Mevalonate Pyrophosphate Decarboxylase is Predominantly Located in the Cytosol of Rat Hepatocytes

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One of the first steps in the biosynthesis of cholesterol from acetic acid is catalyzed by mevalonate pyrophosphate decarboxylase (MPD). This decarboxylase catalyzes a bimolecular reaction between mevalonate pyrophosphate and ATP to form isopentenyl pyrophosphate, inorganic phosphate, adenosine 5'-diphosphate (ADP), and CO2. MPD is found in the 100000×g supernatant fraction of cells or tissues and has been considered a cytosolic protein. Recently, it has been demonstrated by other groups that peroxisomes contain a number of enzymes involved in cholesterol biosynthesis which previously were considered to be cytosolic or located exclusively in the endoplasmic reticulum (ER). Peroxisomes have been shown to contain acetate-CoA thiolase, 1,2 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase, 3 HMG-CoA reductase, 4,5 mevalonate kinase (MVK), 6,7 phosphomevalonate kinase (PMVK), 8 MPD, 8 isopentenyl pyrophosphate isomerase (IPPase), 9 and farnesyl pyrophosphate synthase (FPPase). 10 Recent data have also shown that the activities of enzymes from MVK to FPPase are significantly reduced in liver tissue obtained from patients with peroxisome-deficient diseases (Zellweger syndrome and neonatal adrenoleukodystrophy), thus indicating a peroxisomal localization. 10

The subcellular distribution of MVK, PMVK, IPPase, and FPPase was examined in detail, although there are few reports on the subcellular distribution of MPD. Biardi and Krisans reported that MPD was mainly located in peroxisomes, since the activity of MPD was the same in extracts prepared from intact cells and selectively permeabilized cells, which lack cytosolic enzymes. 5 However, the subcellular distribution of MPD remains unclear.

In this study, we attempted to determine the major subcellular location of MPD in rat hepatocytes using two permeabilized cell and immunofluorescent microscopy. The results indicate that MPD is predominantly located in the cytosol of rat hepatocytes.

MATERIALS AND METHODS

Materials and Animals Mouse anti-hexokinase antibody was purchased from Biogenesis. Fluorescein-conjugated donkey anti-rabbit IgG (H+L) antibody, rhodamine-conjugated donkey anti-mouse IgG (H+L) antibody, and normal donkey serum were purchased from Leinco Technologies. Male Wistar rats (8 weeks old) were used to obtain rat hepatocytes. All other chemicals were of reagent grade and purchased from commercial sources.

Marker Enzyme The activity of catalase (peroxisomal marker) was measured according to a previously reported method. 11 The activity of lactate dehydrogenase (LDH), a cytosol marker was measured according to the method of Abel. 12 The activity of cytochrome-c oxidase, a mitochondria marker was measured according to the method of Polakis et al. 13 Proteins were determined using the method of Lowry et al. using bovine serum albumin (BSA) as the standard. 14

Preparation of Antibody Polyclonal antiserum raised against the lysosome-associated membrane glycoprotein (lamp-2, a lysosome marker) as described by Akasaka et al. 15 was used. Polyclonal antiserum was used against cathepsin B (lysosome marker) as described by Yokota et al. 16 Polyclonal antiserum raised against the phenobarbital-inducible cytochrome P-450 (CYP2B1, a microsome marker) as described by Tsuji et al. 17 was used. MPD was purified from rat liver as described by Michihara et al. 18 and polyclonal antiserum raised against the MPD was used.

Purification of MPD Antibody We previously reported that anti-MPD antiserum contained albumin antibody. 18 Therefore to remove the albumin antibody from anti-MPD antiserum, we carried out the following procedure. Rat serum albumin was coupled to Affigel 15 at a ratio of 2 mg/ml of packed gel. As described by Michihara et al., 18 the purified MPD was coupled to Affigel 15 at a ratio of 0.5 mg/ml of packed gel, and the antiserum was subjected to a column of albumin-Affigel 15 equilibrated with phosphate-buffered saline (PBS). After the column was washed with PBS, the flow-through solution was applied to a column of MPD-Affigel 15 equilibrated with PBS. The column of albumin-Affigel 15 or MPD-Affigel 15 was washed with PBS until the absorbance at 280 nm dropped to the base line level (0.01—0.05 A280). MPD antibody or albumin antibody from each Affigel 15 column was eluted with 0.1 M glycine–HCl (pH
After centrifugation for 5 min at 1000×g, the supernatant solutions were used for marker enzyme or protein assay and immunoblotting. Nonpermeabilized cells were treated with KHM buffer without digitonin and processed in the same way as the digitonin-treated cells. The amount of protein or enzyme activities retained in the nonpermeabilized cells were taken to be 100%.

**Immunofluorescence Microscopy** Rat hepatocytes were plated on coverslips. After 24-h growth at 37 °C, the growth medium was removed and cells were washed twice with PBS. The cells were then fixed with 4% (w/v) paraformaldehyde at room temperature for 20 min, treated with 0.2% Triton X-100 in PBS containing 1% BSA for 10 min, and incubated in 1% glycine in PBS solution for 10 min. Treated cells were incubated with the purified anti-MPD antibody (a final dilution of 1:50 in PBS containing 5% normal donkey serum) at room temperature for 60 min, washed 3×10 min and 1×15 min with PBS containing 0.2% Triton X-100 (PBST), and then incubated for 60 min with mouse anti-hexokinase antibody (diluted 1:50). Cells on coverslips were washed as described above and then incubated with fluorescence-conjugated donkey anti-rabbit IgG (H+L) antibody (diluted 1:200) for 60 min followed by washing with PBST. The cells were then incubated for 60 min with rhodamine-conjugated donkey anti-mouse IgG (H+L) antibody at a final dilution of 1:200. After washing with PBST, the coverslips were mounted on microscope slides with Mowiol 4-88. Fluorescence microscopy was performed using a Zeiss Axiowert 100 microscope.

**Cell Fractionation** Cell fractionation was carried out according to the method of de Duve et al.22) Nonpermeabilized or permeabilized rat hepatocytes (60 mm dish×5) were washed with 100 mM sodium phosphate (pH 7.0) containing 10 mM mercaptoethanol and 1 mM EDTA (buffer A), 1.5 ml of buffer A containing 0.5 mM PMSF, 1 mM leupeptin, 1 mM pepstatin A, 1 mM chymostatin, and 1 mM antipain was added to cells, and then the cells were scraped off using a rubber policeman. The solution of nonpermeabilized or permeabilized rat hepatocytes was homogenized with 5 strokes in a Teflon homogenizer. The homogenate was centrifuged at 1000×g for 10 min. The supernatant was retained, and then pellet was resuspended, homogenized and centrifuged again at 10000×g for 10 min. The two supernatants were then combined and centrifuged at 7000×g for 6 min and at 20000×g for 20 min. The pellet contained the peroxisomal-enriched fraction. After the pellet was resuspended with 3 ml of centrifuge buffer (10 mM glycyglycine, 1 mM EDTA, 0.5 mM PMSF, 1 mM leupeptin, 1 mM pepstatin A, 1 mM chymostatin, and 1 mM antipain), the peroxisomal-enriched fractions were layered on the top of sucrose gradient solutions (7 ml of 23.2%, 8 ml of 37.4%, 10 ml of 45.8%, and 5 ml of 53.9%) containing 10 mM glycyglycine, and then centrifuged at 23000 rpm for 2 h using an SW 28 Beckman rotor. Twenty-three fractions were collected from the top to the bottom. The specific activities of catalase in fraction 19 were approximately 120 times higher than that in rat hepatocyte homogenate. Therefore fraction 19 was designated the peroxisomal fraction in this study.

**Radioactive Assay of MPD** The enzyme activities of the crude extract were measured according to the method of Sawamura et al.23)
RESULTS AND DISCUSSION

Selective Permeabilization of the Plasma Membrane and Release of Cytosolic Components

Digitonin treatment of cells has been reported to permeabilize the plasma membrane reversibly, leaving subcellular organelles intact. Therefore we used digitonin permeabilization of rat hepatocytes to characterize the main subcellular distribution of MPD. The time course of release of cellular enzymes, LDH, catalase, and total cellular protein is shown in Fig. 2. Most protein release from the permeabilized cells occurred within 5 min and was complete after 15 min of treatment. At this time, all of the activity of LDH and about 30% of total cellular protein were released. However, catalase activity was almost all retained in the permeabilized cells, indicating that the peroxisomes remained intact. Based on kinetic studies, we chose a 5-min permeabilization period in which cellular protein loss plateaus (Fig. 2).

To determine whether only the plasma membrane was disrupted and the cell organelles remained intact, we determined various marker enzyme activities or protein in both permeabilized and intact cells. As shown in Fig. 3, total cellular protein, LDH, and catalase activities in medium after digitonin treatment were 30%, 91%, and 8%, respectively. Cytochrome-c oxidase activity, protein of lamp-2, cathepsin B (data not shown), and CYP2B1 were not released in medium from permeabilized cells and were retained in amounts equal to that in intact cells. These results demonstrate that the plasma membrane of rat hepatocytes could be selectively permeabilized with low concentrations of digitonin, resulting in the release of cytosolic proteins while maintaining organelle integrity.

Total Protein and Latency Determinations of MPD in Intact and Permeabilized Rat Hepatocytes

To determine whether MPD was mainly present in the cytosol or peroxisomes (or other organelles), the amount of MPD protein was examined in nonpermeabilized and permeabilized cells or medium using immunoblot analysis. As shown in Fig. 4, nonpermeabilized cells retained most of the MPD protein, while MPD protein from permeabilized cells was found mainly in the medium. Ninety percent of the MPD protein was released after 5-min incubation, and only 5% was retained after 35-min permeabilization. These results suggest that MPD is predominantly located in the cytosol of rat hepatocytes.

Total Protein and Latency Determinations of MPD in Intact and Permeabilized Nonhepatic NRK Cell Lines

To establish whether the release of MPD in medium differs by cell type, we carried out the same experiment using NRK cells. Total cellular protein, LDH, and catalase activity released in medium after digitonin treatment were 40%, 98%, and 0%, respectively (Fig. 5). These results demonstrated that the plasma membrane of NRK cells could be selectively permeabilized with low concentrations of digitonin, similar to rat hepatocytes (Fig. 6). As shown in Fig. 7, nonpermeabilized cells retained most of the MPD protein, while MPD from permeabilized cells was present mainly in the medium. Eighty percent of the MPD protein was released after 5-min incubation, and only 10% was retained after 35-min permeabilization. These results suggest that MPD is predominantly
located in the cytosol of NRK cells as well as of rat hepatocytes. It also found that the amounts of MPD in rat hepatocytes and NRK cells were somewhat different.

Localization of MPD in Cytosol Using Immunofluorescence Microscopy
Since MPD may be solubilized during cell homogenization, cell fractionation studies may not be a reliable method for determining the relative subcellular distribution of MPD. Therefore we investigated the subcellular distribution of MPD by immunofluorescence. Panel A in Fig. 8 shows cytosolic labeling in intact hepatocytes, whereas the permeabilized hepatocytes in panel C are devoid of cytosolic labeling. Double immunofluorescent labeling of cells with both anti-MPD antibody and anti-hexokinase antibody yielded immunofluorescence patterns for both enzymes typical of cytosolic protein, supporting the colocalization of the two in the cytosol (Fig. 8).

Comparison of MPD Activity between Cells
To establish whether our results were due to the methods used, we performed an experiment under the same conditions used by Biardi and Krisans et al.\textsuperscript{8} Seventy percent of LDH activity in permeabilized cells treated with 20 μg/ml of digitonin was released in medium within 5 min and 80% of MPD activity

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Fig. 4. Immunoblot Analysis of MPD in Nonpermeabilized and Permeabilized Rat Hepatocytes
A: Rat hepatocytes were incubated in KHM buffer in the presence (P) or absence (NP) of digitonin for 5 min at 4°C, and the cells (C; 10 μl) and medium (M; 10 μl) were analyzed by immunoblotting. B: Signals of A were quantified using a Gel-Pro analyzer. C: Rat hepatocytes were incubated in KHM buffer in the presence of digitonin for various times at 4°C, and the cells (10 μl) were analyzed by immunoblotting. Signals of the band were quantified using a Gel-Pro analyzer. The amount of MPD retained in the nonpermeabilized cells was taken as 100%. Data are the means of three identical experiments and each varies within 5%.

Fig. 5. Time Course of Release of Protein and Enzymes from NRK Cells during Digitonin Permeabilization
At different incubation times, samples were collected and analyzed for protein (□) and enzymatic activities of LDH (△) and catalase (□) as described in Materials and Methods. Nonpermeabilized cells were treated with buffer without digitonin and samples were processed similarly. After 35 min, the incubation buffers were removed, cell extracts were prepared, and enzyme activities and protein were determined. The amount of protein or enzyme activities retained in cells from control dishes is defined as 100%. The values are expressed as the percentage of total protein or enzyme activities remaining in the nonpermeabilized cells. Data are the means of six identical experiments and each varies within 5%.

Fig. 6. Selective Permeabilization of the Plasma Membrane Releases Cytosolic Components in NRK Cells
Protein or activities (LDH and catalase) in medium (M, □) and protein or activities remaining in cells (C, ■) were measured using nonpermeabilized (NP) or permeabilized (P) cells as described in Materials and Methods. The activities of catalase in cells and medium were measured after concentration by centricon. The amount of protein or enzyme activities retained in the nonpermeabilized cells was taken as 100%. Data are the means of six identical experiments and each varies within 10%.
was released in medium during the same time (Table 1). These data indicate that the difference in distribution of MPD between our results and those of Biardi and Krisans et al. was not due to the difference of the concentration of digitonin or the method based on antibody or activity.

As compared with healthy individuals, the activity of catalase, FPPase, and MPD in peroxisome-deficient patients is 100%, 60%, and 40%, respectively. Since the activity of catalase in peroxisomes is similar to that in healthy people, not all protein in peroxisomes is reduced in peroxisome-deficient patients. Although those findings suggest that MPD is present in peroxisomes, it is not known whether MPD is predominantly located in peroxisomes. In this study, we employed two different methods to study the major subcellular distributions of MPD in rat hepatocytes: 1) comparison of the amounts of MPD in intact and permeabilized cells or medium using immunoblotting (quantitative analysis); and 2) immunofluorescence microscopy. In permeabilized rat hepatocytes treated with digitonin, which lacks cytosolic enzyme, MPD was present mainly in the medium. Double immunofluorescent labeling of cells with both anti-MPD antibody and anti-hexokinase antibody yielded immunofluorescent patterns for both enzymes typical of cytosolic protein. These results indicate that MPD was predominantly located in the cytosol of rat hepatocytes. Also, the major cholesterol synthetic pathway was suggested to be from the cytosol to ER in rat hepatocytes. It is reasonable to assume that enzymatic reactions of cholesterol biosynthesis occur along the pathway from the cytosol to ER rather than from peroxisomes to the ER.

From previous data, it was suggested that MPD exists in peroxisome. Therefore it is highly possible that 5—10% of MPD retained in permeabilized rat hepatocytes after 35-min incubation is in peroxisomes. However, when peroxisomes in intact and permeabilized rat hepatocytes were isolated on sucrose gradients as described in Materials and Methods, MPD in peroxisomes was not detected (data not shown). A small amount of MPD in peroxisomes may be released with rupture of the peroxisomes during cell fractionation.

Our findings are somewhat different from the observations reported by Biardi and Krisans in permeabilized nonhepatic CV-1 (monkey kidney) cells. They reported that the activities of MVK, PMVK, and MPD were almost all retained in permeabilized cells. In this study, we found that the amounts of MPD in the cytosol of rat hepatocytes and NRK cells differed. The difference in distribution of MPD between our results (rat hepatocytes) and other reports (CV-1) may be due to cell type or difference in species. Further studies are necessary to understand the major subcellular localization of enzymes involved in cholesterol synthesis and the physiological role of MPD.

Table 1. Comparison of MPD Activity and the Amount of MPD in Various Cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>Activity (%)</th>
<th>Amount (%)</th>
<th>Activity (%)</th>
<th>Amount (%)</th>
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<tbody>
<tr>
<td>CV-1</td>
<td>100</td>
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<td>Rat hepatocytes</td>
<td>75</td>
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<td>20</td>
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<tr>
<td>NRK cells</td>
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<td>70</td>
<td>35</td>
<td>30</td>
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Data are the means of three identical experiments and each value varies within 2%.
REFERENCES