rapid communication

Lipid droplet targeting domains of adipophilin

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Abstract Adipophilin (ADPH), a prominent protein component of lipid storage droplets (LSDs), is postulated to be necessary for the formation and cellular function of these structures. The presence of significant sequence similarities within an ~100 amino acid region of the N-terminal portions of ADPH and related LSD binding proteins, perilipin and TIP47, has implicated this region, known as the "PAT" domain, in LSD targeting. Here we investigate the role of the PAT domain in targeting ADPH to LSDs by expressing this region, as well as selected N- and C-terminal truncations of mouse ADPH in COS7 cells as epitope-tagged fusion proteins. Our studies show that truncations lacking either the PAT domain or the C-terminal half of ADPH both correctly targeted LSDs and increased the LSD content of transfected cells. Neither the PAT domain nor the C-terminal half of ADPH appeared to target LSDs or affect the LSD number. Instead, targeting fragments encompassed a putative α-helical region between amino acids 189 and 205, implicating this region in both LSD targeting and regulation of LSD formation.—McManaman, J. L., W. Zabaronick, J. Schaack, and D. J. Orlicky. **Lipid droplet targeting domains of adipophilin. *J. Lipid Res.* 2003.** 44: **668–673.**

Supplementary key words lipid storage droplets • protein targeting • functional domains • cell culture • transfection • immunofluorescence

Lipid storage droplets (LSDs) are dynamic subcellular structures that function as storage depots for triglycerides and cholesterol esters (1) and are the immediate precursors of milk lipids, a primary source of nutrients and calories for neonates (2, 3). LSDs are composed of a triglyceride and cholesterol ester core surrounded by a phospholipid monolayer and coated by specific populations of proteins that are thought to be necessary in their formation and function (1, 4, 5). In plants, LSD proteins possess distinctive hydrophobic domains and structural motifs that direct their binding to the lipid droplet matrix (4). Such structural features and motifs, however, have not been detected in animal LSD proteins, and the basis of their association with the lipid droplet matrix is unknown.

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Adipophilin (ADPH) is a prominent LSD-associated protein found in many mammalian cell types, including hepatocytes, adipocytes, muscle cells, and mammary epithelial cells, either during development (6) or in the mature functioning cell (6–8). It has been proposed that ADPH functions in LSD formation (9), fatty acid uptake (9), and milk lipid secretion (2, 3); however, little is known about the structural or biochemical properties involved in its association with LSDs or how it accomplishes its biological functions. Genetic analysis (10, 11) has shown that ADPH is related to a family of lipid droplet associated proteins that include perilipin and the mannose-6-phosphate receptor targeting protein, TIP47, through a conserved region in their N-termini, called the "PAT" domain (perilipin, ADPH, TIP47). The lack of significant sequence conservation outside of this region has led to speculation that it is an important determinant in the association of these proteins with LSDs (10). Here we show that the PAT domain is not required for targeting of ADPH to these structures. However, N- and C-terminal truncations of mouse ADPH that encompassed a putative α-helical region between amino acids 189 and 205 not only targeted LSDs but also increased LSD number in transfected cells. These findings demonstrate that the LSD-targeting and lipid droplet-accumulation functions of ADPH do not require the full-length protein and raise the possibility that a discrete structural region of ADPH possessing α-helical properties is responsible for both LSD association and regulation of LSD formation and, potentially, metabolism.

MATERIALS AND METHODS

Preparation of plasmids expressing epitope-tagged regions of ADPH

A 1,277 bp fragment encoding full-length mouse ADPH (12) was amplified from mouse mammary tissue cDNA by PCR. The upstream primer (ADPH[FL]-upper) 5′-cccaagcttgccaccatggcag-

Abbreviations: ADPH, adipophilin; GRAVY, grand average hydrophobicity; LSD, lipid storage droplet; PAT, perilipin, adipophilin, TIP47.

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cagcagta-3′ (Ransom Hill Biosciences, Ramona, CA) contained the 5′ end of the mADPH coding sequence, the Kozak recognition sequence, and a *Hind*III restriction site. The downstream primer (ADPH[FL]-lower) 5′-ctcgagcctgagctttgacctcagactgct-3′ contained the 3′ end of the mADPH coding sequence flanked by an *Xho*I restriction site. The pADPH[FL] plasmid was generated by inserting this fragment into the multiple cloning site of pcDNA3.1 (Invitrogen, Carlsbad, CA) after digestion with *Hind*III and *Xho*I restriction enzymes. The ADPH[FL]-VSV plasmid was generated by inserting oligonucleotides encoding an 11-amino-acid monoclonal antibody epitope of vesicular stomatitis virus (VSV) glycoprotein (YTDIEMNRLGK) (13) flanked by (5′) *Xho*I and (3′) *Xba*I restriction half-sites into the 3′ end of pADPH[FL]. The upstream primer (VSV-upper-*Xho*) was 5′-tcgagtatacacggacatcgagatgaaccgactagggaaatct-3′; the downstream primer (VSV-lower-*Xba*) was 5′-ctagagatttccctagtcggttcatctcgatgtccgtgtatac-3′. The pADPH[1–103]-VSV plasmid was generated by PCR amplification of a 306 bp region of pADPH[FL] using the ADPH[FL]-upper oligonucleotide described above as the upstream primer and a downstream primer 5′-cacctcgagcctcggacgttggctggttca-3′ that ends at the ADPH sequence encoding amino acid 103 of mADPH and is flanked by a *Xho*I restriction site. The resulting fragment was ligated into the pADPH-VSV after removal of the ADPH coding sequence by restriction digestion with *Hind*III and *Xho*I. Similarly, pADPH[1–220]-VSV was generated by PCR amplification of a 660 bp region of pADPH using the ADPH[FL]-upper oligonucleotide as the upstream primer and a downstream primer 5′-ggtctcgagcggactccagccgttcatagt-3′ that ends at the ADPH sequence encoding amino acid 220 of mADPH and is flanked by a *Xho*I site. This fragment was ligated into pADPH [FL]-VSV after digestion with *Hind*III and *Xho*I to remove the ADPH coding sequence. The pADPH[98–425]-VSV plasmid was generated by amplification of a 984 bp region of pADPH[FL]-VSV. The upstream primer 5′-gaagcttgccaccatgaaccagccaacgtc-3′ includes ADPH sequence beginning at the sequence encoding amino acid 98 of mADPH and is flanked on its 5′ side by a *Hind*III restriction site, a Kozak site, and a methionine translation initiation site. The downstream primer was VSV-lower-*Xba*. The PCR fragment was ligated into pADPH[FL]-VSV after digestion with *Hind*III and *Xba*I to remove the ADPH[FL]-VSV coding sequence. Similarly pADPH[215–425]-VSV was generated by amplification of a 633 bp region of pADPH[FL]-VSV. The upstream primer 5′-gaagcttgccaccatgtatgaacggctgga-3′ includes the ADPH sequence beginning at the sequence encoding amino acid 215 of mADPH and is flanked on its 5′ side by a *Hind*III restriction site, Kozak site, and methionine translation initiation site. The downstream primer was VSV-lower-*Xba*I. The PCR fragment was ligated into pADPH[FL]-VSV after digestion with *Hind*III and *Xba*I to remove the ADPH[FL]-VSV coding sequence. All plasmids were sequenced to confirm that the constructs encoded the desired protein. Expression of exogenous cDNAs in pcDNA3 plasmids is under the control of the cytomegalovirus promoter.

Transfection and cell culture

COS7 (COS) cells were cultured in DMEM supplemented with 10% FBS. Cells were transfected with the indicated plasmids by electroporation at 0.25 kV, 975 ohms using a Gene Pulser electroporation device (BioRad, Richmond, CA). Cells were plated at a density of $1-5 \times 10^6$ cells/ml in 6-well plates containing glass cover slips, incubated in 5% CO_2 at 37°C for 24 h, and then fixed and processed for immunofluorescence. Transfection efficiencies were maintained at a low level to achieve modest overexpression of the constructs and minimize the potential for aberrant construct localization and disruption of cellular function seen with high levels of protein over-expression. To control for the possibility that differences in construct expression levels affected their localization or altered LSD properties, we verified that under our transfection and culture conditions: *a*) similar numbers of cells (5–10%) were transfected with each construct; *b*) the relative fluorescence intensities of expressed constructs were comparable; and *c*) the localization pattern of the protein encoded by a given construct was independent of its relative expression level (data not shown).

Immunofluorescence microscopy

Cells were fixed in 3.7% formalin, washed in PBS, and extracted with methyl alcohol at −20°C. ADPH or ADPH-VSV fragments were detected by binding to antibodies to ADPH (1:500) (3, 7) or VSV (1:250; Roche Biochemicals, Indianapolis, IN) in conjunction with Alexa-488- (Molecular Probes Inc., Eugene, OR) labeled secondary antibodies. Lipid droplets and nuclei were stained Nile red and 4′,6-diamidino-2-phenylindol as described previously (3). Immunofluorescence images were captured using a 100× objective on a Nikon Diaphot fluorescent microscope equipped with a Cooke SensiCam CCD camera (Tonawand, NY) using Slidebook software (Intelligent Imaging Innovations Inc., Denver CO) (3). All images were digitally deconvolved using the No Neighbors algorithm (Slidebook), converted to TIF files, and processed with Photoshop software (Adobe Systems Inc., Mountain View, CA). LSD size and number were quantified manually on digitally deconvolved images using masking functions in Slidebook. Fifteen to twenty microscopic fields were chosen at random for analysis. LSDs were operationally defined as structures that were clearly surrounded by an annulus of ADPH. By this criterion, structures smaller than 200 nm could not be resolved and were therefore not quantified.

RESULTS

Figure 1A shows the specific regions of mouse ADPH (mADPH) that were expressed in COS cells and their relationship to the so-called PAT domain and the predicted α-helical properties of mADPH. ADPH[1–103]-VSV begins at the N-terminus of ADPH and encompasses the PAT domain (10). ADPH[1–220]-VSV begins at the N-terminus of ADPH and encompasses both the PAT domain and the major α-helical region predicted by Chou-Fasman analysis (see arrow). ADPH[98–425]-VSV begins at the C-terminal end of the PAT domain and encompasses the α-helical region and the C-terminal half of ADPH. ADPH[215–425]-VSV begins at the C-terminal end of the α helical region and encompasses the C-terminal half of ADPH. Kyte-Doolittle analysis (Fig. 1B) suggests that neither full-length ADPH nor the truncated forms of ADPH possess extensive regions of hydrophobicity that could account for LSD targeting.

Immunofluorescence analyses of the localization of endogenous ADPH and the exogenously expressed proteins encoded by the ADPH-VSV constructs are shown in **Fig. 2**. Endogenous ADPH, ADPH[FL]-VSV, ADPH[1–220]-VSV, and ADPH[98–425]-VSV selectively localized to Nile red-stained LSDs, forming a green-stained annulus around these structures. In contrast, the anti-VSV immunofluorescence in cells transfected with ADPH[1–103]-VSV and ADPH[215–425]-VSV was not associated with LSDs, but was found primarily in the cytoplasm, although in some

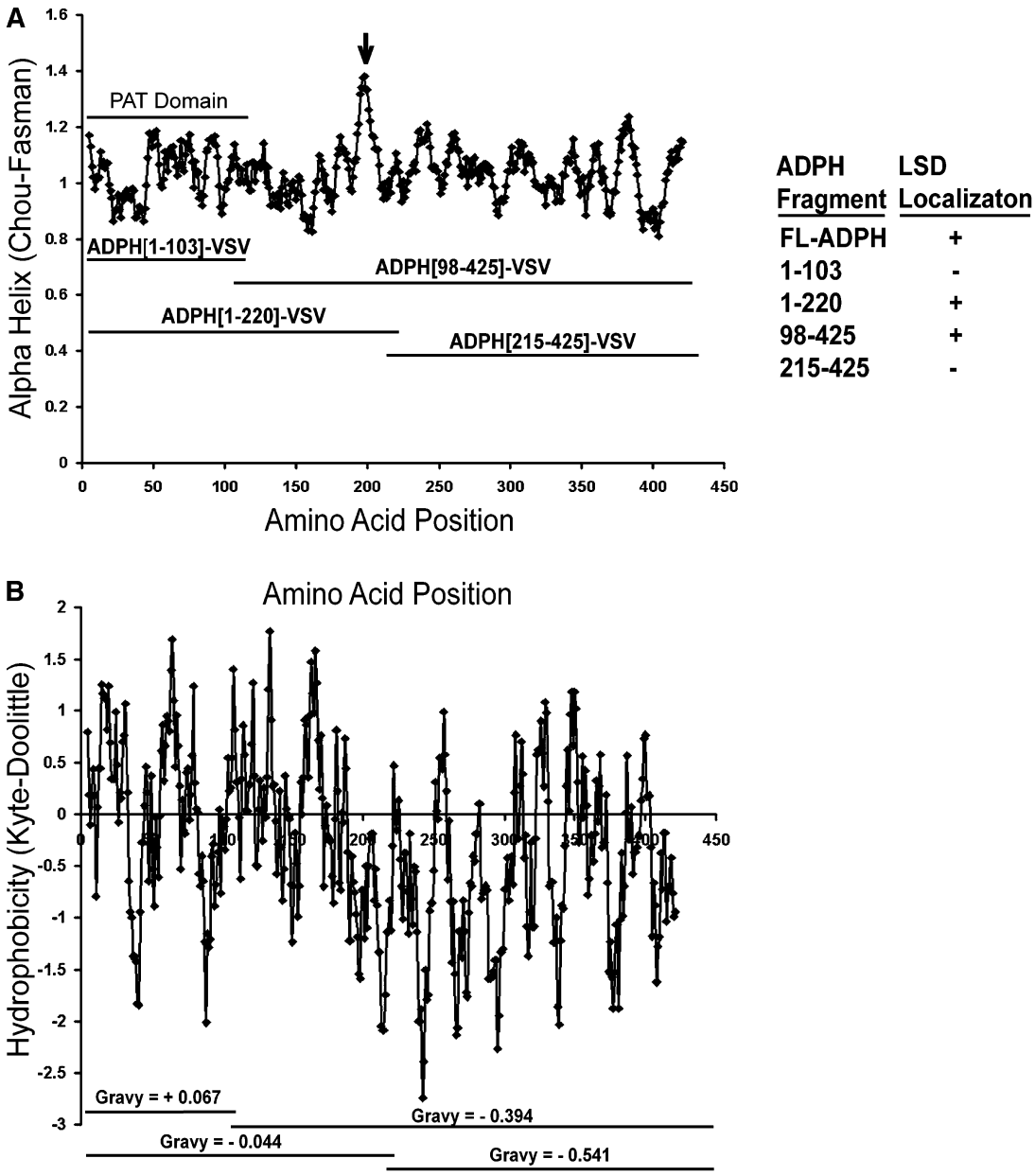

Fig. 1. Hydropathy and α-helical properties of VSV-tagged adipophilin (ADPH) constructs. A: Chou-Fasman (17) α-helical profile of mADPH. The relationship of the expressed mADPH regions to the predicted α-helical properties of mADPH is indicated by the bars below the Chou-Fasman profile. The position of the PAT domain is indicated by the bar above the profile. The arrow indicates the position of the main α-helical region predicted by Chou-Fasman analysis. The lipid storage droplet (LSD) localization results (see Fig. 2) for individual constructs are summarized next to the Chou-Fasman profile. B: Kyte-Doolittle (17) hydropathy profile of mADPH. The grand average hydrophobicity value of each construct is shown above the bar corresponding to its sequence position.

instances immunofluorescence was also detected at cell borders. Figure 2B shows that the apparent failure of ADPH[1–103]-VSV and ADPH[215–425]-VSV to localize to LSDs was not due to a lack of LSDs within cells expressing these constructs. These results confirm that ADPH specifically targets LSDs and demonstrate that this targeting does not require expression of the full-length protein. The apparent inability of ADPH[1–103]-VSV or ADPH[215–425] to target LSDs implies that the biochemical or structural properties of the PAT domain and the C-terminal half of ADPH are insufficient for LSD targeting. Importantly, the ability of ADPH[98–425]-VSV and ADPH[1–220]-VSV, which lack, respectively, the PAT domain and the C-terminal half of ADPH, to target LSDs indicates that LSD targeting is not dependent on the presence of these regions. Although none of the ADPH fragments possessed extensive hydrophobic characteristics (Fig. 1), there were differences in their grand average hydrophobicity (GRAVY) values (Fig. 1B). However, it is unlikely that such differences can account for differences in LSD targeting since ADPH[1–103]-VSV, which had the highest GRAVY value (0.067), appeared to localize to the cyto-

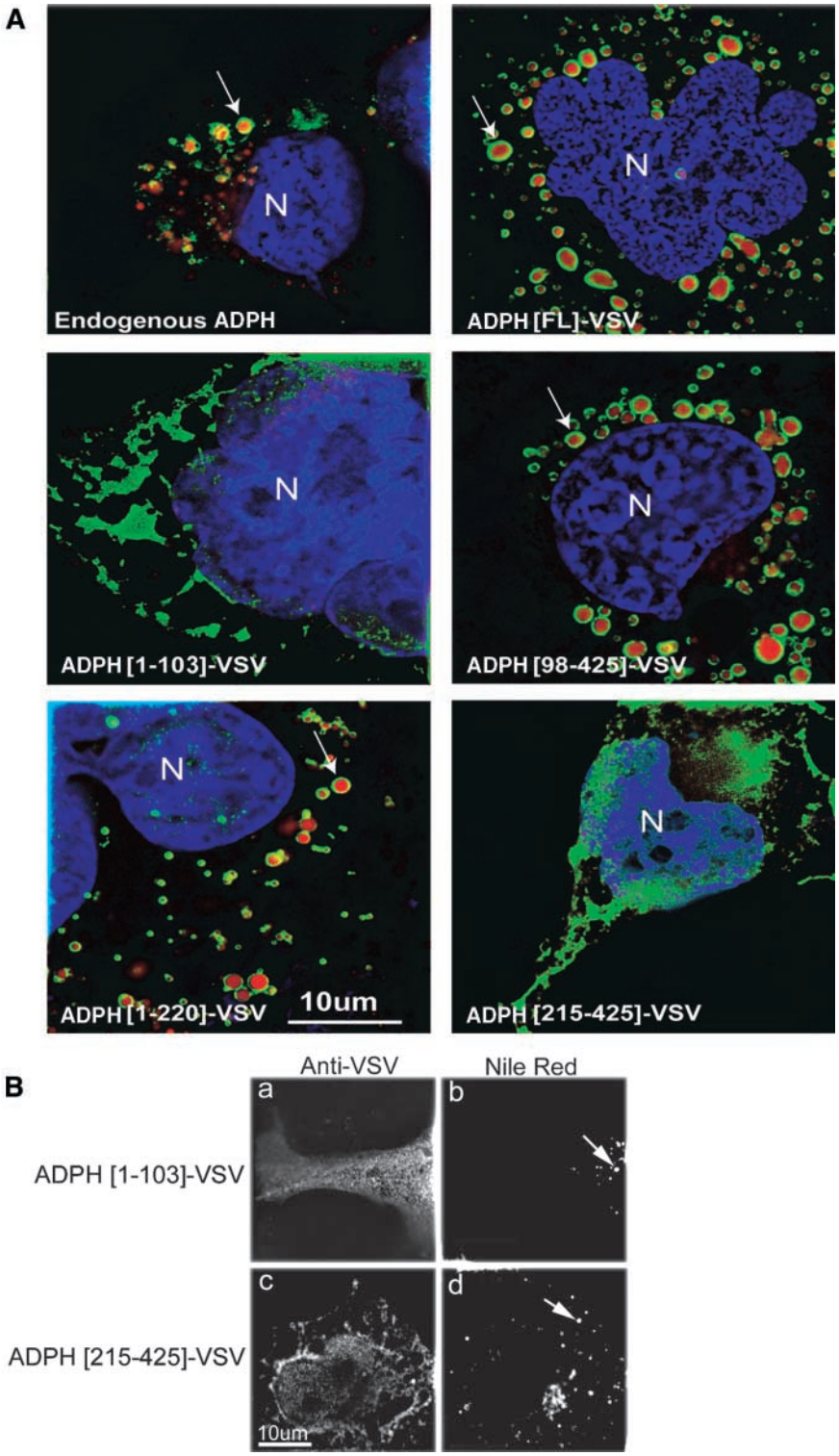

Fig. 2. Localization patterns of ADPH constructs. Immunolocalization patterns of endogenous ADPH and full-length and truncated forms of mADPH-VSV fusion proteins in COS cells are shown in A. Endogenous ADPH in wild-type COS cells was detected by immunostaining with anti-mADPH; VSV-epitope-tagged regions were detected by immunostaining with anti-VSV antibodies. Immunoreactivity was visualized with Alexa-488-coupled secondary antibodies. Endogenous ADPH, ADPH[FL]-VSV, ADPH[98–425]-VSV, and ADPH[1–220]-VSV are shown as green rings around Nile red-stained triglyceride droplets in the cytoplasm (arrows). ADPH[1–103]-VSV and ADPH[215–425]-VSV are shown as diffuse green staining within the cytoplasm. 6-Diamidino-2-phenylindol (blue)-stained nuclei are indicated in each panel. B: The lack of association between the ADPH[1–103]-VSV (a, b) and ADPH[215–425]-VSV (c, d) constructs and Nile red-stained lipid droplets is confirmed. Monochromatic images of VSV staining (a, c) and Nile red staining (b, d) are shown to emphasize differences in their respective localization patterns. Arrows in b and d indicate LSDs.

plasm, whereas ADPH[98–425]-VSV, which had next to the lowest GRAVY value (−0.394), localized to LSDs.

It has been suggested previously that ADPH expression is necessary for LSD formation (9). To test the possibility that regions involved in LSD targeting also influence LSD properties, we determined the effects of expression of full-length and truncated fragments of ADPH on LSD size and number in COS cells. Transfection with ADPH[FL]-VSV, ADPH[1–220]-VSV, and ADPH[98–425]-VSV increased the average LSD number in COS cells 4- to 5-fold over the number found in nontransfected cells (data not shown) or in cells transfected with the empty pCDNA3 vector (**Fig. 3**). In addition, there was a small but significant decrease in the average diameter of LSDs in cells transfected with ADPH[FL]-VSV, ADPH[1–220]-VSV, and ADPH[98–425]-VSV compared with cells transfected with pcDNA3 or untransfected cells (Fig. 3).

DISCUSSION

Our studies show that intact, full-length ADPH is not required for LSD targeting. Importantly, constructs lacking either the PAT domain or the C-terminal half of ADPH correctly targeted LSDs. The LSD targeting constructs instead encompassed a specific region bounded by amino acids 103 and 220 that is predicted to possess a principal α-helical domain of ADPH. Because expression of these constructs also increased cellular LSD content, it is likely that this region also plays a role in regulating LSD accumulation. However, it remains uncertain if the 103–220 region is directly responsible for targeting ADPH to LSDs, as constructs of this region did not localize to LSDs when expressed in COS cells as a VSV-tagged fusion protein (data not shown). The failure of this region to localize to LSDs suggests that LSD targeting is not directed by a simple structural motif within its primary sequence and raises the possibility that additional structural information is required, either for targeting or for proper folding of this construct. It is also unclear at present whether the α-helical region contributes to the LSD-targeting properties of ADPH. α-Helices are known to be lipid binding elements in lipoproteins (15) and important structural features of membrane proteins (16). In addition, Chou-Fasman analysis (17) of the perilipin-A structure also predicts the presence of a prominent α-helical domain within its C-terminal region (data not shown). Thus, it is an attractive possibility that α-helical domains within these proteins function in their binding to the LSD matrix. However, conclusions about the role these putative domains play in the functions ADPH or perilipin will have to await experimental verification of their structural properties and analyses of their functions.

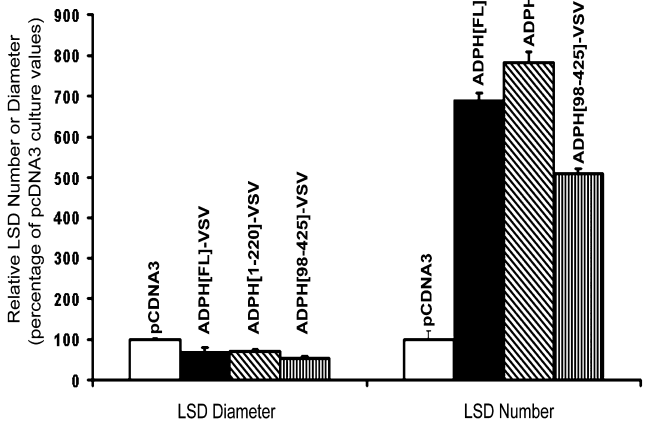

Fig. 3. Effect of ADPH constructs on LSD size and number. The relative LSD diameter and number in COS cells transfected with full-length and truncated regions of mADPH. The values are averages (± SD; N = 15–20) expressed as percentage of the values from cells in pcDNA3-transfected cultures. Similar results were obtained by expressing results as percentage of non-transfected cell values (data not shown).

Earlier studies suggested that exogenously expressed ADPH targeted to the plasma membrane in COS cells (9); however we did not observe plasma membrane association of ADPH[FL]-VSV, ADPH[1–220]-VSV, or ADPH[98–425]-VSV in our study. While the basis of this discrepancy is unclear, endogenous ADPH has been shown to consistently target LSDs in all cell types studied to date. In agreement with the results reported here, recent studies have shown that GFP tagged full length ADPH localized specifically to LSDs in cultured fibroblasts (14). Although the PAT domain and the C-terminal region primarily localized to the cytoplasm in our studies, we occasionally observed staining at the cell border in cells expressing elevated amounts of these regions. Thus, it is possible that ADPH staining previously seen at the plasma membrane in COS cells may have resulted from protein over-expression. Although ADPH can be detected at the plasma membrane in mammary epithelial cells as a result of lipid secretion (3), the weight of evidence favors the concept that ADPH specifically targets LSDs, and the results presented here indicate that specific regions within ADPH are responsible for this targeting.

Finally, the observation that the PAT domain or the C-terminal half of ADPH can be removed without affecting LSD targeting or formation suggests that these regions may participate in other cellular functions. Recent studies have provided evidence that secretion of lipid droplets from mammary epithelial cells during lactation involves the formation of a tripartite complex between ADPH, the mammary-specific transmembrane protein butyrophilin (*Btn*), and xanthine oxidoreductase (XOR) (3). As it is likely that formation of such a complex involves regions of ADPH distinct from those involved in LSD targeting, our results raise the possibility that either the PAT domain or the C-terminal half of ADPH may interact with *Btn* and/or XOR to accomplish lipid droplet secretion.

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