Compartmentalization of Cholesterol Biosynthesis

CONVERSION OF MEVALONATE TO FARNESYL DIPHOSPHATE OCCURS IN THE PEROXISOMES*

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Lenka Biardi and Skaidrite K. Krisans‡

From the Department of Biology, San Diego State University, San Diego, California 92182

We have recently demonstrated that mevalonate kinase and farnesyl diphosphate (FPP) synthase are localized predominantly in peroxisomes. This observation raises the question regarding the subcellular localization of the enzymes that catalyze the individual steps in the pathway between mevalonate kinase and FPP synthase (phosphomevalonate kinase, mevalonate diphosphate decarboxylase, and isopentenyl diphosphate isomerase). These enzyme are found in the 100,000 x g supernatant fraction of cells or tissues and have been considered to be cytoplasmic proteins. In the current studies, we show that the activities of mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase are equal in extracts prepared from intact cells and selectively permeabilized cells, which lack cytosolic enzymes. We also demonstrate structure-linked latency of phosphomevalonate kinase and mevalonate diphosphate decarboxylase that is consistent with a peroxisomal localization of these enzymes. Finally, we show that cholesterol biosynthesis from mevalonate can occur in selectively permeabilized cells lacking cytosolic components. These results suggest that the peroxisome is the major site of the synthesis of FPP from mevalonate, since all of the cholesterogenic enzymes involved in this conversion are localized in the peroxisome.

Recently, it has been demonstrated by our group and others that peroxisomes contain a number of enzymes involved in cholesterol biosynthesis that previously were considered to be cytosolic or located exclusively in the endoplasmic reticulum. Peroxisomes have been shown to contain acetooxyethyl-CoA thiolase (1, 2), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase (3), HMG-CoA reductase (4–6), mevalonate kinase (7, 8), and most recently farnesyl diphosphate (FPP) synthase (9). Both mevalonate kinase and FPP synthase seem to be localized predominantly, if not exclusively, to peroxisomes (8, 9).

The demonstration that mevalonate kinase and FPP synthase are localized predominantly in peroxisomes (8, 9) raises the question regarding the localization of the enzymes that catalyze the individual steps in the pathway between mevalonate kinase and FPP synthase (phosphomevalonate kinase, mevalonate diphosphate decarboxylase, and isopentenyl diphosphate isomerase). Based on results obtained from fractionation studies, these enzymes are believed to be localized in the cytosol. However, recent data have shown that the activities of these enzymes as well as mevalonate kinase and FPP synthase are significantly reduced in liver tissue obtained from patients with peroxisome-deficient diseases (Zellweger syndrome and neonatal adrenoleukodystrophy), thus indicating a peroxisomal localization (9).

We have routinely employed three different methods to study subcellular localization of proteins: (i) analytical subcellular fractionation and measurements of enzyme activities, (ii) immunoblotting of the protein in the isolated fractions with a monospecific antibody, and (iii) immunoelectron and immunofluorescence microscopy. In the studies demonstrating the peroxisomal localization of mevalonate kinase and FPP synthase (8, 9), it was shown that analytical subcellular fractionation of liver and measurements of enzyme activities are not sufficient to determine intracellular localization due to the release of these enzymes in the cytosolic fraction from peroxisomes during the isolation of the organelle. Immunochemistry and immunofluorescence microscopy studies with specific antibodies to these enzymes were critical in determining the correct subcellular localization. To our knowledge antibodies to phosphomevalonate kinase, mevalonate diphosphate decarboxylase, and isopentenyl diphosphate isomerase are not currently available. Hence, in order to study the subcellular localization of these enzymes, we have selected to use permeabilized cells, which retain their organelle integrity yet lack cytosolic components. Permeabilized cells have been used successfully in a number of different studies dealing with subcellular function and localization.

EXPERIMENTAL PROCEDURES

Materials—Biochemicals were purchased from Sigma. (RS)-[5-3H]Mevalonic acid, (R)-[2-14C]mevalonic acid-5-phosphate, (R)-[5-3H]mevalonic acid-5-diphosphate, [1-14C]isopentenyl diphosphate, [1-3H]farnesyl diphosphate, [26–14C]cholesterol, and (R)-mevalonic acid-5-phosphate were purchased from American Radiolabeled Chemicals Inc. AG1-X8 200–400 mesh formate resin was purchased from Bio-Rad. All cell culture media and sera were purchased from Life Technologies, Inc. Monkey kidney cells (CV-1) were obtained from American Type Culture Collection.

Cell Permeabilization—CV-1 cells were seeded at a density of 4.0 x 10⁴ on 60-mm plates and grown to 70% confluence in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Care was taken to seed cells in a manner that resulted in all plates per given experiment to have the same number of cells. This was important since enzyme activities and rates of cholesterol synthesis were determined per plate. As indicated in individual experiments, cells were transferred to media containing lipoprotein-deficient serum 24 h prior to the experiment. The day of the experiment, the media was aspirated off the plates and the plates were then washed twice with ice cold KH buffer (50 m M HEPES, 110 m M KOAc, pH 7.2). The plates were then transferred to ice and the cells were incubated for 5 min in KH buffer containing 20 μM digitonin, 20 μM HEPES, 110 m M KOAc, and 2 m M MgOAc, pH 7.2. The digitonin solution was then aspirated off the cells,
and the cells were washed twice with ice-cold KH buffer and subsequently allowed to incubate in KH buffer for 30 min (10). This procedure allows the cells to remain attached to the plates. Control cells were treated similarly and washed with the same buffer lacking digitonin.

Measurement of Cholesterol Synthesis—CV-1 cells were grown to 80% confluence on 160-mm plates in standard media, and transferred to mevalonate-containing isoprenoid-deficient medium 24 h prior to use. The day of the experiment, the cells were permeabilized as described and each plate was washed with 3 ml of reaction buffer. The reaction buffer consisted of the following: 100 mM phosphate buffer, 4 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, pH 7.4. Control cells were washed with 50 ml of 0.4 M ammonium formate in 4 N formic acid and subsequently with 50 ml of 0.8 M ammonium formate in 4 N formic acid to elute first the substrate and subsequently with 50 ml of 0.8 M ammonium formate in 4 N formic acid to elute the phosphorylated products (9, 14).

Mevalonate Kinase Assay—The reaction buffer consisted of the following: 100 mM phosphate buffer, 4 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, pH 7.4. (R)-[5-3H]Mevalonic acid was added at a specific activity of 625 dpm/nmol and reactions were run on tissue culture plates by adding 200 µl of reaction buffer containing 50,000 dpm of substrate and incubating at 37°C for 10, 20, or 30 min. Reactions were terminated by scraping cells into Eppendorf tubes and boiling the sample for 3 min. [1-14C]Squalene standard was added to each tube and extraction and separation of the non-saponifiable lipids was performed as described (11, 12). The sterol fraction was separated by thin layer chromatography (hexane:diethyl ether, 50:50). The recovery of cholesterol averaged 60–80% and was well resolved from squalene and lanosterol.

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Selective Permeabilization of the Plasma Membrane and Release of Cytosolic Components—Recently, several laboratories have been successful in permeabilizing cells in a manner that allows the cell organelles to remain intact while the plasma membrane is disrupted (10, 19). Digitonin permeabilizes cells by complexing with cholesterol. Since the ER membranes, peroxisomal membranes, and most other cell organelles are almost devoid of cholesterol, digitonin complexes almost exclusively with the plasma membrane. The main consequence of permeabilizing cells is the loss of cytosolic components, while leaving the organelles virtually intact.

To test whether only the plasma membrane was disrupted and the cell organelles remained intact, we determined various marker enzyme activities in both permeabilized and intact cells. Table I illustrates that there was no significant difference between the two groups in the total activity per plate of esterase (marker enzyme for endoplasmic reticulum) and catalase (marker enzyme for peroxisomes), whereas phosphoglucone isomerase (a marker enzyme for cytosolic fraction) was measurable in the control cells and was not detectable in the permeabilized cells. Furthermore, the protein concentration in permeabilized cells was approximately 30–50% less than that of intact cells. This corresponds to the protein content of cell cytosol. However, to further demonstrate that the peroxisomal, ER, and mitochondrial compartments of cells remain intact after permeabilization, we tested the cytosolic contents disappear, we employed an additional method. Control and permeabilized cells grown on coverslips were treated in parallel with antibodies to various marker enzymes as described under “Experimental Procedures.” Fig. 1 illustrates the immunofluorescence pattern obtained for cytosol, peroxisomes, ER, and mitochondria in control cells and digitonin permeabilized cells. Panel A in Fig. 1 shows cytosolic labeling in intact CV-1 cells, whereas the permeabilized CV-1 cells in panel B are devoid of cytosolic labeling. The bright fluorescence in the center of the cell is due to autofluorescence of the nucleus. In panels C and D, the cells were labeled for peroxisomal proteins using an antibody made against the peroxisomal targeting signal (SKL at the C terminus) (20). A uniform punctate pattern characteristic of peroxisomal labeling is observed in both the intact cells (panel C) as well as in the permeabilized cells (panel D). Additionally, the pattern of ER labeling is similar in control cells (panel E) and permeabilized cells (panel F). Cells in panels G (control) and H (permeabilized) demonstrate that the mitochondrial membrane also remains intact during selective permeabilization with digitonin. Taken together, these results demonstrate that the plasma membrane of CV-1 cells can be selectively permeabilized with low concentrations of digitonin, resulting in the release of cytosolic proteins while maintaining organelle

| Protein | Catalase | Esterase | PGI |
|---------|---------|---------|-----|
| mg/plate | millions/plate | millions/plate | millions/plate |
| Control cells | 0.638 | 1.7 | 81.2 | 349.5 |
| Permeabilized cells | 0.394 | 1.6 | 75.0 | 0 |

Rabbit antibody against HMG-CoA reductase (1:30 dilution) was used. For mitochondrial distribution, a rabbit anti-cytochrome c oxidase antibody (1:25 dilution) was used. The secondary label for the peroxisomal, ER, and mitochondrial labels was Texas Red-labeled goat anti-rabbit IgG antibody (1:100 dilution).
Validation of the Experimental Model—

In order to obtain information on the functional characteristics of the peroxisomal membrane and to investigate how long the peroxisomes would maintain their integrity, the latency of catalase was measured as a function of time in permeabilized cells. Fig. 2 shows that catalase remains up to 70% latent (i.e. inside the peroxisomes) during the first 10 min after cell permeabilization. After 30 min, less than 5% of catalase activity is latent. Based on these results, an incubation period of 10 min (or less) was selected for all subsequent experiments dealing with latency determinations.

Total Activity and Latency Determinations of Mevalonate Kinase, Phosphomevalonate Kinase, and Mevalonate Diphosphate Decarboxylase in Control and Permeabilized Cells—In order to determine if mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase are present in the cytosol or in a membrane-bound organelle, the activities

Fig. 1. Selective permeabilization of the plasma membrane releases cytosolic components but maintains the integrity of subcellular organelles. Control and permeabilized cells grown on coverslips were treated in parallel with antibodies to various marker enzymes as described under “Experimental Procedures.” Panels A, C, E, and G illustrate control cell labeling; panels B, D, F, and H represent labeling of permeabilized cells. Panel A shows cytosolic labeling in intact cells; panel B illustrates the absence of cytosolic labeling in permeabilized cells. The bright fluorescence in the center of the cell is due to autofluorescence of the nucleus. In panels C and D, the cells were labeled for peroxisomal proteins, in panels E and F for ER proteins, and in panels G and H for mitochondrial proteins.
of these enzymes were determined in control and in permeabilized cells in the presence and absence of 0.1% Triton X-100. Table II illustrates the results. The activities of all three enzymes were similar in control cells and in permeabilized cells treated with Triton X-100. These results suggest that these enzymes are not predominantly found in the cytosol, since the activities were not increased in control cells containing cytosolic proteins. Similar levels of all three enzyme activities were measured in control cells in the presence of Triton X-100 or in the absence in cells disrupted by homogenization. However, with permeabilized cells in the absence of Triton X-100, the activities of phosphomevalonate kinase and mevalonate diphosphate decarboxylase were significantly lower. These results suggest that the substrates for these two enzymes are not freely permeable across the organelle membrane, whereas the activity of mevalonate kinase was the same in permeabilized cell in the presence or absence of Triton X-100, indicating that the substrate for this enzyme is freely permeable through the peroxisomal membrane. We were unable to measure any isopentenyl diphosphate isomerase activity in these cells (data not shown).

Measurement of Rate of Cholesterol Synthesis in Control and Permeabilized Cells—If indeed the enzymes required for conversion of mevalonate to FPP (i.e. mevalonate kinase, phosphomevalonate kinase, mevalonate diphosphate decarboxylase, and isopentenyl diphosphate isomerase) are not present in the cytosol, then permeabilized and control cells would be expected to have similar rates of cholesterol synthesis, using mevalonate as substrate.

Results of these experiments are shown in Table III. The mean value for control cells was 4287 dpm/plate and for permeabilized cells 3026 dpm/plate. These two means are not significantly different. No conversion of mevalonate to cholesterol was observed if ATP and/or NADPH were omitted from the reaction buffer (data not shown). These results indicate that the cytosolic fraction of cells is not necessary for the biosynthesis of cholesterol from mevalonate.

In summary, the current report demonstrates that mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase activities in extracts prepared from intact cells are equal to those of selectively permeabilized cells that lack cytosolic enzymes. We also demonstrate structure-linked latency of phosphomevalonate kinase and mevalonate diphosphate decarboxylase that is consistent with a peroxisomal localization of these enzymes. These results in combination with the previous observation that mevalonate kinase and FPP synthase are predominantly localized to peroxisomes (8, 9), suggest that all of the cholesterogenic enzymes involved in the conversion of mevalonate to FPP are localized to the peroxisome. This conclusion is further supported by the direct finding that cholesterol biosynthesis from mevalonate can occur in selectively permeabilized cells lacking cytosolic components and indirectly by the previous observation that all the required enzymes for the conversion of mevalonate to FPP are significantly reduced in tissue obtained from patients with Zellweger syndrome and neonatal adrenoleukodystrophy (9).

The isoprenoid biosynthetic pathway is unrivaled in nature for the chemical diversity of the compounds it produces. FPP is a key intermediate that serves as a substrate for a number of critical branch-point enzymes including the synthesis of squalene, cholesterol, farnesylated and geranylgeranylated proteins, dolichols, coenzyme Q, and the isoprenoid moiety of heme a. Thus, the regulation and levels of FPP are important since large perturbations in FPP could alter the flux of isoprenoid compounds down the various branches of the pathway.

If indeed, the majority of the cell’s FPP is produced in the peroxisomes, this means that FPP and/or farnesol has to be transported out of peroxisomes for further metabolism. Since phosphorylated products of mevalonate are not able to cross the peroxisomal membrane, it is likely that FPP may also be impermeable. Therefore, is FPP first converted to farnesol in the peroxisome, and then is freely diffusible out of the organelle? Or is there a transport/binding protein that facilitates the movement of these intermediates? What determines where FPP is utilized? What regulates FPP conversion to farnesol and farnesol conversion to dicarboxylic acids? These are important questions that need to be addressed in order to understand the

![Fig. 2. The release of catalase activity from peroxisomes as a function of time after selective permeabilization of the plasma membrane.](image)

**Table II**

| Enzyme/Condition                      | Activity (nmol/min/plate) | Activity (pmol/min/plate) |
|--------------------------------------|---------------------------|---------------------------|
| Mevalonate kinase                   |                           |                           |
| Control cells + Triton X-100         | 1.62 ± 0.026              | 460 ± 34                  |
| Permeabilized cells + Triton X-100   | 1.54 ± 0.020              | 382 ± 36                  |
| Permeabilized cells − Triton X-100   | 1.20 ± 0.078              | 34 ± 2*                   |
| Phosphomevalonate kinase             |                           |                           |
| Control cells + Triton X-100         | 460 ± 34                  |                           |
| Permeabilized cells + Triton X-100   | 382 ± 36                  |                           |
| Permeabilized cells − Triton X-100   | 34 ± 2*                   |                           |
| Mevalonate diphosphate decarboxylase |                           |                           |
| Control cells + Triton X-100         | 0.014 ± 0.0008            |                           |
| Permeabilized cells + Triton X-100   | 0.017 ± 0.0001            |                           |
| Permeabilized cells − Triton X-100   | 0.003 ± 0.0003*           |                           |

*The means of the groups are significantly different as determined by using Student's t test (p < 0.01).
regulation of FPP/and or farnesol.

It is significant to note two recent studies demonstrating the potential importance of farnesol in regulation of cellular function (21, 22). In the first study, farnesol has been identified as the non-sterol derivative that can initiate and promote the degradation of HMG-CoA reductase in permeabilized cells (21). In the second study, an orphan nuclear receptor named farnesoid X-activated receptor (FXR) is described that is activated by farnesol (22). Thus, FXR provides an example of a vertebrate transcription factor that is regulated by an intracellular metabolite (farnesol) and may indicate the existence of a novel vertebrate-signaling pathway (22). The FXR target genes remain to be identified.

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TABLE III
Compartmentalization of Cholesterol Biosynthesis

| Compartmentalization of Cholesterol Biosynthesis |
|-----------------------------------------------|
| Values represent mean ± S.E. for four experiments (two to four assays per experiment). The protein ranged from 0.865 to 0.991 mg/ml for control cells and from 0.437 to 0.541 mg/ml for permeabilized cells. |
| Control cells | Permeabilized cells |
|----------------|---------------------|
| dpm/plate | 4287 ± 885.8 | 3026 ± 476.8 |