MLDP, a Novel PAT Family Protein Localized to Lipid Droplets and Enriched in the Heart, Is Regulated by Peroxisome Proliferator-activated Receptor α*

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Tomohiro Yamaguchi1, Shuhei Matsushita1, Kiyoto Motojima2, Fumiko Hirose2, and Takashi Osumi1,2

From the 1Graduate School of Life Science, University of Hyogo, 3-2-1 Koto, Kamigori, Hyogo, 678-1297, Japan and the 2Department of Biochemistry, Meiji Pharmaceutical University, Kiyose, Tokyo 204-8588, Japan

Cytosolic lipid droplets (LDs) are multifunctional organelles that exist in all types of eukaryotic cells and control lipid homeostasis. In mammalian cells LDs contain a class of proteins in their surface layers that share a homologous sequence called the PAT domain, including perilipin, adipose differentiation-related protein (ADRP), a tail-interacting protein of 47 kDa (TIP47), and S3-12, which are distributed tissue- or cell type-selectively. Expression in some cases is regulated by peroxisome proliferator-activated receptors (PPARs). In this study we identified a new PAT family member named MLDP (myocardial LD protein) in a murine cDNA database and showed the mRNA and protein to be highly enriched in the heart and also expressed at lower levels in the liver and adrenals. Upon subcellular fractionation, a substantial amount of MLDP was detected in the top fraction enriched with LDs. Furthermore, overexpressed MLDP tagged with green fluorescent protein accumulated at the surfaces of LDs and co-localized with perilipin and ADRP. Deletion analysis demonstrated the N-terminal region containing a PAT-1 domain and the following 33-mer domain to be required for targeting of MLDP to LDs. MLDP was found to be up-regulated at both mRNA and protein levels in the heart and liver by a selective ligand for PPARα, Wy14,643, but not in PPARα knock-out mice. MLDP expression was also increased upon fasting in parallel with ADRP. These results indicate that MLDP is a bona fide new PAT family member localized in LDs. Its expression depends on the physiological conditions and the action of PPARα.

Lipid droplets (LDs)3 are neutral lipid storage facilities surrounded by a phospholipid monolayer (1, 2) that are now recognized to be functional subcellular organelles rather than metabolically inactive lipid depots. They are involved in multiple intracellular processes including lipid metabolism, vesicle traffic, and cell signaling through interactions with other organelles so that important roles in lipid homeostasis are likely (3–5). Although LDs exist in most, or perhaps all types of cells, their sizes and characteristic features considerably differ among tissues. For example, mature adipocytes, typical lipid-storing cells, contain massive LDs that occupy nearly the entire cell volume. Hepatocytes and macrophages have LDs that are highly inducible by the environmental burden. Steroidogenic cells also have numerous small LDs containing cholesterol esters used for steroid hormone synthesis. In contrast, cardiac and skeletal muscle cells with very high rates of lipolytic turnover normally contain minute LDs, which enlarge in response to disease or diet (3). A question thus arises; what is the major determinant of diverse features of LDs among tissues?

It is to be expected that protein components associated with their surfaces would contribute to the specific functions of LDs. Indeed, recent progress of proteomics and microscopic analysis have clarified that there are many proteins on the surfaces of LDs (5–11) and even inside the droplet core (12, 13), and the protein composition varies with the cell type and the physiological condition. Particularly, mammalian LDs contain a specific set of proteins called the PAT domain family that include perilipin, adipose differentiation-related protein (ADRP, also called adipophilin), a tail-interacting protein of 47 kDa (TIP47), and S3-12, sharing regions of conserved sequences, named PAT domains, mainly at their N termini (14). Perilipin is a phosphoprotein involved in the hormone-stimulated lipolysis whose expression is highly restricted to adipocytes and steroidogenic cells (15, 16). In adipocytes, perilipin protects the LDs from lipases under basal conditions. In response to hormonal stimulation, perilipin is multiphosphorylated by CAMP-dependent protein kinase A and recruits hormone-sensitive lipase and other lipases to the LDs, thereby promoting lipolysis (17, 18). S3-12 exhibits a similar tissue distribution but is localized in a separate pool of LDs lacking perilipin, possibly participating in the early events of LD formation in adipocytes (19, 20). ADRP is expressed ubiquitously and functions in incorporation and accumulation of lipid (21–24). Because its expression level is highly dependent on the total fat cell mass, ADRP serves as a marker for lipid storage and disease states of fat-accumulating cells (25, 26). TIP47 was first identified as a binding partner of the mannose 6-phosphate receptor and may be involved in membrane traffic from the trans-Golgi network (27). However, recent work employing proteomics and microscopic analysis has provided evidence that TIP47 is also an LD protein (7, 11, 12, 20). Thus, TIP47 is at least in part located on LDs and possibly functions in lipid metabolism.

Another common feature of PAT family proteins is that expression of the corresponding genes is regulated by peroxisome proliferator-activated receptors (PPARs), ligand-activated transcription factors belonging to the nuclear receptor superfamily. Through heterodimerization with retinoid X receptors, they regulate gene transcription by binding to specific response elements (peroxisome proliferator response elements) (28). The PPAR family comprises three closely related members, α, γ, and δ, each distributing tissue selectively; PPARα and -γ are predomin-
nant in the liver and adipocytes, respectively, whereas PPARα is ubiquitously expressed (28). Corresponding to these distributions, it is known that perilipin and S3-12 genes are up-regulated by PPARγ (29–33), whereas ADRP is controlled by multiple PPARs in a tissue- or cell type-selective manner (34–40). PPARs overall regulate various genes involved in lipid metabolism and are key players in body energy homeostasis. The available data, thus, support the notion that PAT proteins share involvement in lipid metabolism within LDs and that their diversity reflects multiple features of LDs in mammalian tissues.

In line with this conclusion, we report here a novel member of the PAT family named MLDP (myocardial LD protein), also located on the surfaces of LDs and regulated by PPARs. In contrast to the previously described PAT proteins, MLDP is abundant in tissues containing minute LDs such as the heart rather than typical lipid storage organs like adipose tissue.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Reagents**—A cDNA of MLDP was obtained from total RNA of MLTC-1 cells by reverse transcription (RT)-PCR and subcloned into the pcMV5 vector. The entire open reading frame and a series of truncation mutants of MLDP, created by PCR or digestion at appropriate restriction sites, were also inserted into a GFP expression vector, pHGFP (105) (41). PPAR ligands, Wy14,643, rosiglitazone, and PPAR ligands were synthesized from 1 μg of total RNA in a reaction mixture containing Moloney murine leukemia virus reverse transcriptase (Invitrogen) using a downstream primer mixture (2 pmol of each) in a total volume of 20 μl. PCR was performed with 2 μl of the RT product as a template and 10 pmol each of the upstream and downstream primers and rTaq DNA polymerase (Takara). The reaction products were separated in 2% agarose gels and detected with a fluorescence imaging analyzer FLA3000 (Fuji). Intensities of the bands were quantified by densitometry with software installed in FLA3000 imaging analyzer.

**Northern Blotting**—RNA (20 μg) was separated on 1.2% agarose/formaldehyde/MOPS gels and blotted to GeneScreen Plus Nylon membranes (DuPont). After prehybridization for 2 h at 42 °C in hybridization buffer (10% dextran sulfate, 1 mM NaCl, 1% SDS, 50% formamide), membranes were hybridized for 16 h at 42 °C in the same buffer with a radiolabeled probe (32P-labeled cDNA fragment for murine MLDP or rat 36B4, 3 × 10^6 cpm/ml) and then washed with 2× standard saline citrate (SSC (1× SSC = 0.15 mM NaCl and 0.015 mM sodium citrate)), 1% SDS 3 times at 65 °C for 20 min each followed by 2 washes with 0.2× SSC at room temperature for 10 min each. Hybridization signals were identified with the FLA3000 imaging analyzer.

**Subcellular Fractionation**—Fractionation of MLTC-1 cells was carried out according to a previous report (21) with slight modifications. Cells (three 150-mm dishes) were washed with PBS, collected by centrifugation, resuspended in a hypotonic medium (10 mM HEPES/NaOH (pH 7.4), 1 mM EDTA, 10 mM sodium fluoride, protease inhibitor mixture (Roche Applied Science)) and incubated for 10 min on ice followed by 20 strokes with a Teflon/glass homogenizer. The homogenate was centrifuged at 500 × g for 5 min at 4 °C to obtain post-nuclear supernatant (PNS). For gradient centrifugation PNS was adjusted to 30% sucrose and layered beneath a 0–30% sucrose gradient. Centrifugation was carried out at 100,000 × g for 6 h at 4 °C. Twelve fractions were collected from the top, and proteins were sedimented from each fraction with trichloroacetic acid. An equal aliquot (10% for mouse heart or 15% for MLTC-1 cells) of each fraction was analyzed by 10% SDS-PAGE and immunoblotting. For the subcellular fractionation of heart, one mouse heart was minced in a tissue disintegrator and homogenized with a Teflon/glass homogenizer. PNS was obtained by centrifugation at 800 × g for 5 min at 4 °C. Gradient centrifugation was performed as described above.

**Western Blotting**—For immunodetection of MLDP, mouse tissues were homogenized in 0.25 M sucrose containing 1 mM EDTA, 10 mM Tris-HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Sigma). Cultured cells were washed with PBS and directly dissolved in heated SDS-PAGE sample buffer. Aliquots of the extracts were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Proteins were probed with the affinity-purified anti-MLDP antibody and detected by enhanced chemiluminescence (ECL) (Amer sham Biosciences).

**Fluorescence Microscopy**—Transfected MLTC-1 cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and counterstained with 100 ng/ml Nile-Red in PBS for 10 min. After washing with PBS, cells were mounted and observed under a confocal microscope (LSM510, Carl Zeiss). For detection of ADRP and perilipin, cells expressing GFP-MLDP were fixed with 4% paraformaldehyde, PBS. Fixed cells were permeabilized with 0.01% digitonin, PBS for ADRP or 0.2% Triton X-100, PBS for perilipin and blocked with 1% bovine serum albumin in PBS. Cells were then incubated with primary polyclonal antibody against ADRP or perilipin for 1 h, washed with PBS, and incubated with Cy3-conjugated secondary antibody (Jackson Immuno-
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FIGURE 1. MLDP as a new member of PAT family proteins. A comparison of amino acid sequences among PAT family proteins, ADRP, TIP47, MLDP, and perilipin. A highly conserved region at the N terminus is defined as the PAT-1 domain (PAT-1). The following 33-mer motif (33m) is a sequence conserved through all PAT proteins including S3-12 (14). Values indicate the percentage of amino acid sequence homology as compared with ADRP. A phylogenetic tree was predicted by UPGMA (unweighted pair group method with arithmetic mean) method using the full-length protein sequences. Numbers on the right and above each bar indicate the total number and position of amino acid, respectively. a.a., amino acids. B, sequence alignment of the N-terminal regions of murine PAT proteins, MLDP, perilipin (Perl), ADRP, and TIP47. Asterisk, residue conserved throughout the four proteins; period, residue matching in three proteins.

RESULTS

MLDP Is a Novel Member of the PAT Family—PAT family proteins have been identified as proteins mainly associated with LDs and are present in diverse species such as vertebrates, Drosophila, and Dictyostelium but not yeast. In mammalian cells four representative proteins of this family, perilipin, ADRP, TIP47, and S3-12, have hitherto been described and are considered to be primarily involved in intracellular lipid metabolism. In the PAT domain two subdomains have been defined; PAT-1 at the N terminus with ~100 amino acids having high identity (Fig. 1A, PAT-1), and PAT-2, which is less similar at the C terminus, including the 33-mer motif also found in S3-12 (Fig. 1A, 33m). Despite the sequence similarities, individual PAT proteins are likely to have diverse roles in LDs (44). Here, we identified a novel murine protein that contains a PAT domain by NCBI BLAST search. This sequence (gi:27754108, Riken cDNA 2310076L09) has previously been reported with the name PAT1 (14), but the protein was not characterized. Because "PAT1" might easily be confused with the PAT-1 subdomain, we term this protein MLDP (myocardial LD protein, for the reasons described below) to distinguish them. MLDP shares sequence similarity along its entire length with perilipin, ADRP, and TIP47, especially in the PAT-1 domain (Fig. 1, A and B) and the 33-mer motif (Fig. 1A). We also searched for homologous cDNA species in databases and found a putative rat cDNA (gi:62718060) and a human partial cDNA similar to mouse MLDP (gi:61966858), implicating conservation of this sequence in mammals.

Tissue-selective Expression of MLDP—To characterize MLDP, we first examined the relative mRNA levels in various rat tissues by TaqMan RT-PCR, in comparison with those of perilipin and ADRP (Fig. 2A). The MLDP transcript was found in the heart at the highest level followed by skeletal muscle and the adrenals (Fig. 2A, asterisk), with relatively low levels in the liver and adipose tissues. Little or no expression was detected in other tissues. Expression patterns of perilipin and ADRP transcripts were largely consistent with previous reports (15, 21), supporting the fidelity of the results; that is, perilipin expression was highly restricted to adipose tissue and detected to a much lower extent in the adrenal, which contains steroidogenic cells. ADRP expression was nearly ubiquitous but was relatively enriched in the adrenal, liver, and ovary (Fig. 2A). The tissue distribution of MLDP was confirmed by Northern blot analysis (Fig. 2B). The cDNA probe identified two species of transcript, a major one of 2.2 kb corresponding to the cDNA found in data base, and a minor one of 1.4 kb. Similar to the results in Fig. 2A, MLDP was particularly abundant in the heart, adrenal, muscle, and adipose tissue, whereas few or no MLDP transcripts were detected in other tissues. The 36B4, a ribosomal protein gene used as a control, showed a nearly constant expression level across tissues.

Expression of MLDP mRNA was also examined in cultured cells. L6 and 3T3-L1 cells were used as models for muscle and adipocytes, respectively. In L6 myoblasts, MLDP mRNA was expressed at a low level, but on differentiation to myotubes up-regulation was apparent. Perilipin was not expressed, and ADRP mRNA was observed at similar levels irrespective of the differentiation (Fig. 2C). In 3T3-L1 cells, as expected, perilipin and ADRP were up-regulated upon adipocyte differentiation. In 3T3-L1 cells, however, MLDP transcripts were not detected despite the considerable expression in rat adipose tissue (Figs. 2, A and B). This might be due to difference in the natures of adipose tissue and 3T3-L1 cells. In MTL-1 cells, derived from mouse steroidogenic cells that contain LDs storing cholesterol ester, all PAT members including MLDP were expressed (Fig. 2C).
Identification of Endogenous MLDP in LDs—To characterize endogenous MLDP, we raised a polyclonal antibody against a synthetic peptide for the MLDP C terminus for application in an immunoblot analysis of total protein samples from various mouse tissues. As shown in Fig. 3A, the antibody bound to several bands, prominently at the positions of 54 kDa (Fig. 3A, arrow) and 59 kDa (Fig. 3A, asterisk). The lower band corresponded to endogenous MLDP with a calibrated molecular mass of 49 kDa. This was confirmed by electrophoresis of recombinant MLDP protein produced in HeLa cells in parallel (data not shown). Consistent with the mRNA distribution, the most prominent band was observed for the heart. After short exposure times, MLDP was detected only in the heart, but longer exposure uncovered lower levels of expression in the adrenals and liver. Despite the mRNA expression in muscle and adipose tissue, we could not detect MLDP protein in these tissues (Fig. 3A).

To address the intracellular location of MLDP, we next performed subcellular fractionation of mouse heart. PNS was subjected to flotation centrifugation through a 0–30% sucrose density gradient, and fractions were analyzed by immunoblotting for MLDP or other PAT family members. A significant amount of endogenous MLDP was recovered in the top fraction (Fig. 3B, top) and co-fractionated with ADRP but not with PMP70, a peroxisomal marker. We also performed subcellular fractionation using MLTC-1 cells. Again, MLDP was found in the top fraction and co-fractionated with ADRP and perilipin, the latter presenting as multiple bands corresponding to alternatively spliced forms (16) but not
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![Image 349x429 to 529x733](image1)

**FIGURE 3. Detection of endogenous MLDP protein.** A, Western blot analysis of MLDP in various mouse tissues. Aliquots (15 μg) of total proteins from various tissues were analyzed by SDS-PAGE, and the membrane was probed with an antibody raised to a synthetic C-terminal peptide of MLDP. Positions of the molecular weight standards are shown on the left. The arrow and asterisk indicate the position of MLDP and nonspecific material, respectively. B, subcellular fractionation of the mouse heart. PNS from the mouse heart homogenate was subjected to a 0 (Top) to 30 (Bottom) % sucrose density gradient. After centrifugation, fractions were collected from the top (fraction 1). Aliquots of proteins were subjected to SDS-PAGE followed by immunoblotting probed for MLDP, ADRP, and PMP70. PMP70 is a marker of peroxisomes, representing a non-floating organelle. Note that MLDP was found in the top fraction and co-fractionated with ADRP but not PMP70. C, subcellular fractionation of MLTC-1 cells. PNS from the MLTC-1 cell homogenate was analyzed as described in B followed by immunoblotting probed for MLDP, perilipin, and ADRP. The asterisk indicates the position of nonspecific material. Protein disulfide isomerase (PDI) was used as a marker for microsomal fractions. Perilipin displayed multiple bands corresponding to alternatively spliced forms.

with protein disulfide isomerase, a marker of microsomes (Fig. 3C). These results indicate that MLDP, like other PAT family members, is associated with LDs. A significant amount was also detected in lower fractions (no. 8–10) together with considerable quantities of ADRP and a small amount of perilipin. This probably represents a cytosolic form of MLDP because it was not pelleted by centrifugation of PNS at 100,000 × g (data not shown). Furthermore, an upper band of 59 kDa (Fig. 3C, asterisk) was found (see also Fig. 3A), probably due to a nonspecific cross-reactive material.

**Localization of MLDP on LDs as Revealed by Fluorescence Microscopy**—We investigated the subcellular localization of MLDP by introducing an expression vector of GFP-fused MLDP into MLTC-1 cells. GFP-MLDP exhibited ring-shaped subcellular distribution around the LDs revealed by Nile Red staining (Fig. 4A), indicating localization on their surfaces. Endogenous ADRP and perilipin gave ring-shaped distribution patterns, essentially co-localizing with GFP-MLDP (Fig. 4, B and C). However, MLDP was negligible in a small number of LDs where perilipin was particularly abundant (Fig. 4C, arrowhead), and as a whole a stronger GFP-MLDP signal was associated with a weaker perilipin signal and vice versa. Thus, MLDP and perilipin might compete for binding to LDs. On the other hand, intensities of signals of GFP-MLDP and ADRP were in proportionate. Localization of overexpressed GFP-MLDP on the surfaces of LDs was also observed in HeLa and 3T3-L1 cells (data not shown).

We next attempted to determine the region of MLDP that is necessary for the localization to LDs. For this purpose, a series of truncated mutants of MLDP was generated as GFP-fused proteins. After transfection to MLTC-1 cells, distributions of these fusion proteins were observed by fluorescence microscopy, and LD localization was judged by comparison with Nile Red staining. The full-length GFP-MLDP was recruited to LDs (Fig. 5, Full), and truncation of C-terminal region of MLDP maintaining the 33-mer motif (C3, 1–183) intact did not affect the localization. However, a shorter mutant (C4, 1–137) was mostly found to be distributed throughout the cytoplasm. On the other hand, when MLDP was truncated from the N terminus, all mutants failed to be localized to the surface of LDs even with loss of only a short fragment involving the PAT-1 domain (N1, 36–448). These results indicate that the N-terminal region of MLDP including the PAT-1 and 33-mer motifs is necessary for recruitment to LD surfaces.

**Induction of MLDP mRNA and Protein Both in Vitro and in Vivo by PPARα Ligand**—Because most PAT members are transcriptionally regulated by PPARs, we investigated the effect of PPAR ligands (Wy14,643, rosiglitazone, and GW501,516, activating PPARα, -γ, and -δ, respectively) on the level of MLDP mRNA in MLTC-1 cells, estimated by RT-PCR. Induction was observed after 24 h of treatment with Wy14,643...
but not rosiglitazone or GW501,516. Furthermore, only in the Wy14,643-treated cells, increase of MLDP protein was observed upon immunoblotting (Fig. 6B). Unexpectedly, mRNA for perilipin, a known target of PPARγ, was induced with Wy14,643 but not rosiglitazone (Fig. 6A). mRNA of PPARγ was not detectable in Leydig cell lines such as MLTC-1 (data not shown) and MA-10 (45). Thus, perilipin might be regulated by PPARα in these cells. None of the ligands significantly affected the levels of ADRP. To confirm that the MLDP gene is a target of PPARα, we next investigated induction by Wy14,643 in vivo. RT-PCR was performed using cardiac or hepatic RNA from wild-type and PPARα-null mice fed a normal diet (Fig. 7A, Chow) or a diet containing Wy14,643 (Fig. 7A, WY). In the wild-type mice, MLDP mRNA was considerably increased by the ligand in both the heart and liver, consistent with Fig. 6A. However, in the PPARα-null mice, the basal level of MLDP mRNA was extremely low, and no induction was observed upon ligand administration. Results for acyl-CoA oxidase (AOX), a known target gene of PPARα, were similar to those on MLDP, and the level of 36B4 mRNA was not changed under these conditions. It is reported that PPARα activation increases the levels of ADRP in hepatocytes (35). Consistently, ADRP mRNA was increased by Wy14,643 in the liver but not in the heart (Fig. 7A). The effect of PPARα ligand on MLDP protein expression was also investigated using PPARα-null mice (Fig. 7B). Total heart, liver, or muscle lysates were prepared from wild-type and PPARα-null mice fed on diet with or without Wy14,643 and analyzed by immunoblotting. In the heart of normal mice, MLDP protein was clearly increased by Wy14,643 but could not be detected in the PPARα-null mice irrespective of ligand treatment. Interestingly, expression of ADRP protein was decreased by Wy14,643 despite an unchanged mRNA level. This implies that the ADRP protein is susceptible to post-transcriptional regulation such as degradation by the ubiquitin/protesome pathway (46, 47). In the liver, only small amounts of MLDP protein, difficult to detect, were expressed in the absence of ligand (Fig. 3A, liver). However, the liver lysates from Wy14,643-administered wild-type mice exhibited a significant increase in MLDP protein, to an easily detectable level (Fig. 7B). In contrast, even after ligand administration, the PPARα-null mice did not exhibit detectable MLDP protein. Consistent with earlier results (35), expression of ADRP protein in the liver was increased by Wy14,643 in wild-type mice. Significant expression of ADRP protein was constitutively observed also in PPARα null mice despite the lower mRNA expression than in the wild type. MLDP

FIGURE 5. Analysis of MLDP sequences necessary for recruitment to LDs. A, schematic diagram of MLDP truncation mutants and summary of their abilities to be localized to LDs in MLTC-1 cells. MLTC-1 cells were transiently transfected with expression plasmids encoding the full-length and truncated MLDPs tagged with GFP at the N terminus. The subcellular localization of expressed protein was observed by confocal microscopy. The full-length, C1, C2, and C3 constructs were targeted to Nile Red-positive LDs and marked positive (+/+). C4 showed the targeting to LDs in a few cells but not in most of cells and is marked as +/-.. All other mutants failed to be detected on LDs, which are marked negative (−). Values at the right indicate the percentage of cells in which the GFP-protein is predominantly found on LDs. For each construct, the experiment was performed at least twice, and more than 200 cells were scored. Gray and filled areas in the schematic diagrams indicate PAT-1 domain (PAT-1) and 33-mer motif (33m), respectively. Numbers indicate the amino acid positions in mouse MLDP protein. B, distribution of representative MLDP mutants in MLTC-1 cells. Cells labeled with GFP-MLDP and Nile Red are shown. C2 exhibited localization around LDs that is similar to full-length MLDP, whereas C4 was mostly distributed throughout the cells. Bar, 10 μm.

FIGURE 6. Selective induction of mouse MLDP mRNA (A) and protein expression (B) by peroxisome proliferators in MLTC-1 cells. A, MLTC-1 cells were incubated without (−) or with selective agonists of PPARs; 100 nM Wy14,643 (Wy), 10 μM rosiglitazone (Ros), and 100 nM GW501,516 (GW) for PPARα, γ, and δ, respectively, for 24 h. MLDP, ADRP, and perilipin mRNAs were estimated by RT-PCR using total RNA extracted from the cells. 36B4 was used as a control that is not induced by peroxisome proliferators. Values below the panels indicate the relative mRNA levels quantified by densitometry, taking those of non-treated (−) cells as 1. Reproducibility of the results was confirmed by another independent experiment. B, whole cell lysates were prepared from MLTC-1 cells treated with the peroxisome proliferators as indicated. Aliquots of samples were subjected to SDS-PAGE followed by immunoblotting probed with anti-MLDP, ADRP, and perilipin antibodies. Lactate dehydrogenase (LDH) was used as a control. The positions of the molecular mass standards are shown on the left.
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mRNA was also expressed in skeletal muscle (Fig. 2, A and B), although protein expression could not be detected (Fig. 3A) until stimulation by Wy14,643 in normal, but not PPARα-null mice (Fig. 3B). Taken together, these results strongly suggest that PPARα regulates transcription of the MLDP gene in vivo.

MLDP Expression Is Increased by Fasting in the Heart and Liver—It is important how MLDP expression is regulated in vivo under physiological conditions. Starvation is one serious situation encountered by animals that triggers a number of adaptive metabolic responses. For example, it is known that the level of ADRP is increased upon fasting in the heart (48). When MLDP mRNA and protein levels were analyzed by RT-PCR and immunoblotting, respectively, in tissue lysates prepared from mice fed or fasted for 24 h, a marked increase in protein was observed upon fasting, especially drastically in the liver (Fig. 8B, liver), in parallel with increase in ADRP. A small, but significant increase in MLDP protein was also observed in the heart upon fasting (Fig. 8B, heart) along with MLDP mRNA in both tissues (Fig. 8A).

DISCUSSION

In mammalian cells, LDs are coated with a specific set of proteins, perilipin, ADRP, TIP47, and S3-12, constituting the PAT family. In this study, through a data base search we were able to identify another PAT protein, designated MLDP, consisting of 448 amino acids and carrying the PAT domain at the N terminus. MLDP is similar to perilipin, ADRP, and TIP47 over its entire length. As far as we could determine, no other candidate novel PAT member was present. MLDP mRNA was found to be highly enriched in the heart and also expressed at lower levels in liver, skeletal muscle, adrenal, and adipose tissue but not in other organs. Consistent with this, MLDP protein was detected predominantly in the heart. Similar to perilipin and ADRP, MLDP was concentrated in the top fraction by density gradient centrifugation, and GFP-MLDP expressed in MLTC-1 cells was localized at the surface of LDs, indicating a direct association with these cellular bodies. We also tried to examine endogenous MLDP localization in the cells by indirect immunofluorescence analysis but failed to detect any signals in the cells (data not shown). Our antibody, thus, proved useful for immunoblot analysis but is probably not applicable for immunofluorescence. We further showed that expression of MLDP is controlled by PPARα in vivo (mice) and in vitro (MLTC-1 cells).

How is the MLDP gene regulated by PPARα? In the mouse genome (chromosome 17), several genes are positioned closely adjacent to the MLDP gene. The transcriptional initiation site of the MLDP gene is in fact located 2.1 kb downstream from the end of the leucine-rich α-2-glycoprotein 1 (Lrg1) gene. Interestingly, the gene for S3-12, another member of the PAT family, is located 1.8 kb downstream from the end of the MLDP gene, in the same orientation. A similar gene arrangement is conserved in the human genome. In human chromosome 19, a partial sequence highly homologous to the mouse MLDP (gi:61966858) is located between the Lrg1 and predicted S3-12 (KIAA1881) genes, suggesting the presence of a counterpart of MLDP in man. Because the present study indicates that the expression of MLDP is regulated by PPARα, we tried to examine whether the promoter region of MLDP gene contains an active peroxisome proliferator response element. In transfection studies, however, we failed to find any sequence element in the 2.1-kb region upstream of the MLDP initiation site that could confer transactivation by PPARα (data not shown). Sequences in the downstream, structural gene or further upstream region of the MLDP gene may be required for the activation by PPARα. In fact, the downstream region of MLDP corresponds to the promoter region of the S3-12 gene, which does contain functional peroxisome proliferator response elements for PPARγ action (33). Judging from our results, PPARγ does not seem to regulate the transcription of MLDP. However, it is possible that MLDP and S3-12 share binding sites of PPARα as reported to be the case for perilipin and PEX11α genes; these genes are regulated by PPARγ and -α, respectively, through a common cis-element in a tissue-selective manner (30). The functional cis-element for PPARα regulation of MLDP expression should be clarified in the future.

A characteristic common to all PAT family members is localization to LDs. However, the targeting signals for LDs seem quite diverse among PAT members. For example, perilipin is targeted and anchored to LDs via the hydrophobic sequences of its central region (49, 50), which is not conserved in ADRP. The targeting signal of ADRP is not determined by any distinct amino acid sequence. Thus, the amino acid residues important for targeting appear to be spread discontinuously throughout the
entire sequence, and the total three-dimensional structure is critical (51–53). In neither perilipin nor ADRP is the highly conserved PAT domain in the N-terminal region necessary for targeting, in clear contrast to the case with MLDP. Loss of the C-terminal half of MLDP did not affect the LD localization, but deletion of 35 residues at the N-terminal caused mistargeting (Fig. 5). The difference in localization signals among the PAT family members possibly reflects diversity in the mode of association with LDs and, hence, differences in their intracellular functions.

It should be emphasized that MLDP is highly enriched in the heart, a notable feature among PAT family members. Although the heart preferably uses fatty acid as an energy source under normal conditions, it has a relatively limited capacity for storing lipid. Accordingly, cardiomyocytes exhibit a very high rate of lipolytic turnover and abundantly express PPARα, a key regulator of the genes involved in fatty acid oxidation (54, 55). The energy balance of the heart varies depending on physiological conditions such as fasting or disease. The hearts from fasted mice contain numerous LDs, with enlarged sizes compared with those of fed mice (48). During fasting, PPARα has a critical role in stimulating fatty acid oxidation (56, 57). In the present study, fasting caused a marked increase in MLDP expression in the heart. This suggests the involvement of MLDP in the metabolic response to fasting, extending our understanding of how PPARα contributes to this process. LDs accumulate in the heart in certain diseases such as cardiomyopathy or diabetes; hence, leading to lipo-apoptosis. It was reported that activation of PPARα reduces myocardial infarct size and protects the heart from ischemia/reperfusion injury in rodents (58, 59). MLDP might be involved in these protective actions. Based on the observation that MLDP is selectively expressed in the heart under normal conditions and up-regulated by PPARα or fasting, it is likely that MLDP preferentially resides on LDs of tissues with high rates of lipolysis, and hence, facilitates the consumption of intracellular lipids. In contrast, the protein level of ADRP in the heart was decreased by activation of PPARα, raising the possibility that this PAT has opposing functions, that is, accumulation of lipid. This is supported by recent work on ADRP-deficient mice, which display impairment of LD formation and are resistant to diet-induced fatty liver (60). Although fasting led to a small increase in ADRP protein level in the heart, it would reflect the enlarged LDs caused by fasting. On the other hand, it is known that perilipin controls both consumption and accumulation of lipids through regulation of phosphorylation (18). These observations allow us to speculate that PAT family proteins determine LD properties with regard to storage and mobilization of lipids. Furthermore, our current data also suggest that the PAT and PPAR families are more intimately interlinked than is generally recognized. The relative abundance of PPARs as well as selective co-activators and co-repressors for individual PPARs might determine the levels of PAT proteins in individual tissues.

Ligands of PPARs have been applied in clinical treatment for obesity, diabetes, and hyperlipidemia, but the molecular mechanisms underpinning their therapeutic value are not fully understood. PAT proteins may be important mediators of the in vivo effects of these medicines. Thus, further studies of the physiological functions of MLDP along with other PAT family members should not only contribute to elucidating LD multiple functions but also lead to better understanding of the effects of PPAR ligands in the heart and, hence, remedies for heart diseases arising from abnormal lipid accumulation.

**FIGURE 8.** Fasting causes an increase in MLDP mRNA (A) and protein (B) expression in the heart and liver. A, RT-PCR was performed to estimate mRNA levels of MLDP and ADRP with total RNA isolated from the hearts (left) or livers (right) of fed or 24 h-fasted mice. 36B4 was used as a control. Histograms below the panels show the relative mRNA levels determined by densitometry, taking those of fed mice as 1. The experiment was carried out with three mice for each group, and the averages are shown together with the S.D. B, Western blot (WB) analysis was performed for endogenous MLDP and ADRP with tissue lysates from the hearts (left) or livers (right) of fed or 24 h-fasted mice. Experiments were done using two independent mice for each group.
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