Difference in Subcellular Distribution of Mevalonate Pyrophosphohosphate Decarboxylase Occurs by Cell Type

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Recently, it has been questioned whether mevalonate pyrophosphate decarboxylase (MPD) is predominantly located in the peroxisomes or cytosol. We previously reported that MPD was predominantly present in the cytosol of rat hepatocytes, normal rat kidney cells, or mouse melanoma cells. In the present study, we examined whether MPD was predominantly present in the cytosol of HepG2 (human hepatoma) cells and Cos7 (monkey kidney) cells using digitonin permeabilization. In HepG2 cells permeabilized with digitonin, 90 % and 10 % of MPD existed in the cytosol and membrane/organelle (M/O) fraction, respectively, while in Cos7 cells permeabilized with digitonin, 20 % and 80 % of MPD existed in the cytosol and M/O fraction, respectively. These data suggest that the difference in subcellular distribution of MPD is due to the cell type.

Key words— mevalonate pyrophosphate decarboxylase, subcellular distribution, HepG2, Cos7, cytosol

INTRODUCTION

One of the first steps in the biosynthesis of cholesterol from acetic acid is catalysis by mevalonate pyrophosphate decarboxylase (MPD). MPD is found in the 100000 \( \times g \) supernatant fraction of tissues and has been considered to be a cytosolic protein. Recently, it has been demonstrated by other groups that peroxisomes contain a number of enzymes involved in cholesterol biosynthesis, which were previously considered to be cytosolic or located exclusively in the endoplasmic reticulum (ER). Peroxisomes have been shown to contain acetoacetyl-coenzyme A (CoA) thiolase, 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase, HMG-CoA reductase, mevalonate kinase (MVK), phosphomevalonlate kinase (PMVK), MPD, isopentenyl pyrophosphate isomerase (IPPase), and farnesyl pyrophosphate synthase (FPPase). Recent data have also shown that the activity of some enzymes, including MVK and FPPase, is significantly reduced in liver tissue obtained from patients with peroxisome-deficient diseases (Zellweger syndrome and neonatal adrenoleukodystrophy), thus indicating the peroxisomal localization of these enzymes. Hogenboom et al. reported that the activity and protein levels of selected enzymes involved in cholesterol biosynthesis were at least as high in peroxisome-deficient Zellweger mice as in control mice, indicating that the mislocalization of enzymes to the cytosol does not lead to decreased activity or the degradation of these enzymes. They also reported that deficiencies of MVK and PMVK activity in the liver of human Zellweger patients reflect bad conditions (storage conditions or time of storage) of the liver, rather than mislocalization to the cytosol.

Biardi and Krisans reported that MPD was mainly located in membrane/organelles (peroxisomes) of CV-1 (monkey kidney) cells treated with lipoprotein-deficient serum (LDS) for 24 hr, since the activity of MPD was the same in extracts prepared from intact cells and selectively permeabilized cells, which lack cytosolic enzymes; however, the protein level of MPD in steady-state CV-1 cells treated with fetal bovine serum (FBS) was not analyzed. We previously reported that MPD was predominantly present in the cytosol of rat hepatocytes, normal rat kidney (NRK) cells, or mouse melanoma (B16F10 and B16) cells treated with FBS, since MPD was mainly present in the medium (cytosolic fraction) in these cell types.
permeabilized with digitonin. Hogenboom et al. reported that both endogenous MPD in human fibroblasts, human liver, CV-1, and HEK293 (human kidney) cells, and overexpressed human MPD in human fibroblasts, HEK293, and CV-1 cells treated with LDS, were present in the cytosol.\(^{15}\)

Recently, enzymes involved in cholesterol biosynthesis have been shown to contain a peroxisomal targeting signal (PTS-1; [S/A/C][K/H/R] [L/M]), found at the extreme carboxy terminus of most peroxisomal proteins, and PTS-2 ([R/K] [L/V/I]X5[H/Q][L/A]), found within the amino terminal region of a smaller subset of peroxisomal proteins.\(^{16}\) PTSs of MVK and PMVK were identified as PTS-2 [KV(X5)HA] and -1 (SRL), respectively.\(^{16}\) PTSs of HMG-CoA synthase, MPD, and FPPase were identified as SV(X5)QL, SV(X5)QL, and KL(X4)QE as new variations of PTS-2, respectively.\(^{16}\) PTSs of acetoacetyl-CoA thiolase and IP-Pase were identified as QKL and HRM, new variations of PTS-1, respectively; however, the relationship between the new variation sequence (PTS-2) of MPD and transport to peroxisomes or peroxins (which are essential proteins for peroxisome biogenesis) remains unclear.

In the present study, we examined whether endogenous MPD was predominantly present in the cytosol of steady-state HepG2 (human hepatoma) cells and Cos7 (monkey kidney) cells treated with FBS using digitonin permeabilization.

**MATERIALS AND METHODS**

**Materials** —— LDS was obtained from Sigma-Aldrich (Tokyo, Japan). Percoll and the ECL Western blotting detection kit were from Amersham Pharmacia Biotech (Tokyo, Japan). Digitonin was obtained from Wako (Osaka, Japan). Dulbecco’s modified Eagle’s medium (D-MEM) was obtained from Gibco (Tokyo, Japan). Goat antilactate dehydrogenase (LDH)-A (N-14) IgG was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Sheep anti-catalase (bovine or human) IgG was obtained from The Binding Site (Birmingham, U.K.). Rabbit anti-goat IgG conjugated to horseradish peroxidase was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Donkey anti-sheep IgG conjugated to horseradish peroxidase was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). All other chemicals were of reagent grade, and purchased from various commercial sources.

**Cultured Cell Line** —— HepG2 or Cos7 cells were diluted to 1.5 \times 10^6 per 35-mm tissue culture dish or 0.8 \times 10^6 per well in a 12-well multidish with D-MEM containing 10% FBS, and then incubated in humidified air containing 5% CO_2 at 37°C for 24 hr. In some experiments, the cells were shifted to D-MEM in the presence of 10% LDS (from which cholesterol had been removed), and then various concentrations of lovastatin (final concentration, 0–10 \mu M) were added for various periods ranging from 24–120 hr.

**Protein Assay** —— Protein levels were measured by the method of Lowry et al. using bovine serum albumin (BSA) as the standard.\(^{17}\)

**Antibody MPD** —— MPD was purified from rat liver, as described by Michihara et al., and rabbit polyclonal antiserum raised against rat MPD was used.\(^{18}\)

**Permeabilization** —— HepG2 or Cos7 cells incubated on 35-mm dishes or 12-well multidishes were washed several times in cold Hanks’ buffer. On the day of the experiment, the medium was aspirated from the culture dishes, and cells were then washed twice with ice-cold KH buffer [50 mM phosphate buffer (pH 7.2), 110 mM KOAc]. The cells were transferred to ice and then incubated for various times in 1 ml of KH buffer containing 40 \mu g/ml of digitonin, 20 mM phosphate buffer (pH 7.2), 110 mM KOAc, and 2 mM MgOAc. The digitonin solution was retained as a marker enzyme or for protein assay and immunoblotting. After digitonin treatment and washing, 750 \mu l of homogenate buffer containing 50 mM phosphate buffer (pH 7.2), 0.5 mM Phenylmethylsulfonylfluoride (PMSF), 1% Triton X-100, 10 mM 2-mercaptoethanol, 1 mM EDTA, and protease inhibitors (1 \mu M leupeptin, 1 \mu M pepstatin A, 1 \mu M chymostatin, and 1 \mu M antipain) was added to the cells, which were then scraped off using a rubber policeman. The solution of HepG2 or Cos7 cells was homogenized with 5 strokes in a Teflon homogenizer.

After centrifugation for 5 min at 1000 \times g the supernatant solutions were used as marker enzymes or for protein assay and immunoblotting. Non-permeabilized cells were treated with KH buffer without digitonin, and processed in the same way as digitonin-treated cells. The amount of protein retained in non-permeabilized cells was taken to be
Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) —— SDS-PAGE was performed on 10 % slab gels according to the method of Laemmli.19) Immunoblotting —— Proteins on the SDS-slab gel were transferred to a nylon membrane (NEN) by electrophoresis, using a modified version of the procedure of Towbin et al.20) Positive bands were visualized using ECL Western blotting detection kits (Amersham Pharmacia, Amersham, U.K.) that contained a sensitive chemiluminescent substrate for horseradish peroxidase.

Statistics —— Statistical analysis was carried out using Student’s t-test. Data are presented as the means ± S.D.

RESULTS

Identification of MPD in HepG2 and Cos7 Cells

We previously reported that MPD was purified from rat liver, and anti-rat MPD antiserum (MPD antiserum) was produced in a rabbit by multiple injections of purified MPD with a molecular weight of 45 kDa.8) The antiserum reacted with not only 45 kDa protein in various rat tissues but also 46 kDa protein in various mouse tissues.8,21) We examined whether MPD antiserum reacted with MPD in HepG2 and Cos7 cells. When a crude extract of HepG2 and Cos7 cells was subjected to immunoblot analysis using MPD antiserum, this antiserum reacted with the protein of 43 kDa in HepG2 and Cos7 cells (Fig. 1A). The molecular weight of MPD (43 kDa) in HepG2 cells was similar to that in humans reported by Toth et al.22)

There is known to be a marked increase in the enzyme involved in cholesterol biosynthesis after treatment with LDS containing lovastatin, an HMG-CoA reductase inhibitor.14,21,23) To further establish whether the 43-kDa protein was MPD, immunoblot analysis of HepG2 and Cos7 cells treated with lovastatin was performed. When immunoblot analysis was carried out using a crude extract of HepG2 cells treated with various concentrations of lovastatin for 120 hr (medium was changed two times), the level of the 43-kDa protein was markedly higher (10-fold by 1 µM, 18-fold by 5 µM, and 20-fold by 10 µM lovastatin) than that of cells without lovastatin (Fig. 1B). The level was also higher in Cos7 cells treated withLovastatin (2-fold by 1, 5, and 10 µM) than in those not treated (Fig. 1C). From these data, the 43-kDa protein in HepG2 and Cos7 cells detected by MPD antiserum was indicated to be MPD.

Selective Permeabilization of the Plasma Membrane, and Release of Cytosolic Components in HepG2 Cells

Digitonin treatment (digitonin permeabilizes cells by complexing with cholesterol) of cells has been reported to permeabilize the plasma membrane reversibly, leaving subcellular organelles intact.8) The time-course of the release of cellular enzymes, LDH, catalase, and total cellular protein of HepG2 cells is shown in Fig. 2. Most protein release from permeabilized cells occurred within 20 min and did not change between 20 and 40 min of treatment (Fig. 2C). At 20 min, 90 % of LDH and about 25 % of total cellular protein were released (Fig. 2B and 2C); however, 100 % of catalase was retained in permeabilized cells (Fig. 2A and 2C), indicating that the peroxisomes remained intact. Based on kinetic studies and immunoblot analysis, we chose 20 min permeabilization, during which cellular protein loss plateaued. The permeabilization time (20 min) of HepG2 cells permeabilized with digitonin was longer than that of other cell types (rat hepatocytes, B16F10, and B16 cells were 10 min; NRK cells was 5 min).
Localization of MPD in Permeabilized HepG2 Cells

To determine whether MPD was mainly present in the cytosol or other organelles (or peroxisomes), the amount of MPD was examined in the cytosol and membrane/organelle fraction (M/O fraction) of permeabilized HepG2 cells. After 20 min incubation with digitonin, 90 % of MPD existed in the cytosol fraction, and most MPD in the M/O fraction was not retained after 40 min permeabilization (Fig. 3). These results suggest that MPD was predominantly present in the cytosol of HepG2 cells. These results were also similar to those of other cell types, except for the results of CV-1 cells reported by Biardi and Krisans. Furthermore, it is highly possible that MPD was not located in peroxisomes, since the ratio of MPD (10 %) retained in the M/O fractions was similar to that of LDH (10 %) in M/O fractions containing peroxisomes after 20 min permeabilization (Figs. 2 and 3).

Selective Permeabilization of the Plasma Membrane, and Release of Cytosolic Components in Cos7 Cells

The time-course of the release of cellular enzymes, LDH, catalase, and total cellular protein in Cos7 cells permeabilized with digitonin is shown in Fig. 4. Most protein release from permeabilized cells occurred within 5 min and did not change...
between 5 and 15 min of treatment (Fig. 4C). At 5 min, 90% of LDH and about 20% of total cellular protein were released (Fig. 4B and 4C); however, 80% of catalase was retained in permeabilized cells (Fig. 4A and 4C), indicating that the peroxisomes remained intact. After 20 min, less than 40% of catalase is latent (data not shown). Based on kinetic studies and immunoblot analysis, we chose 5 min permeabilization, during which cellular protein loss plateaued. The permeabilization time of Cos7 cells and the release time of catalase to the cytosol in Cos7 cells treated with digitonin were similar to those in CV-1 cells reported by Biardi and Krisans.

**Localization of MPD in Permeabilized Cos7 Cells**

To determine whether MPD was mainly present in the cytosol or other organelles (or peroxisomes), the amount of MPD was examined in the cytosol and M/O fraction of permeabilized Cos7 cells. MPD from permeabilized Cos7 cells was not present mainly in the cytosol fraction; 80% of MPD existed in the M/O fraction after 5 min incubation with digitonin (Fig. 5). At the protein level, the data indicated first that most MPD was located in organelles (peroxisomes), since the ratio of MPD (80%) retained in M/O fractions was markedly higher than that of LDH (10%) in M/O fractions containing peroxisomes after 5 min permeabilization (Figs. 4 and 5). These results were similar to the results of MPD activity using CV-1 cells permeabilized with digitonin as reported by Biardi and Krisans.8) As shown in Table 1, the difference in the subcellular distribution of MPD between the two cell types clearly indicated that MPD was predominantly present in the cytosol of HepG2 cells, but not Cos7 cells.

**Comparison of Subcellular Distribution of MPD**

Biardi and Krisans indicated that MPD was not present in the cytosol as observed by analysis of the MPD activity level using CV-1 cells permeabilized with digitonin (20 µg/ml for 10 min or less, Table 2).8) Hogenboom et al. reported that MPD was predominantly present in the cytosol as observed by an immunoblot using CV-1 cells permeabilized with digitonin (50–150 µg/ml for 5 min for overexpressed human MPD in CV-1 cells, data not shown).15) Although the experiments used the same cells (CV-1), there was a difference in the subcellular distribution of MPD; however, the experimental methods (activity analysis or immunoblotting) were different. The difference in subcellular distribution of MPD may have been caused by the difference in the concentration of digitonin or the time-course of digitonin treatment. As shown in Table 2, MPD was predominantly present in the cytosol as observed by an immunoblot (analysis of protein level) using

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**Table 1. Comparison of Subcellular Distribution of MPD between HepG2 and Cos7 Cells by Digitonin Permeabilization**

<table>
<thead>
<tr>
<th>Protein</th>
<th>HepG2</th>
<th>Cos7</th>
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<tbody>
<tr>
<td></td>
<td>Cytosol (%)</td>
<td>M/O (%)</td>
</tr>
<tr>
<td>LDH</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>CAT</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>MPD</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

LDH, lactate dehydrogenase (cytosol marker); CAT, catalase (peroxisome marker). Digitonin-treatment time of HepG2 or Cos7 cells was 20 or 5 min, respectively (from data in Figs. 2–5).
other permeabilized cells, except for Cos7 and CV-1 cells. Since both Cos7 and CV-1 cells are monkey kidney cells, the subcellular distribution of MPD in monkey kidney cells may be different from that of other cells.

**DISCUSSION**

We and other groups previously reported that MPD was predominantly present in the cytosol using digitonin permeabilization, cell fractionation, and immunofluorescence. Hogenboom et al. also reported that MPD was not present in the peroxisomes of human liver using immunoelectron microscopy. Biardi and Krisans reported only that MPD was not present in the cytosol of CV-1 cells treated with LDS. It was considered that CV-1 cells treated with LDS were not in a steady-state or otherwise normal, since MPD in CV-1 cells treated with LDS was increased by the feedback mechanism. In the present study, we examined whether endogenous MPD was predominantly present in the cytosol of steady-state HepG2 and Cos7 cells treated with FBS using digitonin permeabilization. As shown in Figs. 3 and 5, MPD was predominantly present in the cytosol of HepG2 cells, but not Cos7 cells. In the experiment comparing the two cell types, we found that the difference in subcellular distribution of MPD depended on the cell type.

Biardi and Krisans reported that the cytosolic fraction of CV-1 cells treated with digitonin was not necessary for biosynthesis of cholesterol from mevalonate; however, Hogenboom et al. reported that MVK and PMVK of humans in cholesterol biosynthesis were predominantly present in the cytosol, although these enzymes had PTS-2 and PTS-1 signals, respectively. We suggest that most MVK and PMVK may be located in the peroxisomes of Cos7 cells, when subcellular distribution of these enzymes is examined in detail. If MVK and PMVK exist in the peroxisomes, it may be necessary to reinvestigate the subcellular distribution of cholesterol biosynthetic enzymes in Cos7 cells.

As Cos7 cells are produced by SV40 large T antigen transfection in CV-1 cells, the properties of Cos7 cells are similar to those of CV-1 cells. Therefore, the subcellular distribution of MPD in Cos7 cells may be similar to that in CV-1 cells. As shown in Table 1, MPD was predominantly present in the cytosol of NRK (rat kidney) and HEK293 (human kidney) cells. Although both Cos7 and CV-1 cells are monkey cells derived from the kidney, MPD was not present in the cytosol; therefore, the difference in subcellular distribution of MPD might also be related to the difference in species. We previously reported an additional physiological role of MPD other than the synthesis of cholesterol and isoprenoid in the mouse kidney, since a high level of MPD was observed in the kidney. The difference in the subcellular distribution of MPD may be important to understand the physiological role of MPD in other tissues or cells.

It has been reported that cholesterol biosynthetic enzymes involved in the conversion of mevalonate to farnesyl pyrophosphate exist in peroxisomes, and MVK, PMVK, or IPPase have PTS-2, -1, or -1 signals, respectively. We previously reported that a small amount of MPD in the liver of rats fed a Cholestyramine Pravastatin (CP) diet [5% cholestyramine (inhibitor of small intestine cholesterol adsorption) and 0.1% pravastatin]...
(HMG-CoA reductase inhibitor)] exists in peroxisomes, although MPD is predominantly present in the cytosol in the liver of rats fed normal chow and a CP diet for 12 days; this therefore suggests that the major site of MPD in the M/O fraction of Cos7 cells is the peroxisomes. If this is the case, the PTS of MPD may be found or characterized by elucidating the amino acid sequence of MPD in monkeys.

In conclusion, we found a difference in the subcellular distribution of MPD by cell type. Our results also suggest that the cholesterol biosynthetic pathway may actually be two pathways (from cytosol to ER, and from peroxisome to ER) depending on the cell type or species; therefore, we conclude that the controversy over the subcellular distribution of MPD (i.e., whether the major site of MPD is the cytosol) has occurred because of differences in the cell types or species used in each experiment. Further studies are necessary to understand the physiological role and subcellular distribution of MPD in Cos7 cells in detail.

REFERENCES

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