M-LP, Mpv17-like Protein, Has a Peroxisomal Membrane Targeting Signal Comprising a Transmembrane Domain and a Positively Charged Loop and Up-regulates Expression of the Manganese Superoxide Dismutase Gene*

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M-LP (Mpv17-like protein) has been identified as a new protein that has high sequence homology with Mpv17 protein, a peroxisomal membrane protein involved in the development of early onset glomerulosclerosis. In this study, we verified the peroxisomal localization of M-LP by performing dual-color confocal analysis of COS-7 cells cotransfected with green fluorescent protein-tagged M-LP and DsRED2-PTS1, a red fluorescent peroxisomal marker. To characterize the peroxisomal membrane targeting signal, we examined the intracellular localizations of several green fluorescent protein-tagged deletion mutants and demonstrated that, of the three transmembrane segments predicted, the first near the NH₂ terminus and NH₂-terminal half of the following loop region, which is abundant in positively charged amino acids, were necessary and sufficient for peroxisomal targeting. To elucidate the function of M-LP, we examined the activities of several enzymes involved in reactive oxygen species metabolism in COS-7 cells and found that transfection with M-LP increased the superoxide dismutase activity significantly. Quantitative real-time PCR analysis revealed that the manganese SOD (SOD2) mRNA level of COS-7 cells transfected with M-LP was elevated. These results indicate that M-LP participates in reactive oxygen species metabolism.

The Mpv17-like protein (M-LP)

1 gene was identified on the basis of an expressed sequence tag obtained by differential display screening of age dependently expressed genes in mouse kidneys (1, 2). The M-LP gene is expressed mainly in the kidney and spleen and the amount expressed increases steadily during development, reaches its highest level in adulthood, and decreases gradually with aging. It encodes 194 amino acids of a polypeptide with a sequence and membrane topology markedly similar to those of two peroxisomal membrane proteins, Mpv17 protein (3) (30.4% identity, 66.7% similarity) and PMP22 (4) (25.0% identity, 72.1% similarity). These results suggest that M-LP might be embedded in the peroxisomal membrane in a similar manner to these two proteins.

Peroxisomes are ubiquitous eukaryotic subcellular organelles that play essential roles in a variety of metabolic pathways, including H₂O₂ metabolism and the oxidative degradation of fatty acids (5). Recently, a number of studies have been carried out to investigate the mechanisms of peroxisomal biogenesis and protein import (6–8). Targeting of peroxisomal matrix proteins is mediated by cytosolic receptors Pex5p and Pex7p that recognize the peroxisomal targeting signals PTS1, which consists of the sequence SKL (and conservative variants) at the carboxyl terminus (9, 10), and PTS2, which consists of the consensus sequence (W/K)(L/V/I)X₁(H/Q)X₂(A/L) near the amino terminus (11, 12), respectively. Whereas the targeting of peroxisomal matrix protein import has been well characterized, neither conclusive consensus sequence nor the receptor(s) involved in peroxisomal membrane protein targeting have been determined. Thus, it is important to characterize the membrane peroxisome targeting signals (mPTTs) in as many peroxisomal membrane proteins as possible to understand the biogenesis of peroxisomal membranes.

The Mpv17-negative mouse strain was generated by inserting a defective retrovirus into the germ line of mice and is characterized by progressive glomerulosclerosis and neurosensory deafness at a young age (3, 13). The phenotype results from loss of function of the Mpv17 gene encoding a 20-kDa peroxisomal membrane protein. The molecular function of the Mpv17 protein has yet to be elucidated, but it was recently hypothesized that it plays an important role in peroxisomal reactive oxygen species (ROS) metabolism and that glomerular damage is because of overproduction of ROS. In Mpv17 gene-inactivated mice, a significant increase in ROS and lipid peroxidation adduct production was observed and oxygen radical scavengers prevented glomerular damage (14). Moreover, a recent study showed that the γ-glutamyl transpeptidase enzyme activity and mRNA expression level were higher, whereas plasma glutathione peroxidase (Gpx3) and superoxide dismutase (SOD) activities were lower, in Mpv17 null cells than normal cells (15). These results strongly suggest that the Mpv17 protein is involved in enzymatic antioxidant defense systems. The aims of this study were first, to confirm the peroxisomal localization of M-LP and characterize the mPTTs of this new protein and second, to investigate the activities and expression of antioxidant enzymes to determine whether there is a connection between M-LP and ROS metabolism.
Experimental Procedures

Expression Vectors for the Peroxisomal Forms of Fluorescent Proteins—All the primers used for expression vector construction in this study are listed in Table I. Green fluorescent protein (GFP–PTS1) was amplified by PCR using the primer set exGFPS and GFPPTS1A and pEGFP (Clontech, Palo Alto, CA) as the template. The PCR product was cloned into the EcoRI/BamHI sites of mammalian expression vector, pcDNA3.1 (Invitrogen, Carlsbad, CA). DsRED2-PTS1 was amplified by the PCR using the primer set exGFPS and exGFPA and pEGFP as the template. DsRED2-PTS1 was cloned into the KpnI/HindIII sites of pcDNA3.1/MLP-GFP (Invitrogen, Carlsbad, CA).

Expression Vectors for M-LP—A DNA fragment encoding full-length M-LP was created by PCR amplification using the primer set exMLPS2 and exMLPA2 and first-strand cDNA synthesized using the total RNAs from the kidneys of 9-month-old mice as the template. The PCR products were cloned into the EcoRI/BamHI sites of a mammalian expression vector, pcDNA3.1 (Invitrogen, Carlsbad, CA) containing the coding sequence of GFP behind the KpnI/HindIII site.

Cells and Transfections—COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 1 mm L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) fetal calf serum (Invitrogen) and transiently transfected using LipofectAMINE Plus reagent (Invitrogen), according to the manufacturer's instructions.

Confocal Microscopy—COS-7 cells were plated in 3.5-cm poly-l-lysine-coated glass-bottomed dishes (MatTek, Ashland, MA) and 24 h after transfection, fluorescence images were analyzed by a laser scanning confocal microscope LSM-G2B00 (Olympus, Tokyo, Japan). An argon laser at 488 nm was used for excitation and the fluorescent signals emitted by GFP and DsRed2 were detected using a 535-nm band-pass filter and a 570-nm long pass filter, respectively.

Generation of Anti-M-LP Antibodies—The cDNA encoding NH2-terminal amino acids 1–103 or COOH-terminal amino acids 105–194 of M-LP were cloned in frame with glutathione S-transferase into the pGEX-6P-1 vector (Amersham Biosciences, Tokyo, Japan), introduced into Escherichia coli INVaF (Invitrogen), and induced using standard procedures. The glutathione S-transferase fusion proteins were solubilized from inclusion bodies using 8 m urea and affinity purified using glutathione-Sepharose-4B (Amersham Biosciences). For antibody production, the glutathione S-transferase fusion proteins were emulsified with adjuvant (TiterMax Gold; CytRx, Atlanta, GA), injected intramuscularly into Japanese white rabbits three times and the immunoglobulin G fractions were purified using a protein A column (Hittrap Protein A; Amersham Biosciences).

Enzyme Assays—The activities of enzymes involved in ROS metabolism in COS-7 cells transfected with pcDNA3.1/MLP and pcDNA3.1 as a control were determined. Three days after transfection, culture media and cell lysates were recovered and their enzymatic activities were detected. The cell lysates used for the measurement of SOD and Gpx activities were prepared by mixing them with 250 mm Tris-HCl (pH 7.5) containing 500 μm phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin and subjecting them to three freeze-thaw cycles, after which the SOD and Gpx activities were assayed using a SOD assay kit-WST (Djoin Molecular Technologies, Kumamoto, Japan) and a glutathione peroxidase assay kit (Cayman, Ann Arbor, MI), respectively. To investigate the effects of the anti-M-LP antibody on the SOD activities, cell lysates were incubated with antibody serially diluted with 25 mm Tris-HCl (pH 7.4) containing 8% (w/v) sodium chloride and 0.2% (w/v) potassium chloride at 37 °C for 1 h and the remaining activities were measured.

Cell lysates were prepared for the measurement of catalase (CAT) activity as follows. The cell lysates used for the measurement of SOD and Gpx activities were mixed with 250 mm Tris-HCl (pH 7.4) containing 8% (w/v) sodium chloride and 0.2% (w/v) potassium chloride at 37 °C for 1 h and the remaining activities were measured.

| Name | Sequences (5′–3′) |
|------|------------------|
| exGFPS | GaggattACCAAGGGCATCACCCGCCGAGAG |
| DsREDS | CggagttCAATCTTGGACAGAAGGAGGAGG |
| exMLPS2 | CggtcgcACCTGCTGCTGCTGCTGCTGCTG |
| exMLPA2 | CggtcgcACCTGCTGCTGCTGCTGCTG |
| 55MA | GggtaccGGCCAGAGTGGCCACGCGAC |
| 55MLP | GggtaccGGCCAGAGTGGCCACGCGAC |
| exGFPA | CggtcgcACCTGCTGCTGCTGCTGCTG |
| exGM1A2 | CggtcgcACCTGCTGCTGCTGCTG |
| 35MGS | GggtaccAACATGGTGAGCAAGGGCGAGGAG |
| 34MGS | GggtaccAACATGGTGAGCAAGGGCGAGGAG |
| 91MS | GggtaccAGTCTGATCGCATAGCACCTTG |
| 91MA | GggtaccAGTCTGATCGCATAGCACCTTG |
| 16MGS | GggtaccACCATGCCGTGGCCCACTAACGT |
| 35MGS | GggtaccAACATGGTGAGCAAGGGCGAGGAG |
| 34MGS | GggtaccAACATGGTGAGCAAGGGCGAGGAG |
| 92MGS | GggtaccAACATGGTGAGCAAGGGCGAGGAG |
| 91MA | GggtaccAACATGGTGAGCAAGGGCGAGGAG |
| 15DM5 | GggtaccAACATGGTGAGCAAGGGCGAGGAG |

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Peroxisomal Membrane Targeting

To assess the mPTS of Half of the Loop between TMS1 and TMS2 Are Required for between the mPTS and its receptor(s) was hindered by tagging. Between means were determined using Student's t test and those at p < 0.05 were considered significant.

Measurement of mRNA Levels—Total RNA from mouse kidneys was prepared as described in our previous report (1) and total RNA was extracted from COS-7 cells using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's instructions. The mRNA levels were determined by quantitative real-time PCR analysis with a LightCycler Instrument (Roche Molecular Diagnostics) using the QuantiTect SYBR Green PCR kit (Qiagen, Chatsworth, CA) as previously described (17). The primer sequences and product size for each gene are summarized in Table II. The amount of mRNA was normalized to the internal control, glyceraldehyde-3-phosphate dehydrogenase. All PCR assays were performed in triplicate and the intra-assay variability was <7%.

RESULTS AND DISCUSSION

M-LP Localizes to Peroxisomes—A peroxisomal localization of M-LP was considered highly likely in view of the significant homology with both the amino acid sequences (30.4% identity, 66.7% similarity) and hydrophilicity profile of the peroxisomal membrane protein Mpv17. Accordingly, colocalization analysis of GFP-tagged M-LP and a peroxisomal marker protein was performed. First, the effectiveness of DaRed2-PTS1 as a peroxisomal marker was tested. Punctate fluorescence signals were observed in cells expressing DaRed2-PTS1 and they colocalized completely with the signals because of GFP-PTS1 (Fig. 1A), which has been used as a peroxisomal marker in previous studies (18, 19). The signals were localized exclusively in peroxisomes, whereas diffuse cytoplasmic fluorescence was observed in control cells expressing DaRed2. These results demonstrate that DaRed2-PTS1, as well as GFP-PTS1, is an effective marker for peroxisomal protein. Next, cells were co-transfected with DaRed2-PTS1 and either MLP-GFP or GFP-MLP and their intracellular distributions were examined (Fig. 1B). Punctate green fluorescence because of MLP-GFP was observed in MLP-GFP-expressing cells and was superimposable on the red fluorescence because of DaRed2-PTS1, demonstrating clearly that M-LP was targeted to peroxisomes. However, GFP-MLP was detected in peroxisomes and the cytoplasm. These results could be interpreted as meaning that the mPTS exists around the NH2-terminal and the interaction between the mPTS and its receptor(s) was hindered by tagging GFP onto the NH2 terminus of M-LP.

The Transmembrane Segment 1 (TMS1) and NH2-terminal Half of the Loop between TMS1 and TMS2 Are Required for Peroxisomal Membrane Targeting.—To assess the mPTS of M-LP more closely, we constructed several NH2- and COOH-terminal deletion mutants, as shown in Fig. 2A, and examined their intracellular localizations (Fig. 1C). From the putative topology of M-LP derived from its primary structure, three TMSs, TMS1-(16–34), TMS2-(92–110), and TMS3-(151–168) have been predicted (2). Δ15MLP-GFP, the mutant lacking the NH2-terminal tail consisting of the first 15 amino acids, was targeted properly to peroxisomes, as revealed by its colocalization with DaRed2-PTS1. However, the signals from cells expressing Δ34MLP-GFP, which lacks the NH2-terminal tail and TMS1, did not correspond to peroxisomes, although several punctate signals were observed. In the case of Δ91MLP-GFP, which lacks the NH2-terminal tail, TMS1, and the loop between TMS1 and TMS2, overall diffuse cytosolic fluorescence was observed. Next, in the cases of mutants truncated at the COOH-terminal region, 91MLP-GFP comprising residues 1–91, which include the NH2-terminal tail, TMS1, and the loop between TMS1 and TMS2, and 55MLP-GFP comprising residues 1–55, which include the NH2-terminal tail, TMS1, and half of the loop between TMS1 and TMS2, were located exclusively in peroxisomes, whereas 34MLP-GFP comprising residues 1–34, which include the NH2-terminal tail and TMS1, was partially localized in peroxisomes. These results demonstrate that TMS1 and the NH2-terminal half of the loop between TMS1 and TMS2 are required for the correct peroxisomal localization of M-LP. To confirm this hypothesis, 16/55MLP-GFP, comprising residues 16–55, which include TMS1 and the NH2-terminal half of the loop between TMS1 and TMS2, was not observed and its intracellular localization was examined. As shown in Fig. 1C, the signals were localized exclusively in peroxisomes, suggesting that TMS1 and the NH2-terminal half of the loop between TMS1 and TMS2 are sufficient to function as an mPTS.

Although a great deal of information about peroxisomal matrix protein import has been obtained, our understanding of how peroxisomal membrane proteins are targeted and integrated into peroxisomal membranes is still poor. The sequences involved in targeting or insertion of peroxisomal membrane proteins described so far are as follows: in Pichia pastoris Pas2p (20) and human (21) and Hansenula polymorpha (22) Pex3p, positively charged amino acids at the NH2-terminal region were shown to be involved in targeting or insertion; in Saccharomyces cerevisiae Pex15p, the COOH-terminal luminal tail was required for targeting (23); in rat PMP70, an internal region that roughly corresponded to the TMS3 was necessary for targeting and insertion (24); in S. cerevisiae PMP47, TMS2 plus an adjacent cytoplasmic oriented sequence, a matrix facing basic cluster, and an anchoring TMS were sufficient for targeting (25); in human PMP34, the loop region between TMS4 and TMS5 and three transmembrane segments were required for targeting and insertion (26); and in rat PMP22, the NH2-terminal region and more than one TMS were required for targeting and insertion (27). Although the amino acid se-
Fig. 1. Colocalization analysis of GFP-tagged M-LP with DsRED2-PTS1, a fluorescent marker of peroxisomes. COS-7 cells were cotransfected with GFP-PTS1 and DsRED2-PTS1 (A), GFP-tagged M-LP and DsRED2-PTS1 (B), or GFP-tagged deletion mutants of M-LP and DsRED2-PTS1 (C), respectively. The fluorescent images because of GFP (green) and DsRED (red) were analyzed by a laser scanning confocal microscope.
sequences of M-LP and PMP22 are very similar (25.0% identity, 72.1% similarity), those in the NH2-terminal regions are quite different and the mPTS identified in PMP22 was not found in M-LP. Honsyo and Fujiki (26) pointed out that no conclusive consensus sequence had been observed, but the feature common to most of these proteins was the presence of positively charged amino acids located in the flanking region of a TMS. From this point of view, M-LP has two clusters of positively charged amino acids in the loop between TMS1 and TMS2 (the first cluster at amino acid positions 37, 39, 46, 49, and 50; the second at positions 68, 72, 77, 80, and 85). Our results demonstrate that the presence of the NH2-terminal half of the loop containing the first cluster and adjacent TMS1 were sufficient for M-LP to function as an mPTS. Therefore, it can be said that M-LP satisfies the law these authors suggested.

SOD Activity Is Elevated in COS-7 Cells Transfected with M-LP

The high sequence and membrane topological homologies of M-LP and Mpv17 have led to speculation that M-LP, as well as Mpv17, functions as an ROS scavenger. Accordingly, we determined the activities of enzymes involved in ROS metabolism in COS-7 cells transfected with pcDNA3.1/MLP and pcDNA3.1 as a control. As shown in Table III, there was no significant difference between the Gpx or CAT activities of these cells. However, the SOD activity of the cell lysates prepared from COS-7 cells transfected with M-LP was significantly higher than that of the control cells. The following two mechanisms may be responsible for the elevation of SOD activity: activation of SOD at the protein level and an increase in the amount of SOD enzyme expressed. However, in view of our finding that preincubation with an anti-M-LP antibody did not influence the SOD activity (data not shown), it seems unlikely that the SOD activity is regulated by an...
interaction with M-LP at the protein level. Manganese-SOD (SOD2) mRNA Is Increased in COS-7 Cells Transfected with M-LP—Three SOD isoforms, a cytosolic, copper-zinc SOD (SOD1), a mitochondrial, manganese SOD (SOD2), and an extracellular SOD (SOD3) have been detected in mammalian cells (27). The mRNA levels of these three SODs, cellular Gpx (Gpx1), Gpx3, and CAT in COS-7 cells transfected with M-LP were examined using quantitative real-time reverse transcriptase-PCR (Fig. 3). Whereas the expression levels of five genes (SOD1, SOD3, Gpx1, Gpx3, and CAT) were unchanged, an increase in SOD2 mRNA compared with the control cell level was observed, suggesting that the increase in SOD activity in the M-LP expressing cells was due mainly to up-regulation of the SOD2 gene. Recently, Wagner et al. (15) performed the reverse experiment, i.e. examination of the activities and mRNA levels of antioxidant enzymes in Mpv17 null cells, and found that the absence of the Mpv17 protein reduced expression of all three SOD isoforms and Gpx3 and concomitantly increased γ-glutamyl transpeptidase expression. Therefore, M-LP seems to have the same function as the Mpv17 protein from the point of view that its action leads to a reduction in ROS production.

The Expression Patterns of SOD2 and M-LP Genes in Mouse Kidney during Development and Aging Are Similar—In our previous study, we used comparative reverse transcriptase-PCR to show that M-LP gene expression changed age-dependently (2). Our finding that M-LP affected expression of the SOD2 gene awoke our interest in the expression patterns of the SOD2 gene during development and aging. Therefore, the levels of M-LP and SOD2 mRNAs in the kidneys of 3-day- to 15-month-old mice were measured using quantitative real-time PCR. As shown in Fig. 4A, the amount of M-LP expressed increased steadily during development, reached its highest levels in adulthood and decreased gradually with aging. This observation agreed quite well with our previous results (2). As expected, the expression pattern of the SOD2 gene was very similar to that of M-LP (Fig. 4B), suggesting that M-LP participates in the regulation of SOD2 gene expression.

Age-dependent changes in SOD2 activity or its gene expression have been reported in several mammalian tissues. However, the expression patterns vary depending on species and/or tissues. For instance, SOD2 activity was observed to be lower in human skin fibroblast cell lines derived from fetal skin than those from postnatal skin, although no postnatal age-dependent differences were observed (29). Three-month-old rats had higher hepatic SOD2 activities than 24-month-old rats, whereas there was no significant age-related difference in the SOD2 activities of the lungs (30). In our previous study, we showed that the M-LP gene is expressed mainly in the kidney and spleen. The different expression patterns in various tissues may be attributable to the participation of a tissue-specific molecule like M-LP in the regulation of SOD2.

ROS are generated constantly during oxidative metabolism and cause cellular damage by reacting with proteins, lipids, DNA, and carbohydrates. SOD is considered to be one of the most important constituents of the first line of defense against ROS production by virtue of its ability to convert highly reactive superoxide radicals to hydrogen peroxide and molecular oxygen. Our data demonstrated that, of the three SODs, only the SOD2 gene was up-regulated by the expression of M-LP. The biological importance of SOD2 has been shown in studies on knockout mice (31, 32): homozygous mutant mice died less than 3 weeks after birth, probably because of severe impairment of mitochondrial function because of the elevated level of ROS (28). Analysis of the 5′-flanking region resulted in the identification of several regulatory sequences, including Sp1, NF-κB, CCAAT-enhancer binding protein, and an antioxidant-response element (33). Of the antioxidant enzymes, only SOD2 is induced by various stimuli, such as tumor necrosis factor-α (34), interleukin-1 (35), and X-irradiation (36) that are known to produce ROS or induce intracellular ROS generation. The mechanism responsible for the induction of SOD2 under these conditions has yet to be elucidated, however, it has been proposed that tumor necrosis factor-α and interleukin-1 regulate SOD2 expression by virtue of their ability to convert highly reactive superoxide radicals to hydrogen peroxide and molecular oxygen. M-LP molecule like M-LP in the regulation of SOD2.
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J. Biol. Chem. 2003, 278:6301-6306.
doi: 10.1074/jbc.M210886200 originally published online December 5, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M210886200

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