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Functional Conservation for Lipid Storage Droplet Association among Perilipin, ADRP, and TIP47 (PAT)-related Proteins in Mammals, *Drosophila*, and *Dictyostelium**

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Intracellular neutral lipid storage droplets are essential organelles of eukaryotic cells, yet little is known about the proteins at their surfaces or about the amino acid sequences that target proteins to these storage droplets. The mammalian proteins Perilipin, ADRP, and TIP47 share extensive amino acid sequence similarity, suggesting a common function. However, while Perilipin and ADRP localize exclusively to neutral lipid storage droplets, an association of TIP47 with intracellular lipid droplets has been controversial. We now show that GFP-tagged TIP47 co-localizes with isolated intracellular lipid droplets. We have also detected a close juxtaposition of TIP47 with the surfaces of lipid storage droplets using antibodies that specifically recognize TIP47, further indicating that TIP47 associates with intracellular lipid storage droplets. Finally, we show that related proteins from species as diverse as *Drosophila* and *Dictyostelium* can also target mammalian or *Drosophila* lipid droplet surfaces *in vivo*. Thus, sequence and/or structural elements within this evolutionarily ancient protein family are necessary and sufficient to direct association to heterologous intracellular lipid droplet surfaces, strongly indicating that they have a common function for lipid deposition and/or mobilization.

The intracellular neutral lipid storage droplets (LSDs)[1] of eukaryotes are ubiquitous cellular organelles required for membrane biosynthesis, cholesterol metabolism, lipid trafficking, and energy balance. In the Metazoa, the ADRP and Perilipin (Peri) proteins associate exclusively with the surface of these lipid droplets (1–5). ADRP mRNA is expressed ubiquitously, whereas Peri expression is limited to adipocytes and steroidogenic cells (2, 4, 6). However, the expressions of ADRP and Peri protein are mutually exclusive; ADRP protein is not detected in cells that also express Peri. The common targeting properties of ADRP and Peri to LSDs, nonetheless, suggest a functional linkage for lipid deposition and mobilization (7–9).

Peri is required for lipid storage in adipose tissue. *peri*$^{-/-}$ mice have very reduced adipose tissue mass, and isolated adipocytes from these animals exhibit greatly elevated basal lipolytic activity when compared with wild-type (9, 10). Similarly, Peri confers lipolytic protection to LSDs of unstimulated adipocytes (9, 10) and of various cultured cells that express Peri ectopically (11–13). In addition to this protective function, Peri is required to achieve maximal lipolytic activity in stimulated cells (9, 10), suggesting that Peri is also required to facilitate lipolysis. These antagonistic regulatory functions demonstrate that Peri plays a major role in lipid storage and mobilization. Although the ADRP is less well studied, biochemical studies indicate a role for ADRP in lipid trafficking from lung lipofibroblasts to type 2 epithelial cells for surfactant biosynthesis (14).

Peri and ADRP share extensive sequence similarity (6, 15, 16). The initial ~120 amino acids of ADRP and Peri are ~40% identical (6) and a more limited but statistically significant similarity is observed within the ~150 adjacent amino acids (15). The mannose 6-phosphate/IGF-II receptor (MPR/IGF-IIR) trafficking protein TIP47 has a sequence organization that is also similar to Peri and ADRP (15, 17). Overall, ADRP and TIP47 are ~50% identical in amino acid sequence. Computational analyses (15) also identified proteins in species as diverse as *Drosophila* (LSD-1 and -2) and *Dictyostelium* (LSD1) that are related to Peri, ADRP, and TIP47. Collectively, we termed these PAT proteins, where Peri, ADRP, and TIP47 define the core of the family (15). Further, two PAT subdomains were described; PAT-1 defines the high identity N-terminal region, and PAT-2 defines the more distal region of lesser similarity (15). The conserved splice site junctions between murine *ADRP* and *Drosophila LSD-1* genes emphasize the evolutionary relationships among diverse gene family members (15).

TIP47 was originally identified by its ability to interact with the MPR/IGF-IIR. However, the sequence similarities between TIP47 and ADRP (15, 17) prompted a re-examination of the subcellular localization of TIP47 (18). Indeed, LSD association was detected using antibodies to TIP47 (18), but unfortunately the data were inconclusive. The α-TIP47 antibody used also demonstrated cross-reactivity with the related ADRP, a *bona fide* lipid storage droplet protein (19).

Here we have examined the subcellular localizations of PAT

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[1] The abbreviations used are: LSD, liquid storage droplet; Peri, Perilipin; PAT, Peri, ADRP, and TIP47 proteins; GFP, green fluorescence protein; eGFP, enhanced GFP; CHO, Chinese hamster ovary; IGF, insulin-like growth factor; MPR/IGF-IIR, mannose 6-phosphate/IGF-II receptor.

family proteins TIP47, LSD1 from *Dictyostelium,* and LSD-1 and -2 from *Drosophila*. Using two new reagents, GFP-tagged TIP47 and a TIP47-specific antibody, we have confirmed the ability of TIP47 to co-localize with lipid storage droplets. Finally, we have demonstrated that LSD1 of *Dictyostelium* and LSD-1 and -2 of *Drosophila* also co-localize *in vivo* with lipid storage droplets in mammalian tissue culture cells and, for LSD-1 and -2, in cells of *Drosophila* fat bodies.

EXPERIMENTAL PROCEDURES

GFP Fusion Constructs—Full-length cDNA constructs were fused in-frame with the 3′ end of *eGFP* in pEGFP-C2 (CLONTECH) or pUAST (20). The coding sequences of murine *PeriA* and murine *ADRP* (NM007408) were obtained from previously existing clones (15, 16). Murine *TIP47* (AI892835) cDNA was obtained from the IMAGE consortium (ID no. 571955). *Dictyostelium LSD1* (AU061427) cDNA (SLE217) was obtained from the *Dictyostelium* cDNA project (21). *Drosophila LSD-1* (AF357214) and *LSD-2* (AY060242) cDNA were isolated by RT-PCR using MMLV reverse transcriptase (CLONTECH) and Deep Vent polymerase (New England Biolabs) from adult *Drosophila* head RNA. Expression of full-length eGFP, GFP-Peri, GFP-ADRP, GFP-TIP47, GFP-LSD-1, GFP-LSD-2, and GFP-LSD1 proteins in Chinese hamster ovary (CHO) cells was confirmed by Western blotting using α-GFP. Full-length human TIP47 (O60664) and human ADRP (adipophilin, Q99541) proteins were also expressed in CHO cells. (Accession numbers are from GenBank™.)

α-TIP47 Antibodies—Two unique sequences, MSADGAEADGSTQ and NQKQLQGPEKEPPK, of human TIP47 were selected to generate peptides for antibody production. These are highly divergent regions that have no sequence similarity to ADRP or other proteins in the non-redundant data base of GenBank™. The corresponding sequences from human ADRP are MASVAVDPQPSVV and KRSIGYDDTDESHC. The peptides were cross-linked to KLH and used collectively as antigens in rabbits. The antibodies were affinity-purified to peptide NQKQLQGPEKEPPK of human TIP47. Western blotting confirmed specificity for human TIP47 (see Fig. 4).

Cell Culture—CHO fibroblasts were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 3×10^5 cells per well in 50-mm diameter culture dishes attached with a coverslip. All cells were cultured in 5% CO_2 atmosphere at 37 °C. Intracellular neutral lipid storage was increased by the addition of oleic acid coupled to bovine serum albumin (1).

Transformation and Transgenesis—CHO fibroblasts were transfected with 2 μg of DNA using LipofectAMINE Plus (Invitrogen). Cells were then cultured overnight at 5×10^5 cells per well in a 50-mm diameter culture dish attached with a coverslip. Transgenic *Drosophila* were produced by P-element-mediated transformation using the bipartite *GAL4/UAS* system (22, 23). Expression of *UAS*::transgenes was directed by *hs*::*GAL4* following 1 h of heat shock at 37 °C and a 3-h recovery at 25 °C.

Microscopy—CHO and HeLa cells and *Drosophila* embryos were treated with 400 nM Nile Red (Molecular Probes, Inc.) for 5 min. Neutral lipid droplets were visualized by confocal laser microscopy (LSM 510, Zeiss). HeLa cells were fixed with 3% paraformaldehyde in phosphate-buffered saline for 60 min and blocked prior to immunostaining. Primary rabbit anti-human TIP47 and fluorescein isothiocyanate-conjugated goat anti-rabbit (Jackson ImmunoResearch) were added sequentially to the fixed cells in phosphate-buffered saline containing 0.1% saponin and 28.5 mg/ml Chrompure goat IgG (Jackson ImmunoResearch). The samples were stained with 8 pM Nile red in buffer containing 0.1% saponin and 1× phosphate-buffered saline for 1 min. Images were obtained by confocal laser microscopy using a ×63 water objective lens.

Fractionation of CHO cells—CHO cells were grown, transfected, lipid loaded, and lysed in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 μg/ml leupeptin, 100 μM AEBSF, and 1 mM benzamidine by five passages through a 25-gauge needle. The homogenate was centrifuged at 1000 × *g* at 4 °C. for 10 min. The supernatant was adjusted to 18.46% sucrose and centrifuged at 165,000 × *g* for 1 h at 4 °C. The buoyant fraction was adjusted to 1.5 ml with lysis buffer. 1 μl of sample was mixed with 1 μl of 80 pM Nile Red in 1× phosphate-buffered saline and mounted to a glass slide with 4 μl of 80% glycerol in 1× phosphate-buffered saline. The prepared slide was viewed by confocal laser microscopy using a ×63 water objective lens.

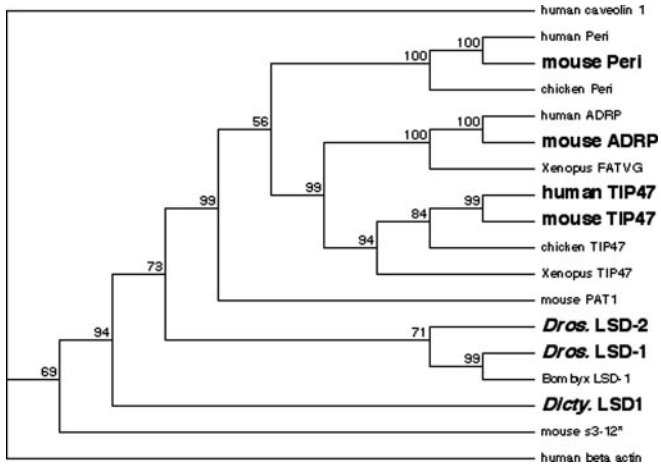

FIG. 1. **Sequence relationships among PAT family proteins.** PAT proteins were identified in an NCBI search (15). A schematic tree was predicted by neighbor-joining using amino acid sequences of the PAT subdomains (15). Numbers at the branch nodes represent bootstrap values (as percentages) obtained in 1000 replications. For the alignment, s3–12* (24) was manually deleted of all but two of its 33-mer repeats (see Ref. 15). Human caveolin 1, an unrelated protein that displays lipid association, and human β-actin were included in the unrooted analyses. Proteins used in this study are in *boldface*.

Comparative Sequence Analyses—PAT proteins were identified previously in an NCBI search (15). Their PAT domain sequences (see Ref. 15) were aligned using ClustalW with the PAM350 matrix provided in the MacVector 7.1 program package. A schematic, unrooted tree was predicted by neighbor-joining methodology. Numbers at the branch nodes represent bootstrap values (as percentages) obtained in 1000 replications. s3–12 is an adipocyte protein with a repeating 33-mer element that shares similarity with the PAT family (24). For the alignment, s3–12 was manually deleted of all but two 33-mer repeats (see Ref. 15). Human caveolin 1 and human β-actin were included in the unrooted tree.

RESULTS AND DISCUSSION

Sequence Relationships among the PAT Protein Family—We had previously identified (15) PAT family proteins in representative species as diverse as vertebrates, *Drosophila,* and *Dictyostelium* that are related to Peri and ADRP. Sequence analyses based upon neighbor-joining comparisons now confirm separate vertebrate groupings for Peri, ADRP, and TIP47. A novel murine family member, PAT1, was also identified. Although the *Drosophila* and *Dictyostelium* proteins are more distantly related, they demonstrate clear PAT group association (Fig. 1).

TIP47 Co-localizes with Lipid Storage Droplets—The subcellular localization of TIP47 has been controversial (18, 19). TIP47 was originally identified in a screen for proteins that interact with the MPR/IGF-IIR (17). Subsequently, antibodies to TIP47 were shown to detect protein at lipid droplet surfaces (18), but because these antibodies cross-reacted with ADRP, it was not possible to assert unequivocally that TIP47 exhibits lipid droplet co-localization (19). We have used two approaches to resolve this controversy. First, to circumvent potential issues of antibody cross-reactivity we visualized TIP47 localization in fusion with GFP. Second, we also generated antibodies to human TIP47 that were directed against peptide sequences that are absent from human ADRP (*i.e.* adipophilin).

CHO cells expressing GFP-TIP47 were grown with oleic acid to increase intracellular neutral lipid stores. Cells expressing GFP-Peri and GFP-ADRP served as positive controls, and cells expressing unfused eGFP served as a negative control. The cells were stained with Nile Red to visualize the clustered neutral lipid droplets (Fig. 2). Images are centered upon clus-

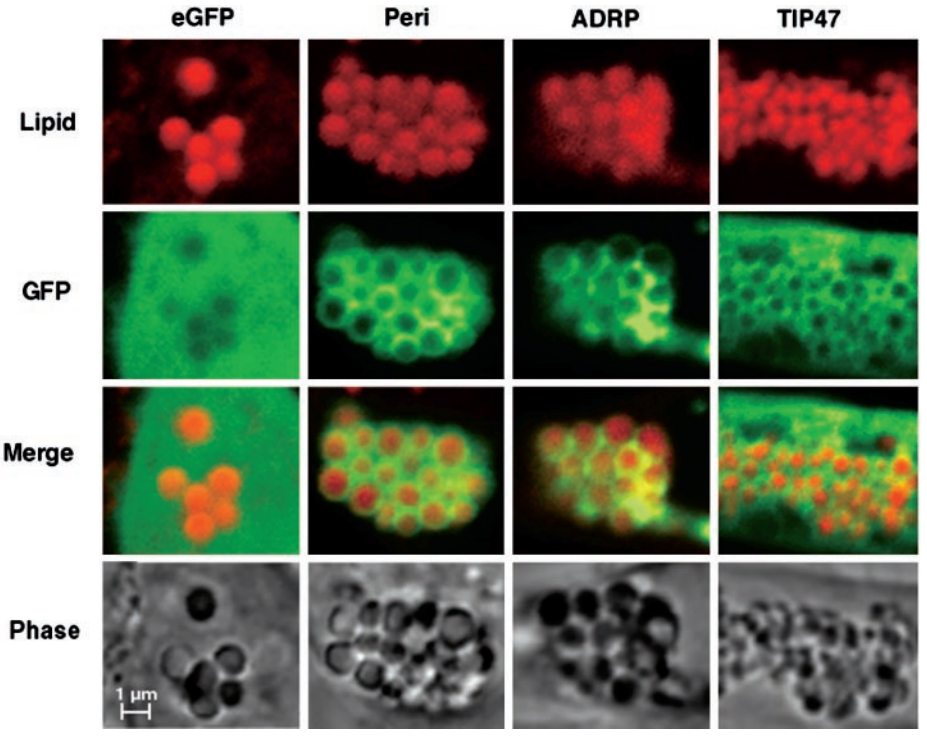

FIG. 2. **GFP-tagged murine Peri, ADRP, and TIP47 target to lipid storage droplets in CHO cells.** CHO cells were transfected with the pEGFP-C2 vector or with the vector containing coding sequences of murine Peri, ADRP, and TIP47. The cells were grown in the presence of oleic acid, and neutral lipids were stained with Nile Red. Fluorescent and phase images of subcellular lipid clusters were generated by confocal laser microscopy.

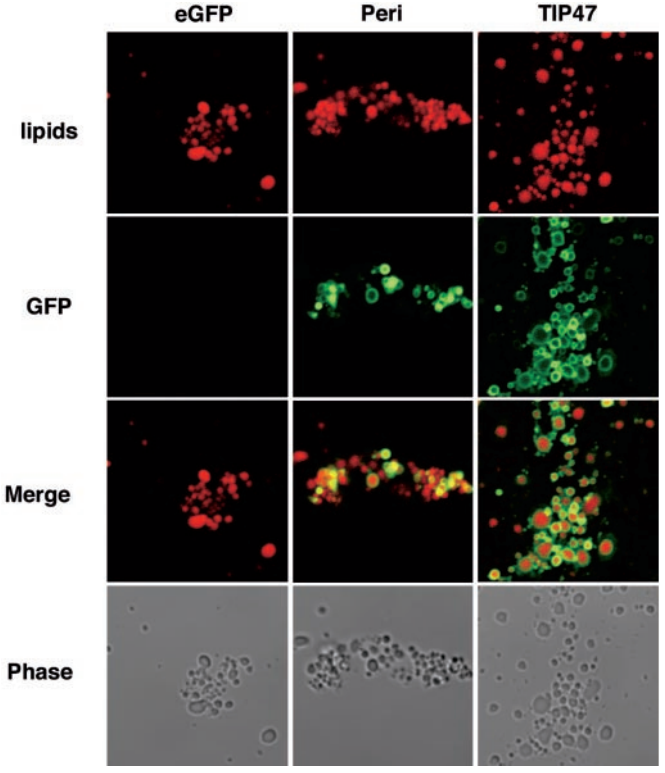

FIG. 3. **GFP-tagged murine Peri and TIP47 associate with isolated lipid storage droplets.** CHO cells were transfected with the pEGFP-C2 vector or with the vector containing coding sequences of murine Peri and TIP47. The cells were grown in the presence of oleic acid, and the intracellular lipid droplets were purified and stained with Nile Red. Fluorescent and phase images were generated by confocal laser microscopy.

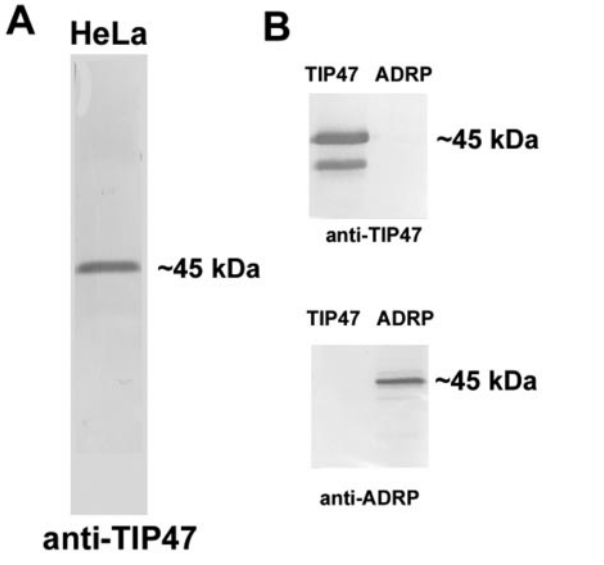

FIG. 4. ***α*-human TIP47 antibodies do not cross-react with human ADRP.** *A*, whole-cell proteins were prepared from HeLa cells, separated by SDS-gel electrophoresis, blotted to filters, and probed with affinity-purified antibody to human TIP47. *B*, whole-cell proteins were prepared from CHO cells that express specifically human TIP47 or human ADRP and separated by SDS-gel electrophoresis. Identical protein blots were probed with affinity-purified antibody to human TIP47 or antibody to human ADRP.

ters of the intracellular neutral lipid droplets and surrounding areas of cytosol. eGFP showed broad and diffuse fluorescence throughout the cytoplasm with clear exclusion from the lipid droplets (Fig. 2). Conversely, GFP-Peri and GFP-ADRP localized exclusively with the surfaces of lipid storage droplets. No overt fluorescence was detected in other compartments (Fig. 2). Confocal cross-sections of lipid droplet clusters showed characteristic rings of Peri and ADRP localization in contact with these neutral lipid stores (Fig. 2).

GFP-TIP47 fluorescence was more complex than for eGFP, GFP-Peri, or GFP-ADRP (Fig. 2). TIP47 appears to be distributed throughout the cytoplasm. However, unlike that observed for eGFP, GFP-TIP47 fluorescence also appears to be associated with the lipid droplets. Indeed, definitive rings of GFP-TIP47 fluorescence are discerned above the general cytoplas-

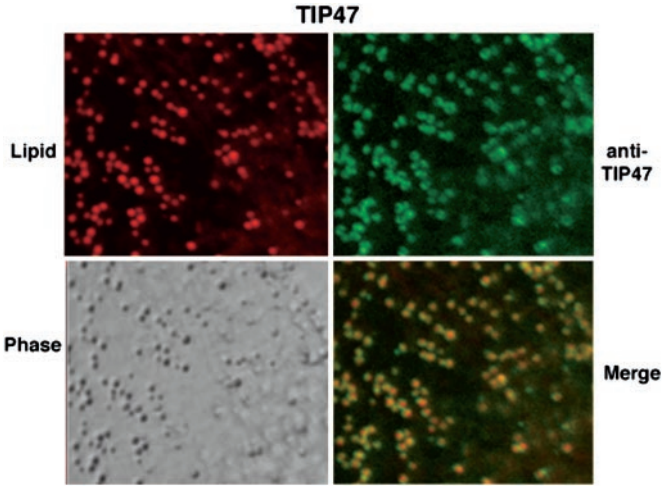

FIG. 5. **Co-localization of human TIP47 with lipid storage droplets of HeLa cells.** HeLa cells were grown in the presence of oleic acid. HeLa cells were stained for neutral lipids with Nile Red and for endogenous TIP47 with affinity-purified antibody to human TIP47 (see Fig. 4). Fluorescent images were obtained by confocal laser microscopy.

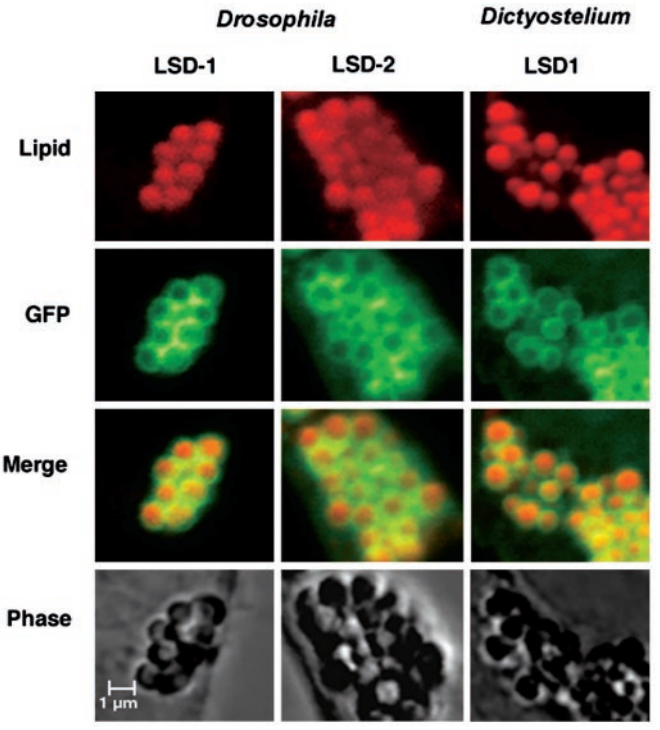

FIG. 6. ***Drosophila* and *Dictyostelium* GFP-tagged LSDs target to lipid storage droplets in CHO cells.** CHO cells were transfected with pEGFP-C2 vector containing coding sequences from *Drosophila LSD-1* and *-2* and *Dictyostelium LSD1*. The cells were grown in the presence of oleic acid and stained with Nile Red. Fluorescent and phase images were generated by confocal laser microscopy.

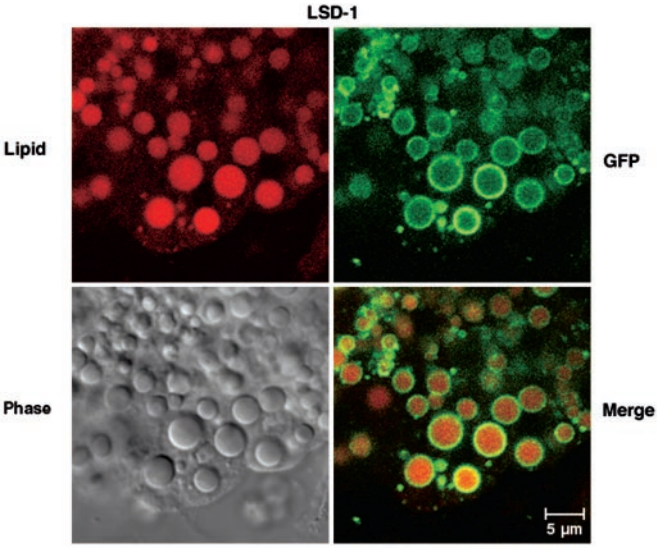

FIG. 7. **GFP-tagged LSD-1 localizes to lipid storage droplets of *Drosophila* adipocytes of first instar larvae.** Fluorescent and phase images of heat-shocked *hs*::*Gal4*/+;*UAS*::*Lsd-1*::*gfp*/+ first instar larvae fat body were generated by confocal laser microscopy.

mic background. The merged Nile Red and GFP images reveal the distinct accumulation of TIP47 around the lipid droplets with the apparent ring structures that are typical in cross-sectional views of protein localization at the surface of lipid droplets. This fluorescence pattern is not the result of simple exclusion from the lipid droplet, because no ring structures were observed in similar views of cells expressing only eGFP (Fig. 2). To further confirm the lipid droplet association of GFP-TIP47, we isolated neutral lipid droplets from these CHO cells and followed the fluorescence of eGFP, GFP-Peri, and GFP-TIP47 (Fig. 3). Images of isolated droplets from GFP-Peri- and GFP-TIP47-expressing cells were effectively indistinguishable, clearly demonstrating the close association of TIP47 at the droplet surface. eGFP did not co-isolate with the lipid droplets. These data indicate that GFP-TIP47 can associate closely with the lipid droplet surface, but they do not exclude an ability of TIP47 to interact with other subcellular components or structures (see Refs. 17, 19, 25).

To exclude the possibility that GFP-TIP47 localization may not accurately reflect the targeting of native protein (see Ref. 25), we examined the intracellular association of endogenous TIP47 by immunofluorescence with antibodies selective for human TIP47. TIP47 and ADRP are closely related in sequence. Accordingly, we selected peptides of human TIP47 that are not related to sequences in human ADRP for antigen production and for affinity purification. Affinity-purified antibodies to human TIP47 only recognized a single protein, of ~45 kDa, in HeLa cells (Fig. 4*A*). However, TIP47 and ADRP proteins are both ~45 kDa. We, therefore, expressed human TIP47 and human ADRP to high levels in CHO cells and probed for cross-reactivity of TIP47 antibody with ADRP. As seen in Fig. 4*B*, the affinity-purified antibody to human TIP47 only recognized protein in the TIP47-expressing CHO cells and not in the CHO cells that express high levels of human ADRP. Thus, the affinity-purified antibody has strong specificity for human TIP47.

We then used the affinity-purified antibody to human TIP47 to examine subcellular localization of endogenous TIP47 in HeLa cells. The small lipid droplets characteristic of HeLa cells were clearly evident by Nile Red fluorescence, and TIP47 was found predominantly in very close juxtaposition (Fig. 5). We did not observe the strong, diffuse cytoplasmic distribution of TIP47 that was apparent when full-length TIP47 was highly expressed as a GFP fusion in CHO cells. Nonetheless, the GFP-TIP47 fusion experiments in conjunction with other reports (17, 19, 25) raise the possibility that TIP47 may be multifunctional, potentially trafficking proteins and/or lipids among several compartments. Perhaps different environmental parameters alter the relative distribution of TIP47 among various intracellular compartments. TIP47 would not be unique in its ability to associate with LSD and non-LSDs, depending upon the physiological state of the cell (26–33).

Drosophila and Dictyostelium PAT Proteins Also Co-localize with LSDs—To determine whether lipid droplet localization is a general quality of the more diverged PAT protein members,

we examined the intracellular targeting of *Drosophila* LSD-1 and -2 and of *Dictyostelium* LSD1 in CHO cells as fusions with GFP. All of the proteins showed selective localization of fluorescence to the lipid droplet surface that was exclusive of any other compartment (Fig. 6). Fluorescent GFP rings were in close apposition with the neutral lipids. These data indicate that lipid droplet targeting is characteristic of PAT family proteins and that sites for their recruitment are highly conserved despite their effective separation through nearly one billion years of evolution.

We also examined the localization of *Drosophila* LSD-1 and -2 proteins in their endogenous environment. Transgenic *Drosophila* were generated that expressed *GFP-LSD-1* or *-2* genes driven with a *GAL4*-inducible promoter. The GAL4 transcriptional regulatory protein was induced by heat shock response. The GFP-LSD-1 (Fig. 7) and -2 proteins (not shown) were clearly shown to associate with lipid droplets within cells of larval (first instar) fat bodies, as evidenced by the rings of GFP fluorescence surrounding the large lipid droplets in these cells. These data confirm that LSD-1 and -2 proteins can associate specifically with lipid droplets in a native environment, as well as in mammalian CHO cells.

In summary, the PAT protein family has ancient progenitors that define a novel protein targeting component for association with intracellular lipid storage droplets. A common sequence and/or structural element among these proteins is necessary and sufficient for this functional conservation even within exogenous cellular environments. Further, structure/function studies of Peri demonstrate its essential role in lipid storage droplet deposition and mobilization (9–13), strongly suggesting related roles for ADRP, TIP47, and other PAT proteins in lipid metabolism and trafficking (7, 8, 14). The PAT family has no apparent sequence relationship with the variety of other proteins capable of lipid droplet association in plants, yeasts, or mammalian cells, including the oleosins, caveolin, and synuclein (25–32). Nonetheless, the confirmation that the distantly related PAT proteins of *Drosophila* and *Dictyostelium* also possess the essential structural elements for LSD targeting emphasizes this functional characteristic. Directed and complementary studies using both mammalian and non-mammalian systems will be required to dissect the molecular mechanisms that are a fundamental property of this important protein family.

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