as lamin B (11, 14−16) and prelamin A (15−17). In the latter case, isoprenylation represents a key step in the proteolytic processing of prelamin A to mature lamin A (17). Several recent studies have shown that p21\textsuperscript{ras} also undergoes isoprenylation in what appears to be the first step in a series of post-translational processing events that occur at the C terminus of the protein (18−21). One of the few structural features common to both the laminins and p21\textsuperscript{ras} is the presence of a C-terminal amino acid sequence consisting of cysteine followed by 3 additional amino acids. Since the first 2 amino acids distal to the cysteine generally have aliphatic or hydroxy side chains, this amino acid sequence is frequently referred to as a Cys-A-A-X motif (where A is an aliphatic residue and X is any amino acid). The Cys-A-A-X sequence is found in several fungal mating factors that undergo farnesylation at C-terminal cysteine residues (22−24), and studies using mutational analysis have established that this is indeed the site of isoprenoid modification in mammalian p21\textsuperscript{ras} (18) and avian nuclear lamin (16).

Mammalian cells contain many low molecular mass (20−30 kDa) GTP-binding proteins in addition to H-, K-, and N-p21\textsuperscript{ras}, e.g. rho (25−27), ypt1/rab1 (28, 29), rab 2,3,4 (29, 30), smg21 (rap1) (31, 32), rul (33, 34), and rac 1,2 (34, 35). Although these proteins are structurally distinct from each other and from p21\textsuperscript{ras}, their sequences all show some degree of homology with that of p21\textsuperscript{ras}, particularly in the regions contributing to the GTP-binding site. We reported recently that cultured MEL cells and fibroblasts contain several low molecular mass GTP-binding proteins that are not recognized by antibodies to p21\textsuperscript{ras} but nevertheless undergo isoprenylation (36). Although some of these may be novel gene products, it seemed likely that others might be previously identified GTP-binding proteins. Among the low molecular mass GTP-binding proteins described in the literature, one particular protein named G25K (formerly Gp) attracted our interest because of its seemingly ubiquitous distribution in normal tissues and a variety of cultured cell lines (37−39).

We now demonstrate that proteins identified as G25K (Gp) by two-dimensional immunoblotting are modified by isoprenoid in cultured MEL cells. In contrast to the mature form of p21\textsuperscript{ras}, which is localized in the cell membrane, a substantial proportion of isoprenylated G25K is concentrated in the soluble fraction. This finding implies that although isopren-

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ylation may promote membrane association of p21P, the stability of this association may vary greatly in the case of other related GTP-binding proteins.

**EXPERIMENTAL PROCEDURES**

**Materials** 8 S [3H]Mevalonolactone was prepared by reduction of mevalonic acid precursor (Sigma) with sodium boro-[3H]hydride (Amersham Corp.) as described by Keller (40). The identity and purity of the tritiated product were confirmed by thin-layer chromatography (41). The specific radioactivity of the [3H]MVA used in the present study was 3.68 Ci/mmol. [3H]-labeled goat anti-rabbit IgG (1 μg/ml) and [3H]-labeled goat anti-mouse IgG (1 μg/ml) were purchased from Dupont-New England Nuclear. Lovastatin (mevastatin) was a gift from Dr. Alberts of the Merck, Sharp and Dohme Research Laboratories (Rahway, N.J.). The lactone form of lovastatin was converted to the sodium salt as described by Kita et al. (43). Affinity-purified antipeptide antibodies to G25K were provided by Alfred Alberts of the Merck, Sharp and Dohme Research Laboratories for 90 min at 4°C. The 100,000 g super- nant solution was obtained after centrifugation at 100,000 × g for 10 min.

**Cell Cultures**—Murine erythroleukemia (MEL) cells (line 745) were originally obtained from Dr. Charlotte Friend, M. Sinai School of Medicine, New York. Lovastatin-resistant MEL cells (LR-MEL) were adapted to grow in the presence of 25 μM lovastatin by sequential passage in the usual medium (see below) containing increasing concentrations of the drug. Normal and C-Ha-ras transformed rat embryo fibroblasts (Rat-6) (44) were provided by Drs. Wendy Hsiao and I. Bernard Weinstein, Columbia University Cancer Center, New York. Simian COS-M6 cells were obtained from Dr. David Russell, University of Texas Southwestern Medical Center, Dallas. HL-60 cells were obtained from the American Type Culture Collection, Rockville, MD. MEL and HL-60 cells were maintained in suspension cultures, and other cell lines were grown as monolayers. HL-60 cells were grown in RPMI medium containing 20% (v/v) fetal calf serum. All other cell lines were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum as described previously (7).

**Metabolic Labeling and Subcellular Fractionation**—Isoprenylated proteins were labeled by incubating cells in medium containing [3H]MVA (200 μCi/ml) and lovastatin (25 μM) for various times as indicated in the figure legends. Cells were harvested and washed three times with cold phosphate-buffered saline. For analysis of total cell protein, the washed cell pellets were dissolved immediately in electrophoresis sample buffer (see below). For studies of the distribution of proteins between the soluble and particulate fractions, cells were homogenized gently in 5 volumes of ice-cold buffer containing 25 mM Tris-HCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, 0.1 μM pepstatin, pH 8.0. The particulate fraction consisted of the pellet obtained after centrifuging the homogenate at 100,000 × g for 90 min at 4°C. The 100,000 × g supernatant solution was designated as the soluble fraction. Prior to electrophoresis, protein was quantitated by the method of Lowry et al. (45).

**Gel Electrophoresis**—One-dimensional SDS-PAGE was performed as described by Laemmli (46), using a 3% polyacrylamide stacking gel and a 12.5% separating gel. In some cases (see Fig. 6) gradient gels consisting of 8–18% polyacrylamide were used. Prior to electrophoresis, intact or particulate fractions were dissolved in Laemmli sample buffer (46) containing 8 M urea and heated at 100°C for 5 min. Soluble proteins were treated identically except that they were first concentrated by precipitation with 4 volumes of ice-cold acetone and centrifugation at 10,000 × g for 10 min.

Two-dimensional electrophoresis was carried out according to the method of Carrera et al. (47), with modifications described previously (36). For the present study, the procedure was modified further as described below. The gel mixture for the first dimension consisted of 0.5 M urea, 4% Nonidet P-40, 4.8% polyacrylamide, 1.5% pH 3–10 Pharmalytes, and 0.5% pH 8–10.5 Pharmalytes. Isoelectric focusing was carried out at 500 V for 7 h and then 1000 V for 2 h. The pH range 3–10 (G25K) in the first dimension was carried out at 40 mA/gel. Upon completion of the two-dimensional separation, proteins in the slab gels were electrophoreted onto nitrocellulose membranes as described previously (36).

**Immunoblotting**—In cases in which [3H]MVA-labeled proteins were to be visualized prior to immunoblotting, the dried blots were dipped in Amplify fluorography reagent (Amersham Corp.), air dried, and exposed to X-Omat AR film at −80°C in cassettes equipped with a Cronex intensifying screen. Alignment of fluorography films with subsequent immunoblots was facilitated by inclusion of fluorescent markers on the blots.

Immunoblotting was performed as described by Munby et al. (48). Blots that had been fluorographed were washed with water for 15 min prior to the initial blocking step. Primary antibodies were diluted to 1/1,000 in buffer containing 5% dry milk, 2% Nonidet P-40, and 0.2% SDS, and incubation was performed at room temperature for 2 h. Secondary antibodies (goat 125I-anti-mouse IgG for anti-p21P primary antibody, or goat 125I-anti-rabbit IgG for anti-G25K or anti-Gp primary antibodies) were added to the same buffer at 500,000 dpm/ml, and incubation was carried out for 1 h. Blots were covered with Saran Wrap and exposed to Kodak X-Omat AR film with a Cronex intensifying screen for various periods of time (see figure legends) at −80°C.

**Transient Expression of H-ras in COS Cells**—The pZIP-ras wild-type leucine 61 plasmid DNA was provided by Dr. Channing Der (La Jolla Cancer Research Foundation, La Jolla, CA). The ras insert was excised with BamHI and subcloned into the polylinker region of the pCMV5 expression vector (49) obtained from Dr. David Russell (University of Texas Southwestern Medical Center, Dallas). As a control for comparison with the ras-transfected cells, parallel cultures were transfected with the pCMV5 plasmid containing a Gαs cDNA insert, provided by Dr. Janet Rohbash (Weis Center for Research, University of Wisconsin, Madison, WI). Expression of the protein migrated at 178,841 daltons and does not undergo isoprenylation despite having a Cys-A-A-X C-terminal sequence (50). Prior to transfection, COS-M6 cells were plated in 60-mm dishes at a density of 9 × 10⁴ cells/dish and grown for 24 h. Cells were transfected with pCMV5 plasmid DNA (4 μg/dish) using a standard DEAE-dextran protocol (51) with the inclusion of 10 μM chloroquine during the initial 4-h incubation with the DNA/DEAE-dextran mixture. Incubation of cells with [3H]MVA to detect the formation of isoprenylated proteins was started 18 h after the transfection.

**RESULTS**

**Antibodies to G25K Detect a Protein Distinct from p21P in MEL Cells**—The presence of G25K (Gp) in MEL cells was established initially by immunoblotting with an affinity-purified antibody against a synthetic peptide derived from the sequence of human placental and platelet G25K (peptide pl described in Ref. 39). The experimental strategy was as follows. LR-MEL cells were incubated with the isoprenylated protein [3H]MVA to label the entire complement of isoprenylated proteins. The soluble and particulate fractions were then subjected to SDS-PAGE, transferred to nitrocellulose membranes, and fluorographed to establish the positions of the isoprenylated proteins. The same blots were incubated subsequently with the antibody to G25K or with a monoclonal antibody (ras-11, pan) which recognizes all H-, K-, and N-p21P proteins (for additional details, see legend to Fig. 1).

In agreement with our previous studies of MEL cells (36), most of the isoprenylated proteins were found in the 21–28-kDa region of the SDS gel and were about equally concentrated in the soluble and particulate fractions (Fig. 1). The soluble fraction contained a protein that reacted strongly with the antibody to G25K. The broad band of immunoreactive material at approximately 23–24 kDa fell within a cluster of proteins (Fig. 1). The antibody to G25K also gave a weak signal with a protein at the same molecular mass in the particulate fraction.

**In a previous study with MEL cells (36), we were unable to detect p21P by immunoblotting with the pan-ras antibody, using a horseradish peroxidase-conjugated secondary antibody. However, in the present study, the increased sensitivity of the 125I-labeled secondary antibody revealed a 26-kDa protein that reacted with the pan-ras antibody in the particulate
fraction of the LR-MEL cells. This protein clearly migrated at a higher molecular mass than the protein recognized by anti-G25K; and contrary to G25K, it was not found in the soluble fraction (Fig. 1).

The 26-kDa protein reacting with the pan-ras antibody in the MEL cells appeared to be different from both p21<sup>ras</sup> and G25K with respect to its electrophoretic mobility. Thus, when COS cells were transfected with cDNA coding for p21<sup>ras</sup> or G25K, using an <sup>3H</sup>MVA-labeled secondary antibody for detection of bound IgG (see "Experimental Procedures"). Exposure time for fluorography of the <sup>3H</sup>MVA-labeled proteins was 5 days. The <sup>3H</sup>-labeled immunoblots were exposed for 4 h. Stds., molecular mass standards.

![Fig. 1. Immunodetection of G25K in MEL cells.](image)

**Fig. 1.** Immunodetection of G25K in MEL cells. A suspension culture of LR-MEL cells was initiated at a density of 200,000 cells/ml and grown for 24 h in medium containing 25 μM lovastatin. Cells were then incubated with [3H]MVA (200 μCi/ml of medium) for 20 h. Cells were lysed in hypotonic buffer, and the soluble and particulate proteins were separated by SDS-PAGE (200 μg of protein/lane). After transfer to nitrocellulose, the isoprenylated proteins were visualized by fluorography. Each of the fluorographed strips of nitrocellulose was cut in half, and the fluor was washed out. The matching halves of the strips were then immunoblotted with antibodies to G25K or p21<sup>ras</sup>, using an '*<sup>125</sup>I-labeled secondary antibody for detection of bound IgG (see "Experimental Procedures"). Exposure time for fluorography of the [3H]MVA-labeled proteins was 5 days. The *<sup>125</sup>I-labeled immunoblots were exposed for 4 h. Stds., molecular mass standards.

![Fig. 2. Comparison of pan-ras immunoreactive proteins in MEL cells with immunoreactive p21<sup>ras</sup> transiently expressed in COS cells.](image)

**Fig. 2.** Comparison of pan-ras immunoreactive proteins in MEL cells with immunoreactive p21<sup>ras</sup> transiently expressed in COS cells. COS cells were transfected with cDNA coding for p21<sup>ras</sup> or G25K, as described under "Experimental Procedures." Beginning 18 h after transfection, the isoprenylated proteins in the COS cells as well as a parallel culture of LR-MEL cells were labeled by incubation for 18 h with [3H]MVA (200 μCi/ml of medium) in medium containing 25 μM lovastatin. Aliquots of whole cell homogenate (90 μg of protein) were resolved by SDS-PAGE, and the proteins were transferred to nitrocellulose. After fluorography (5-day exposure) immunoblotting was performed with pan-ras antibody followed by *<sup>125</sup>I-labeled secondary antibody to detect the bound IgG (8-h exposure). The arrows indicate the position of the transiently expressed p21<sup>ras</sup> in the COS cells transfected with H-ras. Stds., molecular mass standards.

The unexpected subcellular distribution of the immunoreactive protein in the present study led us to consider whether the antipeptide antibody might have been recognizing a soluble protein that shared the G25K p1 peptide sequence but was in fact a protein different from the Gp protein originally isolated by Evans et al. (37). To test this possibility we compared the immunoblot patterns obtained with the anti-p1 peptide antibody with those obtained with a polyclonal antiserum against authentic placental Gp, or platelet G25K. Conversely, the polyclonal antiserum against Gp also reacted with both the placental and platelet proteins. When both antibodies were used in parallel immunoblot assays against soluble and particulate protein fractions from LR-MEL cells, the polyclonal antiserum against Gp gave results that were essentially identical to those obtained with the antipeptide antibody; i.e. a broad immunoreactive band with a mobility corresponding to that of pure G25K, concentrated predominantly in the soluble fraction (Fig. 3, panel b).

Another possible explanation for the high proportion of G25K in the soluble fraction was that this subcellular distribution was a unique feature of the LR-MEL cells. Therefore, we compared the distribution of G25K between the total soluble and particulate compartments in several additional
cell lines (Fig. 4). The salient feature of the results is that a substantial proportion of G25K was present in the soluble fraction in every cell line examined. As in the case of the LR-MEL cells, the protein was predominantly cytosolic in human HL-60 myeloid leukemia cells, simian COS cells, and the parent Friend MEL cell line, which had not been adapted to grow in the presence of lovastatin. In normal Rat-6 fibroblasts, the relative amount of immunoreactive protein detected in the particulate fraction was slightly greater than in the soluble fraction whereas in the H-ras-transformed Rat-6 fibroblasts the G25K appeared to be about equally distributed between the two compartments.

**Demonstration of the Isoprenylation of G25K by Two-dimensional Gel Analysis**—In view of the overlap of proteins recognized by antibodies to G25K with proteins labeled by [3H] MVA on one-dimensional SDS gels (Fig. 1), we performed a two-dimensional immunoblot analysis of MEL cell proteins to establish whether or not G25K was actually one of the isoprenylated proteins. The [3H]MVA-labeled proteins from the soluble and particulate fractions were subjected to two-dimensional electrophoresis, fluorography, and immunoblotting with the anti-G25K p1 antibody. In anticipation of performing further studies on the effects of blocking isoprenoid synthesis on the electrophoretic mobility and subcellular distribution of G25K (see Fig. 6), we elected to perform these studies with the parent MEL cell line, which had not been adapted to grow in the presence of lovastatin and therefore retained full sensitivity to this inhibitor of MVA synthesis. The results, which are shown in Fig. 5, clearly identified three major [3H]MVA-labeled proteins in the soluble fraction which reacted with the antibody to G25K. These proteins were indistinguishable by molecular mass but had varying pl values ranging from approximately 7.5 to 7.8 (determined in a separate experiment in which the proteins were run with carboxylyate standards). In addition to the soluble G25K-reactive proteins, we also detected a doublet of immunoreactive [3H] MVA-labeled proteins in the particulate fraction. These proteins were similar to the soluble proteins with respect to their molecular masses and pl values. Thus far we have observed a similar pattern of [3H]MVA-labeled G25K isoforms in three separate experiments. However, it should be noted that the relative proportions of the three proteins in the soluble fraction, based on the [3H]MVA labeling or immunoblotting, were not always identical. The reasons for this variability remain to be established.

**Effects of Lovastatin on the Subcellular Distribution and**
Isoprenoid Modification of G25K (G\textsubscript{p})

**FIG. 5.** Isoprenylation of G25K demonstrated by fluorography and immunoblotting of proteins separated by two-dimensional electrophoresis. MEL suspension cultures that had reached a density of 10\textsuperscript{6} cells/ml were incubated with [\textsuperscript{3}H]MVA (200 \muCi/ml) and 25 \muM lovastatin for 24 h. In separate experiments, soluble and particulate fractions were prepared and subjected to two-dimensional electrophoresis as described under "Experimental Procedures." The amounts of protein loaded on the gels were 200 \mug (soluble) and 150 \mug (particulate). Following electrophoresis, proteins were transferred to nitrocellulose membranes and fluorographed to visualize the total complement of isoprenylated proteins (upper panels). The regions of the blots containing the 21-28-kDa [\textsuperscript{3}H]MVA-labeled proteins (outlined by boxes) were washed and subjected to immunoblotting with antibody to the G25K p1 peptide using \textsuperscript{125}I-labeled secondary antibody to localize the bound IgG (lower panels). Fluorographs were exposed for 7 days (soluble) or 5 days (particulate). Both of the immunoblots were exposed for 18 h. The arrows indicate [\textsuperscript{3}H]MVA-labeled proteins that were specifically recognized by the antibodies to G25K. IEF, isoelectric focusing.

Electrophoretic Mobility of G25K—Recent studies of p21\textsuperscript{ras} have shown that treatment of cultured cells with inhibitors of isoprenoid biosynthesis prevents the isoprenylation and post-translational proteolytic processing of the protein at its C terminus (i.e. conversion of pro-p21\textsuperscript{ras} to c-p21\textsuperscript{ras}) and the subsequent palmitoylation and membrane localization of the protein (conversion of c-p21\textsuperscript{ras} to m-p21\textsuperscript{ras}) (18, 21). The inhibition of p21\textsuperscript{ras} processing is manifested by the accumulation of pro-p21\textsuperscript{ras}, which can be distinguished from c-p21\textsuperscript{ras} and m-p21\textsuperscript{ras} by virtue of its higher apparent molecular mass on one-dimensional SDS gels (18, 19, 21). In view of these findings, we conducted studies to determine what effect blocking MVA synthesis might have on the subcellular distribution and electrophoretic mobility of G25K. As shown in Fig. 6 (panel a), pretreatment of MEL cells for 24 h with 25 \muM lovastatin resulted in a decrease in the relative amount of immunodetectable G25K in the particulate fraction and an increase in the relative amount of immunodetectable G25K concentrated in the soluble fraction. The broadening of the band of immunoreactive protein in the soluble fraction of MEL cells acutely exposed to lovastatin suggested that forms of G25K with slightly altered electrophoretic mobilities might have accumulated when MVA synthesis was blocked. However, even when gradient gels were used, the resolution afforded by one-dimensional SDS-PAGE was insufficient to delineate clearly a pro-form of G25K analogous to pro-p21\textsuperscript{ras}. To explore this possibility further, two-dimensional electrophoresis was used to resolve the isoforms of G25K (Fig. 6,
Isoprenoid Modification of G25K (Gι)

FIG. 6. Effects of inhibition of MVA synthesis on the subcellular distribution and electrophoretic mobility of G25K in MEL cells. MEL cells in the exponential phase of growth were incubated with 25 μM lovastatin for 24 h. Control cells were incubated without lovastatin. Cells were fractionated into soluble (S) and particulate (P) components, and electrophoresis and immunoblotting were carried out as described under "Experimental Procedures." a, one-dimensional gradient SDS-PAGE. All lanes were loaded with 100 μg of protein, and immunoblotting was performed with antibody to G25K p1 peptide. Blots were exposed for 4.5 h to visualize the lz51-labeled secondary antibody. Using the developed film as a guide, samples of whole cell protein (150 μg) from control and lovastatin-treated cells were subjected to two-dimensional electrophoresis. The panels show identical regions of parallel blots (20–30 kDa, pl approximately 7–8) after reaction with the G25K antibody. In both cases, exposure time was 90 min. IEF, isoelectric focusing.

panel b). Under these conditions, shifts in both the p1 values and the apparent molecular masses of the G25K isoforms were clearly discernible after lovastatin preincubation.

DISCUSSION

The present study demonstrates that in cultured MEL cells a protein that is immunologically indistinguishable from G25K undergoes post-translational modification by isoprenoid groups derived from [1-14C]MVA. G25K (Gι) is a relatively abundant GTP-binding protein in platelets (34), brain (38), and placenta (37), and it was one of the first low molecular mass GTP-binding proteins to be purified from mammalian tissues. Although its function remains to be defined, the studies of Polakis et al. (39) coupled with those in this report imply that G25K is a ubiquitous protein found in a variety of normal and transformed cell lines. G25K is customarily viewed as being a member of the rapidly expanding family of ras-related proteins. However, the available amino acid sequence information derived from four peptide fragments indicates that compared with other low molecular mass GTP-binding proteins, G25K has relatively little homology with p21ras (39). Variation is observed even in the highly conserved Asn-Lys-X-Asp element (where X is an amino acid), which has been proposed to contribute to the guanine nucleotide binding site in the ras-related proteins (52, 53). Therefore, it was not surprising that antibodies generated against the unique p1 peptide sequence of G25K (39) showed no crossreactivity with proteins recognized by the pan-ras antibody in the present study.

As mentioned previously, reports that isoprenoid modification of p21ras, prelamin A, lamin B, and fungal mating factors occurs at a cysteine within a C-terminal Cys-A-A-X sequence have led to speculation that this sequence may represent a consensus motif for isoprenylation. Therefore, a major question is whether the C-terminal sequence of G25K is consistent with this pattern. The full-length cDNA sequence for G25K is not yet available. However, preliminary information indicates that there may be two different forms of G25K, one with a C-terminal sequence fitting the pattern Cys-A-A-X and the other with a variation of this motif, i.e. Cys-Cys-A-X. Although the electrophoretic variants of G25K seen in the present study could have been due to posttranslational modifications of a single protein, it is also conceivable that two or more slightly different G25K gene products are expressed in MEL cells. If the latter is true, then our finding that all of the electrophoretic variants of G25K in MEL cells were isoprenylated (see Fig. 5) raises the possibility that proteins with a Cys-Cys-A-X motif, as well as the usual Cys-A-A-X, may undergo isoprenylation.

Preincubation of MEL cells with an inhibitor of isoprenoid synthesis resulted in changes in the isoelectric points and molecular masses of the G25K-immunoreactive proteins on two-dimensional gels (see Fig. 6). This observation provides some support for the notion that as in the case of p21ras, post-translational processing of G25K is dependent on isoprenoid availability. However, it remains to be determined whether the shifts in electrophoretic mobility of G25K are due solely to accumulation of nonisoprenylated forms of the protein or whether blocking isoprenyl modification prevents additional steps such as proteolytic cleavage or carboxymethylation at the C terminus.

In addition to establishing that G25K is isoprenylated, our results provide the first indication that G25K is normally localized in both the soluble and particulate fractions of several cultured cell lines. Of particular note was the observation that in MEL cells most of the G25K partitioned in the soluble fraction, despite the fact that the protein was isoprenylated. This finding argues against the idea that isoprenylation necessarily results in the stable insertion of proteins into the membrane lipid bilayer and points to a more complex and subtle role for this structural modification. The recent studies with p21ras have established that isoprenylation of Cys190 is sufficient to promote interaction of the protein with the cell membrane and expression of transforming activity (18, 19). However, the presence of an isoprenylated intermediate form of p21ras (c-p21ras) in the soluble fraction of COS cells led Hancock et al. (18) to conclude that although the first steps in p21ras processing (i.e. isoprenylation, proteolytic cleavage, and carboxymethylation) allow some degree of membrane association, this association is not as strong or stable as that which is attained after the protein is palmitoylated. Additional insight into the function of isoprenylation comes from studies of the nuclear lamins. For example, although it is known that prelamin A is isoprenylated (16–17) and that mutations in the Cys-A-A-X motif abolish translocation of the protein to the nuclear envelope (54), forms of prelamin A with point mutations in the α-helical domain remain dispersed in the nucleoplasm despite the fact that they have a normal Cys-A-A-X C-terminal sequence (54). Based on these studies, it has been suggested that isoprenylation alone confers only a weak affinity for membranes and that other structural features or protein-protein interactions are also required for assembly of lamin A into the nuclear envelope (54). A similar situation may exist in the case of lamin B, in which isoprenylation at a C-terminal cysteine (11, 14–16) and interaction with a specific receptor (55) may both contribute to the stable asso-

2 Dr. Richard Cerione, Cornell University, Ithaca, NY, personal communication.

3 Dr. Paul Polakis, Cetus Corp., Emeryville, CA, personal communication.
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Consistent with the foregoing concepts, our studies suggest that although isoprenylation has some influence on the localization of G25K to the particulate fraction, it is not the exclusive determinant of the proportion of G25K associated with the membrane or other organelles. One possibility is that isoprenylation of G25K constitutes a permissive event for subsequent post-translational modifications such as fatty acylation (56) or carboxymethylation (57, 58), which confer additional hydrophobicity and thereby increase the affinity of the protein for the cell membrane. This could explain why blocking isoprenoid synthesis with lovastatin resulted in a decreased concentration of G25K in the particulate fraction of MEL cells (see Fig. 6). Alternatively, weak membrane interactions promoted by isoprenylation may serve to bring G25K into proximity with a membrane receptor protein in which other factors (e.g. GTP binding and hydrolysis, phosphorylation) ultimately control the release of the protein into the soluble fraction. Several lines of evidence suggest that the latter possibility merits serious consideration. For instance, Nagata et al. (59) found that a membrane-bound small GTP-binding protein in platelets could be phosphorylated by cyclic AMP-dependent protein kinase whereas a similar cytoplasmic GTP-binding protein was incapable of being phosphorylated by the same enzyme unless it was first treated with phosphatase. Hart et al. (60) reported recently that purified platelet G25K undergoes epidermal growth factor stimulated tyrosine phosphorylation when reconstituted into phospholipid vesicles with purified epidermal growth factor receptor and speculated that phosphorylation might serve to stimulate release of the protein from membranes in vivo. In this regard, it is noteworthy that several of the 20-30-kDa isoprenylated proteins have been shown previously to be phosphorylated in murine lymphoma cells (9). It is not yet known if any of these proteins correspond to G25K, but it is conceivable that phosphorylation could account for the multiple isoforms of G25K observed in the present study.

The discovery that both p21 replacement and G25K are isoprenylated strongly suggests that other ras-related GTP binding may undergo similar modifications. Although proteins bearing the Cys-A-A-X C-terminal sequence (e.g. rho, rap1) remain the most likely candidates, our studies with G25K suggest that proteins with variations in this general motif should not be ruled out. In this regard, we demonstrated recently that when MEL cell proteins were separated by two-dimensional electrophoresis, several [3H]MVA-labeled proteins in the 20-28-kDa region of the gel were able to bind [alpha-32P]GTP after transfer to nitrocellulose membranes (36). The GTP-binding capacity of two isoprenylated proteins migrating at 26-28 kDa was particularly striking since it was retained after long term fluorography. Although it is known that G25K does not bind [32P]GTP after transfer to nitrocellulose, another related protein, H-ras, has been reported to do so with high affinity (34). Interestingly, H-ras migrates at approximately 28 kDa on SDS gels, and its amino acid sequence deduced from a cDNA cloned from a placental library (34) contains a C-terminal motif (Cys-Cys-A-X) similar to that of one form of G25K. Studies aimed at determining if this protein represents one of the major isoprenylated GTP-binding proteins in MEL cells are currently underway.

An important area for future investigation concerns the structure of the isoprenoid moiety attached to G25K and other GTP-binding proteins. Initial studies of lamin B (11) and p21 replacement (19) suggested that these proteins were modified by farnesyl (C15) groups. In a preliminary characterization of isoprenoids released from the low molecular mass isoprenylated proteins of MEL cells by sulfonium salt cleavage, we found material with chromatographic characteristics of farnesol, nerolidol, and additional unidentified rearrangement products (10). More recent studies of isoprenylated proteins from Chinese hamster ovary cells (12) and HeLa cells (13), employing gas chromatography-mass spectrometry to characterize products released by Raney-Nickel-catalyzed desulfurization, have demonstrated the existence of a second class of C20 (geranylgeranyl) isoprenoids. Thus, it is possible that C20 isoprenoid alcohols might have accounted for some of the unidentified material released from MEL cell proteins with methyl iodide cleavage (10). In view of the apparent heterogeneity of the modifying isoprenoid groups, it will be necessary to determine whether or not specific proteins such as G25K can be modified interchangeably by farnesyl or geranylgeranyl groups, depending on substrate availability or prenyltransferase activity in a given tissue. An alternative possibility is that variations in the C-terminal motifs may dictate which proteins will act as substrates for particular prenyltransferases. Clarification of these issues should be forthcoming as additional isoprenylated proteins are identified and their modifying groups are characterized.

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