Mevalonate Pyrophosphate Decarboxylase is Predominantly Located in the Cytosol of both B16 and B16F10 Cells in Mouse Melanoma Treated with Lovastatin

Akihiro Michihara,* Sachiyo Morita, Ken Toda, Kenji Akasaki, and Hiroshi Tsuji

Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729–0292, Japan

(Received September 25, 2007; Accepted December 25, 2007; Published online January 11, 2008)

Recently, it has been questioned whether mevalonate pyrophosphate decarboxylase (MPD) is predominantly located in the peroxisomes or cytosol. We previously reported that a small amount of MPD in the liver of rats fed a CP diet (5% cholestyramine and 0.1% pravastatin) existed in the peroxisomes, although MPD is predominantly located in the cytosol in the liver of rats fed normal chow and a CP diet for 12 days. In the present study, we examined the subcellular distribution of MPD in mouse melanoma cells (B16 and B16F10) treated with or without lovastatin, using digitonin permeabilization and immunoblotting. In permeabilized B16 by digitonin after treatment with or without lovastatin, 95% and 5%, or 98% and 2% of MPD existed in the cytosol and membrane/organelle (M/O) fraction, respectively. Using B16F10 under the same conditions, 80% and 20%, or 91% and 9% of MPD existed in the cytosol and M/O fraction, respectively. These results indicated that MPD was predominantly located in the cytosol in both mouse melanoma cells treated with or without lovastatin.

Key words —— mevalonate pyrophosphate decarboxylase, subcellular distribution, melanoma, mouse, cytosol

*To whom correspondence should be addressed: Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729–0292, Japan. Tel.: +81-849-36-2111; Fax: +81-849-36-2024; E-mail: mitihara@fupharm.fukuyama-u.ac.jp

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 tissue 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 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,[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 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.[10] 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.[11] They also reported that deficiencies of MVK and PMVK activity in the liver of human Zellweger patients reflect the bad condition of the liver, rather than mislocalization to the cytosol. The relationship between peroxisome proliferation and the enzyme involved in cholesterol biosynthesis of mice remains unclear.

Biardi and Krisans reported that MPD was mainly located in peroxisomes of CV-1 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.[8] We previously reported that MPD was predominantly located in the cytosol of rat hepatocytes,[12] normal rat kidney cells (NRK),[12] or mouse melanoma cells (B16F10)[13] treated with fetal bovine serum (FBS), respectively, since in permeabilized rat hepatocytes, NRK, or B16F10 treated with digitonin, which lack cytosolic enzymes, MPD was mainly present in the

medium (cytosolic fraction).

Recently, the enzymes involved in cholesterol have been shown to contain a peroxisomal targeting signal (PTS-1; [S/A/C][K/H/R][L/M]), found at the extreme carboxy terminal of the 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).[14] Peroxisomal targeting signals (PTS) of MVK and PMVK were identified as PTS-2(KV(X_5)HA) and-1(SRL), respectively.[14] Peroxisomal targeting signals of HMG-CoA synthase, MPD, and FPPase were identified with SV(X_5)QL, SV(X_5)QL, and KL(X_4)QE, as new variations of PTS-2, respectively.[14] Peroxisomal targeting signals of acetoacetyl CoA thiolase and IPPase 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.

We also reported that a small amount of MPD in the liver of rats fed a CP diet (5% cholestyramine [inhibitor of small intestine cholesterol adsorption] and 0.1% pravastatin [inhibitor of liver cholesterol biosynthesis, which is an HMG-CoA reductase inhibitor]) existed in peroxisomes isolated by cell fractionation, although MPD in the liver of rats fed normal chow and a CP diet is predominantly located in the cytosol.[15] From these data, we suggested that a small amount of MPD was transported to peroxisomes from cytosol, when the total amount of MPD increased or cholesterol content decreased by a CP diet. To establish whether a small amount of MPD was transported to peroxisomes from the cytosol when the increase of MPD or the decrease of cholesterol content in cells was caused by lovastatin (HMG-CoA reductase inhibitor), we examined the change of the subcellular distribution of MPD in mouse melanoma cells (B16, which is black cell with a lot of melanin, and B16F10, which is white cell without melanin) treated with or without lovastatin using digitonin permeabilization and immunoblotting.

MATERIALS AND METHODS

Materials —— Lovastatin was obtained from Merck (Tokyo, Japan). LDS was obtained from Sigma-aldrich (Tokyo, Japan). Percoll and the ECL Western blotting detection kit were from Amersham Pharmacia Biotech (Tokyo, Japan). Digitonin and Cholesterol E-test Wako were obtained from Wako (Osaka, Japan). Dulbecco's modified Eagle's medium (D-MEM) was obtained from Gibco (Tokyo, Japan). B16 were obtained from RIKEN (Ibaraki, Japan). B16F10 cells were kindly provided by Dr. J. T. Augast (Johns Hopkins University). Goat anti-lactate dehydrogenase (LDH)-A (N-14) IgG was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Sheep anti-catalase (Bovine) IgG was obtained from The Binding Site (Birmingham, U.K.). Rabbit anti-goat IgG conjugated to horseradish peroxidase was obtained from American Qualex Antibodies (San Clemente, CA, U.S.A.). Goat anti-rabbit IgG conjugated to horseradish peroxidase was obtained from Invitrogen Corporation (Carlsbad, CA, U.S.A.). Donkey anti-sheep IgG conjugated to horseradish peroxidase was obtained from Cortex Biochem, Inc. (Sanleandro, CA, U.S.A.). All other chemicals were of reagent grade, and purchased from various commercial sources.

Cultured Melanoma Cell Line —— B16 and B16F10 were diluted to 3×10^6 per 60 mm tissue culture dish 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, 0–30 μM) were added for various periods ranging from 0 to 48 hr.

Enzyme Activity —— The activity of LDH, a cytosol marker, was measured according to the method of Abei.[16] The activity of catalase, a peroxisomal marker, was measured according to the method of Coudrier *et al.*[17]

Protein Assay —— Protein levels were measured by the method of Lowry *et al.*, using bovine serum albumin (BSA) as the standard.[18]

Antibody MPD —— MPD was purified from rat liver, as described by Michihara *et al.*, and rabbit polyclonal antiserum raised against rat MPD was used.[19]

Permeabilization —— B16 or B16F10 cells incubated on 35 mm dishes 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 KHM buffer containing 40 μg/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, 1 ml of phosphate-buffered suline (PBS) containing 0.5 mM Phenylmethylsulfonylfluoride (PMSF), 1% Triton X-100, 10 mM mercaptoethanol, 1 mM EDTA, and protease inhibitors (1 μM leupeptin, 1 μM pepstatin A, 1 μM chymostatin, and 1 μM antipain), was added to the cells, which were then scraped off using a rubber policeman. The solution of B16 or B16F10 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 KHM buffer without digitonin, and processed in the same way as digitonin-treated cells. The amount of protein or enzyme activity retained in the nonpermeabilized cells was taken to be 100%.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) —— SDS-PAGE was performed on 10% slab gels according to the method of Laemmli.[20]

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.*[21] Positive bands were visualized using ECL Western blotting detection kits (Amersham Pharmacia, Amersham, U.K.) that contained a sensitive chemiluminescent substrate for horseradish peroxidase.

Cholesterol Content of Cells —— Two hundred microliters of postnuclear supernatant (PNS) was mixed with 5 ml of Folch extract (chloroform [2]: methanol [1]), and the mixture was incubated for 10 min at 37°C with shaking. After the mixture was centrifuged at 3000 rpm for 10 min, 3 ml of the supernatant was evaporated dry by boiling at 100°C and then dissolved in 200 μl of isopropylalchol containing 1% Triton X-100. The cholesterol content of the solution was determined using the Cholesterol E-test Wako.

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

RESULTS

Selective Permeabilization of the Plasma Membrane, and Release of Cytosolic Components in B16

We previously reported that MPD is predominantly located in the cytosol of B16F10 without melanin;[13] however, no subcellular distribution of MPD in B16 containing a lot of melanin was found. In order to identify whether the subcellular distribution of MPD changed by differences in melanin biosynthesis, the subcellular distribution of MPD in B16 was examined using permeabilized cells, which retain their organelle integrity yet lack cytosolic components. Digitonin treatment of cells has been reported to permeabilize the plasma membrane reversibly, leaving subcellular organelles intact. The time course of the release of cellular enzymes, LDH, catalase, and total cellular protein, is shown in Fig. 1A, B. Most protein release from permeabilized cells occurred within 10 min and did not change between 10 and 20 min of treatment. At 10 min, 80% of the activity of LDH and about 39% of total cellular protein were released (Fig. 1A); however, 90% of catalase activity was retained in the permeabilized cells (Fig. 1B), indicating that the peroxisomes remained intact. In order to further confirm that peroxisomes remained intact, under the same condition (at 10 min), when band signals were quantified using an Intelligent Quantifier after immunoblot analysis of LDH and catalase, 80% of the protein level of LDH, and 90% of the protein level of catalase were retained in the permeabilized cells (Fig. 1C). Based on kinetic studies and immunoblot analysis, we chose 10 min permeabilization during which cellular protein loss plateaued.

Localization of MPD in Permeabilized B16

To determine whether MPD was mainly present in the cytosol or peroxisomes (or other organelles), the amount of MPD protein was examined in the cytosol and membrane/organelle (M/O) fraction of permeabilized B16. As shown in Fig. 2, MPD protein from permeabilized B16 was found mainly in the cytosol fraction. Ninety-five percent of MPD protein existed in the cytosol fraction after 10 min incubation of digitonin, and was not retained after 30 min permeabilization. These results suggest that MPD was predominantly located in the cytosol of B16. Also, it is highly possible that MPD does not exist in peroxisomes, since the ratio of MPD (5%) retained in the M/O fractions was lower than that

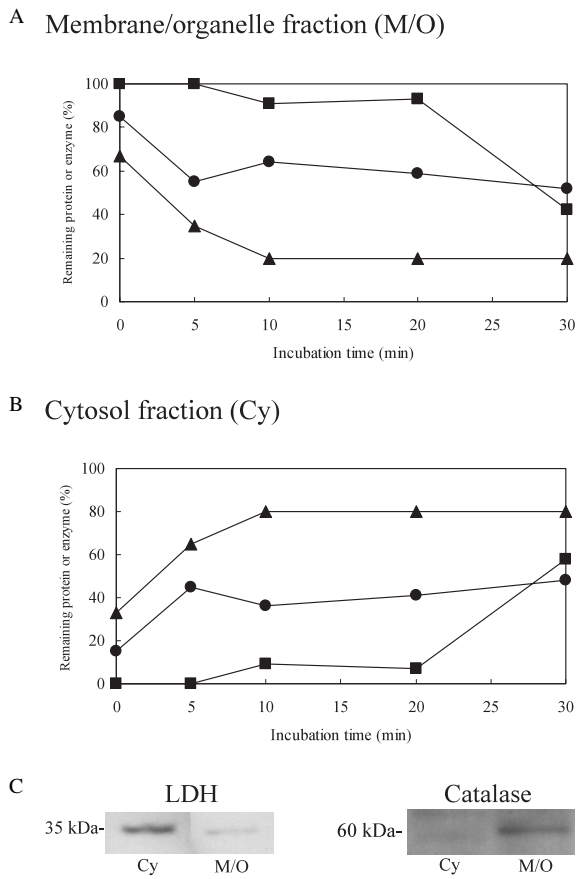

Fig. 1. Time Course of the Release of Protein and Enzymes from B16 during Digitonin Permeabilization

A, B: At different incubation times for digitonin, samples (cell; membrane/organelle fraction [M/O], buffer containing digitonin and the cytosol fraction [Cy]) were collected and analyzed for protein (●) and enzymatic activities of LDH (▲) and catalase (■), as described in Materials and Methods. Protein or enzyme activity combined in Cy and M/O are expressed as 100%, respectively. C: B16 were incubated in KHM buffer in the presence of digitonin at 4°C for 10 min, and the cells [M/O (■); 10 μl] and medium [Cy (●); 10 μl] were analyzed by immunoblotting using anti-LDH or anti-catalase antibody, respectively. Data are the means of three identical experiments and each varied within 5%.

of LDH (20%) in M/O fractions containing peroxisomes after 10 min permeabilization (Figs. 1, 2).

Induction of MPD and Reduction of Cholesterol Content

When the amount of cholesterol in cells is reduced by lovastatin, an HMG-CoA reductase inhibitor, the levels of enzymes involved in the synthesis of cholesterol, including MPD, in cells are increased by a feedback mechanism. Also, we previously reported that the cholesterol content in B16F10 significantly decreased, when the amount of MPD was maximally increased by lovastatin.[22] Therefore, the increase in the level of MPD in cells

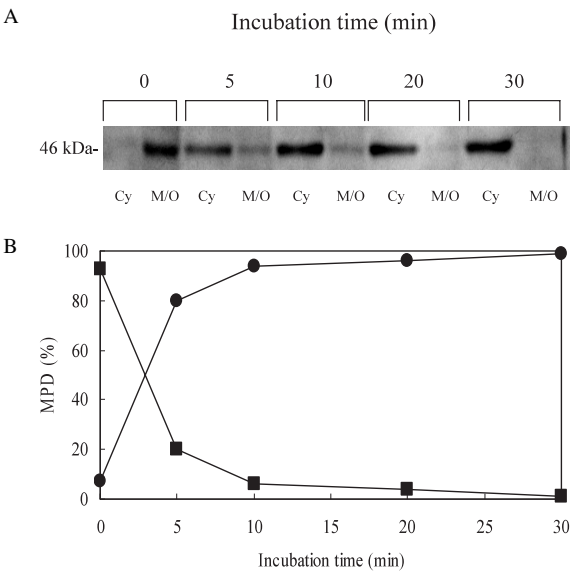

Fig. 2. Immunoblot Analysis of MPD in Permeabilized B16

A: B16 were incubated in KHM buffer in the presence of digitonin at 4°C for the indicated periods, and the cells [membrane/organelles fraction; M/O (■); 10 μl] and medium [cytosol fraction; Cy (●); 10 μl] were analyzed by immunoblotting. B: Signals of A were quantified using an Intelligent Quantifier. Data are the means of three identical experiments.

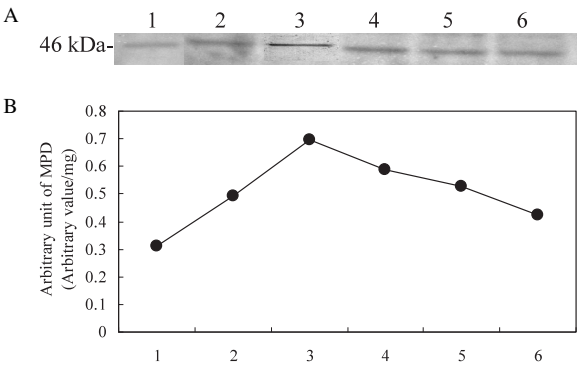

Fig. 3. Dose-Dependency of the Effect of Lovastatin on the Level of MPD of B16

Crude extracts were prepared from B16 incubated for 24 hr with various concentrations of lovastatin in medium containing 10% LDS or with 10% FBS. Crude extract (10 μg) was subjected to immunoblotting using anti-rat-MPD antiserum (A), and then these signals were measured using an Intelligent Quantifier (B). Lane 1, cells incubated in medium containing FBS; lane 2, cells incubated in medium containing LDS; lane 3, cells incubated in medium containing LDS and 10 μM lovastatin; lane 4, cells incubated in medium containing LDS and 30 μM lovastatin; lane 5, cells incubated in medium containing LDS and 50 μM lovastatin; lane 6, cells incubated in medium containing LDS and 100 μM lovastatin. Data are the means of three identical experiments.

was measured to examine the marked decrease of the cholesterol level in cells or the maximum effect by lovastatin. Figure 3 shows the typical increase

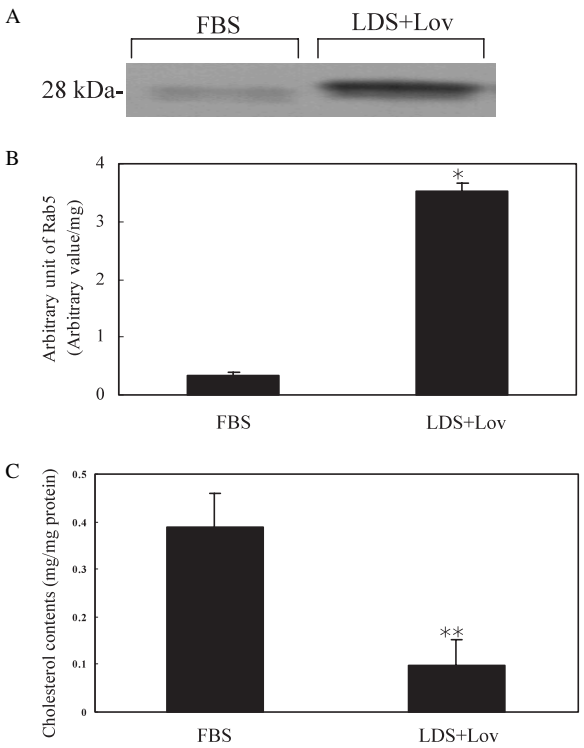

Fig. 4. Induction of Rab5 and Reduction of Cholesterol Contents in B16 by Lovastatin

Crude extracts were prepared from B16 incubated for 24 hr with 10% FBS or 10% LDS containing 10 μM lovastatin. Crude extract (10 μg) was subjected to immunoblotting using an anti-Rab5 antibody (A), and then these signals were measured using an Intelligent Quantifier (B). C: Cholesterol content was measured under the above conditions. FBS: cells incubated in medium containing 10% FBS, LDS + Lov: cells incubated in medium containing 10% LDS and 10 μM lovastatin. Data are the means of three identical experiments. Significantly different: $^{*}p < 0.000005$, $^{**}p < 0.05$ ($n = 3$).

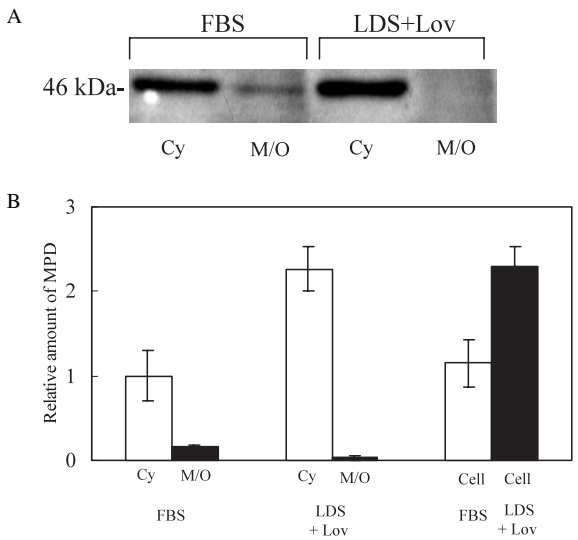

Fig. 5. Immunoblot Analysis of MPD in Permeabilized B16 after Treatment with Lovastatin

A: B16 were incubated in KHM buffer in the presence of digitonin at 4°C for 10 min, and the cells (membrane/organelles fraction; M/O; 10 μl) and medium (cytosol fraction; Cy; 10 μl) were analyzed by immunoblotting. B: Signals of A were quantified using an Intelligent Quantifier. Data are the means of three identical experiments.

of MPD in lovastatin-treated B16. When B16 was treated with 10% LDS containing 10 μM of lovastatin for 24 hr, a maximum of up to 2.2-fold of MPD in B16 treated with 10% FBS was observed.

Other groups reported that Rab5 and Rab7 proteins, which are involved in the regulation of the endocytic pathway, were significantly increased in FRTL-5 thyroid cells treated with lovastatin;[23] therefore, the increase in the level of Rab5 and the decrease of cholesterol in cells were measured to further establish whether the action by lovastatin and marked decrease of the cholesterol level in cells by lovastatin occurred under the above conditions. When B16 was treated with 10% LDS containing 10 μM of lovastatin for 24 hr, up to 10-fold of Rab5 in B16 treated with 10% FBS (Fig. 4A, B) and a significantly decrease of cholesterol (Fig. 4C) were observed. These data indicate that the conditions causing the maximal increase of MPD and significant decrease of cholesterol content were treatment with 10% LDS containing 10 μM lovastatin for 24 hr.

Localization of MPD in B16 Treated with Lovastatin

To determine whether MPD was mainly present in the cytosol or peroxisomes (or other organelles) of B16 treated with lovastatin, the amount of MPD protein was examined in the cytosol and M/O fraction of B16 treated with 10% FBS or 10% LDS containing 10 μM lovastatin for 24 hr. As shown in Fig. 5, MPD protein from permeabilized B16 after treatment with or without lovastatin was found mainly in the cytosol fraction. Also, 15%, 90%, and 15%, or 10%, 95%, and 2% of LDH, catalase, and MPD were retained in the M/O fraction of B16 treated with or without lovastatin, respectively (data not shown). Since the ratio of MPD (15%) retained in the M/O fraction was similar to that of LDH (15%) retained in the M/O fraction of nontreated B16, we consider that MPD detected in the M/O fraction was cytosol protein retained in the M/O fraction as well as LDH. The amount of MPD (2%) in the M/O fraction of B16 treated with lovastatin was lower than that (15%) of nontreated B16, and the ratio of MPD (5%) was lower than that of LDH (10%) retained in the M/O fraction of B16 treated with lovastatin. Therefore, it is highly pos-

sible that MPD does not exist in the peroxisomes of B16 treated with lovastatin. We concluded that MPD was predominantly located in the cytosol of B16 treated with lovastatin and non-treated B16.

Localization of MPD in B16F10 Cells Treated with Lovastatin

We previously reported that MPD is predominantly located in the cytosol of B16F10 and is not located in peroxisomes, and the level of MPD (3-fold) in B16F10 was markedly increased and the level of cholesterol content in B16F10 was significantly decreased by treatment with 10% LDS containing 10 μM lovastatin for 24 hr;[22] however, it was not confirmed whether MPD is predominantly located in the cytosol of B16F10 treated with lovastatin, so we examined the subcellular distribution of MPD using B16F10 treated with or without lovastatin. As shown in Fig. 6, MPD protein from permeabilized B16F10 after treatment with or without lovastatin was found mainly in the cytosol fraction. Also, 20%, 90%, and 20%, or 10%, 90%, and 9% of LDH, catalase, and MPD were retained in the M/O fraction of B16F10 treated with or without lovastatin, respectively (data not shown). Since the ratio of MPD (20%) retained in the M/O fraction was similar to that of LDH (20%) retained in the M/O fraction of nontreated B16, we consider that MPD detected in the M/O fraction was cytosol protein retained in the M/O fraction in addition to LDH. The amount of MPD (9%) in the M/O fraction of B16 treated with lovastatin was lower than that (20%) of nontreated B16, and the ratio of MPD (9%) was similar to that of LDH (10%) retained in the M/O fraction of B16 treated with lovastatin. Therefore, it is highly possible that MPD does not exist in the peroxisomes of B16F10 treated with lovastatin. We concluded that MPD was predominantly located in the cytosol, and MPD was not transported to peroxisomes from cytosol in both B16 and B16F10 treated with lovastatin.

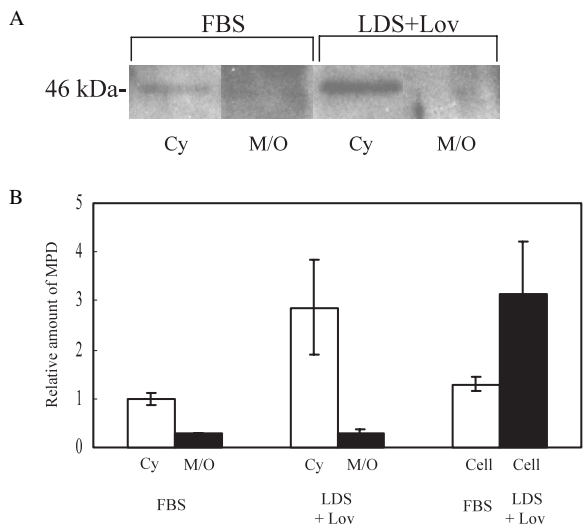

Fig. 6. Immunoblot Analysis of MPD in Permeabilized B16F10 after Treatment with Lovastatin

A: B16F10 were incubated in KHM buffer in the presence of digitonin at 4°C for 10 min, and the cells (membrane/organelles fraction; M/O; 10 μl) and medium (cytosol fraction; Cy; 10 μl) were analyzed by immunoblotting. B: Signals of A were quantified using an Intelligent Quantifier. Data are the means of three identical experiments.

DISCUSSION

We indicated in the present study (Fig. 4C) and a previous report[22] that the cholesterol content in B16 and B16F10 treated with 10% LDS containing 10 μM lovastatin at 24 hr was significantly decreased. Therefore, it was suggested that the transport of MPD to peroxisomes from cytosol in mouse melanoma was not responsible for the significant decrease of cholesterol contents in cells. We also previously reported a marked increase (10-fold or more) of MPD in the cytosol of the liver of rats fed a CP diet for 12 days as compared with nontreated rats, and a small amount of MPD in the peroxisomes of the liver of rats fed a CP diet for 12 days isolated by cell fractionation.[15] The increase of MPD was 1.8- or 2.3-fold in B16 (Fig. 5) or B16F10 (Fig. 6) treated with lovastatin, respectively. *In vivo*, as melanocytes or melanoma are not major sites of cholesterol biosynthesis like the liver or hepatocytes, melanocytes mostly maintain the cholesterol contents of cells by the uptake of cholesterol from outside. As the liver is the major organ of cholesterol biosynthesis supplying cholesterol to other tissues, there is much more cholesterol or cholesterol biosynthetic enzymes in the liver than in other tissues or cells. Therefore, there is difference in cholesterol biosynthesis potency between melanocytes or melanoma and the liver. When the ratio of MPD induction was compared between melanoma and the liver, the increase of MPD in mouse melanoma treated with lovastatin was lower than the increase of MPD (10-fold) in the liver of rats fed a CP diet for 12 days. Therefore, the difference in the transport or distribution of MPD between melanoma (*in vitro*) and liver (*in vivo*) treated with HMG-CoA reductase inhibitor might be due to

the cell type, but not the difference in the experimental conditions of *in vitro* and *in vivo*. Namely, the difference in the transport of MPD between the liver and melanoma treated with HMG-CoA reductase inhibitor might be due to the different induction potency of cholesterol biosynthetic enzyme between melanoma and the liver.

In conclusion, we suggested that the transport of MPD to peroxisome from cytosol might markedly increase MPD in cells not involved in the decrease of cholesterol in mouse melanoma. Further study is necessary to understand the transport mechanism of MPD to peroxisomes.

REFERENCES

1) Thompson, S. L. and Krisans, S. K. (1990) Rat liver peroxisomes catalyze the initial step in cholesterol synthesis. The condensation of acetyl-CoA units into acetoacetyl-CoA. *J. Biol. Chem*., **265**, 5731–5735.
2) Hovik, R., Brodal, B., Bartlett, K. and Osmundsen, H. (1991) Metabolism of acetyl-CoA by isolated peroxisomal fractions: formation of acetate and acetoacetyl-CoA. *J. Lipid Res*., **32**, 993–999.
3) Olivier, L. M. and Krisans, S. K. (2000) Peroxisomal protein targeting and identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes. *Biochim. Biophys. Acta*, **1529**, 89–102.
4) Keller, G. A., Barton, M. C., Shapiro, D. J. and Singer, S. J. (1985) 3-Hydroxy-3-methylglutaryl-coenzyme A reductase is present in peroxisomes in normal rat liver cells. *Proc. Natl. Acad. Sci. U.S.A*., **82**, 770–774.
5) Keller, G. A., Pazirandeh, M. and Krisans, S. (1986) 3-Hydroxy-3-methylglutaryl coenzyme A reductase localization in rat liver peroxisomes and microsomes of control and cholestyramine-treated animals: quantitative biochemical and immunoelectron microscopical analyses. *J. Cell. Biol*., **103**, 875–886.
6) Stamellos, K. D., Shackelford, J. E., Tanaka, R. D. and Krisans, S. K. (1992) Mevalonate kinase is localized in rat liver peroxisomes. *J. Biol. Chem*., **267**, 5560–5568.
7) Biardi, L., Sreedhar, A., Zokaei, A., Bozeat, R. L., Vartak, N. B., Shackelford, J. E., Keller, G. A. and Krisans, S. K. (1994) Mevalonate kinase is predominantly localized in peroxisomes and is defective in patients with peroxisome deficiency disorders. *J. Biol. Chem*., **269**, 1197–1205.
8) Biardi, L. and Krisans, S. K. (1996) Compartmentalization of cholesterol biosynthesis. Conversion of mevalonate to farnesyl diphosphate occurs in the peroxisomes. *J. Biol. Chem*., **271**, 1784–1788.
9) Paton, V. G., Shackelford, J. E. and Krisans, S. K. (1997) Cloning and subcellular localization of hamster and rat isopentenyl diphosphate dimethylallyl diphosphate isomerase. A PTS1 motif targets the enzyme to peroxisomes. *J. Biol. Chem*., **272**, 18945–18950.
10) Krisans, S. K., Ericsson, J., Edwars, P. A. and Keller, G. A. (1994) Farnesyl-diphosphate synthase is localized in peroxisomes. *J. Biol. Chem*., **269**, 14165–14169.
11) Hogenboom, S., Romeijin, G. J., Houten, S. M., Baes, M., Wanders, R. J. and Waterham, H. R. (2002) Absence of functional peroxisomes does not lead to deficiency of enzymes involved in cholesterol biosynthesis. *J. Lipid Res*., **43**, 90–98.
12) Michihara, A., Sawamura, M., Yamori, Y., Akasaki, K. and Tsuji, H. (2001) Mevalonate pyrophosphate decarboxylase is predominantly located in the cytosol of rat hepatocytes. *Biol. Pharm. Bull*., **24**, 1235–1240.
13) Michihara, A., Akasaki, K., Yamori, Y. and Tsuji, H. (2003) Subcellular distribution of mouse mevalonate pyrophosphate decarboxylase. *Biol. Pharm. Bull*., **26**, 579–584.
14) Olivier, L. M., Kovacs, W., Masuda, K., Keller, G. A. and Krisans, S. K. (2000) Identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes. AA-CoA thiolase, hmg-coa synthase, MPPD, and FPP synthase. *J. Lipid Res*., **41**, 1921–1935.
15) Michihara, A., Sawamura, M., Yamori, Y., Akasaki, K. and Tsuji, H. (2001) Difference in subcellular distribution between 45- and 37-kDa mevalonate pyrophosphate decarboxylase in rat liver. *Biol. Pharm. Bull*., **24**, 1347–1350.
16) Coudrier, E., Reggio, H. and Louvard, D. (1983) Characterization of an integral membrane glycoprotein associated with the microfilaments of pig intestinal microvilli. *EMBO J*., **2**, 469–475.
17) Abei, H. (1974) *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.), Vol. 2, Verlag Chemie, Wienheim, pp. 673–684.
18) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., **193**, 265–275.
19) Michihara, A., Sawamura, M., Nara, Y., Ikeda, K. and Yamori, Y. (1997) Purification and characterization of two mevalonate pyrophosphate decarboxylases from rat liver: a novel molecular species of 37 kDa. *J. Biochem*. (*Tokyo*), **122**, 647–654.
20) Laemmli, U. K. (1970) Cleavage of structural pro-

teins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
21) Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350–4354.
22) Michihara, A., Toda, K., Suenobu, M., Akasaki, K. and Tsuji, H. (2007) Decrease of cholesterol in mouse melanoma causes secretion of lysosomal enzymes. *J. Biochem.* (Tokyo), **141**, 239–250.
23) Laezza, C., Bucci, C., Santillo, M., Bruni, C. B. and Bifulco, M. (1998) Control of Rab5 and Rab7 expression by the isoprenoid pathway. *Biochem. Biophys. Res. Commun.*, **248**, 469–472.