Isoprenoid Requirement for Intracellular Transport and Processing of Murine Leukemia Virus Envelope Protein*

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Lovastatin blocks the biosynthesis of the isoprenoid precursor, mevalonate. When Friend murine erythroleukemia (MEL) cells are cultured in medium containing lovastatin, the precursor of murine leukemia virus envelope glycoprotein (gPr90') fails to undergo proteolytic processing, which normally occurs in the Golgi complex. Consequently, newly synthesized envelope proteins are not incorporated into viral particles that are shed into the culture medium. gPr90' appears to be localized in a pre-Golgi membrane compartment, based on its enrichment in subcellular fractions containing NADPH-cytochrome c reductase activity and the sensitivity of its carbohydrate chains to digestion by endoglycosidase H. Arrest of gPr90' processing occurs at concentrations of lovastatin that are not cytostatic, and the effect of the inhibitor is prevented by addition of mevalonate to the medium. The low molecular mass GTP-binding proteins, rab1p and rab6p, which are believed to function in early steps of the exocytic pathway, are normally modified posttranslationally by geranylgeranyl isoprenoids. However, in MEL cells treated with 1 μM lovastatin, nonisoprenylated forms of these proteins accumulate in the cytosol prior to arrest of gPr90' processing. These observations suggest that lovastatin may prevent viral envelope precursors from reaching the Golgi compartment by blocking the isoprenylation of rab proteins required for ER to Golgi transport.

The low molecular mass GTP-binding protein, YPT1p, plays an essential role in early steps of the secretory pathway in yeast (1, 2). Increasing evidence indicates that mammalian homologs of YPT1p encoded by the rab gene family (e.g. rab1Ap, rab1Bp, and rab2p) are similarly involved in facilitating the vectorial transport of exocytic vesicles between the endoplasmic reticulum (ER) and Golgi complex in mammalian cells (3-7). In cells infected with retroviruses, the envelope glycoproteins encoded by the viral env genes undergo proteolytic processing and oligosaccharide maturation upon translocation from the ER to the Golgi apparatus (reviewed in Refs. 8 and 9). Hence, it is reasonable to suspect that these maturation events may be dependent on vesicular transport pathways mediated by the rab proteins. Recent studies have shown that cysteine residues at or near the carboxyl termini of several rab proteins are posttranslationally modified by 20-carbon geranylgeranyl isoprenoids (10-13). As in the case of p21 and other low molecular mass GTP-binding proteins, isoprenylation appears to be essential for association of the rab proteins with intracellular membrane systems (12, 13). All cellular isoprenoids, including the farnesyl- and geranylgeranylpyrophosphates that serve as substrates for isoprenyltransferases, are derived from a common precursor, mevalonate (MVA) (14-16). The cholesterol-lowering drug, lovastatin, blocks MVA synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (17). Thus, in the present study we used this highly specific inhibitor to examine the potential relationship between isoprenoid biosynthesis and the processing of murine leukemia virus (MuLV) envelope glycoprotein in erythroleukemia cells. The results demonstrate that cells treated with noncytostatic concentrations of lovastatin are unable to convert the MurLV envelope glycoprotein precursor, gPr90', to the mature envelope glycoprotein, gp70'. Under these conditions, no incorporation of newly synthesized envelope protein into viral particles is detected. Preliminary evidence suggests that the perturbation of viral envelope protein transport and processing in cells exposed to lovastatin may be related to the accumulation of nonisoprenylated rab proteins in the cytosol.

EXPERIMENTAL PROCEDURES

Materials—Lovastatin was provided by A. Alberts of Merck Sharp and Dohme Research Laboratories (Rahway, NJ). L-[3-3H]Methionine (Trans-3-label, 1147 Ci/mmol) was purchased from ICN Biochemicals and [methyl-3H]thymidine (6.7 Ci/mmol) was obtained from Du Pont-New England Nuclear. Endoglycosidase H (Endo H) was purchased from Boehringer Mannheim.

Metabolic Labeling of Proteins—Murine erythroleukemia cells (line 745), originally obtained from Dr. Charlotte Friend, were grown in suspension in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as described previously (18). Suspension cultures were seeded at 2 x 10⁶ cells/ml and grown for 24 h prior to starting each experiment. After preincubation for varying periods of time in medium with or without lovastatin, cells were incubated in methionine-free medium containing 10% dialyzed fetal calf serum and 25 μCi/ml [35S]methionine Trans-3-label (with or without lovastatin) for 6 h. Cells were then collected by centrifugation, washed three times with ice-cold phosphate-buffered saline, and prepared for immunoprecipitation assays as described below. Measurements of [35S]methionine incorporation into trichloroacetic acid-insoluble material (mean ± S.E. of three parallel cultures), and assessment of cell viability by Trypan blue dye exclusion were performed as described previously (18, 19).

Immunoprecipitation of Viral Envelope Proteins—Cells pellets were lysed in 0.5 ml of 1.0% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), and the lysates were centrifuged at 15,000 x g for 10 min. The supernatant...
solution was combined with 0.5 ml of 0.4% SDS, 20 mM dithiothreitol, 50 mM Tris-HCl (pH 7.5) and heated for 10 min at 100 C. Samples were brought to 50 mM with iodoacetamide and incubated at 37 C for 30 min. After addition of 0.5 ml of 2.0% Triton X-100, samples were placed on ice for 15 min and mixed with 5 l of antiserum to gp70' (32). Immunoprecipitation was carried out after overnight incubation at 4 C, using protein A-Sepharose beads as described by Ullmer and Palade (20). The proteins were released from the beads by boiling in Laemmli's sample buffer (21) and subjected to SDS-PAGE and fluorography (22). In some cases, the immunoprecipitated proteins were incubated for 2 h at 37 C with 10 milligrams of Endo H in 50 l of 0.75% SDS, 30 mM sodium citrate, pH 5.5, prior to elution from the protein A-Sepharose. The relative amounts of radiolabeled gp90' and gp70' on the autoradiograms were determined by densitometry, using a Technology Resources Microscan 1000 area scanner.

Analysis of Viral Particles—Virions shed from MEL cells during a 6-h incubation with [35S]methionine were recovered from the medium by centrifugation at 1 for 1 h at 40,000 x g. Viral particles were solubilized in 0.5% Nonidet P-40, 2% SDS, 10 mM dithiothreitol, 50 mM Tris-HCl, pH 7.5, and immunoprecipitation of envelope proteins was carried out as described above. Parallel immunoprecipitations of the viral gag proteins were carried out with polyclonal antiserum to MuLV p30' (1:250 dilution).

Subcellular Distribution of Viral Envelope Proteins—Cells were suspended in 5 volumes of homogenization buffer (25 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM PMSF, 1 mM leupeptin, 1 mM pepstatin, 50 mM phenylmethylsulfonyl fluoride, 50 l of 10% Triton X-100) and centrifuged at 4 C for 10 min. The supernatant was removed and the pellets were resuspended in 5 volumes of homogenization buffer and pelleted by centrifugation at 100,000 x g for 10 min. The pellets from both steps were pooled and designated the particulate fraction (P,). Each fraction was assayed for ouabain-sensitive Na+,K+-ATPase (25), NADPH-dependent cytochrome c reductase (26), and cytochrome c oxidase (26), using established assays. For detection of viral envelope proteins, samples of each fraction (10 l) were subjected to SDS-PAGE and transferred to nitrocellulose. Immunoblotting was carried out for 1 h with goat antiserum to MuLV gp70' (12,000 dilution), followed by a 1-h incubation with 0.5 l of rabbit anti-goat IgG. Immune complexes were then detected by incubating the blots with [125I]-labeled goat antirabbit IgG (33). Antibodies to rab Proteins—Polyclonal antiserum against rab6p was provided by Dr. A. Zaharai (Institut National de la Santé et de la Recherche Médicale, Paris, France). Polyclonal antiserum to rab1p was raised against recombinant rab1B as follows: The full-length cDNA for rab1B was cloned into the vector pMAL-C (New England Biolabs) and expressed in Escherichia coli TMB 1 as an MBP-rab1B fusion protein (13). The recombinant protein was purified on amylose resin, digested with Factor X, to remove the MBP, and separated from free MBP by SDS-PAGE. Rab1B eluted from the SDS gel was mixed with RIBI adjuvant (ImmunoChem Research, Inc.) and used for immunization of rabbits according to the protocol recommended by the supplier of the adjuvant. Affinity-purified anti-rab1p IgG was obtained by incubating aliquots of crude antiserum with preblocked strips of Immobilon-P containing gel-purified recombinant rab1B and then eluting the bound IgG with 0.2 M glycine.

Subcellular Distribution of rab Proteins—To determine the proportion of rab proteins in the cytosolic and particulate fractions of MEL cells, cells were lysed in 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 l of 10% Triton X-100, and 0.1 l per ml of antiserum to(gp70') raised previously (22). Samples (50 l) of cytosol (50 l) were pooled from the resulting supernatant solution at 100,000 x g for 1 h. The pellets from both steps were pooled and designated the particulate fraction. Proteins in the soluble and particulate fractions were subjected to SDS-PAGE, transferred to Immobilon-P (Millipore Corp.), and immunoblotted with affinity-purified anti-rab6p IgG (1:100 dilution) or antiserum to rab6p (1:500 dilution). Immune complexes were detected by incubating the blots with [125I]-labeled goat anti-rabbit IgG as described previously (22). To determine the percentage of isoprenylated versus nonisoprenylated rab6p in cytosols from cells grown with or without lovastatin, soluble fractions prepared as described above were subjected to a Triton X-114 partitioning assay which has been used previously to distinguish between isoprenylated and nonisoprenylated forms of preS1 (27, 28) and YFP proteins (29). Aliquots of cytosol (50 l) were mixed with 10 l of 10% Triton X-114 and incubated on ice for 10 min. Phase separation was promoted by incubation of the mixture at 37 C for 2 min, and the detergent phase was separated from the aqueous phase by centrifugation for 2 min at top speed in an Eppendorf microfuge. Proteins were collected from the aqueous and detergent phases by addition of 1 ml of ice-cold 10% trichloroacetic acid, followed by centrifugation at 10,000 x g for 10 min. Proteins were solubilized in SDS sample buffer, resolved by SDS-PAGE, and immunoblotted with anti-rab6p IgG as described above. The relative amounts of rab6p in the aqueous and detergent phases were determined by densitometric scanning of the resulting autoradiograms.

RESULTS

Inhibition of Isoprenoid Synthesis Prevents Proteolytic Processing of MuLV Envelope Protein Precursor—MEL cells are infected with a replication-defective spleen focus forming virus (SFFV) and a helper virus, MuLV (8). Both viruses encode genes for envelope proteins that are glycosylated in the ER (30). However, while only a small percentage (3–5%) of the SFFV gp55' leaves the ER (31, 32), the MuLV gp70' is efficiently translocated to the plasma membrane, where it combines with viral cores as they are shed from the cell (33). The MuLV envelope protein is synthesized as a precursor, gp90', which is concentrated in ER membranes (23, 33) and contains high-mannose carbohydrate chains that are sensitive to digestion with Endo H (34, 35). Proteolytic processing of gp90' to form gp70' appears to occur in a late Golgi compartment (35) and is blocked when the Golgi membranes are disrupted with brefeldin A (20). MuLV gp70' is found in the plasma membrane (23, 33) and contains complex sialylated oligosaccharides (35, 36). Because of the well-defined changes in molecular mass and oligosaccharide maturation that occur as the MuLV envelope protein is transported from the ER to the plasma membrane, this protein is regarded as a useful marker for intracellular protein trafficking in MEL cells.

Previous studies have shown that addition of 25 l of lovastatin to MEL cells blocks the synthesis of MVA, the obligate precursor for all cellular isoprenoids, including those required for protein isoprenylation (18). The results depicted in Fig. 1 indicate that at this concentration lovastatin has a profound impact on proteolytic processing of the MuLV envelope glycoprotein. Thus, when [35S]methionine-labeled viral envelope proteins were immunoprecipitated from MEL cells cultured without lovastatin, both the precursor (gp90') and the mature (gp70') forms of the MuLV protein were readily detected. However, when 25 l of lovastatin was added for varying periods of time prior to labeling the proteins with [35S]methionine, there was a marked decline in the ratio of radiolabeled gp70' to gp90', beginning after 12 h (Fig. 1A). By 21–24 h it was not possible to detect significant production of radiolabeled gp70' to gp90', since gp70' contains only two of the seven isoprenines present in gp90' (33).

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polyclonal antibody against MuLV gp70<sup>env</sup> and gPr90<sup>env</sup> also protein, consistent with the finding that cells treated with the proteins were immunoprecipitated from lysates prepared from equal numbers of control and lovastatin-treated cells. The percentage of the ratio determined for the parallel control culture. The inset shows the actual fluorograms obtained at 24 h, with the molecular masses of standard proteins indicated at the right of the panel. MEL cells were incubated with or without 25 μM lovastatin as described above. 

Inhibition of Viral Envelope Protein Processing Occurs at Concentrations of Lovastatin That Are Not Cytostatic—Lovastatin was prevented when exogenous MVA was added to the culture medium, suggesting that the effect was specifically due to depletion of MVA-derived products (Fig. 1B). The polyclonal antibody against MuLV gp70<sup>env</sup> and gPr90<sup>env</sup> also recognizes the related SFFV gp55<sup>env</sup>, which is not efficiently transported out of the ER (31, 32). Lovastatin treatment did not reduce the incorporation of [35S]methionine into this protein, consistent with the finding that cells treated with the inhibitor for 24 h were greater than 95% viable by Trypan dye exclusion and did not show a marked decline in incorporation of [35S]methionine into total cellular protein (control, 12,097 ± 333 dpm/μg protein; +lovastatin, 10,472 ± 1,639 dpm/μg protein).

Inhibition of Viral Envelope Protein Processing Occurs at Concentrations of Lovastatin That Are Not Cytostatic—Lovastatin has previously been shown to be a potent inhibitor of cell proliferation at concentrations above 5 μM, whereas lower concentrations of the drug have diminishing effects on cell cycling in serum-supplemented cultures (37). Therefore, we wished to establish the minimum concentration at which lovastatin affects gPr90<sup>env</sup> processing. The data presented in Fig. 2 indicate that 1 μM lovastatin was sufficient for maximal inhibition of gp70<sup>env</sup> production, while 0.5 μM lovastatin caused partial inhibition after 21 h. The identity of the labeled 60-62-kDa protein appearing in the immunoprecipitates from the lovastatin-treated cells is unknown. It may represent a degradation product derived from accumulated gPr90<sup>env</sup>, since a similar protein has been observed when processing of gPr90<sup>env</sup> is blocked by disruption of the Golgi complex with brefeldin A (20). It is noteworthy that MEL cells exposed to 1 μM lovastatin for 21 h showed no decline in their capacity to incorporate [3H]thymidine into trichloroacetic acid-precipitable material (1,261 ± 9 dpm/μg protein), relative to parallel untreated controls (1,164 ± 126 dpm/μg protein). Thus, at concentrations that inhibit viral envelope protein processing, lovastatin does not appear to act as a general cytostatic agent in MEL cells.

**Fig. 1.** Inhibition of proteolytic processing of MuLV gPr90<sup>env</sup> in MEL cells treated with lovastatin. A, parallel cultures of MEL cells were incubated with or without 25 μM lovastatin (Lov) for the indicated periods of time. At each point [35S]methionine-labeled proteins were immunoprecipitated from lysates prepared from equal numbers of control and lovastatin-treated cells (1.6 × 10<sup>6</sup>), using antiserum to MuLV gp70<sup>env</sup> (see “Experimental Procedures”). Proteins were subjected to SDS-PAGE and fluorography, and the ratio of radiolabeled gp70<sup>env</sup> to gPr90<sup>env</sup> was determined by densitometry. The gp70<sup>env</sup>/gPr90<sup>env</sup> ratio for each lovastatin-treated culture is expressed as a percentage of the ratio determined for the parallel control culture. The inset shows the actual fluorograms obtained at 24 h, with the molecular masses of standard proteins indicated at the right of the panel. B, MEL cells were grown for 24 h in medium containing 10 μM lovastatin, with supplemental mevalonate (MVA) added as indicated. Immunoprecipitation of radiolabeled proteins with antiserum to gp70<sup>env</sup> was carried out as described above.

**Fig. 2.** Processing of MuLV gPr90<sup>env</sup> in MEL cells treated with increasing concentrations of lovastatin. Parallel cultures were incubated for 21 h with lovastatin added to the medium at the indicated concentrations. Following a 6-h incubation with [35S]methionine, viral envelope proteins were immunoprecipitated and subjected to SDS-PAGE and fluorography as described under “Experimental Procedures.” The gp70<sup>env</sup>/gPr90<sup>env</sup> ratios, determined by densitometry, are displayed beneath the corresponding lanes of the fluorogram.

gPr90<sup>env</sup> Is Sensitive to Digestion with Endo H and Is Localized in Membrane-enriched Subcellular Fractions—The ability of lovastatin-treated MEL cells to produce MuLV gPr90<sup>env</sup>, but not gp70<sup>env</sup>, raised the possibility that inhibition of isoprenoid synthesis might interfere with processing indirectly, by preventing the translocation of the envelope precursor protein from the ER to the Golgi compartment where proteolytic cleavage occurs. To explore this possibility, we digested the immunoprecipitated viral envelope proteins from control and lovastatin-treated MEL cells with Endo H, which cleaves high-mannose N-linked oligosaccharide chains of the type found on glycoproteins that have not yet reached the...
Golgi apparatus (38). As shown in Fig. 3, all of the gPr90<sup>env</sup> synthesized in both the control and lovastatin-treated MEL cells was sensitive to Endo H. The 66-kDa product resulting from the Endo H digestion of gPr90<sup>env</sup> corresponds to the fully digested form of the protein observed in previous studies (20, 34, 35). In the control cells, the 66-kDa Endo H derivative of gPr90<sup>env</sup> comigrated with gp70<sup>env</sup>, which shows a small increase in mobility when exposed to Endo H, due to its content of both sensitive and resistant side chains (20, 35). 

As expected, the SFFV gp55<sup>env</sup>, which is localized predominantly in the ER (31, 32), was also completely sensitive to Endo H in both the control and lovastatin-treated cells. 

Previous subcellular fractionation studies have indicated that gPr90<sup>env</sup> normally resides in an ER or early Golgi membrane compartment (23, 33). When MEL cells were treated with 1 μM lovastatin for 21 h and fractionated by differential centrifugation, gPr90<sup>env</sup> was absent from the S2 (cytosol) and was concentrated in the P2 fraction (Fig. 4), which was enriched in marker enzymes for mitochondria (cytochrome c oxidase), plasma membrane (ouabain-sensitive Na<sup>+</sup>,K<sup>+</sup>-ATPase), and ER (NADPH-cytochrome c reductase) (Table I). Immunoblotting revealed a significant amount of gp70 in the homogenate and P2, indicating that not all of the mature envelope protein produced before the onset of the block in gPr90<sup>env</sup> processing is immediately packaged into virions and exported from the lovastatin-treated cells. Upon further fractionation on a discontinuous sucrose gradient, the P2 material yielded two major bands. The activity of the enzymatic marker for plasma membrane was highest in the lighter band (0.8/1.2 m sucrose boundary), whereas the activity of the mitochondrial marker was localized predominantly in the heavier band (1.2/1.6 m boundary). The specific activity of the ER marker was similar in both fractions (Table I). When equal amounts of each fraction were subjected to immunoblotting, the MuLV gp70<sup>env</sup> was most concentrated in the 0.8/1.2 m sucrose band, consistent with the enrichment of plasma membrane in this fraction (Fig. 4). In contrast, the gPr90<sup>env</sup> was equally concentrated in the 0.8/1.2 and 1.2/1.6 m sucrose bands (Fig. 4), paralleling the distribution of the ER marker enzyme (Table I). A similar subcellular distribution of gPr90<sup>env</sup> was observed in MEL cells that were not exposed to lovastatin (not shown). These observations, together with the results of Endo H digestion (Fig. 3), support the assumption that in lovastatin-treated MEL cells gPr90<sup>env</sup> is localized primarily in pre-Golgi membranes.

**Lovastatin-treated MEL Cells Do Not Incorporate Newly Synthesized Envelope Proteins into Virions**—To further explore the relationship between intracellular transport of viral envelope proteins and isoprenoid biosynthesis, MEL cells were grown for 21 h with or without 1 μM lovastatin, and [<sup>35</sup>S]methionine was then added to the medium for 6 h to label newly synthesized proteins. Viral particles extruded into the medium during the labeling period were collected by ultracentrifugation, and the solubilized viral proteins were immunoprecipitated with antiserum to MuLV gp70<sup>env</sup> or antiserum to p30<sup>env</sup>, a protein that is assembled into the virion core from the cytoplasmic pool, independent of vesicular transport pathways (8, 33). As shown in Fig. 5, viruses shed from control and lovastatin-treated cells contained comparable amounts of radiolabeled gag core protein, but were markedly different with respect to their contents of radiolabeled envelope protein. Whereas [<sup>35</sup>S]methionine-labeled gp70<sup>env</sup> was clearly evident in virus collected from untreated cells, neither radiolabeled gp70<sup>env</sup> nor the gPr90<sup>env</sup> precursor was detected in viral particles that were shed from cells treated with lovastatin. Since MuLV gp70<sup>env</sup> is packaged into budding virions at the cell surface (8, 33), this observation provides further evidence that the capacity for transport of newly synthesized gPr90<sup>env</sup> through the exocytic pathway is impaired when cellular isoprenoid synthesis is blocked.

**Changes in Subcellular Distribution of rab Proteins Precede the Arrest of Viral Envelope Protein Processing in Lovastatin-treated Cells**—Our recent studies with COS cells have established that blocking MVA synthesis and protein isoprenylation with 25 μM lovastatin results in accumulation of newly synthesized rab proteins in the cytosol (13). Therefore, we asked whether a lower concentration of lovastatin (e.g. 1 μM), which effectively arrests the Golgi-dependent processing of

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**FIG. 3.** Endo H digestion of viral envelope glycoproteins. Parallel MEL cell cultures were incubated for 21 h with or without 10 μM lovastatin in the growth medium. Cells were then labeled with [<sup>35</sup>S]methionine for 6 h and the viral envelope proteins immunoprecipitated with antiserum to MuLV gp70<sup>env</sup>. Prior to SDS-PAGE and fluorography, the immunoprecipitated proteins were incubated for 2 h with (+) or without (−) Endo H (see "Experimental Procedures").

**FIG. 4.** Immunoblot detection of viral envelope proteins in subcellular fractions from lovastatin-treated MEL cells. MEL cells were grown for 21 h in medium containing 1 μM lovastatin, and the following subcellular fractions were prepared as described under "Experimental Procedures": H, homogenate; S<sub>1</sub>, 100,000 × g supernatant; P<sub>2</sub>, 250,000 × g pellet; P<sub>3</sub>, 100,000 × g pellet; 0.8/1.2, P<sub>2</sub> material separated on discontinuous gradient, 0.8/1.2 m sucrose interface; 1.2/1.6, P<sub>2</sub> material separated on discontinuous gradient, 1.2/1.6 m sucrose interface. 10 μg of protein from each fraction was subjected to SDS-PAGE and transferred to nitrocellulose. All lanes were immunoblotted with antiserum to gp70<sup>env</sup> (see "Experimental Procedures"), and the autoradiogram was exposed for 4.5 h.

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**TABLE I**

<table>
<thead>
<tr>
<th>Fraction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Na&lt;sup&gt;+&lt;/sup&gt;K&lt;sup&gt;+&lt;/sup&gt;-ATPase</th>
<th>Cytochrome c oxidase</th>
<th>NADPH-cytochrome c reductase</th>
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<td>11.5</td>
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<td>392</td>
<td>36.5</td>
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<td>47</td>
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<td>950</td>
<td>66.6</td>
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</tbody>
</table>

<sup>a</sup>Subcellular fractions are defined under "Experimental Procedures" and in the legend to Fig. 4.

<sup>b</sup>One unit of enzyme activity is defined as follows: Na<sup>+</sup>,K<sup>+</sup>-ATPase, 1 nmol of inorganic phosphate released/min at 37 °C; cytochrome c oxidase, 1 nmol of cytochrome c oxidized/min at 30 °C; NADPH-cytochrome c reductase, 1 nmol of cytochrome c reduced/min at 30 °C.

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Fig. 5. Effects of lovastatin on incorporation of gp70env and p30env into viruses shed by MEL cells. Parallel cultures containing 1.6 x 10^6 cells were incubated with (+) or without (−) 1 μM lovastatin for 21 h, and proteins were labeled with [35S]methionine for 6 h. The indicated proteins were immunoprecipitated from viral particles collected from the culture medium and subjected to SDS-PAGE and fluorography (10-day exposure). Molecular masses of standard proteins run in adjacent lanes of the gel are indicated at the right of the fluorogram.

Fig. 6. Inhibition of isoprenoid synthesis alters the subcellular distribution of rab proteins in MEL cells. Immunoblot assays for detection of rab1p and rab6p were performed on aliquots of soluble (S) and particulate (P) fractions obtained from identical parallel cultures that had been incubated with (+) or without (−) 1 μM lovastatin for 6 or 24 h. At 6 h the entire S and P protein fractions were loaded on the gel. At 24 h, one-third of the total protein in each fraction was loaded. The panels show regions of the blots between the 21.5- (bottom) and 31-kDa (top) marker proteins. Exposure times for the rab1p and rab6p blots were 24 and 27 h, respectively. For each culture, the ratio of immunodetectable protein in S vs. P was determined by densitometry. The lower band in the P fractions immunoblotted with antiserum to rab6p appears to be nonspecific and was therefore excluded from the calculation.

MuLV gPr90^env might cause alterations in the subcellular distributions of ER- and Golgi-associated rab proteins in MEL cells. As shown in Fig. 6, approximately 75% of the total rab1p in untreated MEL cells was found in the soluble fraction at 6 h, with the proportion of soluble rab1p declining to 51% at 24 h. The distribution of rab6p in untreated cells was noticeably different at 6 h, with most of the protein localized in the particulate fraction. However, by 24 h the distribution of rab6p in untreated cells resembled that of rab1p, with approximately 40% of the immunodetectable protein in the soluble fraction. The cause of these time-dependent shifts in rab protein distribution in untreated MEL cells is presently unclear. Nevertheless, when MEL cells that had been treated with 1 μM lovastatin for 24 h were compared to the controls, striking increases in the ratios of soluble versus particulate rab1p and rab6p were clearly evident (Fig. 6). It is particularly noteworthy that elevations in the relative amounts of soluble rab protein were discernible within 6 h after addition of 1 μM lovastatin (Fig. 6), well before changes in MuLV gPr90^env processing were observed (Fig. 1).

When the cytosols from control and lovastatin-treated cells were partitioned into aqueous and detergent phases (Fig. 7), nearly all of the cytosolic rab1p from control cells partitioned with the detergent phase, consistent with the behavior of other isoprenylated proteins (27–29). However, in cells treated with lovastatin for 6 h, 73% of the cytosolic rab1p partitioned with the aqueous phase (Fig. 7), suggesting that the increase in the amount of cytosolic rab1p (see Fig. 6) may be accounted for by accumulation of rab1p that is not modified by hydrophobic isoprenyl groups.

Discussion

The results of the present study demonstrate that inhibition of MVA synthesis in MEL cells prevents the proteolytic processing of MuLV gPr90^env to gp70^env, which normally occurs within the Golgi apparatus. Under these conditions, newly synthesized envelope proteins are not incorporated into viral particles that are shed into the culture medium (Fig. 5), and the oligosaccharide chains on gPr90^env fail to undergo Golgi-dependent maturation (Fig. 3). Thus, MVA synthesis appears to be required for early events in the exocytic transport of viral envelope proteins in MEL cells.

Because MVA serves as a precursor for a number of isoprenoid products (14–16), it is not yet possible to define the precise molecular basis for the effect of lovastatin on viral envelope protein transport and processing. Nevertheless, several observations suggest that a direct causal link to depletion of isoprenoids such as cholesterol, ubiquinone, or dolichol is unlikely. First, lovastatin-treated cells grown in medium containing fetal calf serum are able to maintain a normal sterol/phospholipid ratio (37), presumably because they can compensate for diminished endogenous cholesterol synthesis by receptor-mediated uptake of sterol-rich low density lipoprotein (39). Second, although ubiquinone levels have been shown to fall by 50% in cultured cells treated with 25 μM lovastatin for 24 h, this decline does not impair mitochondrial oxidative function (40). Finally, in the lovastatin-treated cells, both the MuLV gPr90^env and SFFV gp55^env were metabolically labeled...
with [3H]mannose to an extent comparable to that obtained in controls (not shown), and their electrophoretic mobilities did not increase as they do when glycosylation is blocked with tunicamycin (30). Therefore, we conclude that the available pool of dolichol phosphates was adequate to support the addition of high mannose oligosaccharides to these proteins.

Several studies have established that when MVA synthesis is blocked by lovastatin at concentrations ranging from 1 to 25 μM, cultured cells are unable to generate sufficient amounts of farnesyl- or geranylgeranylpyrophosphates to sustain the isoprenylation of proteins (18, 22, 41, 42). Among the proteins currently known to undergo isoprenoid modification in mammalian cells, the members of the rab protein family have been most clearly implicated as potential mediators of intracellular protein trafficking. For example, previous work has shown that rab1Bp can functionally replace YPT1p in the yeast exocytic pathway (3) and that monoclonal antibodies to rab1Bp can disrupt ER to Golgi transport of vesicular stomatitis virus G-protein in semi-intact cells (7). Rab6p, by virtue of its localization in the medial Golgi, has been suggested as a possible regulator of transport through the Golgi membrane stack (43). Thus, although suppression of isoprenoid synthesis with lovastatin may affect isoprenylation of many proteins, it is reasonable to speculate that the arrest of MuLV GPr90™ processing could be closely linked to inhibition of geranylgeranylation of rab proteins in the ER or Golgi compartments. Our observations concerning the subcellular distribution of rab1Bp and rab6Bp in MEL cells (Fig. 6) lend support to the foregoing hypothesis. For example, in untreated MEL cells these rab proteins were found in both the particulate and cytosolic fractions, in accord with a model depicting the reversible association of GTPase proteins with vesicular membranes during the transport cycle (44). However, when isoprenoid synthesis was blocked with lovastatin, the relative amounts of rab1Bp and rab6Bp in the cytosol increased dramatically during the hours preceding arrest of GPr90™ processing. In the case of rab1Bp, the results of the Triton X-114 partitioning studies suggest that most of the additional immunoreactive rab proteins in the cytosol of lovastatin-treated cells is not isoprenylated. Along with the changes in cytosolic rab proteins, the net amounts of rab1Bp and rab6Bp in the particulate fraction declined by 60–75% within 24 h after addition of lovastatin (Fig. 6), possibly due to turnover of the preexisting geranylgeranylated proteins. Therefore, it is possible that the arrest of GPr90™ processing is precipitated by a decline in the concentration of rab1Bp in vesicular membranes below a threshold needed to support protein transport to the Golgi compartment.

The ability of cytosolic rab1Bp from untreated MEL cells to partition into Triton X-114 (Fig. 7) suggests that this protein is isoprenylated but is not firmly anchored to intracellular membranes. In this regard, the behavior of rab1Bp is similar to that of many other low molecular mass GTP-binding proteins which exhibit dual localization in cytosol and membrane fractions even though their isoprenylation can be demonstrated by metabolic labeling with [3H]MVA (15, 22). These findings raise the possibility that membrane association of rab proteins and related members of the ras superfamily may involve specific protein-protein interactions instead of, or in addition to, direct insertion of the carboxyl-terminal isoprenoids into the membrane lipid bilayer. In support of this concept, Takai and coworkers have recently reported that carboxyl-terminal geranylgeranylated-modified cysteines are required for the interactions of rap1Bp with smg921B (45), rhoA (46), and smg25A/rab6A (47) proteins with GDI or GDS proteins that regulate guanine nucleotide dissociation and promote interactions with membranes in vitro. If rab proteins interact with such proteins in intact cells, it is conceivable that nonisoprenylated rab proteins are functionally disabled as a result of an inability to exchange GDP for GTP. In any case, the role of the carboxyl-terminal geranylgeranyl groups in facilitating associations between rab proteins and other components of the vesicular transport apparatus promises to be an important area for future study.

The observation that blocking isoprenoid synthesis in MEL cells impairs their ability to incorporate newly synthesized envelope proteins into virus particles (Fig. 5) is particularly intriguing when considered in light of a report that an envelope-deficient MuLV virus produced by cells harboring a mutant proviral clone is significantly less infectious than the wild-type virus (48). Thus, an important direction for future research will be to determine whether inhibition of GPr90™ processing in lovastatin-treated cells eventually becomes a limiting factor for production of infectious virus, once the intracellular pool of preexisting gp70™ is depleted.

The general features of MuLV GPr90™ processing resemble those reported for other retroviral envelope proteins, including those encoded by human T-cell leukemia virus type I (49) and human immunodeficiency virus type 1 (HIV-1) (9). For example, the HIV envelope protein precursor, gp160, is translocated from the ER to the Golgi apparatus, where proteolytic processing yields a disulfide-linked complex of gp120 and a carboxyl-terminal gp41 fragment (reviewed in Ref. 9). Similarities in the amino acid sequences at the cleavage sites of the MuLV and HIV envelope precursors further suggest that related host cell proteases may be involved in processing these glycoproteins (50). Therefore, it will be of considerable interest to determine whether inhibition of the isoprenylation of proteins in general, and the rab proteins in particular, may prevent the proteolytic maturation of the HIV envelope protein.

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Isoprenoids and Viral Envelope Protein Processing


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