Hydrophobic sequences target and anchor perilipin A to lipid droplets

Vidya Subramanian, Anne Garcia, Anna Sekowski, and Dawn L. Brasaemle[1]

Department of Nutritional Sciences, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901

Abstract **Perilipins regulate triacylglycerol storage and hydrolysis in adipocytes. The central 25% of the perilipin A sequence, including three hydrophobic sequences (H1, H2, and H3) and an acidic region, targets and anchors perilipins to lipid droplets. Thus, we hypothesized that H1, H2, and H3 are targeting and anchoring motifs. We now show that deletion of any single hydrophobic sequence or combinations of H1 and H3 or H2 and H3 does not prevent targeting of the mutated perilipin to lipid droplets. In contrast, mutated perilipin lacking H1 and H2 showed reduced targeting, whereas perilipin lacking H1, H2, and H3 targeted poorly to lipid droplets; thus, H3 is a weak targeting signal and either H1 or H2 is required for optimal targeting. Complete elimination of perilipin targeting was observed only when all three hydrophobic sequences were deleted in combination with either the acidic region or N-terminal sequences predicted to form amphipathic β-strands. Unlike intact perilipin A, mutated perilipin lacking either H1 and H2 or H1, H2, and H3 was released from lipid droplets after alkaline carbonate treatment, suggesting that these forms are loosely associated with lipid droplets. The three hydrophobic sequences play a major role in targeting and anchoring perilipins to lipid droplets.**—Subramanian, V., A. Garcia, A. Sekowski, and D. L. Brasaemle. **Hydrophobic sequences target and anchor perilipin A to lipid droplets.** ***J. Lipid Res.*** **2004.** 45: **1983–1991.**

Supplementary key words adipocyte • triacylglycerol • neutral lipid • immunofluorescence microscopy • immunoblotting

Perilipins are lipid droplet-associated phosphoproteins that function as key regulators of triacylglycerol storage and hydrolysis in adipocytes (1–8). Perilipins are members of the PAT family of lipid droplet-associated proteins (9), which includes adipophilin (also called adipose differentiation-related protein) (10, 11), TIP47 (9, 12), and S3-12 (13, 14) as well as proteins in species as diverse as *Drosophila* (Lsd1 and Lsd2) and *Dictyostelium* (9). Three perilipin isoforms, perilipins A, B, and C, are encoded by alternatively spliced forms of mRNA transcribed from a single gene (15, 16). Perilipins A, B, and C share a common N terminus but have distinct C termini. Perilipin A is the longest isoform with a unique C-terminal sequence of 112 amino acids (aa) and is the most abundant lipid droplet-associated protein in adipocytes.

The current model for lipid droplet assembly hypothesizes the nucleation of a lens of neutral lipids within the membrane bilayer of the endoplasmic reticulum, which upon accumulating a critical mass of neutral lipid is released into the cytoplasm (17, 18). Thus, lipid droplet-associated proteins may be synthesized on membrane-bound ribosomes and localize cotranslationally to nascent lipid droplets, as has been demonstrated for oleosins in the seeds of plants (18–22). Alternatively, proteins may be synthesized on cytoplasmic ribosomes and then assembled onto lipid droplets. Because perilipins traffic posttranslationally to lipid droplets (23), one or more signals within the primary amino acid sequence directs the targeting of nascent perilipin to lipid droplets. We recently reported that the central 25% of the perilipin A sequence, which includes three moderately hydrophobic sequences of 16–23 aa at positions 242–260 (H1), 320–342 (H2), and 349–364 (H3) and an acidic sequence from aa 291–318 (**Fig. 1**), contains all of the information necessary to target and anchor nascent perilipin A to lipid droplets (24). Deletion of the entire central region eliminated targeting of the mutated perilipin to lipid droplets; conversely, fusion of this central region to Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* targeted the GFP chimera to the surfaces of lipid droplets (24). However, although it was clear from our study that the central region of the perilipin sequence contains partially redundant targeting signals, the exact identity of these signals, and their relative importance, were unresolved.

In the current study, we tested the hypothesis that the central sequences of hydrophobic amino acids are the critical determinants for targeting perilipin A to lipid droplets. We have stably expressed forms of perilipin with internal deletion mutations in 3T3-L1 fibroblasts and charac-

Manuscript received 2 August 2004 and in revised form 19 August 2004.

Published, JLR Papers in Press, September 1, 2004.
DOI 10.1194/jlr.M400291-JLR200

Abbreviations: aa, amino acids; GFP, Green Fluorescent Protein; H1, H2, and H3, hydrophobic sequences of perilipin A from amino acids 243–260 (H1), 320–342 (H2), and 349–364 (H3).

[1] To whom correspondence should be addressed.
e-mail: brasaemle@aesop.rutgers.edu

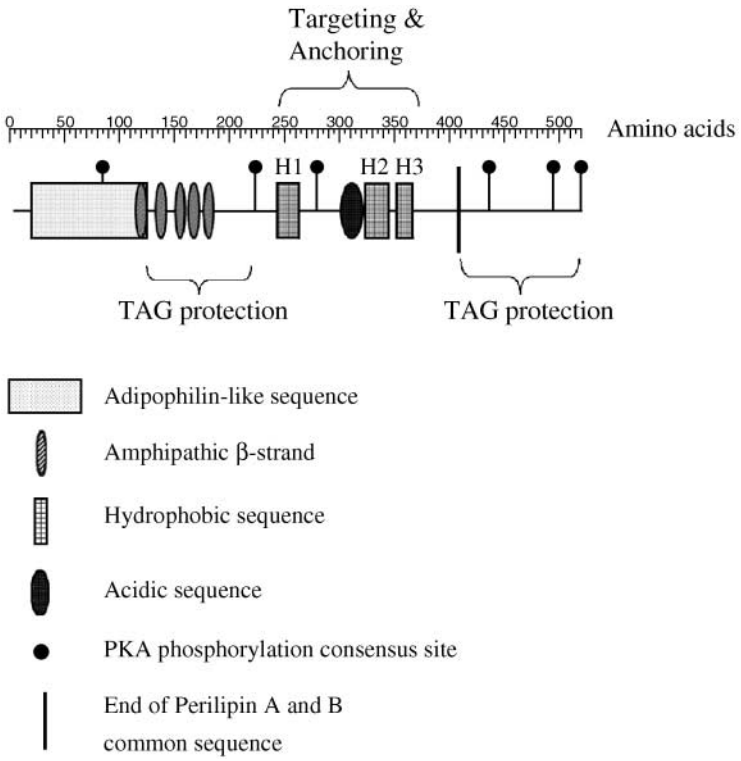

Fig. 1. Schematic diagram depicting predicted structural motifs of mouse perilipin A. The 517 amino acid (aa) sequence of perilipin A includes an N-terminal sequence of 105 aa that is conserved among the PAT family proteins (shaded adipophilin-like sequence), followed by five 10 aa sequences (aa 111–182; shaded ovals) that are predicted to form amphipathic β-strands (34). The central region that mediates the targeting of perilipin A to lipid droplets contains three moderately hydrophobic sequences (aa 242–260, H1; aa 320–342, H2; and aa 349–364, H3; shaded rectangles) and a highly acidic region (aa 291–318; dark oval) (24). Six consensus sites for phosphorylation of serine by cAMP-dependent protein kinase (PKA) are distributed throughout the perilipin sequence (small dark circles). The sequences before the vertical line are common to both perilipin A and B; perilipin A has a unique C terminus of 112 aa after aa 405, whereas the C terminus of perilipin B is 17 aa in length. Sequences that play a role in shielding stored triacylglycerol (TAG) from cytosolic lipases include aa 122–222 and aa 406–517 (33).

terized the localization of the mutated perilipins using immunofluorescence microscopy and immunoblotting of subcellular fractions. We have found that multiple partially redundant targeting signals include not only H1, H2, and H3 but also the central acidic region and N-terminal sequences predicted to form amphipathic β-strands. Because the anchoring of perilipins into lipid droplets is resistant to alkaline carbonate treatments that disrupt electrostatic interactions (24) and is disrupted by detergents such as SDS (25), we further tested the hypothesis that perilipins anchor into lipid droplets by embedding the three hydrophobic sequences into the triacylglycerol core.

EXPERIMENTAL PROCEDURES

Materials

Pfu DNA polymerase was purchased from Stratagene. Alexa Fluor 488-conjugated goat anti-rabbit IgG and Bodipy 493/503 were obtained from Molecular Probes, Inc. (Eugene, OR). Lissamine rhodamine-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Geneticin was purchased from Mediatech, Inc. (Herndon, VA).

Cell culture

3T3-L1 preadipocytes and 293T cells were cultured as described previously (4).

Expression of intact and mutated forms of perilipin A in 3T3-L1 fibroblasts

The coding sequence of mouse perilipin A cDNA subcloned into the pSRαMSVtkneo retroviral expression vector was reported previously (4). To generate mutated forms of perilipin A, nucleotide sequences encoding H1, H2, and H3 were replaced with sequences for the restriction sites *Sal*I, *Xba*I, and *Bgl*II, respectively; the resulting perilipin A cDNA was amplified using *Pfu* DNA polymerase and 5′ and 3′ oligonucleotide primers with added *Hin*dIII sites and subcloned into pSRαMSVtkneo (26). Perilipin cDNAs lacking the nucleotide sequences for H1, H2, or H3 were then used as templates to generate additional constructs lacking all combinations of two hydrophobic sequences. The perilipin cDNA lacking H1 and H3 was then used as a template to generate constructs lacking the nucleotide sequences for *1*) H1, H2, and H3 and *2*) H1, H2, H3, and the acidic region from aa 291 to 318 by replacing the nucleotides encoding aa 291–342 (including the acidic region and H2) with an *Xba*I restriction site. Mutated perilipin cDNA lacking the sequences for H1, H2, and H3 was also used as a template to replace the nucleotide sequence encoding aa 111–182, which is predicted to form five amphipathic β-strands with an *Spe*I restriction site. A summary of all constructs is shown in **Table 1**. The amplified intact and mutated perilipin cDNAs were ligated into the unique *Hin*dIII site of the pSRαMSVtkneo retroviral expression vector (26). All mutated forms of perilipin cDNA were sequenced to confirm the fidelity of amplification. The procedures used to assemble the retrovirus in 293T cells, transduce 3T3-L1 fibroblasts, and select cells stably expressing the various cDNAs were described previously (4). Control cells were selected to stably express the retroviral vector lacking a cDNA insert. All experiments were conducted on cells stably expressing the various mutated forms of perilipin from two or more transduction procedures.

Immunofluorescence microscopy

Cells were grown on glass coverslips in culture media without additions or media supplemented with 400 μM oleic acid (Sigma) complexed to fatty acid-free BSA (Biocell Laboratories, Inc.) at a 6:1 molar ratio for 24 h before fixation to increase triacylglycerol synthesis and storage and, hence, increase protein levels of perilipin by increasing its stability (23). Cells were fixed with 3% paraformaldehyde in PBS and prepared for indirect immunofluorescence microscopy, as described previously (27). Cells were stained with polyclonal antisera raised against a recombi-

TABLE 1. Summary of mutated perilipin constructs

Name of Construct	Amino Acids Deleted
Intact perilipin A	0
Δ1H	242–260
Δ2H	320–342
Δ3H	349–364
Δ1,2H	242–260 320–342
Δ2,3H	320–342 349–364
Δ1,3H	242–260 349–364
Δ1,2,3H	242–260 320–342 349–364
AΔ	242–260 291–342 349–364
βΔ	111–182 242–260 320–342 349–364

nant N-terminal peptide of perilipin A (10) diluted 1:1,500. Secondary antibodies used were either Alexa Fluor 488-conjugated goat anti-rabbit IgG or Lissamine rhodamine-conjugated goat anti-rabbit IgG when costaining for neutral lipids with Bodipy 493/503. Cells were viewed with a Nikon Eclipse E800 fluorescence microscope equipped with a Hamamatsu Orca digital camera interfaced with a Power Macintosh G4. Images were captured in monochrome and processed using Improvision Openlab software; for aesthetic reasons, doubly stained cells are depicted in the opposite colors to those observed. The intracellular localization of ectopically expressed forms of mutated perilipin or intact perilipin A was quantified by scoring at least 100 cells per transduction experiment; classification of the mutated perilipins as clearly targeting to lipid droplets, poorly targeting to lipid droplets, or failing to target to lipid droplets was compared with values acquired for intact perilipin A.

Subcellular fractionation

Confluent monolayers of 3T3-L1 fibroblasts stably expressing intact perilipin A or mutated forms of perilipin were incubated with 600 μM oleic acid complexed to fatty acid-free BSA for 24 h. Cells were harvested, homogenized, and fractionated to collect lipid droplets, as described previously (24).

Alkaline carbonate treatments of isolated lipid droplets

Lipid droplet fractions recovered from subcellular fractionation were adjusted to 100 mM sodium carbonate, pH 11.5, with 10 μg/ml leupeptin, 1 mM benzamidine, 100 μM [4-(2-aminoethyl)-benzenesulfonylfluoride] hydrochloride, and 20% sucrose (final concentration) and layered beneath 100 mM sodium carbonate, pH 11.5, with protease inhibitors in centrifuge tubes. The tubes were centrifuged for 30 min at 26,000 *g* at 4°C in a Sorvall TH660 rotor, and the floating lipid droplets were recovered by slicing off the tops of the tubes with a Beckman tube slicer.

Immunoblotting of lipid droplet proteins

Isolated lipid droplets were delipidated by precipitation with cold acetone overnight at −20°C; precipitated proteins were solubilized in 2× concentrated Laemmli's sample buffer (28). Proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were probed with anti-perilipin polyclonal antisera and a horseradish peroxidase-conjugated secondary antibody (Sigma); protein bands were detected using enhanced chemiluminescence reagents from Amersham Biosciences.

Northern blot analysis

Total RNA was extracted from cells stably expressing intact perilipin A and mutated perilipins using RNAeasy (Qiagen) and separated by electrophoresis on 1% agarose gels, as described previously (24). RNA was transferred electrophoretically to MagnaCharge nylon membranes (Osmonics) and probed with ^{32}P-labeled cDNA for the coding sequence of perilipin A. Membranes were reprobed for β-actin to correct for variations in RNA load. The mass of perilipin mRNA relative to β-actin mRNA was determined by densitometric scanning of exposed X-ray films using a Personal Densitometer from Molecular Dynamics.

RESULTS

Patterns of localization of mutated forms of perilipin on lipid droplets

When using immunofluorescence microscopy to observe the localization of mutated forms of perilipin, we consistently noted three patterns of fluorescence in transduced 3T3-L1 fibroblasts (**Fig. 2**). Intact perilipin A and some mutated forms of perilipin clearly targeted to lipid droplets and were detected as bright, uninterrupted rings of fluorescence around the lipid droplets when cells were stained with polyclonal antisera raised against perilipin A (Fig. 2A). Cells expressing some of the mutated forms of perilipin displayed weaker, interrupted rings of fluorescence around lipid droplets (Fig. 2B); we interpreted this pattern to represent less efficient targeting of the mutated perilipins to lipid droplets. Control cells lacking perilipins and cells expressing some of the mutated forms of perilipin lacked a specific staining pattern around lipid droplets and displayed only diffuse background fluorescence (Fig. 2C). Using this classification scheme, 95% of 3T3-L1 fibroblasts expressing intact ectopic perilipin A showed strong localization, 4% of cells showed weak localization, and 1% showed no specific staining on lipid droplets (Figs. 2, 3, **Table 2**). In contrast, 100% of cells stably expressing neomycin resistance conferred by the retroviral vector lacking an inserted cDNA sequence showed no specific staining on lipid droplets (Figs. 2, 3, Table 2).

The hydrophobic sequences are individually dispensable for the targeting of perilipin A to lipid droplets

Based on our previous findings, we hypothesized that the hydrophobic sequences of perilipin A are important for the targeting of nascent perilipins to lipid droplets. To systematically dissect the functional roles of these hydrophobic sequences, we first deleted H1, H2, or H3 individually from otherwise intact perilipin A; mutated perilipins Δ1H (lacking H1; aa 242–260), Δ2H (lacking H2; aa 320–342), and Δ3H (lacking H3; aa 349–364) clearly localized to lipid droplets by immunofluorescence microscopy of fixed cells (**Fig. 3**) and by immunoblotting of proteins from isolated lipid droplets (**Fig. 4**). Ninety-six percent of cells expressing mutated perilipin Δ1H, 92% of cells expressing mutated perilipin Δ2H, and 97% of cells expressing mutated perilipin Δ3H showed strong staining of peri-

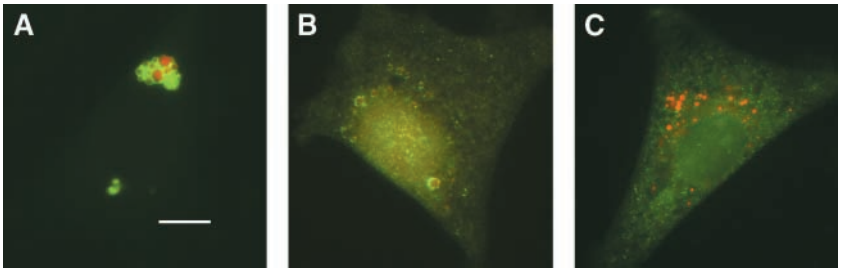

Fig. 2. Classification of the targeting of mutated forms of perilipin A. 3T3-L1 fibroblasts stably expressing intact perilipin A (A), mutated perilipin lacking H1 and H2 (perilipin mutation Δ1,2H) (B), and the retroviral vector lacking a cDNA insert (C) were fixed with 3% paraformaldehyde and costained for perilipin A using anti-perilipin polyclonal antisera followed by Lissamine rhodamine-tagged secondary IgG and neutral lipid using Bodipy 493/503. A: Cell with strong localization of perilipin A to lipid droplets. Bar = 10 μm. B: Example of a cell with weak localization of the mutated perilipin to lipid droplets. C: Example of a cell with no specific staining of lipid droplets.

lipin on lipid droplets (Table 2). Furthermore, the mass of mutated perilipins Δ1H, Δ2H, and Δ3H detected on immunoblots of lipid droplet fractions was similar to that of intact perilipin A (Fig. 4A). Thus, perilipin A lacking any one of the three hydrophobic sequences targets to lipid droplets.

Deletion of combinations of two of the hydrophobic sequences reveals unequal roles for these sequences in targeting perilipin A to lipid droplets

We next asked whether deletion of more than one hydrophobic sequence alters targeting of the mutated perilipin to lipid droplets. Mutated forms of perilipin lacking H1 and H3 (Δ1,3H) or H2 and H3 (Δ2,3H) targeted efficiently to lipid droplets, as assessed by immunofluorescence microscopy (Fig. 3); 99% of cells showed either strong or weak staining of perilipin on lipid droplets (Table 2). In contrast, mutated perilipin lacking H1 and H2 (Δ1,2H) targeted less efficiently to lipid droplets (Fig. 3); only 9% of cells showed strong staining of perilipin on lipid droplets, 81% of cells showed weak staining, and 10% of cells displayed only background staining (Table 2). Correspondingly, levels of Δ2,3H and Δ1,3H mutated perilipins isolated in lipid droplet fractions were comparable to that of intact ectopic perilipin A, whereas the mass of Δ1,2H mutated perilipin on lipid droplets was significantly reduced (Fig. 4B). Thus, perilipin A lacking the combination of H1 and H2 targets less efficiently to lipid droplets, indicating that H3 is a relatively weak targeting signal.

TABLE 2. Summary of the targeting of mutated forms of perilipin to lipid droplets

	Percentage of Cells (Mean ± SD)		
Name of Construct	Strong Localization	Weak Localization	No Specific Staining
Perilipin A (n = 7)	95 ± 4	4 ± 3	1 ± 2
Control cells lacking perilipins (n = 7)	0	0	100
Δ1H (n = 1)	96	1	3
Δ2H (n = 1)	92	3	5
Δ3H (n = 1)	97	3	0
Δ1,2H (n = 4)	9 ± 8	81 ± 3	10 ± 9
Δ2,3H (n = 3)	89 ± 9	10 ± 8	1 ± 1
Δ1,3H (n = 3)	73 ± 11	26 ± 11	1 ± 1
Δ1,2,3H (n = 3)	0	36 ± 2	64 ± 2
AΔ (n = 3)	0	0	100
βΔ (n = 3)	0	0	100

n, number of repetitions of 100 cell counts.

Mutated perilipin lacking all three hydrophobic sequences targets poorly to lipid droplets

Cells expressing mutated perilipin lacking H1, H2, and H3 (Δ1,2,3H) displayed either weak staining of perilipin on lipid droplets (36% of cells) or no specific staining pattern (64% of cells; Table 2, Fig. 3). Correspondingly, immunoblots showed only a trace amount of Δ1,2,3H mutated perilipin on lipid droplets (Fig. 4C). Surprisingly, the deletion of H1, H2, and H3 did not completely eliminate targeting of the mutated perilipin to lipid droplets, suggesting that additional sequences may play a role in targeting. Thus, we further deleted the N-terminal sequences from aa 111 to 182 that are predicted to form amphipathic β-strands (βΔ) and, separately, the acidic region from aa 291 to 318 (AΔ) from mutated perilipin that lacks H1, H2, and H3. Both the βΔ and AΔ mutated forms of perilipin failed to target to lipid droplets when assessed by immunofluorescence microscopy or immunoblotting of lipid droplet fractions (Figs. 3, 4C, Table 2). Furthermore, these nontargeting forms of perilipin were not detected in supernatant or membrane fractions (data not shown), suggesting that the proteins were rapidly degraded. Interestingly, deletion of either the sequence from aa 111 to 182 (our unpublished observation) or from aa 291 to 318 (24) from otherwise intact perilipin did not inhibit targeting of the mutated forms of perilipin to lipid droplets.

Levels of mRNA of intact and mutated forms of perilipin A are similar in transduced cells

We used Northern blotting to assess transduction efficiency in the experiments. Levels of mRNA of mutated forms of perilipin lacking hydrophobic sequences were consistently comparable to or greater than those of cells expressing intact perilipin A (**Fig. 5**); thus, the reduced targeting of some of the mutated forms of perilipin was not attribut-

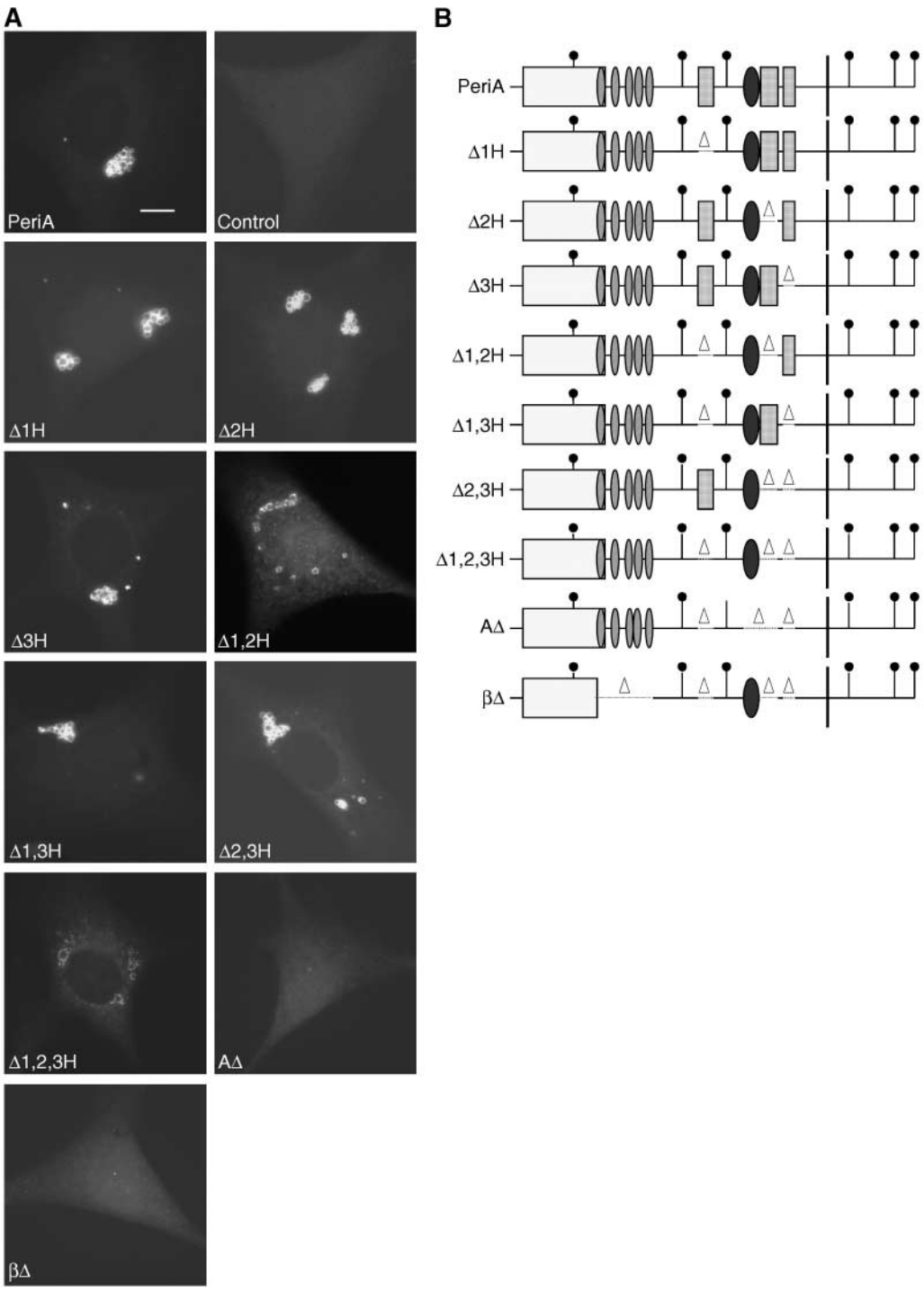

Fig. 3. Targeting of mutated perilipins to lipid droplets in 3T3-L1 fibroblasts. A: 3T3-L1 fibroblasts stably expressing intact perilipin A (PeriA), control retroviral vector lacking a cDNA insert (Control), mutated perilipins lacking individual hydrophobic sequences (Δ1H, Δ2H, Δ3H), combinations of two hydrophobic sequences (Δ1,2H, Δ1,3H, Δ2,3H), all three hydrophobic sequences (Δ1,2,3H), or all three hydrophobic sequences and either the acidic region (AΔ) or N-terminal sequences predicted to form amphipathic β-strands (βΔ) were stained with anti-perilipin polyclonal antisera and an Alexa Fluor 488-tagged secondary antibody. Each panel shows a single representative cell from more than 100 observed in each of two to three transduction experiments. Bar = 10 μm. B: Schematic diagrams of the expressed regions of intact perilipin A and the mutated forms of perilipin. Δs above dotted lines indicate the positions of the deleted sequences. See legend to Fig. 1 for details of the depicted structural motifs.

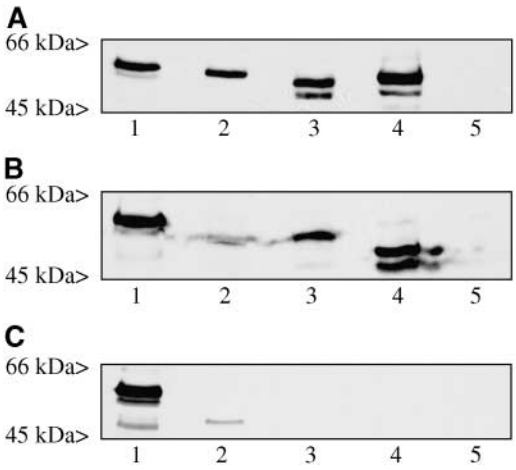

Fig. 4. Immunoblots of proteins in lipid droplet fractions. 3T3-L1 fibroblasts ectopically expressing mutated forms of perilipins were lipid-loaded before the isolation of lipid droplets. Lipid droplet proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes; membranes were probed with polyclonal antisera raised against perilipin. A: Intact perilipin A (lane 1), mutated forms of perilipin Δ1H (lane 2), Δ2H (lane 3), Δ3H (lane 4), and control cells lacking perilipins (lane 5). B: intact perilipin A (lane 1), mutated forms of perilipin Δ1,2H (lane 2), Δ1,3H (lane 3), Δ2,3H (lane 4), and control cells lacking perilipins (lane 5). C: or intact perilipin A (lane 1), mutated forms of perilipin Δ1,2,3H (lane 2), AΔ (lane 3), βΔ (lane 4), and control cells lacking perilipins (lane 5). Each lane contains lipid droplet proteins collected from an equivalent mass of cells.

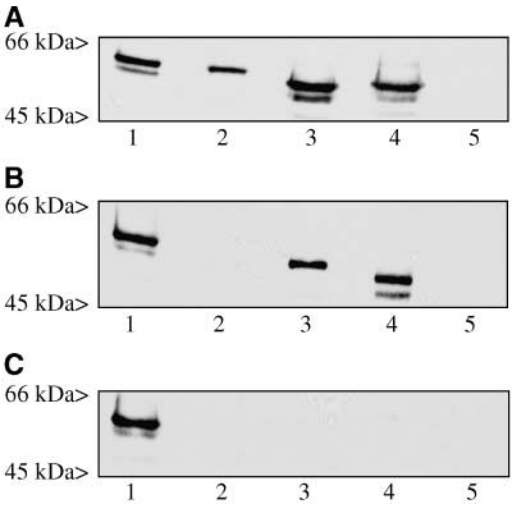

Fig. 6. Perilipin A requires hydrophobic sequences to anchor to lipid droplets. Confluent 3T3-L1 fibroblasts stably expressing mutated forms of perilipin were lipid-loaded before the isolation of lipid droplets. Lipid droplet fractions were centrifuged through alkaline carbonate solutions, and proteins that remained associated with lipid droplets were resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were probed with polyclonal antisera raised against perilipin. A: Intact perilipin A (lane 1), mutated forms of perilipin Δ1H (lane 2), Δ2H (lane 3), and Δ3H (lane 4), and control cells lacking perilipins (lane 5). B: Intact perilipin A (lane 1), mutated forms of perilipin Δ1,2H (lane 2), Δ1,3H (lane 3), and Δ2,3H (lane 4), and control cells lacking perilipins (lane 5). C: Intact perilipin A (lane 1), mutated forms of perilipin Δ1,2,3H (lane 2), AΔ (lane 3), and βΔ (lane 4), and control cells lacking perilipins (lane 5).

able to poor expression. However, levels of mRNA of the βΔ and AΔ forms of mutated perilipin were consistently lower, suggesting that the mRNA may be unstable, leading to reduced expression of these proteins.

The hydrophobic sequences anchor perilipins to lipid droplets

Intact perilipin A adheres to lipid droplets during incubations with alkaline carbonate solutions (24) that disrupt electrostatic interactions between proteins and membrane phospholipids or other proteins (29). Thus, we tested for the adherence of the mutated forms of perilipin to lipid droplets after incubations with alkaline carbonate solutions. Like intact perilipin A, the Δ1H, Δ2H, and Δ3H forms of mutated perilipins remained associated with carbonate-washed lipid droplets (**Fig. 6A**), suggesting that

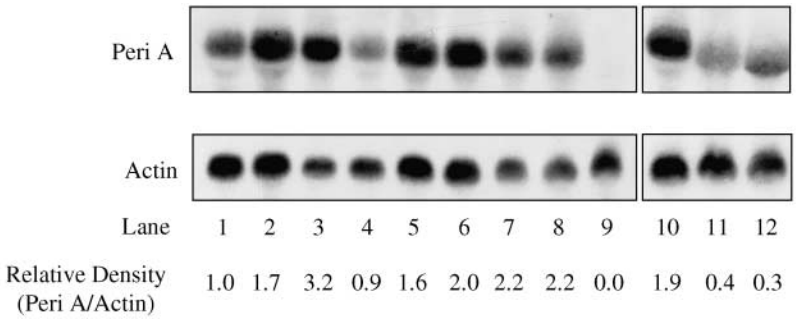

Fig. 5. Northern blots of mRNA for ectopic intact or mutated forms of perilipin A. Total RNA was isolated from 3T3-L1 cells stably expressing intact or mutated forms of perilipin A (Peri A) and was separated electrophoretically on agarose gels before transfer to charged nylon membranes. The membranes were probed with a radiolabeled perilipin A cDNA probe (top panel) and then stripped and reprobed for β-actin (bottom panel) as a control for sample load. Northern blots show samples from cells expressing intact perilipin A (lane 1), mutated forms of perilipin Δ1H (lane 2), Δ2H (lane 3), Δ3H (lane 4), Δ1,2H (lane 5), Δ2,3H (lane 6), Δ1,3H (lane 7), and Δ1,2,3H (lane 8), control cells lacking perilipins (lane 9), intact perilipin A (lane 10), and mutated forms of perilipin AΔ (lane 11) and βΔ (lane 12). Each lane contained 10 μg of total RNA; the relative density of perilipin signal was calculated relative to the actin signal.

the loss of a single hydrophobic sequence does not significantly compromise perilipin anchoring to lipid droplets. Although the Δ1,3H and Δ2,3H forms of mutated perilipin remained adherent to lipid droplets during incubations with alkaline carbonate solutions, Δ1,2H mutated perilipin did not (Fig. 6B), suggesting that H3 in conjunction with the remaining sequences is not sufficient to anchor perilipins to lipid droplets. As expected, mutated perilipin Δ1,2,3H was also released from lipid droplets after incubation with alkaline carbonate solutions (Fig. 6C). The βΔ and AΔ mutated forms of perilipin were absent from carbonate-washed lipid droplets (Fig. 6C), consistent with our finding that these forms of mutated perilipins fail to target to lipid droplets.

DISCUSSION

The main finding of this study is that the three hydrophobic sequences of perilipin A play important roles in targeting and anchoring perilipin to lipid droplets. The deletion of each of the three hydrophobic sequences individually does not hinder either targeting or anchoring of the mutated perilipin to lipid droplets, whereas the deletion of H1, H2, and H3 in combination severely compromises targeting. These observations suggest that there is functional redundancy within the targeting signals for nascent perilipins. Furthermore, the individual hydrophobic sequences appear to be nonequivalent. Mutated perilipin containing H3 in the absence of H1 and H2 fails to efficiently target and anchor to lipid droplets, whereas mutated forms of perilipin that include either H1 or H2 in the absence of the other hydrophobic sequences clearly target to and firmly embed into lipid droplets. Thus, either H1 or H2 is required for optimal targeting and stable association of perilipin with lipid droplets.

The mechanism by which the hydrophobic sequences target and anchor perilipin to lipid droplets is unknown. The secondary structure of the hydrophobic sequences has not yet been elucidated, so it is unclear whether these sequences form α-helices analogous to sequences in apolipoproteins that shallowly embed these proteins into the hydrophobic environment of lipoproteins (30–32) or fold into an alternative structure. The current study and two additional studies from our laboratory (24, 33) have demonstrated the remarkable plasticity of the perilipin sequence; deletion of small or large segments of the N-terminal, C-terminal, or central sequences of perilipin A has little or no effect on the capacity of mutated forms of perilipin to both target to lipid droplets and embed deeply enough into the hydrophobic core of the droplet to withstand incubation with alkaline carbonate solutions. Consequently, the folding of perilipin into a tertiary structure that is competent for targeting and assembly onto lipid droplets is permissive of a variety of mutations. It is clear that the hydrophobic sequences are an important component of the anchoring of perilipin into the lipid droplet, but N-terminal sequences that have been predicted to form amphipathic β-strands (34) may also serve to position perilipin onto lipid droplets. Interestingly, although the deletion of the β-strand sequences from otherwise intact perilipin A has no effect on targeting of the mutated perilipin (our unpublished observation), the deletion of these sequences from perilipin that also lacks sequences for H1, H2, and H3 eliminates all residual targeting of the mutated perilipin to lipid droplets.

Several other proteins target to lipid droplets using signals present within hydrophobic sequences. A long hydrophobic spike of 72 aa is required to target oleosins to lipid droplets in the seeds of plants; deletion of this sequence abolishes targeting (18, 21). Furthermore, targeting of the hepatitis C core protein (35) and the GB virus-B protein (36) to lipid droplets in mammalian cells requires signals contained within hydrophobic sequences. Caveolins, a family of proteins that localize to sphingolipid- and cholesterol-enriched domains in the plasma membrane, can also associate with lipid droplets in cultured cells when the proteins are overexpressed with either a KDEL sequence for retrieval to the endoplasmic reticulum (37) or an N-terminal deletion mutation (38), when cells are treated with brefeldin A (38), or when caveolin-2 is overexpressed in the absence of caveolin-1 (38). Mutagenesis studies of both caveolin-1 and caveolin-2 show that hydrophobic sequences contain the signals that specify targeting to lipid droplets (38, 39). Although hydrophobic sequences are important in the targeting of perilipins and a variety of other proteins to lipid droplets, there is no obvious consensus sequence that is common to all of these proteins that can be identified as the specific targeting signal.

The perilipins are members of the PAT family of proteins that also includes the ubiquitously expressed lipid droplet-associated protein, adipophilin. Adipophilin, like perilipins, has been found to localize to lipid droplets and to no other subcellular compartment (10). The targeting of adipophilin to lipid droplets is mediated by multiple, partially redundant signals that are discontinuous and spread throughout the primary amino acid sequence (40–42). Interestingly, the targeting signals for adipophilin are distinct from those that we have identified for perilipin in that they are not particularly hydrophobic and are not conserved between the two proteins. No targeting signals for either protein have been identified within the N-terminal sequences that are most highly conserved between members of the PAT family. TIP47 is the PAT family member that is most highly related to adipophilin and is also ubiquitously expressed in tissues, but unlike perilipin and adipophilin, it localizes to both lipid droplets and the cytoplasm (9, 12). Although targeting studies have not been reported yet for TIP47, the crystal structure for a portion of the C-terminal domain of TIP47 has been solved (43). Sequences within the C terminus of TIP47 fold into a four helix bundle of amphipathic α-helices that is remarkably similar to a four helix bundle formed by N-terminal sequences of apolipoprotein E, an apolipoprotein that maintains both a stable soluble conformation and a lipoprotein-bound conformation. Thus, in analogy to the exchangeable apolipoproteins, opening and closing of the four helix bundle of TIP47 may mediate the exchangeability of TIP47

between a lipid droplet-bound form and a stable soluble conformation in the cytosol. Interestingly, the targeting signals identified for adipophilin reside outside of the putative four helix bundle sequences, although it is as yet unknown whether the C terminus of adipophilin folds into a comparable structure. Furthermore, the C-terminal sequence of TIP47 that has been modeled is not conserved in the more specialized family members, perilipin and S3-12. Thus, despite an evolutionary relationship, the mechanisms by which members of the PAT family of proteins target to lipid droplets are clearly distinct.

This work was supported by National Institutes of Health Grant DK-54797. The authors thank Boris Rubin, Ashleigh Naczkowski, Florence Fudalan, Maryellen Sepelya, Sucharita Bhattacharyya, Alexis Rothenberg, Denise Gauthier, and Terry Yin for technical support. The authors thank Dr. Yanxin Wang and Amy Marcinkiewicz for helpful discussions.

REFERENCES

1. Martinez-Botas, J., J. B. Anderson, D. Tessier, A. Lapillonne, B. H. Chang, M. J. Quast, D. Gorenstein, K. H. Chen, and L. Chan. 2000. Absence of perilipin results in leanness and reverses obesity in Lepr(db/db) mice. *Nat. Genet.* **26:** 474–479.
2. Souza, S. C., L. M. de Vargas, M. T. Yamamoto, P. Lien, M. D. Franciosa, L. G. Moss, and A. S. Greenberg. 1998. Overexpression of perilipin A and B blocks the ability of tumor necrosis factor alpha to increase lipolysis in 3T3-L1 adipocytes. *J. Biol. Chem.* **273:** 24665–24669.
3. Tansey, J. T., C. Sztalryd, J. Gruia-Gray, D. L. Roush, J. V. Zee, O. Gavrilova, M. L. Reitman, C. X. Deng, C. Li, A. R. Kimmel, and C. Londos. 2001. Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proc. Natl. Acad. Sci. USA.* **98:** 6494–6499.
4. Brasaemle, D. L., B. Rubin, I. A. Harten, J. Gruia-Gray, A. R. Kimmel, and C. Londos. 2000. Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *J. Biol. Chem.* **275:** 38486–38493.
5. Souza, S. C., K. V. Muliro, L. Liscum, P. Lien, M. T. Yamamoto, J. E. Schaffer, G. E. Dallal, X. Wang, F. B. Kraemer, M. Obin, and A. S. Greenberg. 2002. Modulation of hormone-sensitive lipase and protein kinase A-mediated lipolysis by perilipin A in an adenoviral reconstituted system. *J. Biol. Chem.* **277:** 8267–8272.
6. Sztalryd, C., G. Xu, H. Dorward, J. T. Tansey, J. A. Contreras, A. R. Kimmel, and C. Londos. 2003. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *J. Cell Biol.* **161:** 1093–1103.
7. Tansey, J. T., A. M. Huml, R. Vogt, K. E. Davis, J. M. Jones, K. A. Fraser, D. L. Brasaemle, A. R. Kimmel, and C. Londos. 2003. Functional studies on native and mutated forms of perilipins. A role in protein kinase A-mediated lipolysis of triacylglycerols. *J. Biol. Chem.* **278:** 8401–8406.
8. Zhang, H. H., S. C. Souza, K. V. Muliro, F. B. Kraemer, M. S. Obin, and A. S. Greenberg. 2003. Lipase-selective functional domains of perilipin A differentially regulate constitutive and protein kinase A-stimulated lipolysis. *J. Biol. Chem.* **278:** 51535–51542.
9. Miura, S., J. W. Gan, J. Brzostowski, M. J. Parisi, C. J. Schultz, C. Londos, B. Oliver, and A. R. Kimmel. 2002. Functional conservation for lipid storage droplet association among perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, Drosophila, and Dictyostelium. *J. Biol. Chem.* **277:** 32253–32257.
10. Brasaemle, D. L., T. Barber, N. E. Wolins, G. Serrero, E. J. Blanchette-Mackie, and C. Londos. 1997. Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J. Lipid Res.* **38:** 2249–2263.
11. Heid, H. W., R. Moll, I. Schwetlick, H. R. Rackwitz, and T. W. Keenan. 1998. Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. *Cell Tissue Res.* **294:** 309–321.
12. Wolins, N. E., B. Rubin, and D. L. Brasaemle. 2001. TIP47 associates with lipid droplets. *J. Biol. Chem.* **276:** 5101–5108.
13. Scherer, P. E., P. E. Bickel, M. Kotler, and H. F. Lodish. 1998. Cloning of cell-specific secreted and surface proteins by subtractive antibody screening. *Nat. Biotechnol.* **16:** 581–586.
14. Wolins, N. E., J. R. Skinner, M. J. Schoenfish, A. Tzekov, K. G. Bensch, and P. E. Bickel. 2003. Adipocyte protein S3-12 coats nascent lipid droplets. *J. Biol. Chem.* **278:** 37713–37721.
15. Greenberg, A. S., J. J. Egan, S. A. Wek, M. C. Moos, Jr., C. Londos, and A. R. Kimmel. 1993. Isolation of cDNAs for perilipins A and B: sequence and expression of lipid droplet-associated proteins of adipocytes. *Proc. Natl. Acad. Sci. USA.* **90:** 12035–12039.
16. Lu, X., J. Gruia-Gray, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, C. Londos, and A. R. Kimmel. 2001. The murine perilipin gene: the lipid droplet-associated perilipins derive from tissue-specific, mRNA splice variants and define a gene family of ancient origin. *Mamm. Genome.* **12:** 741–749.
17. Murphy, D. J., and J. Vance. 1999. Mechanisms of lipid-body formation. *Trends Biochem. Sci.* **24:** 109–115.
18. Murphy, D. J. 2001. The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Prog. Lipid Res.* **40:** 325–438.
19. Beaudoin, F., and J. A. Napier. 2002. Targeting and membrane-insertion of a sunflower oleosin in vitro and in *Saccharomyces cerevisiae*: the central hydrophobic domain contains more than one signal sequence, and directs oleosin insertion into the endoplasmic reticulum membrane using a signal anchor sequence mechanism. *Planta.* **215:** 293–303.
20. Hills, M. J., M. D. Watson, and D. J. Murphy. 1993. Targeting of oleosins to the oil bodies of oilseed rape (*Brassica napus* L.). *Planta.* **189:** 24–29.
21. Huang, A. H. 1996. Oleosins and oil bodies in seeds and other organs. *Plant Physiol.* **110:** 1055–1061.
22. Loer, D. S., and E. M. Herman. 1993. Cotranslational integration of soybean (*Glycine max*) oil body membrane protein oleosin into microsomal membranes. *Plant Physiol.* **101:** 993–998.
23. Brasaemle, D. L., T. Barber, A. R. Kimmel, and C. Londos. 1997. Post-translational regulation of perilipin expression. Stabilization by stored intracellular neutral lipids. *J. Biol. Chem.* **272:** 9378–9387.
24. Garcia, A., A. Sekowski, V. Subramanian, and D. L. Brasaemle. 2003. The central domain is required to target and anchor perilipin A to lipid droplets. *J. Biol. Chem.* **278:** 625–635.
25. Londos, C., D. L. Brasaemle, J. Gruia-Gray, D. A. Servetnick, C. J. Schultz, D. M. Levin, and A. R. Kimmel. 1995. Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells. *Biochem. Soc. Trans.* **23:** 611–615.
26. Muller, A. J., J. C. Young, A. M. Pendergast, M. Pondel, N. R. Landau, D. R. Littman, and O. N. Witte. 1991. BCR first exon sequences specifically activate the BCR/ABL tyrosine kinase oncogene of Philadelphia chromosome-positive human leukemias. *Mol. Cell. Biol.* **11:** 1785–1792.
27. Blanchette-Mackie, E. J., N. K. Dwyer, T. Barber, R. A. Coxey, T. Takeda, C. M. Rondinone, J. L. Theodorakis, A. S. Greenberg, and C. Londos. 1995. Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *J. Lipid Res.* **36:** 1211–1226.
28. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
29. Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* **93:** 97–102.
30. Bolanos-Garcia, V. M., and R. N. Miguel. 2003. On the structure and function of apolipoproteins: more than a family of lipid-binding proteins. *Prog. Biophys. Mol. Biol.* **83:** 47–68.
31. Frank, P. G., and Y. L. Marcel. 2000. Apolipoprotein A-I: structure-function relationships. *J. Lipid Res.* **41:** 853–872.
32. Segrest, J. P., M. K. Jones, H. De Loof, and N. Dashti. 2001. Structure of apolipoprotein B-100 in low density lipoproteins. *J. Lipid Res.* **42:** 1346–1367.
33. Garcia, A., V. Subramanian, A. Sekowski, S. Bhattacharyya, M. W. Love, and D. L. Brasaemle. 2004. The amino and carboxyl termini of perilipin A facilitate the storage of triacylglycerols. *J. Biol. Chem.* **279:** 8409–8416.
34. Londos, C., D. L. Brasaemle, C. J. Schultz, J. P. Segrest, and A. R. Kimmel. 1999. Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Semin. Cell Dev. Biol.* **10:** 51–58.

35. Hope, R. G., and J. McLauchlan. 2000. Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. *J. Gen. Virol.* **81:** 1913–1925.
36. Hope, R. G., D. J. Murphy, and J. McLauchlan. 2002. The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. *J. Biol. Chem.* **277:** 4261–4270.
37. Ostermeyer, A. G., J. M. Paci, Y. Zeng, D. M. Lublin, S. Munro, and D. A. Brown. 2001. Accumulation of caveolin in the endoplasmic reticulum redirects the protein to lipid storage droplets. *J. Cell Biol.* **152:** 1071–1078.
38. Fujimoto, T., H. Kogo, K. Ishiguro, K. Tauchi, and R. Nomura. 2001. Caveolin-2 is targeted to lipid droplets, a new "membrane domain" in the cell. *J. Cell Biol.* **152:** 1079–1085.
39. Ostermeyer, A. G., L. T. Ramcharan, Y. Zeng, D. M. Lublin, and D. A. Brown. 2004. Role of the hydrophobic domain in targeting caveolin-1 to lipid droplets. *J. Cell Biol.* **164:** 69–78.
40. McManaman, J. L., W. Zabaronick, J. Schaack, and D. J. Orlicky. 2003. Lipid droplet targeting domains of adipophilin. *J. Lipid Res.* **44:** 668–673.
41. Nakamura, N., and T. Fujimoto. 2003. Adipose differentiation-related protein has two independent domains for targeting to lipid droplets. *Biochem. Biophys. Res. Commun.* **306:** 333–338.
42. Targett-Adams, P., D. Chambers, S. Gledhill, R. G. Hope, J. F. Coy, A. Girod, and J. McLauchlan. 2003. Live cell analysis and targeting of the lipid droplet-binding adipocyte differentiation-related protein. *J. Biol. Chem.* **278:** 15998–16007.
43. Hickenbottom, S. J., A. R. Kimmel, C. Londos, and J. H. Hurley. 2004. Structure of a lipid droplet protein: the PAT family member TIP47. *Structure.* **12:** 1199–1207.