Dynamic and differential regulation of proteins that coat lipid droplets in fatty liver dystrophic mice

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Abstract Lipid droplet proteins (LDPs) coat the surface of triglyceride-rich lipid droplets and regulate their formation and lipolysis. We profiled hepatic LDP expression in fatty liver dystrophic (fld) mice, a unique model of neonatal hepatic steatosis that predictably resolves between postnatal day 14 (P14) and P17. Western blotting revealed that perilipin-2/ADRP and perilipin-5/OXPAT were markedly increased in steatotic fld liver but returned to normal by P17. However, the changes in perilipin-2 and perilipin-5 protein content in fld mice were exaggerated compared with relatively modest increases in corresponding mRNAs encoding these proteins, a phenomenon likely mediated by increased protein stability. Conversely, cell death-inducing DFFA-like effector (Cide) family genes were strongly induced at the level of mRNA expression in steatotic fld mouse liver. Surprisingly, levels of peroxisome proliferator-activated receptor γ, which is known to regulate Cide expression, were unchanged in fld mice. However, sterol-regulatory element binding protein 1 (SREBP-1) was activated in fld liver and CideA was revealed as a new direct target gene of SREBP-1. In summary, LDP content is markedly increased in liver of fld mice. However, whereas perilipin-2 and perilipin-5 levels are primarily regulated posttranslationally, Cide family mRNA expression is induced, suggesting that these families of LDP are controlled at different regulatory checkpoints.—Hall, A. M., E. M. Brunt, Z. Chen, N. Viswakarma, J. K. Reddy, N. E. Wolins, and B. N. Finck. Dynamic and differential regulation of proteins that coat lipid droplets in fatty liver dystrophic mice. J. Lipid Res. 2010. 51: 554–563.

Supplementary key words hepatic steatosis • lipodystrophy • perilipin family • lipin • lipid droplet

Lipid droplets (LDs) are metabolically active structures that play important roles in lipid transport, sorting, and signaling cascades (1). Although adipose tissue is the predominant site of fat storage in higher organisms, most tissues have at least some capacity to store triglyceride (TG) in small LDs that can be used for an immediate energy source. However, several pathologic conditions are associated with marked ectopic fat deposition. For example, fatty liver disease is characterized by striking accumulation of neutral lipid in the cytosol of hepatocytes. The accumulation of LDs in hepatocytes is often, at least overtly, a non-progressive condition. However, in some individuals, lipotoxicity can result in pathologic changes in hepatic cytoarchitecture, including hepatocyte apoptosis and ballooning and inflammation (steatohepatitis) that subsequently can result in fibrosis and parenchymal remodeling with cirrhosis.

The surface layer of LDs is coated by numerous proteins collectively known as lipid droplet proteins (LDPs) (reviewed in ref. 2). Although a variety of proteins associate with LDs, the best-studied is the family of PAT proteins (3). This family was named after the original three constituents, perilipin, adipocyte differentiation-related protein (ADRP), and tail interacting protein 47 (TIP47). Recently, a standardized nomenclature for PAT family proteins has been adopted (4) and this revised naming system for perilipin-1 (perilipin), perilipin-2 (ADRP or adipophilin), and perilipin-3 (TIP47) is used hereafter. Two additional

Abbreviations: Acacb, acetyl-coenzyme A carboxylase β; ChIP, chromatin immunoprecipitation; Cide, cell death-inducing DFFA-like effector; Elov16, elongation of very long chain fatty acids-16; fld, fatty liver dystrophic; Gpam, glycerol-3-phosphate acyltransferase, mitochondrial; LD, lipid droplet; LDP, lipid droplet protein; OA, oleic acid; P14, postnatal day 14; PAT, perilipin-ADRP-TIP47; PPARγ, peroxisome proliferator-activated receptor γ; Scd1, stearoyl-coenzyme A desaturase 1; SREBP-1, sterol-regulatory element binding protein 1; TG, triglyceride, WT, wild-type.

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The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one table and one figure.

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proteins, perilipin-4 (S3-12) and perilipin-5 (also known as OXPAT, MLDP, PAT-1, and LSDP5) (5, 6), have now been added to the perilipin family based on sequence and functional similarities. Another family of proteins, known as the cell death-inducing DFFA-like effector (Cide) family of proteins (CideA, CideB, and CideC), has also recently emerged as LDPs that regulate LD metabolism (7). The mouse homolog of CideC is known as Fsp27 and will be referenced as such henceforth. Interestingly, work from several groups has convincingly demonstrated that Cide proteins are also physically associated with LDs and modulate LD size and metabolism (8, 9).

LDPs allow LDs to maintain a dynamic communication with the endoplasmic reticulum and the plasma membrane (1). Several lines of evidence suggest that perilipin-1 and perilipin-2 are physical barriers to lipolytic enzymes under basal conditions yet can facilitate interactions with lipases and enhance lipolysis in response to lipolytic stimuli (10–12). The significant alterations in lipid metabolism observed in mouse models with targeted deletion of LDPs are strong evidence for important roles for these proteins in regulating metabolism (13–15).

Although LDPs were originally studied from the perspective of their effects in adipose tissue, more recent work has shown critical roles for LDPs in regulating fat metabolism in liver, especially in the context of lipid overload such as occurs in obesity-related fatty liver disease. Specifically, the expression of perilipin-2, CideA, and Fsp27 is induced in liver of ob/ob mice, which exhibit marked hepatic steatosis (16–18). Moreover, mice deficient in perilipin-2, CideB, or Fsp27 are protected from developing obesity-related fatty liver disease (15, 19), suggesting that these proteins play a role in driving hepatic lipid accumulation or enhancing the capacity for storing lipid.

We sought to evaluate the expression of lipid droplet proteins in liver of fatty liver dystrophic (fld) mice, a lipodystrophic model of fatty liver (20). The complex and severe metabolic phenotype of fld mice is caused by a mutation in the gene encoding lipin 1 (Lpin1) (21). The fld mice appear normal at birth, but rapidly develop an enlarged fatty liver (20). Hepatic lipid accumulation in fld mice spontaneously and rapidly resolves prior to weaning (20), making this model a unique and interesting system in which to study the expression of the LDP proteins. Herein, we demonstrate that expression of several LDPs is markedly increased in the steatotic liver of fld mice, and decreases as the fatty liver phenotype resolves. Our studies also revealed that the PAT and Cide families of LDPs are controlled by distinct regulatory mechanisms in steatotic hepatocytes.

MATERIALS AND METHODS

Animal studies

All studies were conducted with matched littermate mice. Homozygous fld (fld/fld) mice were compared with littermate heterozygous (fld/+ or wild-type (+/+) control mice. Eighteen-week-old female ob/ob and lean littermate (ob+/+) were sacrificed for tissue collection. All animal experiments were approved by the Washington University School of Medicine Animal Studies Committee and conformed to criteria outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tissue histology

Freshly obtained liver samples were fixed overnight in 10% buffered formalin and embedded in paraffin. Hematoxylin and eosin staining was carried out by the Digestive Disease Research Core Center at Washington University School of Medicine.

Determination of liver TG levels

TG concentration was determined by a commercial colorimetric method (Wako Chemicals, Richmond, VA) with solvent extracted lipid, as described (22). Total liver protein was extracted using tissue protein extraction reagent (number 78510; Pierce) and TG concentration is expressed as milligrams of TG/g of liver protein.

mRNA isolation and gene expression analyses

Liver RNA was extracted with RNAzol Bee (Isotexdiagnostics, Friendswood, TX) according to the manufacturer’s instructions. Real time RT-PCR was performed using the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA) and the SYBR green kit. Using the standard curve method, the relative amount of specific PCR products for each primer set was generated. For normalization, 36B4 was amplified from each sample, and arbitrary units of target mRNA were corrected to the corresponding level of the 36B4 mRNA. The sequences of the primers used in these studies can be found in supplementary Table I.

Protein isolation and cellular fractionation of mouse livers

For Western blot studies in Fig. 2, protein extracts were obtained from liver by using the following lysis buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% sodium deoxycholate, and 1% Triton X-100) containing a protease inhibitor cocktail. For sterol-regulatory element binding protein (SREBP)-1 Western blots, nuclear proteins were isolated using the NTRACT nuclear protein isolation kit (Sigma Chemical Co., St. Louis, MO). Protein concentrations were determined with the Micro BCA protein assay kit (Thermo Scientific, Rockford, IL).

For subcellular fractionation studies, livers from 12-week-old fed fld and wild-type (WT) mice were minced and then homogenized by a Teflon pestle tissue homogenizer in lysis buffer (10 mM HEPES, 1 mM EDTA, pH 7.4). The nuclear fraction was obtained by centrifugation for 5 min. The supernatant was weighed with sucrose to 40% (w/v) with 65% sucrose and was then overlaid with successive layers of 5 ml 35% sucrose and 2,000 g centrifugation for 5 min. The 2,000 g supernatant was then overlaid with successive layers of 5 ml 35% sucrose and 5 ml 10% sucrose. The tubes were then filled to capacity with lysis buffer. The gradients were centrifuged at 172,000 × g for 3 h at 4°. Fractions were harvested as described previously and stored at −80°C until Western blot analyses (23).

Western blot analysis

Western blotting studies were performed with whole-cell lysates (40 µg) or nuclear lysates (20 µg) as indicated. Proteins from sucrose gradient isolation were loaded by volume rather than protein content. Proteins were separated on 4–12% gradient gels by SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes that were then blocked with 5% (w/v) nonfat dry milk in TBS-Tween (20 mM Tris-HCl pH 7.5, 0.9% Tween-20) and incubated with antibodies at 4°C overnight. Blots were then washed with TBS-Tween and incubated in secondary antibodies (anti-rabbit IgG or anti-mouse IgG) for 1 h. Blots were washed again and detected with enhanced chemiluminescence (ECL) reagent (Pierce).
NaCl, 0.05% Tween 20) prior to immunoblotting. Generation of rabbit-derived antibodies directed to carboxy-terminus of perilipin-5, the amino-terminus of perilipin-3, and the amino-terminus of perilipin-2 has been described previously (5, 6). Antibodies to glycogen synthase (ProteinTech Group, Chicago, IL), peroxisome proliferator-activated receptor γ (PPARγ) (Santa Cruz, San Diego, CA), SREBP-1 (Santa Cruz), CideA (Genway, San Diego, CA), Fsp27 (generous gift of Dr. Vishwajeet Puri), and actin (Sigma) were used according to the manufacturer’s instructions.

Primary mouse hepatocyte isolation

Primary mouse hepatocytes were isolated from mice as previously described (24). Briefly, mice were anesthetized and then perfused through the portal vein with warmed HBSS containing collagenase. Livers were mechanically disrupted with forceps. Released cells were washed extensively and plated onto collagen coated dishes and grown in culture in DMEM supplemented with 5% FBS.

Pulse-chase experiment

After plating and adherence, hepatocytes from adult WT mice were washed three times with PBS and incubated in Met- and Cys-free DMEM for 1 h to deplete the cellular pool of Met and Cys. Thereafter, the medium was replaced with 1 ml of Met- and Cys-free DMEM containing 200 µCi of [35S]Promix with or without oleic acid (OA) (0.4 mM; to give an OA/BSA ratio of 6) for 1 h. After the 1 h pulse, the cells were washed twice with PBS and incubated in 1 ml of DMEM containing 10 mM Met and 3 mM Cys for 12 h with or without the specified OA.

Immunofluorescence microscopy

Hepatocytes from P14 WT or fld mice were isolated, plated, and then fixed with formaldehyde after adhering for 1 h. Fixed hepatocytes were then stained as described previously (25) with antibodies below. The cells were stained with anti-perilipin-2 (Fitzgerald Industries International, Flanders NJ, CAT# 20R-AP002), perilipin-3 (6), or perilipin-5 (5) antibodies. The secondary antibodies were goat Anti-Guinea Pig Alexa 488 and Donkey Anti-Rabbit Alexa 594 (Invitrogen, CAT# A-11073, CAT# A-21207), respectively. Images were captured on a Nikon Eclipse TE2000U by using a 60x oil objective lens on a photometric cool-snap camera (Nikon Instruments) driven by Metamorph software (UIC, Downingtown, PA). Appropriate filters were used to image the signals from the two secondary antibodies separately.

Adenovirus studies

To produce an adenovirus expressing a constitutively active form of human SREBP-1a (26), a cDNA fragment (~1.5 kb) encoding the amino acids 1–460 of human SREBP-1a was generated from poly-A RNA isolated from HepG2 cells by RT-PCR using the following primers: 5′ primer, 5′GGGAAAGCTGCTCCC-TAGGAAAGGCGGTACGGGCCG-3′; and 3′ primer, 5′GTCGAC-GACTGAGATGCTGCTGCTGCGAC-3′. The resultant PCR product was subcloned into a TA-cloning vector and subcloned into the Ad-track shuttle vector. For overexpression of PPARγ, a full-length, FLAG-tagged, mouse PPARγ1 cDNA was cloned into the Adtrack vector. The final adenovirus constructs were produced using the AdEasy system as described (27). Mouse hepatocytes were infected with the specified adenovirus at an MOI of 8 as described previously (28). RNA was isolated 48 h after adenoviral infection.

Promoter-luciferase reporter studies

HEK-293 cells were maintained in DMEM-10% fetal calf serum. Cells were transfected using calcium-phosphate coprecipitation and luciferase activity in cell lysates determined by Dual-Glo (Promega) assays 60 h after transfection. The Cidea promoter constructs have been previously described (29) and contain -1385 (pCD2), -888 (pCD3), or -578 (pCD4) of the 5′-flanking sequence of the Cidea gene (all notations are relative to the transcriptional start site). Cidea promoter constructs were cotransfected with expression constructs driving expression of the caSREBP-1 ((26), generous gift of Jay Horton) or empty vector control and SV40-driven renilla luciferase expression construct. All values are normalized to 1.0 and firefly luciferase values were corrected to renilla luciferase activity.

ChIP analyses

Chromatin immunoprecipitation (ChIP) experiments were performed as previously described (27). Hepatocytes from WT mice were dissociated with collagenase, plated, and infected with adenovirus to express caSREBP-1 and/or GFP. After 24 h of infection, hepatocytes were cross-linked in 1% formaldehyde for 15 min. Chromatin-bound proteins were immunoprecipitated using antibodies directed against SREBP-1 or IgG control. PCR primers (supplementary Table I) were designed to amplify a region of the Cidea gene promoter identified in the promoter deletion series as being responsive to SREBP-1 or an exon of the Acedm gene (27, 30).

Statistical analyses

Statistical comparisons were made using ANOVA or t-test. All data are presented as means ± SEM, with a statistically significant difference defined as a P-value < 0.05.

RESULTS

Time-course of hepatic steatosis in neonatal fld mice

We sought to evaluate the hepatic histological and biochemical phenotype of young fld mice at 3 day intervals. Histological examination of hepatic sections from WT and fld mice at P8 and P11 revealed virtual replacement of the parenchyma by hepatocytes distended with tiny fat droplets. Hepatocytes had a “foamy” appearance, which is a morphologic appearance consistent with microvesicular steatosis (Fig. 1). Hepatic triglyceride content was strikingly elevated 30-fold and 40-fold at P8 and P11, respectively, in fld mice compared with WT mice (Table 1). By P14, microvesicular LD were greatly reduced in fld mice. However, occasional large lipid droplets remained present and hepatic TG content remained elevated 8-fold compared with WT. At P17, fld and WT liver sections were indistinguishable in biochemical TG and histological analyses

PAT protein expression is increased in steatotic fld mouse liver

LDL expression is induced in many models of hepatic steatosis secondary to obesity or lipodystrophy (16, 18, 31–33), but whether these genes are regulated in fld mice is unknown. Of the five PAT genes, only the hepatic expression of perilipin-2 mRNA (Plin2) was significantly affected in fld mice compared with control WT littersmates (Fig. 2A). Plin2 mRNA expression was increased in fld mice at P8 and P11 but then declined by P14 during the resolution of fatty liver. Perilipin-2 protein levels were also

http://www.jlr.org/content/suppl/2009/09/11/jlr.M000976.DC1
levels declined coincident with the resolution of hepatic steatosis. Perilipin-1 and perilipin-4 protein and mRNA were undetectable in all livers (data not shown), consistent with low expression of these proteins in liver. These data suggest that levels of the PAT proteins, perilipin-2, perilipin-5, and to a lesser extent, perilipin-3, are increased in fdl liver coincident with elevated hepatic TG levels, but are regulated independent of changes in their corresponding RNAs.

To confirm that the observed disconnect between mRNA protein was applicable to other models of hepatic steatosis, we measured the expression of Plin2 and Plin5 mRNA in ob/ob mouse liver and compared the levels of the corresponding proteins. We found that perilipin-2 and perilipin-5 protein levels were markedly induced in ob/ob liver compared with littermate controls (supplementary Fig. 1). Although Plin2 and Plin5 mRNA expression was also upregulated, the observed increase in protein was out of proportion to the mRNA induction. These data also suggest posttranscriptional regulation of PAT proteins possibly related to the increased stability with fatty acid overabundance.

Fat loading increases PAT protein stability in hepatocytes

Previous work has shown that fatty acid abundance can increase the stability of perilipin-2 and perilipin-1 in immortalized cell lines (34, 35). Given the disconnect between PAT mRNA and protein in steatotic liver, we sought to evaluate protein stability of perilipin-2, perilipin-3, and perilipin-5 after loading WT hepatocytes with 400 μM oleate for 1 h. As we hypothesized, oleate loading increased the quantity of 35S-labeled perilipin-2 6-fold 12 h after chasing with cold methionine (Fig. 2C). Similarly, oleate loading increased perilipin-5 content (3-fold) after a 12 h chase, whereas perilipin-3 content was not significantly increased (Fig. 2C). These data suggest that lipid overabundance stabilizes perilipin-2 and perilipin-5 and enhances their half-lives.

Perilipin-2, perilipin-3, and perilipin-5 coat LDs but may mark distinct lipid pools

We next determined the subcellular distribution of the PAT proteins that were induced in fdl liver by using isolated hepatocytes from WT and fdl mice and performing immunofluorescent staining. Perilipin-2 was localized primarily to large LDs in both WT and fdl hepatocytes (Fig. 3). An increase in the quantity of perilipin-2 in fdl hepatocytes compared with WT cells was also evident. In contrast, perilipin-5 and perilipin-3 are observed in a diffuse pattern throughout the cytosol in both fdl and WT hepatocytes with more intense staining in the fdl hepatocytes (Fig. 3). Perilipin-5 antibodies also illuminate a punctate pattern in both WT and fdl hepatocytes. Although the identity of the structures marked by perilipin-5 and perilipin-3 is beyond the technical capability of this technique to visualize, based on the biochemical studies below, we believe that these two proteins are soluble in WT mice and may be marking small LDs in fdl hepatocytes.

TABLE 1. Hepatic TG content in WT and fdl mice at indicated postnatal day

<table>
<thead>
<tr>
<th>TG content</th>
<th>WT</th>
<th>fdl</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg liver TG/g</td>
<td>mg liver TG/g</td>
<td></td>
</tr>
<tr>
<td>P8</td>
<td>4.97 ± 3.22</td>
<td>159.62 ± 71.54*</td>
</tr>
<tr>
<td>P11</td>
<td>5.42 ± 3.45</td>
<td>195.63 ± 100.83*</td>
</tr>
<tr>
<td>P14</td>
<td>4.94 ± 0.67</td>
<td>37.50 ± 16.43*</td>
</tr>
<tr>
<td>P17</td>
<td>3.71 ± 1.07</td>
<td>5.31 ± 3.27</td>
</tr>
</tbody>
</table>

*P < 0.05.
PAT protein localization was confirmed by subcellular fractionation and Western blotting. We subjected fld or WT liver homogenates to sucrose gradient centrifugation and determined the distribution of endogenous PAT proteins across the gradient (Fig. 4). In the WT liver homogenates, perilipin-2 was primarily detected in the LD fraction whereas perilipin-3 and perilipin-5 were detected primarily in the cytosolic fractions, which were marked by glycogen synthase (Fig. 4). Importantly, we also observed redistribution of perilipin-5 and perilipin-3 protein to the LD fraction in fld liver homogenates compared with WT controls. Together with the immunofluorescence data (Fig. 3), these data suggest that perilipin-3 and perilipin-5 proteins are primarily soluble in normal mouse liver, but a significant portion of the protein redistributes to coat small LDs in steatotic liver.

**Cide mRNA expression is increased in fld liver**

Given emerging evidence that the Cide family of LDPs plays important roles in regulating hepatic fat metabolism, we also examined the expression of this family of genes. Cidea and Fsp27 were strongly induced in fld livers at P8, P11, and P14 but then declined to control levels at P17 (Fig. 5). Cideb expression was unchanged between WT and fld mice at indicated postnatal days. These data parallel observed increases in Cidea and Fsp27 expression in ob/ob liver (supplementary Fig. I). Western blotting analyses using antibodies against CideA and Fsp27 and lysates from LD fractions from P8 WT and fld mice demonstrated that changes in Cide mRNA levels were accompanied by coordinate increases in protein levels.

**PPARγ levels are not increased in fld liver**

The induction of PPARγ is believed to be a primary driving force in the development of hepatic steatosis as well as the increased expression of perilipin-2 and Cide genes (18). PPARγ is expressed at very low levels in normal liver, but the hepatic expression of this transcription factor is induced in nearly every animal and human model of fatty liver disease examined thus far (36). Interestingly, PPARγ mRNA (Pparg) and protein were not increased in fld mice at any time point and were actually reduced in P8 fld mice compared with littermate controls (Fig. 6). This is a unique feature of fld liver that differentiates it from other fatty liver models.
from isolated P14 WT (A, C, E) or cellular structures in primary hepatocytes. Images were captured by immunofluorescent microscopy. Perilipin-2, perilipin-3, and perilipin-5 mark distinct subcellular structures in primary hepatocytes. Images were captured from isolated P14 WT (A, C, E) or fld (B, D, F) hepatocytes fixed and then stained with antibodies directed against perilipin-2, perilipin-3, or perilipin-5. Proteins were then visualized using immunofluorescent microscopy.

We also evaluated the nuclear content of SREBP-1, which is known to be activated in steatotic liver and to regulate lipogenic gene expression (37). Nuclear content of the active SREBP-1 cleavage product was increased in P8 fld mice (Fig. 7A), suggesting that SREBP-1 is activated in fld mouse liver. Consistent with an activation of SREBP-1, several well-defined SREBP-1 target genes [Scd1, Elovl6, Gpat, Acacb (38)] were increased in P8 fld mice compared with littermate controls (Fig. 7B). Collectively, this evidence supports the idea that SREBP-1 is activated in fld mouse liver.

Cidea is a direct target gene of SREBP-1

Although SREBP-1 regulates many genes involved in lipid metabolism, to our knowledge, Cide genes have not been identified as SREBP-1 targets. To address this, we overexpressed a constitutively active form of SREBP-1a (26) in WT hepatocytes using an adenovirus and assessed Cide gene expression. Cidea expression was robustly induced, whereas Fbp27 and Cideb were not significantly affected by constitutively-active SREBP-1 (Fig. 8). In addition, expression of Plin2, Plin3, and Plin5 were not induced by SREBP-1 (Fig. 8).

To determine whether SREBP-1 directly regulates Cidea transcription, a series of Cidea promoter-luciferase reporter constructs (29) was transfected into 293 cells, which are virtually null for SREBP-1, with a caSREBP-1 expression construct. As predicted, SREBP-1a overexpression induced Cidea promoter activity more than 30-fold in 293 cells (Fig. 9). A deletion series of Cidea promoter constructs was used to map the SREBP1-responsive regions of the Cidea promoter. Loss of nucleotides -888 to -578 relative to the transcriptional start site significantly blunted the SREBP-1 response. However, the previously defined minimal promoter remained responsive to SREBP-1 and was activated 10-fold. This is consistent with the presence of a canonical SREBP-1 response element (SRE) in this region. Congruent with this, ChIP studies demonstrated that endogenous SREBP-1 protein was directly associated with chromatin in the Cidea promoter (Fig. 9). Collectively, these data identify Cidea as a direct target of SREBP-1 signaling.

DISCUSSION

The profile of proteins that coat LDs plays important roles in regulating LD formation, morphology, and lipolysis. Herein, we characterized the expression of LDP in fld mice; a unique model of lipodystrophy-related fatty liver that spontaneously and rapidly resolves. A loss of function mutation in the gene encoding lipin 1 causes the conspicuous hepatic steatosis and global metabolic abnormalities of fld mice (20, 21). The physiologic mechanisms whereby lipin 1 deficiency causes hepatic steatosis are still unclear, but the molecular functions of lipin 1 provide some possible explanations. Lipin 1 acts in the nucleus to coactivate lipid metabolism, to our knowledge, Cide genes have not been identified as SREBP-1 targets.

![Fig. 3](http://www.jlr.org/content/suppl/2009/09/11/jlr.M000976.DC1)

![Fig. 4](http://www.jlr.org/content/suppl/2009/09/11/jlr.M000976.DC1)
The evidence for a key role for PPAR\(\gamma\) in the development of hepatic steatosis and ectopic induction of lipogenic gene expression is extensive. Liver-specific inactivation of PPAR\(\gamma\) markedly ameliorates fatty liver in several obesity-related fatty liver models (46, 47) whereas PPAR\(\gamma\) overexpression drives hepatic steatosis.

Hepatic steatosis is usually characterized by macrovesicular fat droplets. Thus, the microvesicular morphology of the LD may be another indication of severe defects in hepatic fatty acid oxidation and/or mitochondrial dysfunction in \(\text{fld}\) liver that result in hepatic steatosis.

On the other hand, the lack of adipose tissue (lipodystrophy) is also likely to play a role in the development of hepatic steatosis in these mice. Most models of lipodystrophy are accompanied by hepatic steatosis (reviewed in ref. 42) probably because there is insufficient capacity to store TG in adipose tissue, which increases the flux of fatty acids to the liver. Lipin 1 is a bi-functional protein that also catalyzes a key step in TG synthesis [phosphatidate phosphohydrolase (43)]. \(\text{fld}\) mice completely lack Mg\(^{2+}\)-dependent phosphatidate phosphohydrolase activity in adipose tissue (44) leading to a failure of adipocytes to differentiate and store TG (45). It is our opinion that the hepatic steatosis in \(\text{fld}\) mice is a product of both the hepatic oxidative insufficiency and lipodystrophy, which synergize to cause severe hepatic steatosis. The explanation for the recovery remains unclear. Because lipodystrophy is a lifelong aspect of the \(\text{fld}\) mouse, the resolution suggests compensatory changes in liver metabolism. A developmental and adaptive normalization of oxidative capacity (our unpublished observations) or increased VLDL tri-glyceride export (28) could explain the resolution of the neonatal fatty liver. However, additional mouse models are needed to probe this idea further and to understand the sudden, predictable, and apparently genetically-programmed resolution of the fatty liver in this model.

The \(\text{fld}\) mouse model is also unique in that PPAR\(\gamma\) levels are not increased as has been reported in nearly every model of obesity- or lipodystrophy-related hepatic steatosis (reviewed in ref. 36). The evidence for a key role for PPAR\(\gamma\) in the development of hepatic steatosis and ectopic induction of lipogenic gene expression is extensive. Liver-specific inactivation of PPAR\(\gamma\) markedly ameliorates fatty liver in several obesity-related fatty liver models (46, 47) whereas PPAR\(\gamma\) overexpression drives hepatic steatosis.
Hepatic LDP expression in fatty liver dystrophic mice

We found that the nuclear content of SREBP-1 and the expression of multiple known SREBP-1 target genes was induced in liver of P8 fld mice, which is strong evidence for increased SREBP-1 activity. We show herein that Cidea expression is induced by SREBP-1 via a direct effect of SREBP-1 occupying the Cidea promoter. To our knowledge, Cidea has not previously been identified as a target of SREBP-1 transcriptional control. Based on consensus sequence matching, the original characterization of the Cidea promoter identified two putative canonical SREBP-1 response elements, including a site within the minimal promoter (pL-Cid4) (29). Indeed the pL-Cid4 construct responds 10-fold to SREBP-1 overexpression and this region would be immunoprecipitated in our ChIP studies. The promoter deletion studies also suggest an SREBP-1-responsive region between -888 and -578. However, our searches have not revealed a canonical SREBP-1 response element within this region. Due to the proximity of the canonical site in the minimal promoter, ChIP studies cannot distinguish whether SREBP-1 binds directly to DNA in the -888/-578 region. Nevertheless, our studies indicate that Cidea is a new and direct target of SREBP-1 signaling and future work will be needed to determine the metabolic consequences of SREBP-1-mediated Cidea activation.

Whereas the Cide genes are regulated at the transcriptional level, PAT protein levels were governed by post-translational mechanisms. Although Plin5 mRNA expression was unchanged, levels of its corresponding protein were increased several-fold in liver of fld mice compared with controls. Perilipin-2 was also upregulated at the level of its mRNA but the increase in its protein levels was significantly greater. Degradation of perilipin proteins can occur by proteosomal and lysosomal pathways (35, 49, 50). In immortalized cell lines, previous work has shown that perilipin-1 and perilipin-2 protein stability is enhanced by the presence of fatty acids and that the increased stability is due to inhibition of ubiquitin-mediated degradation (35, 49). Herein, we demonstrate that perilipin-2 stability is regulated by fat loading in primary hepatocytes and present data that perilipin-5 stability is

Fig. 7. Nuclear content of active SREBP-1 and the expression of SREBP-1-target genes are increased in fld mice. A: Representative Western blotting analysis of cleaved SREBP1 using hepatic nuclear protein preparations from WT and fld mice at postnatal day 8. B: Elevated expression of known SREBP-1 target genes in fld mice. The graph depicts the expression of established SREBP-1 target genes in fld liver (WT normalized to 1.0). *P < 0.05 versus WT control.

Fig. 8. Forced-expression of SREBP-1 leads to increased expression of CideA. Hepatocytes isolated from adult WT mice were infected with adenovirus expressing a constitutively active form of SREBP-1a (Ad-caSREBP-1) or control adenovirus expressing green fluorescent protein (GFP). The graph depicts the results of quantitative RT-PCR analyses of Cide and Plin family genes. *P < 0.05 versus GFP control.
also subject to regulation by fatty acid availability. This seems to be a key mechanism of regulation and demonstrates the importance of quantifying PAT protein levels, rather than mRNA content, in future studies.

CONCLUSIONS

Our work has shown that the microvesicular hepatic steatosis of fld mice is accompanied by increased levels of several LDPs. These studies also reveal that the PAT and Cide families of LDPs are controlled at different regulatory levels and that the acute hepatic steatosis in fld mice is not accompanied by an increased in PPARγ, a putative master regulator of hepatic steatosis. Further study of the mechanisms that regulate LD levels in steatotic liver, in particular the marked and rapid egress of steatosis, is needed to determine whether targeting these pathways might be a viable treatment for fatty liver disease.

REFERENCES


