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The Central Domain Is Required to Target and Anchor Perilipin A to Lipid Droplets*

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The perilipins are the most abundant proteins coating the surfaces of lipid droplets in adipocytes and are found at lower levels surrounding lipid droplets in steroidogenic cells. Perilipins drive triacylglycerol storage in adipocytes by regulating the rate of basal lipolysis and are also required to maximize hormonally stimulated lipolysis. To map the domains that target and anchor perilipin A to lipid droplets, we stably expressed fragments of perilipin A in 3T3-L1 fibroblasts. Immunofluorescence microscopy and immunoblotting of proteins from isolated lipid droplets revealed that neither the amino nor the carboxyl terminus is required to target perilipin A to lipid droplets; however, there are multiple, partially redundant targeting signals within a central domain including 25% of the primary amino acid sequence. A peptide composed of the central domain of perilipin A directed a fused green fluorescent protein to the surfaces of lipid droplets. Full-length perilipin A associates with lipid droplets via hydrophobic interactions, as shown by the persistence of perilipins on lipid droplets after centrifugation through an alkaline carbonate solution. Results of the mutagenesis studies indicate that the sequences responsible for anchoring perilipin A to lipid droplets are most likely domains of moderately hydrophobic amino acids located within the central 25% of the protein. Thus, we conclude that the central 25% of the perilipin A sequence contains all of the amino acids necessary to target and anchor the protein to lipid droplets.

All proteins contain sequences of amino acids that specify their ultimate subcellular localization. These molecular zip codes may consist of a short motif such as the KDEL sequence that serves as a retention signal to hold proteins in the endoplasmic reticulum (1, 2) or may be quite complex like mitochondrial targeting signals that lack amino acid identity, but are enriched in hydrophobic, hydroxylated, and positively charged amino acids, and have the potential to form amphipathic α-helices (3). Proteins specifically associated with lipid droplets have only recently been described, and the amino acid sequences required to target nascent proteins to lipid droplets are uncharacterized. The purpose of this study is to identify the structural motifs responsible for directing the targeting of perilipins, the major proteins coating the prominent lipid droplets of adipocytes, to lipid droplets.

Lipid droplets are spherical organelles found in many types of eukaryotic cells that are composed of a core of neutral lipids covered by a monolayer of phospholipids, free cholesterol, and proteins. Depending on the cell type, the number of lipid droplets, the relative mass of stored triacylglycerol and cholesterol esters, and the protein composition of the droplet vary. Adipocytes store almost exclusively triacylglycerol in enormous lipid droplets that may exceed 100 μm and constitute the major energy storage depot of the body. Many other cells store cholesterol ester in tiny droplets; this cholesterol is used to maintain cellular cholesterol levels for membrane synthesis, and, in specialized cells in the adrenal cortex, testes, and ovaries, it serves as a source of substrate for steroid hormone synthesis (4). To date, few lipid droplet-associated proteins have been identified in mammals, yet recent functional studies show that these proteins serve essential roles in regulating neutral lipid storage and release (5–9).

The perilipins are a family of three protein isoforms (10, 11) encoded by a single gene that are localized exclusively to lipid droplets in adipocytes (12, 13) and steroidogenic cells (14). Perilipin A is the most abundant isoform in both cell types, whereas perilipin C is unique to steroidogenic cells; low levels of perilipin B can be found in both types of cells. The perilipins share a common amino-terminal region, and each isoform has a unique carboxyl-terminal end (10, 11, 14). Perilipin A is a relatively abundant protein on adipocyte lipid droplets and functions to increase cellular triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis (5–8); thus, is required to maximize the storage of triacylglycerols in adipose tissue (6, 7). Furthermore, perilipin A is multiply phosphorylated by cAMP-dependent protein kinase (PKA)[1] following the stimulation of lipolysis in adipocytes (15), and serves an additional role in controlling the release of triacylglycerol at times of need (6, 9).

Current models for lipid droplet assembly favor the nucleation of lipid droplets as a lens of neutral lipid within the membrane bilayer of the endoplasmic reticulum that pinches off and enters the cytosol following the accumulation of sufficient neutral lipid (4). Perilipins are synthesized on free ribosomes rather than on endoplasmic reticulum-bound ribosomes (16, 17); thus, nascent perilipins must travel to and assemble onto lipid droplets post-translationally. The processes that control the directing of nascent proteins to lipid droplets and the

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; α-COT, polyclonal antibodies raised against the carboxyl terminus of perilipin A; α-PAT, polyclonal antibodies raised against the amino terminus of perilipin A; BSA, bovine serum albumin; GFP, green fluorescent protein; H1, H2, and H3, first, second, and third hydrophobic domains of perilipin A, respectively; PBS, phosphate-buffered saline; aa, amino acid(s).

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assembly of these proteins onto the droplet have not been elucidated. The purpose of this study is to identify the structural domains that mediate the targeting and anchoring of perilipin A to lipid droplets. Deletion mutations of perilipin A were stably expressed in 3T3-L1 fibroblasts, a cell line that does not express endogenous perilipins. Targeting of mutated perilipins to lipid droplets was assessed by immunofluorescence microscopy and immunoblotting of subcellular fractions; effective anchoring of the mutated proteins into the droplet was assayed by subjecting isolated lipid droplets to alkaline carbonate solutions, in a classic test for hydrophobic interactions. The studies show that the central 25% of perilipin A contains all of the amino acid sequences required to target and anchor the protein to lipid droplets.

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). Rhodamine Red X-conjugated goat anti-guinea pig IgGs, fluorescein isothiocyanate-conjugated goat anti-guinea pig IgGs, and lissamine rhodamine-conjugated goat anti-rabbit IgGs were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). RNase inhibitor was from 5 Prime → 3 Prime, Inc. (Boulder, CO). Geneticin was purchased from Mediatech, Inc. (Herndon, VA).

Cell Culture—3T3-L1 preadipocytes and 293T cells were cultured as previously described (5).

Expression of Perilipin A in 3T3-L1 Fibroblasts—The coding sequence of the cDNA for mouse perilipin A was amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase and oligonucleotide primers corresponding to the ends of the regions of the perilipin A cDNA sequence that needed to be amplified, with added *Hin*dIII sites. When the perilipin A cDNA was amplified to truncate the 5′ end, an ATG codon was inserted into the 5′ primer; when perilipin A cDNA was amplified to truncate the 3′ end, a TGA stop codon was inserted into the 3′ primer. Internal deletions were created by replacing the cDNA sequence to be deleted by a *Bgl*II or *Xba*I site, introduced by PCR oligonucleotide primers. The amplified cDNA sequences were ligated into the unique *Hin*dIII site of the pSRαMSVtkneo retroviral expression vector (18). The procedure used to assemble the retrovirus, transduce 3T3-L1 fibroblasts, and select cells stably expressing the cDNAs was described previously (5). Cells used for control conditions were selected to stably express the retroviral vector lacking perilipin A cDNA. Stably selected cells from multiple transduction experiments were used for each of the mutated perilipin constructs in all of the experiments.

Expression of Green Fluorescent Protein (GFP)-Perilipin Fusion Proteins—The cDNA sequence for a mutated GFP engineered to fluoresce more efficiently at the wavelength for fluorescein was used (19) (mutation 2). The GFP mutation 2 cDNA was amplified from the pGAL-GFP plasmid (gift of Dr. Joseph Nickels, MCP Hahnemann School of Medicine) by PCR, using primers matching the 5′ and 3′ ends of the coding sequence of the GFP cDNA with added *Hin*dIII sites at both ends, and an added *Xba*I site proximal to the 3′ end to aid in subsequent subcloning steps; the amplified cDNA was then ligated into the *Hin*dIII site of the pSRαMSVtkneo retroviral expression vector. The resulting expression vector was named pSRαMSVtkneo-GFP. After amplification by PCR using 5′ and 3′ primers containing added *Xba*I sites, full-length or truncated perilipin A cDNA sequences were ligated in frame into the *Xba*I site of pSRαMSVtkneo-GFP. GFP-perilipin fusion constructs in the retroviral expression vector were stably or transiently expressed in 3T3-L1 fibroblasts following the previously described procedure (5). Cells for control conditions stably expressed GFP. The GFP fusion proteins consist of GFP separated at its carboxyl terminus from the amino terminus of perilipin A by a hinge sequence of 5 prolines.

Fluorescence Microscopy—Cells were grown on glass coverslips and fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS). 3T3-L1 fibroblasts expressing GFP constructs were observed under the microscope without fixation. Cells expressing amino-terminally truncated perilipin A were probed with polyclonal antibodies raised against the carboxyl-terminal domain of perilipin A (α-COT) (20). Cells expressing carboxyl-terminally truncated, or perilipin A mutated by internal deletions, were probed with antibodies directed against the amino terminus of perilipin A (α-PAT) (20). The α-PAT antibodies do not detect the structurally similar protein, adipophilin (20). When cells were simultaneously stained for perilipins and neutral lipids, Bodipy 493/503 was added to the solutions containing the fluorescently labeled secondary antibodies at a final concentration of 10 μg/ml (21). 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 acquired and processed using Improvision Openlab software.

Subcellular Fractionation—Confluent monolayers of 3T3-L1 fibroblasts stably expressing full-length or mutated perilipin A were incubated with either 400 μM oleic acid complexed to fatty acid-free bovine serum albumin (BSA) at a 6:1 molar ratio, when the BSA was purchased from Sigma, or with 600 μM oleic acid complexed to fatty acid-free BSA (6:1) when the BSA was purchased from Biocell Laboratories, Inc. (Rancho Dominguez, CA) for 24 h to increase triacylglycerol synthesis and storage (5); the two different levels of fatty acid-albumin complexes were necessary to obtain approximately the same level of lipid loading of the cells. Cells were harvested by scraping into cold PBS and pelleted by low speed centrifugation. Pelleted cells were resuspended and lysed in a hypotonic solution containing 10 mM Tris, pH 7.4, 1 mM EDTA, 10 mM sodium fluoride, 10 μg/ml leupeptin, 1 mM benzamidine, and 100 μM [4-(2-aminoethyl)benzenesulfonylfluoride] hydrochloride, for 10 min at 4 °C, followed by 10 strokes in a Teflon/glass Dounce homogenizer. The homogenate was centrifuged for 30 min at 26,000 × *g* at 4 °C, and the floating lipid layer was recovered after slicing off the tops of the tubes with a Beckman tube-slicer. The infranatant and pellet fractions were also collected.

Analysis of Cellular Fractions—The lipid droplet-containing fractions were concentrated and delipidated by overnight precipitation with cold acetone at −20 °C, followed by solubilization in 2× concentrated Laemmli's sample buffer (22); the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose membranes. Immunoblots were probed with the primary antibodies described above, with the inclusion of polyclonal antibodies raised against full-length rat perilipin A (12) for some samples, and horseradish peroxidase-conjugated secondary antibodies (Sigma), and developed using enhanced chemiluminescence reagents from Amersham Biosciences.

Polysome Profile Sucrose Gradients—Confluent monolayers of 3T3-L1 fibroblasts were harvested on ice by scraping the cells into cold PBS and were pelleted by low speed centrifugation at 4 °C. Cells were resuspended in lysis buffer (10 mM Tris, pH 7.4, 15 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X-100, 20 mM dithiothreitol, 53 mM cycloheximide, 0.025% heparin, and 2 units/ml RNase inhibitor), incubated for 10 min on ice, and homogenized with 10 strokes of a Teflon/glass homogenizer. The homogenate was centrifuged at 12,000 × *g* at 4 °C for 15 min, and the resulting supernatant was layered over a 10–50% sucrose gradient placed on a 1-ml 60% sucrose cushion. The sucrose solutions contained 20 mM Hepes, pH 7.2, 0.25 M KCl, 10 mM $MgCl_2$, 20 mM dithiothreitol, 53 mM cycloheximide, 0.5 mg/ml heparin, and 3.3 units/ml RNase inhibitor. As a control for the fractionation of untranslated mRNAs, half of each post-mitochondrial supernatant fraction was adjusted to 20 mM EDTA and layered onto a gradient containing 20 mM EDTA. Gradients were centrifuged for 3 h at 180,000 × *g* at 4 °C in a Beckman SW40Ti rotor. 12 1-ml fractions were harvested on ice and stored at −80 °C for subsequent RNA extraction and analysis.

Northern Blot Analysis—Total RNA was extracted from cultured cells using RNeasy minicolumns (Qiagen), and from polysome profile fractions with TRIzol LQ (Invitrogen) according to the protocols of the manufacturers. RNA was separated by electrophoresis in 1% agarose gels using NorthernMax™-Gly reagents (Ambion, Inc.). RNA was transferred electrophoretically to MagnaCharge nylon membranes (Osmonics), and the membranes were probed with ^{32}P-labeled cDNA probes corresponding to the full-length coding sequence of perilipin A using the ExpressHyb hybridization solution from Clontech.

RESULTS

Predicted Structural Motifs of Murine Perilipin A—The predicted amino acid sequence of murine perilipin A includes 517 amino acids containing several notable domains (Fig. 1; see also Ref. 17). Sequences from amino acids 17 to 121 of both rat and murine perilipin A are 32% identical and 65% similar to the amino-terminal region of adipophilin (10) and 38% identical and 60% similar to the amino-terminal region of TIP47; adipophilin (20, 23) and TIP47 (21) are ubiquitously distributed lipid droplet-associated proteins. Between amino acids 111 and 182, perilipin A contains five sequences of 10–11 amino acids that are predicted by the LOCATE program to

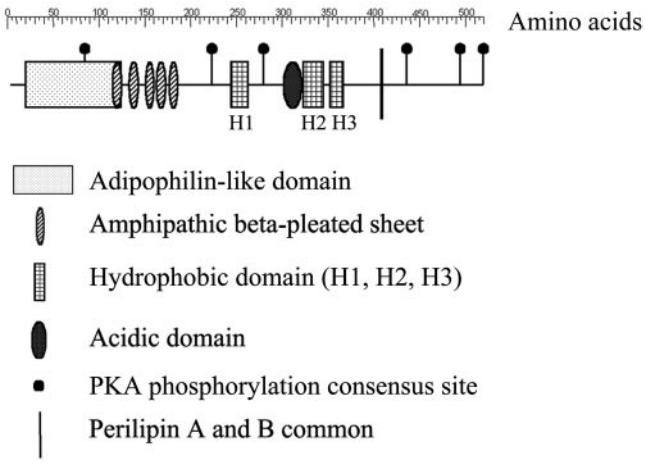

FIG. 1. **Schematic diagram of mouse perilipin A structural domains.** Perilipin A contains 517 amino acids. Starting from the amino terminus, perilipin A contains a sequence of 105 amino acids similar to adipophilin and TIP47 (amino acids 17–121), five 10-amino acid domains with amphipathic β-pleated sheet character (between amino acids 111 and 182), three sequences of moderate hydrophobicity (amino acids 243–260, 320–342, and 349–364), a highly acidic region (amino acids 291–318), and six consensus sites for phosphorylation by PKA at the indicated positions. The *vertical line* indicates the limit of the amino-terminal region common to perilipins A and B; the carboxyl terminus following amino acid 405 is unique to perilipin A.

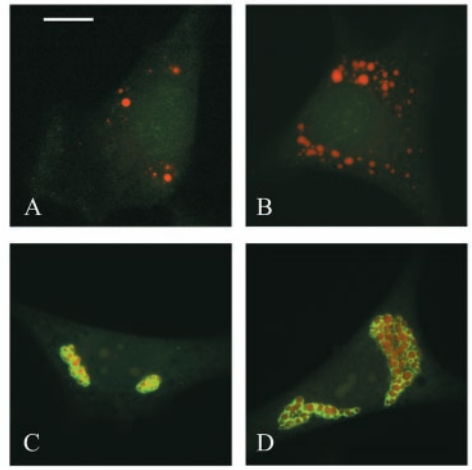

FIG. 2. **Ectopic perilipin A localizes to lipid droplets in 3T3-L1 fibroblasts.** Control 3T3-L1 fibroblasts (*A* and *B*) and cells stably expressing perilipin A (*C* and *D*) were lipid-loaded (*B* and *D*) for 24 h with 400 μM oleic acid complexed to albumin. Cells were prepared for immunofluorescence microscopy and probed for perilipin (*green*) using α-PAT. Neutral lipids (*red*) were simultaneously stained with Bodipy 493/503. Each *panel* depicts a single cell. The background fluorescence of the cells has been enhanced to make the cells more visible. *Bar* = 10 μm.

form amphipathic β-pleated sheets (17). Three domains of moderate hydrophobicity of 18 (aa 243–260; H1), 23 (aa 320–342; H2), and 16 (aa 349–364; H3) amino acids are located in the center of the sequence surrounding a highly acidic domain (aa 291–318) where 19 of 28 consecutive amino acids are glutamic or aspartic acid. Six consensus phosphorylation sites for PKA (consensus: R(R/K)*X*S) are located throughout the predicted protein sequence; the PKA consensus site serines are in positions 81, 222, 276, 433, 492, and 517. It is not known whether all of these serines are phosphorylated when adipocytes are lipolytically stimulated, thus activating PKA. The first 405 amino acids are common to perilipins A and B; the unique carboxyl terminus of 112 amino acids of perilipin A is depicted, whereas perilipin B contains a different sequence of 16 amino acids (10, 11). The aim of this study is to elucidate the roles that the depicted motifs may play in targeting perilipin A to lipid droplets and in mediating the anchoring of perilipin A to lipid droplets.

Ectopically Expressed Perilipin A Targets to Lipid Droplets in Cultured Fibroblasts—To elucidate the targeting motifs responsible for directing nascent perilipin A to lipid droplets, we used cultured cell lines that lack endogenous perilipins, but form lipid droplets when incubated with exogenous fatty acids. Previous studies indicated that perilipin A expressed in 3T3-L1 fibroblasts (5) or CHO-K1 fibroblasts (not shown) targets to lipid droplets and tethers to the droplets in a manner indistinguishable from the association of endogenous perilipins with droplets in differentiated adipocytes; furthermore, the ectopic perilipin is found only on lipid droplets in subcellular fractionation experiments, and in no other cellular compartment (5). Cultured 3T3-L1 fibroblasts ectopically expressing perilipin A were stained simultaneously for neutral lipids and for perilipins and were compared with cells expressing the retroviral expression vector lacking an inserted cDNA (referred to as control cells) (Fig. 2). Although both cell types contained lipid droplets in the presence (Fig. 2, *B* and *D*) or absence (Fig. 2, *A* and *C*) of excess oleic acid in the culture medium, control cells did not express perilipin A (Fig. 2, *A* and *B*). Expression of perilipin A caused an increase in the numbers and sizes of intracellular lipid droplets, and altered the localization of the droplets from a dispersed to a clustered arrangement (Fig. 2, *C* and *D*), when compared with control cells (Fig. 2 and Ref. 5). Furthermore, perilipin A was only found on the surfaces of lipid droplets (Fig. 2 and Ref. 5), as in adipocytes (13), although the droplets were much smaller in the fibroblasts.

Identification of the Domain(s) of Perilipin A Required for Its Targeting to Lipid Droplets—To identify the regions of perilipin A that are necessary for its targeting to lipid droplets, truncation and internal deletion mutations of the murine perilipin A cDNA were ligated into a retroviral expression vector and used to stably transfect 3T3-L1 fibroblasts that lack endogenous perilipins. A summary of data collected from cells stably expressing selected mutations of perilipin A is shown in Table I. Sites for truncation were designed to test potential requirements for the various notable domains (Fig. 1) in mediating the targeting of perilipin A to lipid droplets. Targeting of the mutated perilipins was assessed by immunofluorescence microscopy (Figs. 3–5) using antibodies directed against either amino- or carboxyl-terminal peptides of perilipin A. Three or more microscopy experiments were conducted for each mutated perilipin construct; each experiment involved the observation of all of the densely subconfluent cells on three or more coverslips per construct. When the results indicate that a mutated perilipin has targeted to lipid droplets, then greater than 95% of the stably selected cells displayed a perilipin signal on lipid droplets. Furthermore, cells on additional coverslips in each experiment were lipid-loaded to increase the storage of triacylglycerol in lipid droplets, thus facilitating the observation of perilipin targeting; under these conditions, the targeting of the indicated mutated perilipins to lipid droplets was observed in 100% of the cells. In all cases, mutated perilipins that were scored as failing to target to lipid droplets were not observed on lipid droplets in any cells, whether or not the cells were lipid-loaded.

To confirm the localization of the mutated perilipins, lipid droplets were isolated from stably transfected cells and the proteins contained in the lipid droplet fractions were delipidated, separated by SDS-PAGE, and immunoblotted for perilipins (Fig. 6); additionally, supernatant fractions and the membrane pellets were probed for the presence of perilipins (data

TABLE I
Summary of experiments

Deletion mutations of perilipin A were stably expressed in 3T3-L1 fibroblasts. For deletion mutations in the N and C series, the expressed amino acids are indicated; for deletion mutations in the Δ series, deleted amino acids are indicated. A "+" in the Targets? column indicates the detection of the mutated perilipins on lipid droplets by immunofluorescence microscopy and immunoblotting; a "−" indicates the failure to detect the protein on lipid droplets. A "+" in the Anchors? column indicates the association of the mutated perilipins with lipid droplets following an alkaline carbonate wash. A "+" in the Clusters? column indicates the appearance of clustered lipid droplets in cells following the stable expression of the mutated perilipin; a "+/−" indicates the appearance of limited clusters of lipid droplets. NA, not applicable.

Name of the construct	Amino acids expressed	Targets?	Anchors?	Clusters?
Full-length	1–517	+	+	+
N1	82–517	+	+	+
N2	122–517	+	+	+
N3	183–517	+	+	+
N4	223–517	+	+	+
N5	233–517	+	−	+
N6	249–517	−	NA	NA
C1	1–489	+	+	+
C2	1–429	+	+	+/−
C3	1–405	+	+	+/−
C4	1–364	+	+	−
C5	1–344	+	+	−
C6	1–324	+	−	−
C7	1–302	−	NA	NA
Name of the construct	**Amino acids deleted**	**Targets?**	**Anchors?**	**Clusters?**
Δ1	291–318	+	+	+
Δ2	291–342	+	+	+/−
Δ3	233–290	+	+	+/−
Δ4	233–342	+	+	−
Δ5	233–364	−	NA	NA

not shown). For the immunoblotting experiments, the cells were grown in the presence of 400 μM oleic acid to increase the number of lipid droplets per cell, and hence the amount of expressed perilipin (16); we observed an increase in the protein mass of the expressed mutated perilipins that targeted to lipid droplets under lipid-loading conditions (data not shown), suggesting that these truncated perilipins are stabilized by binding to lipid droplets similarly to full-length perilipin A (16). By contrast, no protein mass was observed for non-targeting mutated perilipins in any subcellular fraction (data not shown).

Identification of Potential Amino-terminal Targeting Sequences of Perilipin A—To identify potential targeting motifs within the amino terminus of perilipin A, a series of amino-terminal truncation mutations of perilipin A were stably expressed in 3T3-L1 fibroblasts (Table I, Figs. 3 and 6). Removal of the first 81 amino acids from the amino terminus of perilipin A (mutation N1) does not impair the ability of the mutated protein to target to lipid droplets in 3T3-L1 fibroblasts (Figs. 3 and 6, Table I); hence, the consensus site for phosphorylation by PKA (aa 78–81) does not play an essential role in perilipin A localization. The amino-terminal domain is shared by perilipins, adipophilin, and TIP47, making it a logical sequence to contain information required for the targeting of lipid droplet-associated proteins; however, truncation of this entire amino-terminal sequence (removal of the first 121 aa; mutation N2) does not prevent the targeting of perilipin A to lipid droplets (Table I, Figs. 3 and 6). Therefore, this conserved amino-terminal domain does not appear to contain essential targeting information. The amino acids immediately following this conserved domain contain five short sequences that are predicted to form amphipathic β-pleated sheets (17); these sequences were likely candidates for motifs that could be involved in directing or anchoring perilipins to lipid droplets. Surprisingly, mutation N3, which further deletes these five sequences (removal of the first 182 aa), targets to lipid droplets. Removal of an additional 40 amino acids (to aa 222; mutation N4), which includes a second PKA consensus phosphorylation site, yielded a mutated perilipin that also targeted to lipid droplets (Table I, Figs. 3 and 6); however, removal of the following 26 amino acids (to aa 248; mutation N6), including a portion of H1, eliminated the localization of the mutated perilipin to lipid droplets. The expression of mutated perilipin N6 in cells was not detected by immunofluorescence microscopy (Fig. 3) or by the immunoblotting of proteins from lipid droplets (Fig. 6), or other subcellular fractions including pelleted membranes and supernatants containing microsomes and soluble proteins (data not shown); hence, the non-targeting mutated perilipin was likely rapidly degraded within the cells. Mutated perilipin N5 (protein expressed from aa 233 to 517) was the shortest amino-terminally truncated perilipin that targeted to lipid droplets and was visualized by immunofluorescence microscopy (Fig. 3) and by the immunoblotting of proteins from lipid droplets (Fig. 6, Table I). Thus, removal of the amino acid sequences preceding H1 (to aa 233) failed to prevent targeting of mutated perilipin to lipid droplets, whereas removal of the following 15 amino acids including the disruption of H1 yielded a mutated perilipin that failed to target to lipid droplets and was not detected anywhere else in the cell.

Identification of Potential Carboxyl-terminal Targeting Sequences of Perilipin A—Cells stably expressing mutated perilipins containing successive carboxyl-terminal truncations were studied to map potential carboxyl-terminal targeting motifs. The extreme carboxyl-terminal region of perilipin A contains three consensus sites for PKA phosphorylation. To test the importance of these sites in the targeting of perilipin to lipid droplets, mutated perilipins C1 (expressing aa 1–489) and C2 (expressing aa 1–429) were studied and shown to target to lipid droplets (Figs. 4 and 6, Table I), thus indicating that the consensus sites for phosphorylation by PKA are dispensable. Mutated perilipin expressing only the sequences common to perilipins A and B (mutated perilipin C3, expressing aa 1–405) also targeted to lipid droplets (Figs. 4 and 6, Table I), thus showing that the unique carboxyl-terminal domains of perilipins A and B are not necessary for the targeting of perilipins to lipid droplets. Furthermore, truncation of the entire carboxyl terminus following H3 (mutation C4; aa 1–364) yielded a mu-

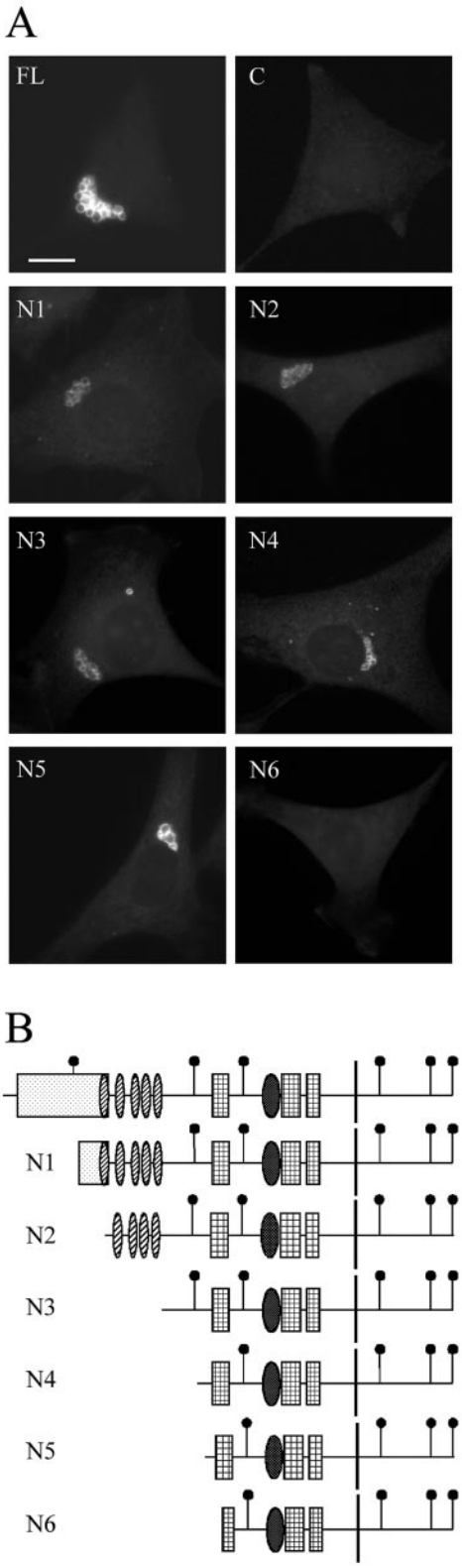

FIG. 3. **Localization of amino-terminal truncation mutations of perilipin A in 3T3-L1 fibroblasts.** *A*, 3T3-L1 fibroblasts stably expressing full-length perilipin A (*FL*), vector control (*C*), and amino-terminal truncation mutations of perilipin A (*N1–N6*) were prepared for microscopy and probed for perilipin using the α-PAT antibody (*FL* and *C*) or α-COT (*N1–N6*). *Bar* = 10 μm. Differences in the relative intensity of lipid droplet staining for the *FL panel* relative to the other *panels* reflects the use of different antibodies, and not the relative levels of expression of the N1–N6 mutated perilipins when compared with full-length perilipin. *B*, schematic diagrams of the expressed portions of full-length and amino-terminal truncation mutations of perilipin A labeled N1–N6.

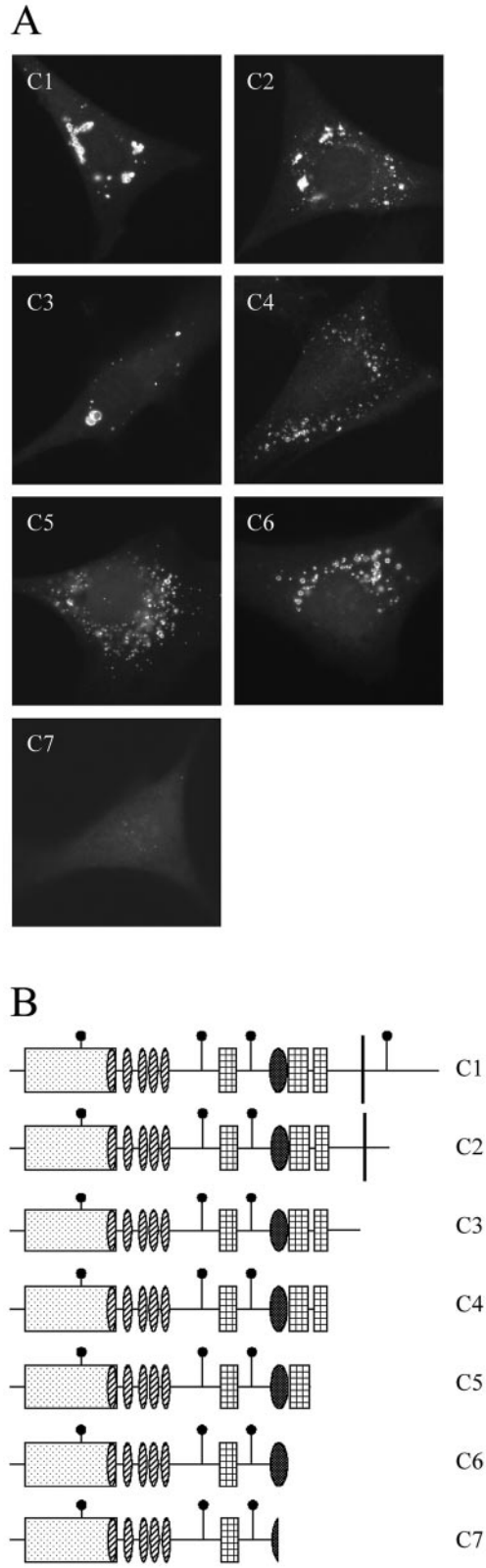

FIG. 4. **Localization of carboxyl-terminal truncation mutations of perilipin A in 3T3-L1 fibroblasts.** *A*, 3T3-L1 fibroblasts stably expressing carboxyl-terminal truncation mutations of perilipin A (*C1–C7*) were prepared for microscopy and probed for perilipin using the α-PAT antibody. *B*, schematic diagrams of the expressed portions of carboxyl-terminal truncation mutations of perilipin A labeled C1–C7.

tated perilipin that targeted to lipid droplets. Finally, mutated perilipin C7 (expressing aa 1–302), which includes the amino terminus of perilipin A through the first third of the acidic

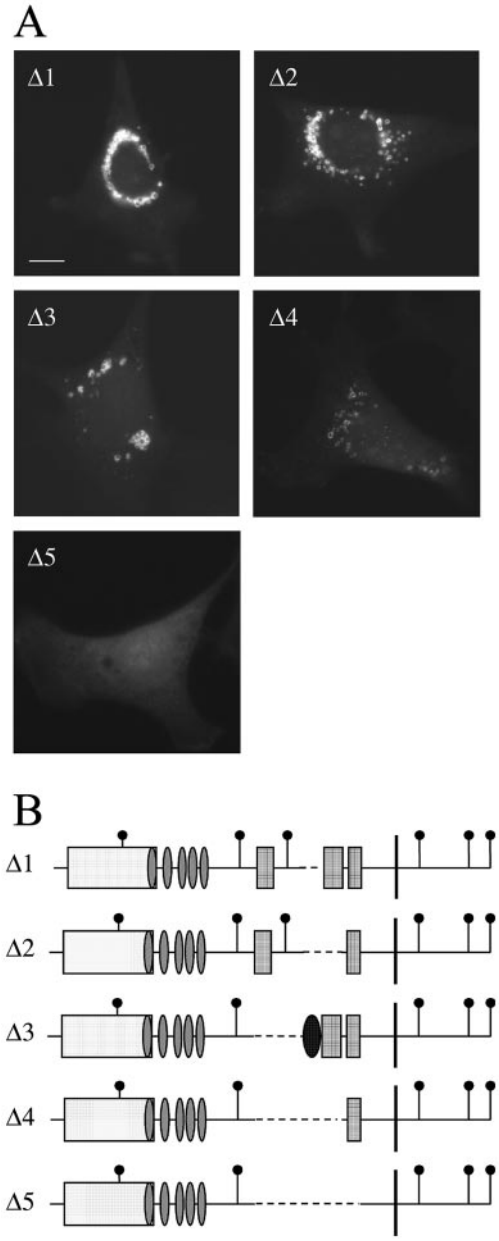

FIG. 5. **Localization of internal deletion mutations of perilipin A in 3T3-L1 fibroblasts.** *A*, 3T3-L1 fibroblasts stably expressing internal deletion mutations of perilipin A (*Δ1–Δ5*) were prepared for microscopy and probed for perilipin using the α-PAT antibody. *Bar* = 10 μm. *B*, schematic diagrams of internal deletion mutations of perilipin A labeled Δ1–Δ5. The deleted portion of perilipin A is represented by a *dotted line*.

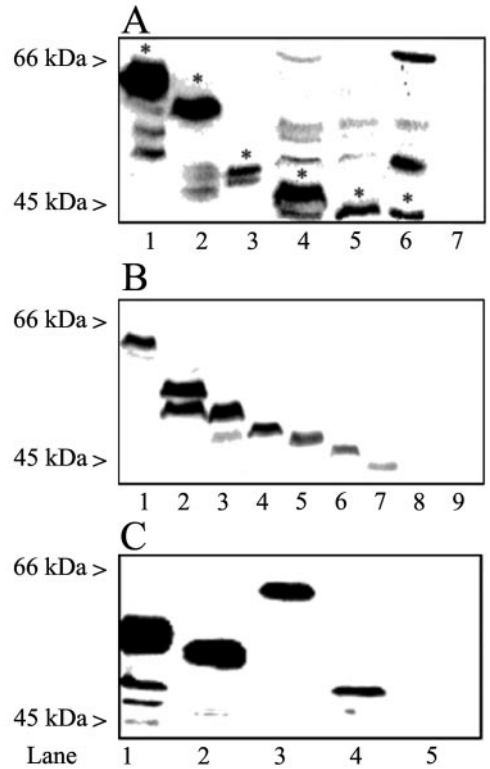

FIG. 6. **Immunoblots depicting the localization of mutated perilipins on isolated lipid droplets.** Confluent 3T3-L1 fibroblasts ectopically expressing mutated perilipin A were cultured in the presence of oleic acid for 24 h. Cells were harvested and fractionated; the lipid droplet fraction was delipidated with acetone, and the component proteins were solubilized in Laemmli's sample buffer, separated by SDS-PAGE, transferred electrophoretically to nitrocellulose membranes, and probed for perilipin using either the α-PAT antibody (*panels B* and *C*) or a polyclonal antibody raised against full-length rat perilipin A (*panel A*). *A*, *lane 1*, full-length perilipin A; *lane 2*, N1; *lane 3*, N2; *lane 4*, N3; *lane 5*, N4; *lane 6*, N5; *lane 7*, N6. *Asterisks* identify the major perilipin bands for full-length and mutated perilipins. Lower molecular weight bands likely represent either degradation products or nonspecific proteins bound by the antibodies; higher molecular weight bands are nonspecific proteins. *B*, *lane 1*, full-length perilipin A; *lane 2*, C1; *lane 3*, C2; *lane 4*, C3; *lane 5*, C4; *lane 6*, C5; *lane 7*, C6; *lane 8*, C7; *lane 9*, control cells (no perilipin). Full-length perilipin A generally migrates as a doublet; C1 and C2 also show this pattern. *C*, *lane 1*, Δ1; *lane 2*, Δ2; *lane 3*, Δ3; *lane 4*, Δ4; *lane 5*, Δ5. Each lane contains lipid droplet proteins from an equivalent mass of cells.

domain, fails to target to lipid droplets (Fig. 4, Table I) and cannot be found in the lipid droplet (Fig. 6), soluble, or membrane-containing fractions of lysed cells (data not shown). In summary, truncation of the final 193 carboxyl-terminal amino acids (or 38% of the sequence) does not alter the intracellular localization of perilipin A, whereas the additional disruption of the acidic domain eliminates the targeting of perilipin to lipid droplets. These experiments implicate the central acidic domain as a potential determinant for the targeting of perilipin A to lipid droplets. Interestingly, lipid droplets in cells expressing mutated perilipins C2–C6 (Fig. 4) are arranged differently than those found in cells expressing full-length perilipin A (Fig. 2); as more of the carboxyl terminus is successively removed, the lipid droplets gradually lose the tightly clustered appearance and become dispersed throughout the cytoplasm.

Identification of Core Targeting Motifs Using Internal Deletion Mutations of Perilipin A—Because both the amino and carboxyl termini appear to be dispensable in targeting perilipin A to lipid droplets, the role of the central domain, comprising ~25% of the protein, was investigated. Mutated perilipins containing internal deletions, in which a defined central portion of the protein was deleted from otherwise intact perilipin A, were stably expressed in cells. Because the central acidic domain was implicated in the targeting of perilipin A to lipid droplets in studies of cells expressing carboxyl-terminal truncated perilipins, cells were transfected with a mutated perilipin lacking only the acidic domain (mutated perilipin Δ1). Surprisingly, mutated perilipin Δ1 targeted to lipid droplets in 3T3-L1 fibroblasts (Figs. 5 and 6, Table I), thus indicating that the acidic domain is dispensable for targeting nascent perilipins to lipid droplets when the remainder of the carboxyl terminus is intact. Additionally, the mutated perilipin created by removing both the central acidic domain and the adjacent H2 (mutated perilipin Δ2, lacking aa 291–342) targeted to lipid droplets (Figs. 5 and 6, Table I). Amino-terminal truncation studies suggested a role for H1 in directing the targeting of perilipin A to lipid

droplets. Hence, a mutated perilipin was constructed that contained a deletion from H1 up to the acidic domain (lacking aa 233–290, mutated perilipin Δ3); this mutated perilipin targeted to lipid droplets (Figs. 5 and 6, Table I), as did the mutated perilipin lacking this entire sequence and H2 (lacking aa 233–342, mutated perilipin Δ4; Figs. 5 and 6, Table I). We reasoned that the targeting of the Δ4 mutated perilipin to lipid droplets may be directed by the remaining H3, so we expressed a mutated perilipin lacking the entire central region (lacking aa 233–364, mutation Δ5) in 3T3-L1 fibroblasts; the Δ5 mutated perilipin failed to target to lipid droplets (Figs. 5 and 6, Table I). Like other non-targeting perilipin mutations, the Δ5 mutated perilipin was not detected in cells by immunofluorescence microscopy (Fig. 5) or by the immunoblotting of proteins from lipid droplets (Fig. 6) or other subcellular fractions (data not shown). These results suggest that there are multiple and somewhat redundant targeting signals in the central domain comprising 25% of the amino acid sequence; motifs such as the hydrophobic domains may be individually dispensable in the presence of the remaining sequences, and to remove all targeting signals requires the deletion of the entire central domain. Furthermore, the deletion of some of the central sequences of perilipin A led to dispersion of the lipid droplets throughout the cells.

Cells Stably Transfected with Non-targeting Mutated Perilipins Express mRNA for the Mutated Constructs—The non-targeting mutated perilipins were undetectable by immunofluorescence microscopy of stably transfected cells, and by immunoblotting of proteins from subcellular fractions isolated from these cells. These results implied that either the mutated perilipin constructs were not expressed, or the nascent proteins were unstable and rapidly degraded. To test the possibility that the constructs that yielded non-targeting mutated proteins were not expressed, total RNA was isolated from cells stably expressing constructs from both targeting and non-targeting perilipin mutations; the RNA was separated electrophoretically on agarose gels, transferred to charged nylon membranes, and probed with radiolabeled perilipin cDNA probes. Cells transfected with either targeting or non-targeting mutated perilipin cDNAs generally had comparable levels of mRNA for the mutated perilipin constructs (Fig. 7), thus implying similar levels of transcription of all constructs.

mRNAs from Mutated Perilipins That Fail to Target to Lipid Droplets Are Translated—Because the cDNAs for the non-targeting mutated perilipin constructs were transcribed (Fig. 7), the failure to observe protein encoded by these constructs was the result either of the failure of the cells to translate the corresponding mRNAs or of instability and rapid degradation of the nascent mutated perilipins. To address the possibility that the mRNAs for these mutated perilipins were not translated, we fractionated post-mitochondrial supernatants from cells stably transfected with the non-targeting C7, N6, or Δ5 mutated perilipin constructs on sucrose gradients. Northern blot analysis of RNA extracted from the gradient fractions showed that mRNAs for the three mutated perilipins were efficiently translated (Fig. 8), because the majority of perilipin mRNA was isolated from dense fractions, thus indicating the association of multiple ribosomes. Although these data are qualitatively comparable with results obtained for the translation of unmodified perilipin A (data not shown, and in Ref. 16), they provide no information regarding the relative rates of protein synthesis of the non-targeting mutated perilipins relative to the rates of synthesis of either unmodified perilipin A or the targeting mutated perilipins. It is possible that differences in the rates of translational extension and termination for the non-targeting mutated perilipins are slower, resulting in lower levels of protein expression.

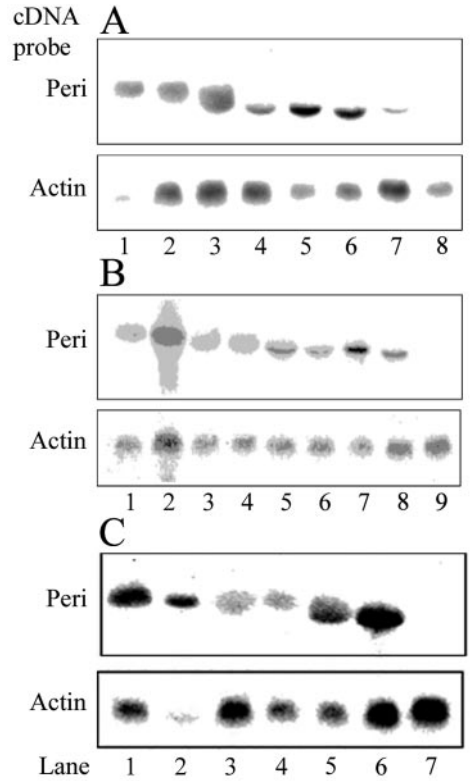

FIG. 7. **Cells transfected with both targeting and non-targeting mutated perilipins express mRNAs for perilipin.** Total RNA isolated from confluent 3T3-L1 fibroblasts ectopically expressing mutated perilipins was separated electrophoretically on agarose gels and transferred to charged nylon membranes. Membranes were probed with radiolabeled full-length perilipin A cDNA probes (*top panels* of *A–C*); blots were stripped and re-probed for β-actin (*lower panels* of *A–C*). *A*, *lane 1*, full-length perilipin A; *lane 2*, N1; *lane 3*, N2; *lane 4*, N3; *lane 5*, N4; *lane 6*, N5; *lane 7*, N6; *lane 8*, control cells (no perilipin). *B*, *lane 1*, full-length perilipin A; *lane 2*, C1; *lane 3*, C2; *lane 4*, C3; *lane 5*, C4; *lane 6*, C5; *lane 7*, C6; *lane 8*, C7; *lane 9*, control cells. *C*, *lane 1*, full-length perilipin A; *lane 2*, Δ1; *lane 3*, Δ2; *lane 4*, Δ3; *lane 5*, Δ4; *lane 6*, Δ5; *lane 7*, control cells.

The Central Domain of Perilipin A Is Sufficient to Redirect the Soluble GFP to Lipid Droplets—The analysis of cells expressing deletion mutations of perilipin A indicated that the essential domains that direct the targeting of nascent perilipin to lipid droplets are potentially redundant sequences found within the central 25% of the protein that is limited by H1 and H3. To determine whether portions of this domain are sufficient to target proteins to lipid droplets, we created a fusion construct of the cDNA sequence of the soluble GFP ligated 5′ to the cDNA sequence encoding the amino acids from the start of H1 to the end of H2 (aa 233–345; mutated perilipin GP2) of perilipin A. Additional fusion constructs of the GFP cDNA ligated 5′ to 1) the cDNA encoding full-length perilipin A (perilipin construct GP1), 2) the cDNA encoding mutated perilipin A lacking the first 248 amino acids (mutated perilipin GP3), 3) the cDNA encoding the first 232 amino acids of perilipin A (mutated perilipin GP4), 4) the cDNA encoding the first 302 amino acids of perilipin A (mutated perilipin GP5; data not shown), and 5) the cDNA encoding the last 153 amino acids (mutated perilipin GP6; data not shown) were also prepared. Data collected for these perilipin-GFP fusion proteins are shown, in part, in Fig. 9 and are summarized in Table II. Fluorescence microscopy demonstrated that the full-length perilipin A-GFP fusion protein was efficiently targeted to lipid

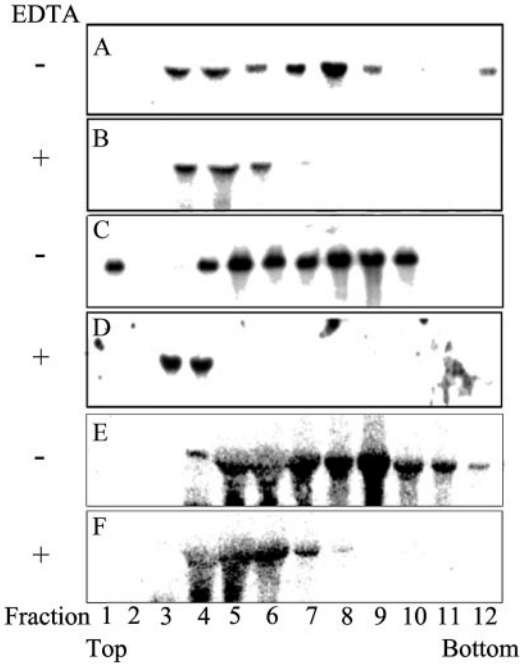

FIG. 8. **mRNAs from mutated perilipins that fail to target to lipid droplets are translated.** Confluent 3T3-L1 fibroblasts expressing non-targeting mutated perilipins were harvested, and post-mitochondrial supernatants were fractionated on 10–50% sucrose gradients in the presence (*B*, *D*, and *F*) or absence (*A*, *C*, and *E*) of EDTA; EDTA releases mRNAs from ribosomes and serves as a control to identify fractions containing non-translated mRNA. Twelve fractions were collected from each gradient following centrifugation. Total RNA was extracted from these fractions and was analyzed by Northern blotting; blots were probed for perilipins using a full-length perilipin A cDNA probe. *A* and *B* are samples from cells expressing mutated perilipin N6; *C* and *D* are samples from cells expressing mutated perilipin C7; *E* and *F* are samples from cells expressing mutated perilipin Δ5. The high density of fractions containing abundant mRNA for the mutated perilipins (*A*, *C*, and *E*) indicates efficient translation of the mRNAs.

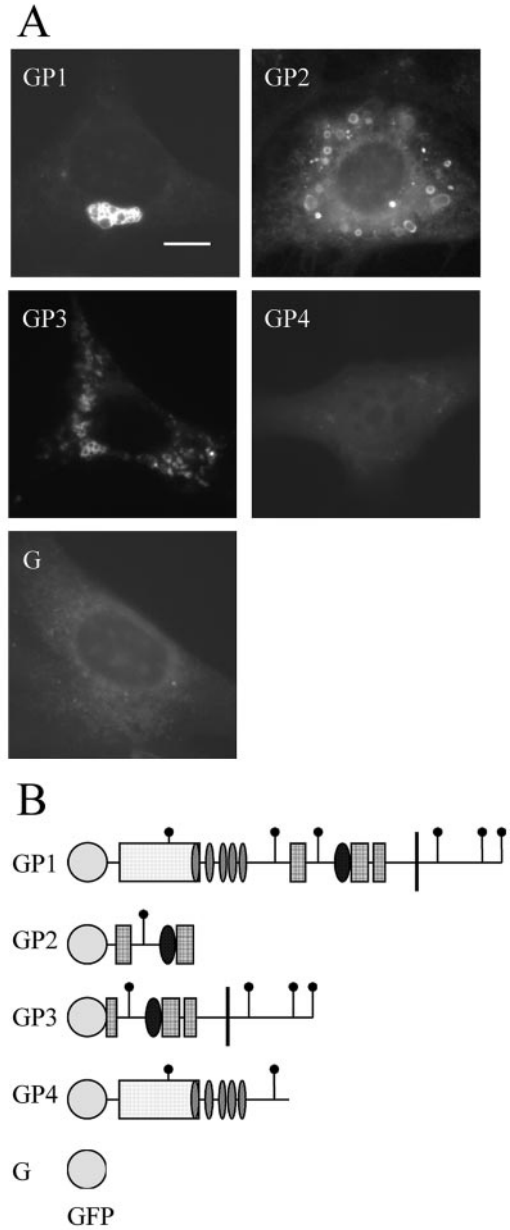

FIG. 9. **The central domain of perilipin A is sufficient to redirect the soluble green fluorescent protein to lipid droplets.** *A*, 3T3-L1 fibroblasts stably or transiently expressing GFP fused to full-length perilipin A (*GP1*), GFP fused to the central region of perilipin including amino acids 233–345 (*GP2*), GFP fused to mutated perilipin A lacking the amino-terminal 248 amino acids (*GP3*), GFP fused to a perilipin peptide containing amino acids 2–232 (*GP4*), and GFP alone (*G*) and growing on coverslips were mounted on glass slides in PBS and visualized with a light microscope. Full-length perilipin A (*GP1*), the central domain of perilipin (*GP2*), and mutated perilipin lacking the amino terminus (*GP3*) altered the localization of GFP from a diffuse cytoplasmic staining pattern (*G*) to localization at the surfaces of lipid droplets; lipid droplets coated with full-length perilipin A fused to GFP were clustered, whereas droplets coated with the mutated perilipins fused to GFP (*GP2* and *GP3*) were dispersed in the cytoplasm. By contrast, the amino-terminal peptide of perilipin failed to direct GFP to lipid droplets (*GP4*). The background fluorescence of the cells was enhanced to make the cells more visible. *Bar* = 10 μm.

droplets (Fig. 9, *GP1*). Furthermore, lipid droplets coated with the GFP-perilipin A fusion protein were arranged in clusters (Fig. 9, *GP1*) comparable with the pattern observed in cells expressing full-length perilipin A (Fig. 2*C*, and Ref. 5). The fusion protein containing a portion of the central region of perilipin A fused to GFP also targeted to lipid droplets (Fig. 9, *GP2*), thus suggesting that portions of the central domain are sufficient to redirect a soluble protein to the surfaces of lipid droplets; however, the lipid droplets coated with the truncated perilipin-GFP fusion protein labeled GP2 retained a dispersed arrangement (Fig. 9, *GP2*) comparable with that of control cells lacking perilipins (Fig. 2, *A* and *B*). A GFP fusion protein of perilipin lacking the amino-terminal 248 amino acids also targeted to lipid droplets (Fig. 9, *GP3*), but lipid droplets retained a mostly dispersed arrangement in the cytoplasm. The targeting of GP3 to lipid droplets is interesting, because a comparable mutated perilipin lacking the fused GFP (mutated perilipin N6; Fig. 3 and Table I) fails to target to lipid droplets. These findings support the concept that a short sequence of amino acids (*e.g.* aa 234–248) is not required to direct perilipin to lipid droplets, but instead, that potentially redundant sequences within the central domain, which may be stabilized by the additional GFP sequence, are critical. The remaining mutated perilipin-GFP fusion proteins including those containing short amino-terminal (Fig. 9, *GP4*) and carboxyl-terminal (GP6; data not shown) perilipin peptides, and a longer perilipin peptide lacking the carboxyl terminus (GP5; data not shown) failed to target, and cells displayed only a diffuse faint fluorescent signal. Although the GP4 and GP6 mutated perilipins lacked all sequences within the central putative targeting domain and, hence, gave the expected results of failing to target to lipid droplets, the GP5 construct contained approximately half of the central domain, but was unable to support the targeting of the mutated perilipin-GFP fusion protein to lipid droplets. Control cells expressing soluble GFP displayed diffuse green fluores-

TABLE II
Summary of experiments with GFP-perilipin A fusion proteins

Fusion proteins of full-length perilipin A (GP1) or portions of perilipin A (GP2–6) fused to GFP were stably or transiently expressed in 3T3-L1 fibroblasts. The amino acid sequence of perilipin A contained in the fusion protein is indicated. A "+" in the Targets? column indicates the detection of the mutated perilipin-GFP fusion protein on lipid droplets by fluorescence microscopy; a "−" indicates the failure to detect the fusion protein on lipid droplets. A "+" in the Clusters? column indicates the appearance of clustered lipid droplets in cells following the expression of the mutated perilipin-GFP fusion protein in cells. NA, not applicable.

Name of the construct	Amino acids fused to GFP	Targets?	Clusters?
GP1	2–517	+	+
GP2	233–345	+	−
GP3	249–517	+	−
GP4	2–232	−	NA
GP5	2–302	−	NA
GP6	365–517	−	NA

cence throughout the cytoplasm, and no specific staining of lipid droplets (Fig. 9 *G*).

The Central Hydrophobic Domains Are Essential to Anchor Perilipin A to Lipid Droplets—Previous observations have suggested that perilipins are very tightly associated with lipid droplets, probably via hydrophobic interactions; the release of perilipins from isolated lipid droplets requires detergents such as sodium dodecyl sulfate (12). Furthermore, the flotation of lipid droplets in 100 mM carbonate, pH 11.5, fails to remove perilipins; this classic technique was used initially by Fujiki *et al.* (24) to remove peripherally associated proteins from microsomes, thus leaving integral membrane proteins associated with the phospholipid bilayers. Although lipid droplets lack a membrane bilayer, the resistance of full-length perilipin A to removal by alkaline carbonate treatments suggests that perilipin A associates with lipid droplets through hydrophobic interactions. We used this classic protocol to study the nature of the association between the mutated perilipins and lipid droplets. Lipid droplets were isolated by centrifugation from 3T3-L1 cells stably transfected with the different mutated perilipin constructs. The isolated lipid droplets were further centrifuged through an alkaline carbonate solution, and the presence of mutated perilipins was assessed by immunoblotting of delipidated proteins from the buoyant lipid droplet fractions that floated in the second centrifugation step. Perilipin mutations N1–N4 were detected on lipid droplets that had been centrifuged through carbonate, thus indicating that amino acids 1–222 are not required to embed perilipin A into lipid droplets (Fig. 10*A*). The further removal of 10 amino acids (mutation N5) eliminated the association of the mutated perilipin with carbonate-washed lipid droplets. Perilipin mutations C1–C5 were detected on lipid droplets that had been centrifuged through carbonate (Fig. 10*B*), but the further removal of H2 (mutation C6) impaired the ability of the mutated perilipin to embed into lipid droplets. These observations imply that the central portion of perilipin with its hydrophobic domains is required for the anchoring of the protein to lipid droplets. Interestingly, although the Δ2, Δ3, and Δ4 mutated perilipin containing two, two, or one hydrophobic domains, respectively, adhere to carbonate-washed lipid droplets (Fig. 10*C*), the C6 and N5 mutated perilipins do not, suggesting that the individual hydrophobic domains may not be equally effective in anchoring perilipins to lipid droplets in the absence of the remaining amino- and carboxyl-terminal sequences. We cannot exclude the possibility that sequences in addition to the hydrophobic domains are essential in positioning and anchoring perilipins to lipid droplets.

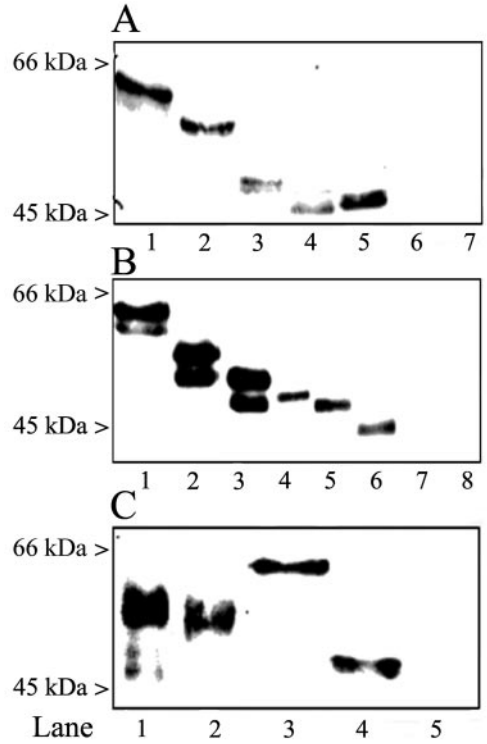

FIG. 10. **The central hydrophobic domains are essential to anchor perilipin A to lipid droplets.** Confluent 3T3-L1 fibroblasts ectopically expressing mutated perilipin A were cultured in the presence of oleic acid for 24 h. Cells were harvested and fractionated; lipid droplets were centrifuged through an alkaline carbonate solution, the floating lipid droplet fractions were delipidated, and the solubilized proteins were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and immunoblotted with either α-PAT antibodies (*panels B* and *C*) or antibodies raised against full-length rat perilipin A (*panel A*). *A*, *lane 1*, full-length perilipin A; *lane 2*, N1; *lane 3*, N2; *lane 4*, N3; *lane 5*, N4; *lane 6*, N5; *lane 7*, N6. *B*, *lane 1*, full-length perilipin A; *lane 2*, C1; *lane 3*, C2; *lane 4*, C3; *lane 5*, C4; *lane 6*, C5; *lane 7*, C6; *lane 8*, C7. *C*, *lane 1*, Δ1; *lane 2*, Δ2; *lane 3*, Δ3; *lane 4*, Δ4; *lane 5*, Δ5.

DISCUSSION

The major finding of this study is that the central domain of perilipin A, representing 25% of the amino acid sequence, contains all of the sequences that are required to target the protein to lipid droplets. A portion of this region, comprising H1 through H2, is sufficient to redirect soluble GFP to lipid droplets. There appear to be redundancies among a combination of targeting signals within this central region. The removal of various small portions of the central region fails to eliminate the targeting of perilipin to lipid droplets, but removal of the entire sequence prevents targeting. Hence, we suggest that the signals required to target perilipin A to lipid droplets are a combination of hydrophobic domains in conjunction with the acidic domain. When these targeting domains are present within the entire protein, they are dispensable individually or in some combinations, but the presence of at least the sequence from H1 through H2 is sufficient to redirect GFP to lipid droplets, and the absence of the entire sequence from H1 through H3 is necessary to eliminate the targeting of perilipin A to lipid droplets. Thus, we conclude that perilipin A does not contain a single short amino acid "zip code" responsible for directing its targeting to lipid droplets.

The domains mediating the anchoring of perilipin A to lipid droplets are similar to those responsible for targeting perilipins to lipid droplets. Results obtained from subjecting lipid droplets to alkaline carbonate solutions suggest that perilipin A

associates with lipid droplets via hydrophobic interactions; this observation implies that the hydrophobic domains of perilipin may embed into the core of the lipid droplet to anchor the protein. Our results indicate that H3, in the absence of the sequence comprising H1 through H2, but in the context of the remaining amino- and carboxyl-terminal sequences (mutation Δ4), is sufficient to mediate the targeting and anchoring of perilipin to lipid droplets, but results obtained with other mutations indicate that H3 is dispensable. However, if H3 and the remainder of the carboxyl terminus are deleted, then both H1 and H2 are necessary to tether the protein to the lipid droplets. It is likely that the context of the amino acids surrounding the hydrophobic domains plays a role in positioning the protein with respect to the droplet, and that the individual hydrophobic domains may not play equivalent roles in embedding the protein into the droplet. This idea gains support from the observation that perilipin mutation N5 targets to lipid droplets but fails to withstand the carbonate wash conditions despite the presence of three hydrophobic domains; in the absence of the adjacent amino acids, the positioning of this mutated protein at the surface of the droplets may be insufficient to support firm anchoring into the droplet.

We hypothesize that perilipin A is anchored onto lipid droplets through the three hydrophobic domains, which embed into the neutral lipid-filled core, whereas the central highly charged acidic domain loops away from the surface of the lipid droplet. The sequences with the characteristics of amphipathic β-pleated sheets may be shallowly embedded into the surface phospholipids of the droplets; this positioning of the amino terminus may help perilipin A to serve its function of stabilizing the lipid droplet. Alternatively, these regions could be folded in upon themselves with the hydrophobic faces in close proximity, in which case the "globular" arrangement would limit the contact of the protein with the surface of the lipid droplet and may lead to a less stabilizing configuration. In our hypothetical model of perilipin association with lipid droplets, the extreme amino and carboxyl termini may be positioned near the surface of the lipid droplet, without being embedded. These sequences contain dispersed charged amino acids that might engage in interactions with other cellular proteins. Deletion of the carboxyl terminus or portions of the central domain leads to the dispersion of lipid droplets (Figs. 4 and 5) that appear in a clustered arrangement when full-length perilipin A or amino-terminal deletion mutations (Fig. 3) are expressed; thus, residues within the carboxyl terminus and central domain may participate in interactions that promote the clustering of lipid droplets in one or two regions of the cytoplasm.

TIP47 and adipophilin share a high degree of sequence similarity with the perilipins and, furthermore, like perilipins, target to and associate with lipid droplets, most likely through hydrophobic interactions, because the proteins resist removal by the extraction of lipid droplets with alkaline carbonate solutions. Although a recent study has questioned the association of TIP47 with lipid droplets (25), further studies by Miura and co-workers (26), and in our laboratory,[2] have confirmed the localization of TIP47 to lipid droplets. To date, no studies have addressed the molecular basis of the targeting of TIP47 or adipophilin to lipid droplets. Surprisingly, the amino-terminal domain of 105 amino acids that is highly conserved within this family of proteins is not required to mediate the targeting of perilipin A to lipid droplets, thus suggesting that this domain may play another, as yet uncharacterized role in the function of these lipid droplet-associated proteins. Furthermore, the dispensability of the amino terminus of perilipin is remarkable in light of the observation that the earliest synthesized sequences in the amino termini of many different proteins contain essential targeting information. TIP47 and adipophilin are less highly conserved in the central sequences that target and anchor perilipins to lipid droplets. None of the members of this family of proteins contain extensive stretches of hydrophobic amino acids, although hydropathy plots of the proteins show short moderately hydrophobic sequences for all members. An alternative mechanism of anchoring these proteins to lipid droplets may be through the acylation of cysteine residues; Heid and co-workers (23) have shown that adipophilin is likely acylated. Future studies are needed to determine the mechanism required to direct these additional family members to lipid droplets. A comparison of the sequences of TIP47 and adipophilin may also be instructive to explain some significant differences in the targeting behavior of TIP47 relative to the other family members; in addition to localizing to lipid droplets, TIP47 can be found in the cytosol of most cells (21, 27), and has been proposed to play a role in endosomal trafficking of the mannose 6-phosphate receptor as part of a complex with Rab 9 (28–30). Finally, additional members of this protein family have been identified in *Drosophila*, *Bombyx*, and *Dictyostelium* (11); both *Drosophila* proteins, as well as the *Dictyostelium* protein, target to lipid droplets when expressed in Chinese hamster ovary cells in culture (26).

While perilipins, adipophilin, and TIP47 are naturally occurring lipid droplet-associated proteins, several additional proteins have recently been demonstrated to localize to lipid droplets under some conditions in laboratory experiments. Although caveolins normally associate with small invaginations along the cytoplasmic face of the plasma membrane, ectopically expressed caveolins have been shown to localize to lipid droplets in cultured cells when plasma membrane targeting signals are deleted, endoplasmic reticulum retrieval signals are added, or when cells are treated with oleic acid or brefeldin A (31–33). Deletion mutagenesis experiments described in these studies suggest that caveolins associate with lipid droplets via a central hydrophobic domain. Additionally, when expressed in cultured cell lines, the core proteins of both the hepatitis C virus and the GB virus-B associate with lipid droplets via a central highly hydrophobic domain (34), and localize to the cytoplasm and several other membranes within the cell. Thus, in all lipid droplet-associated proteins studied to date, hydrophobic domains have been implicated in targeting of the proteins to lipid droplets.

Mutated perilipins that are not found on lipid droplets cannot be found in any other subcellular compartments and appear to be synthesized but rapidly degraded. Because perilipins are stabilized upon association with lipid droplets (16), we hypothesize that the removal of essential targeting sequences produces mutated perilipins in the cytoplasm that fail to become stabilized by anchoring to lipid droplets and, hence, are rapidly degraded. Alternatively, the removal of specific sequences may either prohibit the proper folding of the mutated perilipins, leading to failure to target and subsequent degradation, or promote much slower synthesis or more rapid degradation of the mutated protein, thus eliminating subsequent targeting to droplets. Interestingly, although the removal of a few extra amino acids from an already truncated perilipin construct prevented targeting of the translated product and promoted degradation (as seen for mutated perilipins N6 and C7), the deletion of these few amino acids from otherwise intact perilipin had no effect on targeting or on the stability of the protein whatsoever (perilipin mutations Δ1 and Δ3); these observations suggest that the perilipin sequence lacks short seg-

[2] D. L. Brasaemle, N. E. Wolins, and B. Rubin, unpublished data.

ments that are required to prevent rapid degradation. The only mutated perilipins that failed to target lacked large portions of the protein sequence. Furthermore, the addition of oleic acid to the culture medium, which has been demonstrated to stabilize overexpressed perilipin A (16), failed to stabilize the non-targeting mutated perilipins.

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