Evidence for Post-translational Incorporation of a Product of Mevalonic Acid into Swiss 3T3 Cell Proteins*

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Previous studies have identified several cellular requirements for mevalonic acid that appear unrelated to cholesterol, dolichol, or ubiquinone. To search for other products of mevalonic acid that might account for these requirements we cultured Swiss 3T3 cells in the presence of mevinolin, an inhibitor of mevalonic acid biosynthesis, then labeled the cells with exogenous radioactive mevalonic acid. Upon analyzing the radioactive material formed, we found that 40–50% of it was not extractable into lipid solvents, and that most of the lipid-insoluble material behaved like protein when treated with sodium dodecyl sulfate:chloroform:phenol, RNase, or proteinase K. Further analysis by electrophoresis revealed that radioactivity was associated with a few specific proteins that had apparent molecular weights of 13,000–58,000. Control experiments indicated that authentic radioactive (R)-mevalonic acid was the active precursor. Other lines of evidence suggested that mevalonate was first converted to an isoprenoid compound, then covalently incorporated into proteins by way of a cycloheximide-insensitive mechanism. These results suggest that Swiss 3T3 cells possess novel metabolic products of mevalonic acid metabolism that are formed by post-translational incorporation of isoprenoids into specific cell proteins.

Compactin and mevinolin are competitive inhibitors of HMG-CoA reductase that can effectively block the biosynthesis of MVA in cultured cells. Compactin, in particular, has recently demonstrated that MVA is required independently of cholesterol for maintenance of the cell shape of Swiss 3T3 cells. For each of these three requirements the unidentified active products of MVA metabolism are likely to be quantitatively minor cell constituents. When cholesterol is present in the cell medium, none of the requirements become manifest until sufficiently high concentrations of compactin or mevinolin are added to inhibit residual MVA biosynthesis almost completely. Then, uptake of tiny amounts of exogenous MVA suffices to overcome the effects of the inhibitors.

To search for the active products we studied the metabolic fate of radioactive MVA under culture conditions that were chosen to detect relevant, quantitatively minor products. We cultured large quantities of Swiss 3T3 cells in the presence of cholesterol and amounts of mevinolin sufficient to induce MVA-deficient cell rounding. We then added concentrations of radioactive MVA that we previously found were barely sufficient to prevent (50–100 μM) or reverse (600 μM) the shape change. Upon analyzing the intracellular products that were formed, we obtained evidence for what appears to be a hitherto undescribed pathway of MVA metabolism in mammals (8).

MATERIALS AND METHODS

Except as noted, all radioisotopes were obtained from New England Nuclear and all reagents and enzymes from Sigma. (R)-[1-3H]Mevalonic acid (10 mCi/mmol) was a generous gift from Dr. J. Watson (University of California, San Francisco). Mevinolin was the kind gift of Dr. A. Alberts (Merck, Sharp & Dohme Research Laboratories, Rahway, NJ) and was converted to its Na+ salt before use (2). PBS without Ca2+ and Mg2+ was prepared by the method of Dubeczko (9). Tissue culture reagents were from Gibco Laboratories (Grand Island, NY).

Cell Culture—Mass cultures of Swiss 3T3 cells (2) were obtained by inoculating approximately 2 × 106 cells into 850 cm2 roller bottles (Corning Scientific Products, Corning, NY) in 100 ml of Dulbecco’s modified Eagle’s medium containing 10% calf serum, 30 μM mevinolin, and 67 μM unlabeled MVA. After 24 h the cells were washed twice with cold PBS, then resuspended in PBS containing 1.6 mM phenylmethylsulfonyl fluoride, 4.5 mM diisopropyl fluorophosphate, 2.4 mM EDTA, 2.4 mM EGTA at 37 °C for 60 min. Cells from half a roller bottle were then sequentially extracted three times each with 5 ml of cold acetone, 5 ml of cold chloroform:methanol (2:1), and twice with 5 ml of cold 96% EtOH. The delipidated residues were dissolved.

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‡ The abbreviations used are: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.34); MVA, mevalonic acid; PBS, phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate.
in 2.2 ml of 1.4% SDS, 0.1 M NaCl, 10 mM EDTA, 50 mM Tris, pH 7.4, and extracted with phenol and CHCl₃ according to the method of Penman (10). Macromolecules were precipitated from the final aqueous phase with 2 volumes of 95% EtOH (>95% efficiency). CHCl₃ was evaporated from the pooled organic phases and macromolecules precipitated by adding 5 volumes of 95% EtOH (>95% efficiency). All samples were treated with 0.1% diethyl pyrocarbonate and autoclaved before use, and all glassware was heated to 110 °C for at least 1 h prior to use.

The preceding delipidation procedure appears to be complete with respect to MVA-labeled lipids because further extractions with either the same solvents or with CHCl₃methanol/water (10:10) failed to release significant amounts of MVA-derived radioactivity. At least 95% of cellular cholesterol was extracted by this procedure (4). The labeled lipids have not all been identified but approximately 50% of the acetone-soluble material was in a non-saponifiable lipid that co-migrated with cholesterol during thin-layer chromatography on Silica Gel H in heptane-ether-methanol (96:9:15) (4) while 10-30% behaved as MVA or more polar lipids (not shown).

In other experiments we incubated confluent cultures with 30 μM mevinolin in fresh medium for 24 h, then scraped the cells directly into the medium. Aliquots of cells were spun down, resuspended in a small volume of mevalonol-containing medium, and incubated with [3H]MVA (200 μCi/ml) for 3 h at 37 °C in a 5% CO₂ atmosphere with occasional agitation. The labeled cells were washed twice with PBS, incubated with protease inhibitors, extracted three times with 2 ml of acetic acid, then dissolved in electrophoresis sample buffer.

Partial Purification of [3H]MVA on DEAE-cellulose—For competition experiments we partially purified radioactive and non-radioactive mevalonate on DEAE-cellulose (11). (RS)-[5-3H]MVA was loaded on a column (0.9 x 30 cm) of acid base washed DEAE-cellulose (CO₂ form) in 1 mM ammonium carbonate, pH 8. After washes with 1 mM ammonium carbonate and distilled PBS (1:200 in buffer), [3H]MVA was eluted with full strength PBS containing penicillin-streptomycin and stored at -75 °C. Non-labeled MVA was spiked with trace amounts of [3H]MVA, then DEAE-purified in the same way.

Electrophoresis and Fluorography of Radioactive Cell Material—Samples from equal numbers of cells (approximately 160 μg of protein/gel lane) were dissolved in electrophoresis sample buffer containing 5% 2-mercaptoethanol, 2 M urea, and 2% SDS, heated at 100 °C for 10 min, and subjected to electrophoresis in 0.1% SDS, 2 M urea through a 1.5 mm thick 12.5% polyacrylamide slab gel with a 45,000-Da band were due to an effect of N-ethylmaleimide which was investigated with t3H]MVA (200 pCi/ml) for 0.1 M Tris, pH 8 (pretreated with diethyl pyrocarbonate), for 1 h at 37 °C. Remaining macromolecules were precipitated with 2 ml of cold 95% EtOH, then dissolved in electrophoresis sample buffer.

Similar samples were suspended in 200 μl of 50 mM Tris, pH 7.4 (pretreated with diethyl pyrocarbonate), sonicated briefly in a sonicating bath, then incubated with or without 20 μg of RNase A (Calbiochem-Behring) in the same buffer (previously boiled for 10 min) at 37 °C for 60 min. Remaining macromolecules were precipitated with 2 ml of cold 95% EtOH and redissolved in electrophoresis sample buffer.

Extensive Proteolysis of MVA-labeled Proteins and Subsequent Purification—MVA-labeled proteins from the organic phase of the SDS/CHCl₃phenol extraction were suspended in 50 mM Tris acetate, 3 M urea, 5% CHCl₃ (final concentration), 16% CHCl₃, and 3 HCl. Containing 0.5 μg/ml of [3H]MVA (50 μCi/ml) then analyzed the radioactive intracellular products that had been formed. We found typically (Table I) that about half of the cell-associated radioactivity could not be extracted with lipid solvents. By this criteria, most of the non-lipid radioactivity seemed to be associated with protein rather than RNA (19). First, when we employed an SDS: CHCl₃:phenol extraction technique to separate nucleic acids from proteins, we found (Table I) that about 80% of the non-lipid radioactivity was soluble in the protein-containing SDS:CHCl₃:phenol phase, whereas only 16% of the radioac-

### RESULTS

**Evidence That MVA or One of Its Products Is Incorporated into Specific 3T3 Cell Proteins—**In three separate experiments we incubated roller bottle cultures of Swiss 3T3 cells for 24-25 h in the presence of 17 μM mevinolin and 50 μM [3H]MVA (5 μCi/ml), then analyzed the radioactive intracellular products that had been formed. We found typically (Table I) that about half of the cell-associated radioactivity could not be extracted with lipid solvents. By this criteria, most of the non-lipid radioactivity seemed to be associated with protein rather than RNA (19). First, when we employed an SDS: CHCl₃:phenol extraction technique to separate nucleic acids from proteins, we found (Table I) that about 80% of the non-lipid radioactivity was soluble in the protein-containing SDS:CHCl₃:phenol phase, whereas only 16% of the radioac-

### Table I

| Component | Acetonate extracts | CHCl₃MeOH (2:1) extracts | Ethanol extracts | Phenol extraction | Aqueous phase | Organic phase |
|-----------|-------------------|--------------------------|-----------------|-------------------|---------------|--------------|
| MVA       | 51.1              | 5.5                      | 0.3             | 8.5               | 42.0          | 108.1        |
| Uridine   | 1.0               | 1.3                      | 2.0             | 9.2               | 83.5 × 0.9    | 98.0 × 0.9   |
| Leucine   | 9.9 × 0.9          | 22.7 × 0.9               | 20.0 × 0.9      | 99.1 × 0.9        | 89.0 × 0.9    | 118.8        |

*Initial cell-associated radioactivity for MVA-, uridine-, and leucine-labeled cells was 846,930, 385,570, and 894,400 cpm, respectively.

*Numbers refer to percent recovery of non-lipid radioactivity in the two phases of the phenol extraction.
tivity was soluble in the RNA-containing aqueous phase. This distribution of radioactivity was similar to that obtained when corresponding non-lipid material from [3H]leucine-labeled control cells was fractionated by the same technique, but differed dramatically from that obtained using comparable [3H]uridine-labeled material (Table I).

Second, SDS-gel electrophoresis revealed that the MVA-derived non-lipid radioactivity was associated with discrete macromolecules (Fig. 1). Most labeled macromolecules were present primarily or exclusively in the organic phase, although prominent bands of less than 14 kDa were found only in the organic phase, although demonstrated by gel slicing and counting, was in macromolecules that had apparent $M_n = 20,000-30,000$. Individual leucine-labeled bands from control cells also were found primarily in the organic phase, but most of them were macromolecules larger than 30 kDa.

Third, MVA-labeled macromolecules were degraded under conditions of selective proteolysis. In three experiments, MVA-labeled macromolecules from both the aqueous and organic phases were completely hydrolyzed by proteinase K in 1% SDS, but not by boiled RNase (not shown). The sole exception was the broad MVA-labeled band of approximately 14 kDa that appeared to be completely resistant to proteinase K. Control digestions of leucine and uridine-labeled material showed that the digestion conditions were completely selective for protein and RNA. Taken together, these results strongly suggest that most of MVA-derived non-lipid radioactivity is tightly linked to protein.

A minor fraction of the MVA-labeled, non-lipid material, however, probably consisted of isopentenylated tRNA. We found previously (20) that some MVA-labeled, aqueous phase material co-migrates with tRNA in an electrophoretic system for protein and RNA. Eighty percent of the organic phase label, as determined by gel slicing and counting, was in macromolecules larger than 30 kDa.

Evidence That the Radioactivity Incorporated into 3T3 Cell Protein Is Derived from Authentic Labeled (R)-MVA—Although the experiments described above provided strong evidence that much of the cell-associated radioactivity in our experiments was present in protein, it remained to be demonstrated that this radioactivity was actually derived from radioactive MVA. To exclude the possibility that a radioactive contaminant in the preparations of [3H]MVA might have labeled the proteins, we labeled the proteins with two different radioisomers of MVA that had been synthesized from different starting materials. Whenever we added either [2,14C]MVA or [5-3H]MVA to separate cell cultures, we always found labeled proteins of similar molecular weights, and in two experiments where we concomitantly added them to the same cell culture we found the same ratio of $^3$H to $^{14}$C in delipidated, SDS:CHCl$\_3$:phenol-extracted cell proteins of 20–30 kDa as in lipids that co-migrated with cholesterol and ubiquinone on thin-layer chromatography (not shown). Moreover, the naturally occurring (R)-stereoisomer of MVA appeared to be active because similar proteins were labeled with (R)-[3$^3$H]MVA as with either (RS)-[5-3H] or (RS)-[2,14C]MVA (not shown). These results strongly suggest that authentic radioactive (R)-MVA is indeed the source of the labeled moiety that becomes incorporated into 3T3 cell proteins.

Using a modified labeling system we obtained evidence suggesting that unlabeled MVA can compete with radioactive MVA for incorporation into cell proteins whether the unlabeled MVA is derived from endogenous or exogenous sources. Thus, when we incubated cells with or without mevinolin for 24 h, then labeled them with radioactive MVA and examined the proteins by SDS gel electrophoresis, we found (Fig. 2) detectable radioactivity in macromolecules only in the cells that had been pretreated with mevinolin. In the same experiment we also added increasing concentrations of unlabeled MVA to the culture medium of mevinolin-pretreated cells at the same time that we added radioactive MVA, and found that concentrations of unlabeled MVA greater than 100 $\mu$M competitively prevented the incorporation of radioactivity into cell protein. Below 100 $\mu$M unlabeled MVA, the amount of protein-bound radioactivity was unaffected, presumably because insufficient MVA was taken up by the cells during the relatively short labeling period to fill all potential protein acceptor sites.

**Evidence That Radioactivity from MVA Is Incorporated into Proteins by a Post-translational Mechanism**—We tested the abilities of cycloheximide (12 $\mu$g/ml) and chloramphenicol (60 or 300 $\mu$g/ml) to prevent mevinolin-treated cells from incorporating radioactive MVA into proteins. Neither inhibitor affected the incorporation of labeled MVA into cell proteins (Fig. 3) even though cycloheximide dramatically reduced the incorporation of labeled leucine into the proteins of control cells (not shown). Similar results were obtained both times the experiment was repeated. Cycloheximide had no effect on the conversion of labeled MVA into lipids which co-migrated with cholesterol, cholesteryl oleate, dolichol, or ubiquinone during thin-layer chromatography in heptane:ether:methanol (90:9:15) (not shown). Thus, it appears that the labeling of...
cell proteins by MVA occurs by a post-translational mechanism rather than a pre- or co-translational one.

Partial Purification of Proteolytic Fragments of MVA-labeled Proteins—We have begun to purify proteolytic fragments of MVA-labeled proteins in order to determine the structure of the MVA-derived moiety and its link to protein. In order to monitor our purification we first prepared delipidated, phenol-extracted proteins that had been labeled either with \(^{14}C\)MVA (as in Table I) or with \(^3H\)-amino acids (by growing cells for 4 days in medium that contained either labeled phenylalanine, tyrosine, tryptophan, cysteine or a labeled algal protein hydrolysate). After separately incubating these proteins with Pronase for 3 days, we found in eight experiments that 96–99% of the \(^3H\)-labeled material became water-soluble whereas 88–92% of \(^14C\)-labeled material remained insoluble. By centrifuging the hydrolysate and decanting the supernatant we thus effected an approximately 30-fold enrichment of the MVA-labeled material. We then dissolved the insoluble residues in SDS, pooled them, and further incubated them with proteinase K and carboxypeptidase Y. Although these digestions had little effect on MVA-labeled material, \(^3H\)-labeled material was degraded further (not shown). By passing the final hydrolysate over an AG 1-X2 (formate) column in 1 M ammonium formate, we separated water-soluble fragments (which washed through the column) from SDS and MVA-labeled material (which bound). We then eluted MVA-labeled products (substantially free of SDS) with formic acid:EtOH (1:4). In seven experiments, this step caused a further 2.2–15-fold purification. Analytic chromatography of the MVA-labeled eluate over Sephadex LH-20 in formic acid:EtOH (1:4) revealed that it contained at least two major components with apparent sizes of 1000 and 500 Da (Fig. 4A). By preparative LH-20 chromatography we separated these components from each other (Fig. 4B) and simultaneously achieved an additional 3-fold purification. The MVA-labeled material in the resulting pools showed an overall enrichment of 400–750-fold, with recoveries of up to 70%. Nevertheless, it still appeared to contain substantial amounts of other fragments that were prelabeled by the amino acids (not shown).

Both the material of 1000 Da and that of 500 Da were extremely hydrophobic and even insoluble in 6 M guanidine hydrochloride. They also were almost insoluble in hexane but appeared to be very soluble in acidic polar organic solvents. Upon being chromatographed on thin-layer plates of silicic acid in solvent systems for neutral lipids (4, 22), they remained at the origin, unlike cholesterol, dolichol, and ubiquinone. On the other hand, they migrated near the solvent front in a phospholipid solvent system (23). These properties, and the results of our other work, are consistent with the possibility that the MVA-labeled, proteolytic fragments are small, hydrophobic peptides that contain polyisoprenoid side chains.

**DISCUSSION**

In this investigation we studied the metabolic fate of radioactive MVA in mevinolin-treated cultures of 3T3 cells and...
obtained results strongly suggesting that a product of authentic (R)-MVA was converted to a protein-bound form. Since no protein-bound products of MVA in mammalian cells have been described previously, several important questions arise. Could the labeled proteins be experimental artifacts? In what form is the radioactivity bound to protein? Are the modified proteins likely to be biologically important?

We considered two potential artifacts: 1) spurious labeling of proteins by a radiolabeled contaminant, and 2) protein "labeling" as a result of nonspecific complex formation between proteins and labeled lipids. Our experiments provided strong evidence that authentic (R)-MVA serves as the active precursor (see "Results"). Moreover, they made it seem very unlikely that the MVA-labeled proteins represent noncovalent complexes of unlabeled proteins with either MVA or labeled isoprenoids (including isopentenylated tRNA). Thus, the radioactive products released by extensive proteolytic digestion clearly differed from MVA during chromatography on Sephadex LH-20, and also behaved differently from cholesterol, dolichol, ubiquinone, and cholesteryl oleate during thin-layer chromatography. Furthermore, we were not able to separate the radioactive moiety from proteins using any of the following methods: extraction with organic solvents, extraction with SDS:CHCl₃:phenol, SDS-gel electrophoresis, or chromatography in formic acid:EtOH (1:4). We were also unable to liberate the radioactivity using extractions for farnesyl-containing heme (24-26) or solvents that disrupt tight, noncovalent binding of polyphosphorylated lipids to proteins (27). These results strongly suggest, therefore, that the MVA-derived moiety is covalently linked to protein. Since, to the best of our knowledge, this type of linkage has never been described in mammalian cells, it seems likely that MVA-labeled proteins represent novel products of MVA metabolism, either known products linked in novel ways or entirely novel metabolites of MVA.

Radioactivity in MVA-labeled proteins might have reflected the presence of MVA itself, a product of a degradative pathway such as the transmethylgluconurate shunt, or an isoprenoid product of MVA. One type of experiment strongly suggests, however, that radiolabeled MVA was not involved. When we incubated mevinolin-treated 3T3 cells with either [1-¹⁴C] or [2-¹⁴C]MVA for 24 h then measured radioactivity in proteins by electrophoresis and fluorography, we found that 1-¹⁴C was incorporated into proteins at least 91-fold less efficiently than 2-¹⁴C.² Because C₁ is selectively lost from MVA during the formation of isopentenyl pyrophosphate, we postulate the MVA-labeled proteins are actually labeled by either isopentenyl pyrophosphate or one of its metabolic products.

Our results make it seem unlikely that labeled isoprenoid pyrophosphates are first degraded to smaller labeled intermediates, then incorporated into proteins. Most importantly, 5-H, 3-¹⁴C-, and 2-¹⁴C-labeled MVA were each incorporated into the same proteins and [2-¹⁴C]- and [5-³H]MVA, when added to cells concomitantly, were both incorporated into proteins in the same ratio as into lipids. This strongly suggests that the bonds between the labeled atoms were not cleaved during the conversion of labeled MVA to labeled proteins. It also specifically argues against a role of the transmethylgluconurate shunt (28) because the shunt converts [5-³H]- and [2-¹⁴C]MVA to labeled acetyl-CoA and acetoacetate, respectively. In addition, the conversion of labeled MVA to long chain fatty acids by the shunt is not a quantitatively important pathway under our culture conditions. Less than 2.5% of the radioactivity in the lipid or protein extracts of MVA-labeled cells was in a saponifiable form that co-migrated with fatty acids in a neutral lipid chromatography system.³ Taken together, these results strongly suggest that the fragment of MVA that is incorporated into proteins is not an end product of the shunt pathway or any other degradative pathway. On the other hand, they do not rule out the possibility that labeled proteins are formed from an early intermediate in the shunt (e.g. dimethyl acrylic acid).

A final possibility is that the MVA-labeled proteins contain isoprenoid products of MVA. At least three mechanisms can be envisioned whereby an isoprenoid compound might become covalently bound to protein. In one, a cysteine residue might condense across the double bond in isoprenoids, as may occur in feline (29). In a second, isoprenoid fatty acids such as have been found in bovine retinas (30) might be linked to proteins through amide or ester bonds, although the latter

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1 R. A. Schmidt, C. J. Schneider, and J. Glomset, unpublished observations.
2 R. A. Schmidt, C. J. Schneider, and J. Glomset, unpublished results.
proteins are related to cellular requirements for MVA such as labeled protein contained within 3T3 cells (see below) and we account for the biosynthesis of isopentenyl adenosine (32), which then couples to electron-rich sites in proteins via its C, carbon atom. The latter mechanism is thought to underlie the polymerization of isoprenoids (reviewed in Ref. 31), and may account for the turnover of isopentenyl adenosine (32), dimethylallyl tryptophan (33), heme a (34), and yeast peptidal sex hormones (35). In order to choose among these mechanisms, we shall need to determine the structure of the MVA-derived moiety and its linkage to protein from fragments of MVA-labeled proteins. We have made progress toward this goal and have purified labeled proteolytic fragments of 3T3 cell proteins by approximately 400–750-fold. This approach has been limited, however, by the small amount of MVA-labeled protein contained within 3T3 cells (see below) and we are currently investigating alternative starting materials that contain more modified protein.

Three arguments raise the possibility that MVA-modified proteins are related to cellular requirements for MVA such as DNA synthesis, shape control, and HMG-CoA reductase regulation. First, appropriate amounts of MVA-labeled protein are present within 3T3 cells. Thus, when we cultured 3T3 cells for four population doublings in the presence of 32 μM compactin and 92 μM radiolabeled MVA, we calculated the total protein-bound label to be equivalent to 265 × 10^-19 mol of MVA per cell (approximately 265 pmol of MVA/mg of cell protein). This estimate is quite similar to our previous finding (4) that the uptake of as little as 250 × 10^-18 mol of MVA/cell is sufficient to reverse the cellular shape change due to MVA deficiency. Second, a major fraction (26–46% in five experiments) of the MVA that partially prevents cell rounding in the roller bottle culture system is converted ultimately to protein-bound form. Finally, unpublished results in our PDGF-stimulated cell culture system (2, 4) indicate that MVA-labeled proteins are major metabolic products of MVA that turn over very slowly but nonetheless become deficient at the same time that mevinolin-treated cells change shape and are prevented from synthesizing DNA. Taken together, these results support the existence of MVA-labeled proteins as important novel metabolic products of MVA. Future studies into the identity of the individual labeled proteins, their function within cells, and their relation to cellular requirements for MVA will be of great interest.

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