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Multiple functions encoded by the N-terminal PAT domain of adipophilin

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Summary

Adipophilin (ADPH), a member of the perilipin family of cytoplasmic lipid droplet (CLD)-binding proteins, is crucially dependent on triglyceride synthesis for stability. We have used cell lines expressing full-length or N-terminally modified forms of ADPH to investigate the role of the N-terminus in regulating ADPH stability and interactions with CLD. Full-length ADPH was unstable and could not be detected on CLDs unless cultures were incubated with oleic acid (OA) to stimulate triglyceride synthesis, or were treated with MG132 to block proteasomal degradation. By contrast, ADPH lacking amino acids 1–89 (Δ 2,3 ADPH), or N-terminally GFP-tagged full-length ADPH, was stable in the absence of OA or MG132, as was the closely related protein TIP47. However, none of these proteins localized to CLDs unless OA was added to the culture medium. Furthermore, immunofluorescence analysis showed that TIP47 localization to CLDs was prevented by full-length ADPH, but not by Δ 2,3 ADPH. These results suggest that the N-terminal region of ADPH mediates proteasomal degradation and access of TIP47 to the CLD surface and possibly contributes to CLD stability. Chimeras of ADPH and TIP47, generated by swapping their N- and C-terminal halves, showed that these properties are specific to ADPH.

Keywords

Adipophilin (ADPH; ADFP); TIP47 (M6PRBP1); Lipid droplets; Proteasome; Domain

Introduction

Nearly all eukaryotic cells are capable of forming cytoplasmic lipid droplets (CLDs), which function as storage depots for triglycerides (TAGs) and cholesterol esters (CEs), and serve as a source of lipid to be used in membrane synthesis and secretion (Murphy, 2001). CLDs

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consist of a neutral lipid core of TAGs and CEs, immediately surrounded by a phospholipid monolayer (Murphy, 2001; Tauchi-Sato et al., 2002) into which are embedded certain associated proteins important for the formation and physiological functions of CLD (Murphy, 2001).

Members of the perilipin (PAT) family of CLD-binding proteins have been shown to be prominent components of the CLDs of adipose and non-adipose cells in mammals and lower organisms (Brasaemle et al., 1997; Greenberg et al., 1991; Lu et al., 2001; Murphy, 2001; Wu et al., 2000). In mammals, the principal PAT members are perilipin, adipophilin (ADPH; also known as ADFP), mannose-6-phosphate receptor-binding protein 1 (TIP47; M6PRBP1), S3-12 and OXPAT (Wolins et al., 2006). Unlike apolipoproteins and caveolins (Knott et al., 1986; Ostermeyer et al., 2004; Schlegel and Lisanti, 2000), PAT family members do not have obvious hydrophobic domains that would target them to CLDs. However, perilipin, ADPH and TIP47 share a high degree of sequence similarity within their first 110 amino acids, which is known as the PAT domain (Lu et al., 2001; Miura et al., 2002). Amino acid sequence conservation within the PAT domain, along with structural considerations which predict that it is composed of amphipathic α -helical repeats (Nielsen et al., 1999), led to suggestions that it mediates CLD binding (Lu et al., 2001; Miura et al., 2002; Nielsen et al., 1999). However, the PAT domains of ADPH and perilipin do not appear to be capable of independently targeting CLDs, and the respective PAT domains of ADPH and perilipin can be removed without affecting the ability of the remaining portions of each molecule to bind to CLDs (Garcia et al., 2003; Garcia et al., 2004; McManaman et al., 2003; Targett-Adams et al., 2003). Moreover, members of the perilipin family that lack the PAT domain, such as S3-12, nevertheless localize to CLDs (Wolins et al., 2006; Wolins et al., 2005). Thus, it is unclear what functions, if any, the PAT domain encodes.

ADPH is implicated in the formation and secretion of CLDs by mammary epithelial cells (McManaman et al., 2002; McManaman et al., 2007; Russell et al., 2007). During lactation, CLDs in mammary epithelial cells are secreted as membrane-bound structures known as milk fat globules (MFGs). ADPH is a major protein component of CLDs isolated from lactating mouse mammary glands and from the membrane surrounding secreted MFGs (Heid et al., 1998; Heid et al., 1996; Wu et al., 2000). Recently, we demonstrated that mice thought to be *Adph*-null in fact produce an N-terminally truncated form of ADPH in their mammary tissue that appears to mediate CLD formation and secretion, albeit less efficiently (Russell et al., 2008). The mutant gene is missing exons 2 and 3, which encode amino acids 1–75 of mouse ADPH and include most of the PAT domain. Stable expression of plasmids containing only exons 4–8 of mouse *Adph* (Δ 2,3 ADPH) in HEK 293 cells confirmed that amino acids 1–75 are not required for ADPH to bind to CLDs (Russell et al., 2008). These results suggest that a large part of the PAT domain of ADPH can be removed without dramatically interfering with its physiological functions. During the course of characterizing the *Adph* mutant mice, we noticed that transcript levels from the truncated gene were at least an order of magnitude lower than those from the intact gene in wild-type animals, whereas the amounts of truncated protein in mammary glands of mutant animals appeared to be only slightly lower than the amounts of full-length protein in wild-type mammary glands. This observation prompted us to explore the possibility that loss of the N-terminal region might increase the stability of the ADPH protein. Using cell lines that stably express full-length or N-terminally modified forms of ADPH, we have obtained evidence that the N-terminal region of mouse ADPH encodes multiple functions, including mediating proteasomal degradation, contributing to CLD stabilization, controlling access of TIP47 to CLDs and specifying the cytoplasmic distribution of CLDs.

Results

N-terminal modification enhances ADPH stability

We tested the hypothesis that the N-terminal region of ADPH mediates its intracellular stability by stably transfecting HEK 293 (293) cells with plasmids expressing: (1) non-tagged full-length mouse ADPH (ADPH[fl]); (2) full-length mouse ADPH with a C-terminal VSV epitope tag (ADPH[fl]-VSV); (3) full-length mouse ADPH fused at its N-terminus to GFP and at its C-terminus to the VSV epitope (GFP-ADPH[fl]-VSV); and (4) a variant of mouse ADPH lacking the amino acids encoded by exons 2 and 3 (amino acids 1–75) with a VSV tag at the C-terminus (Δ 2,3 ADPH-VSV) (Russell et al., 2008). The 293 cells were chosen for these studies as they endogenously express TIP47 (Listenberger et al., 2007) but lack detectable amounts of ADPH, perilipin and S3-12 (data not shown). The effects of incubation in oleic acid (OA), to stimulate triglyceride synthesis, on the relative levels of each of the ADPH variants are shown in Fig. 1. ADPH[fl] and ADPH[fl]-VSV were not detected in extracts of cultures grown in the absence of added OA, but significant amounts of both proteins were detected when cultures were cultured in the presence of 300 μ M OA. GFP-ADPH[fl]-VSV was detected in cultures grown in the absence of added OA; however, its levels increased following incubation in OA suggesting that it was further stabilized by stimulating triglyceride synthesis. By contrast, significant amounts of Δ 2,3 ADPH-VSV were present in cultures grown in the absence of added OA and its levels did not appear to increase appreciably by inclusion of OA in the culture medium. Similar levels of endogenous TIP47 were detected in cultures of parental 293 cells in the absence or presence of OA (Fig. 1), as well as in clonal derivatives expressing ADPH variants (data not shown). Importantly, the plasmid constructs employed in these studies make use of the pcDNA3 vector, which contains a strong CMV promoter in front of the cDNA of interest. We have verified that the transcript levels of all stably transfected genes are highly expressed in the respective clones (data not shown); thus, the lack of accumulation is entirely consistent with the notion that ADPH levels are tightly controlled by degradation in the absence of triglyceride synthesis (Xu et al., 2005). These results indicate that modification, or loss, of the N-terminal region of ADPH increases its intracellular stability under fatty acid-deficient conditions.

To determine whether the apparent increased stability of Δ 2,3 ADPH-VSV reflects differential responsiveness to fatty acid levels, we quantified the effects of OA concentration on the levels of ADPH[fl]-VSV and Δ 2,3 ADPH-VSV protein. As shown in Fig. 1B,C, the levels of Δ 2,3 ADPH-VSV remained relatively constant over a wide range of OA concentrations; the levels of Δ 2,3 ADPH-VSV in cultures treated with 1200 μ M OA were not significantly different from control levels ($P < 0.478$). By contrast, ADPH[fl]-VSV levels increased with increasing OA concentrations up to 1200 μ M, suggesting that its stability was related to the concentration of OA in the media (Fig. 1B,C). Indeed, we found that concentrations as low as 15 μ M OA were capable of increasing ADPH[fl]-VSV levels (data not shown). The results shown in Fig. 1C further suggest that the effects of OA on ADPH[fl]VSV levels are biphasic. ADPH[fl]VSV levels in cultures incubated with 150 μ M OA were not significantly different from those incubated with 300 μ M ($P < 0.37$) or 600 μ M ($P < 0.46$) OA. Increasing OA concentrations to 900 μ M, however, increased ADPH[fl]VSV levels by ~100% as compared with levels in 150 μ M OA ($P < 0.02$). ADPH[fl]VSV levels in cultures incubated with 1200 μ M OA were not significantly different from those incubated with 900 μ M OA ($P < 0.43$).

Stabilization of ADPH by fatty acids has been shown to depend on TAG formation (Xu et al., 2005); however, other studies have suggested that fatty acids are capable of directly preventing ADPH proteolysis (Gao et al., 2000). To distinguish between these two possibilities, we incubated cell lines expressing ADPH[fl]-VSV or Δ 2,3 ADPH-VSV with

elevated concentrations of OA in the presence of triacsin C to inhibit TAG synthesis (Igal et al., 1997). In our cultures, 5 μ M triacsin C completely blocked formation of Nile Red-stained lipid droplets (supplementary material Fig. S1). As seen in Fig. 1D, incubation with triacsin C blocks the ability of OA to increase the levels of ADPH[fl]-VSV, but it does not affect Δ 2,3 ADPH-VSV levels, suggesting that like endogenous ADPH, fatty acid stabilization of ADPH[fl]-VSV requires TAG synthesis. By contrast, the inability of either elevated OA concentrations or triacsin C to alter Δ 2,3 ADPH-VSV levels indicates that neither elevated fatty acid levels nor TAG formation is required for its stability.

N-terminal modification reduces proteasomal degradation of ADPH

Elegant studies by Xu et al. suggest that endogenous ADPH is degraded by the ubiquitin/proteasome pathway (Xu et al., 2005). However, the region of ADPH that mediates proteasomal turnover was not identified. To test the hypothesis that the N-terminal region of ADPH mediates this function, we investigated the effects of inhibitors of various intracellular proteolytic pathways on the levels of intact full-length and N-terminally modified forms of ADPH. Fig. 2A shows the effects of increasing concentrations of the proteasomal pathway inhibitor MG132 on levels of full-length untagged ADPH, ADPH[fl]-VSV, GFP-ADPH[fl]-VSV, Δ 2,3 ADPH-VSV and endogenous TIP47. We found that inhibition of proteasomal activity was required to detect either untagged full-length ADPH or ADPH[fl]-VSV in the absence of added OA, and that maximal stabilization of both proteins occurred at 3 μ M MG132. Consistent with the data in Fig. 1, we found low levels of GFP-ADPH[fl]-VSV in cultures grown in the absence of OA or MG132 (Fig. 2A); however, incubation in 3 μ M MG132 increased the levels of this protein ~600% over controls ($P<0.0001$). Compared with its effects on the levels of full-length forms of ADPH, MG132 produced relatively modest increases in the levels of Δ 2,3 ADPH-VSV (124% over control, $P<0.02$). At 3 μ M, MG132 produced variable but modest increases in the levels of endogenous TIP47 relative to controls ($P<0.02$).

To examine whether other mechanisms contribute to ADPH or TIP47 degradation in cultures of 293 cells, we first determined if the effects of OA and MG132 on ADPH[fl]-VSV, Δ 2,3 ADPH-VSV and TIP47 were synergistic (Fig. 2B). Again, we did not detect ADPH[fl]-VSV in the absence of treatment with MG132 or OA. The levels of ADPH[fl]-VSV in cultures incubated with MG132 were ~450% ($P<0.0009$) greater than those in cultures treated with OA. Incubation with OA in combination with MG132, however, did not increase the levels of ADPH[fl]-VSV above that achieved by MG132 alone ($P<0.41$). The differences in the effects of OA and MG132 on Δ 2,3 ADPH-VSV were more modest than those observed for ADPH[fl]-VSV. The levels of Δ 2,3 ADPH-VSV in cultures incubated with OA were not different from controls ($P<0.45$). In cultures incubated with MG132, the levels of Δ 2,3 ADPH-VSV were ~120% greater than those incubated with OA ($P<0.005$). As with ADPH[fl]-VSV, the levels of Δ 2,3 ADPH-VSV in cultures incubated with both OA and MG132 were not different than those treated with MG132 alone ($P<0.2$). The levels of endogenous TIP47 in 293 cultures incubated with MG132 were ~80% greater than controls ($P<0.02$). The levels of TIP47 in cultures treated with both OA and MG132 were not significantly different from those incubated in either OA ($P<0.14$) or MG132 ($P<0.2$) alone.

We next determined whether the lysosomal proteases contribute to the degradation of these molecules. As shown in Fig. 2C, ADPH[fl]-VSV was detectable in cultures incubated with 300 μ M OA but was not detectable in control cultures, or cultures incubated with calpeptin, leupeptin or chloroquine in the absence of OA. Lysosomal inhibitors had modest but variable effects on Δ 2,3 ADPH-VSV and TIP47 levels. Calpeptin increased Δ 2,3 ADPH-VSV by ~50% over controls ($P<0.005$), but did not significantly alter TIP47 levels relative to controls ($P<0.22$). Leupeptin increased Δ 2,3 ADPH-VSV by ~90% ($P<0.0004$) and

TIP47 by ~80% ($P<0.0009$) over controls, whereas chloroquine increased $\Delta 2,3$ ADPH-VSV by ~80% ($P<0.004$) and TIP47 by ~40% ($P<0.04$) over controls.

The effects of ADPH stabilization on CLD formation

Preventing ADPH degradation has been shown to induce formation of ADPH-coated CLDs in the absence of OA-stimulated TAG synthesis in embryonic fibroblasts (Xu et al., 2005), raising the possibility that CLD accumulation is directly linked to ADPH stability. To examine the role of ADPH and TIP47 stability on CLD accumulation, we investigated CLD formation in the absence and presence of added OA in parental 293 cultures, in cultures expressing ADPH[fl]-VSV and in cultures expressing $\Delta 2,3$ ADPH-VSV. As shown in Fig. 3Aa–c, we did not detect TIP47-, ADPH[fl]-VSV- or $\Delta 2,3$ ADPH-VSV-coated CLDs in the absence of added OA. Under these conditions, TIP47 was found in small, diffusely distributed punctate structures in parental 293 cells as well as in each cell line. These structures did not stain with Nile Red and were not sensitive to triacsin C treatment (supplementary material Fig. S2), suggesting that they were not small CLDs. ADPH[fl]-VSV, by contrast, was undetectable by immunofluorescence, whereas $\Delta 2,3$ ADPH-VSV exhibited a diffuse cytosolic appearance (Fig. 3Ab). All three proteins localized to CLDs following incubation with OA (Fig. 3Ad–f); enlargements of selected regions of these cells are shown in Fig. 3Ba–c. As described in more detail below, the presence of ADPH[fl]-VSV on CLDs appears to exclude endogenous TIP47 from these structures, whereas $\Delta 2,3$ ADPH-VSV does not.

To determine whether inhibiting proteasomal degradation induced CLD accumulation in cells expressing these proteins in the absence of added OA, we incubated them overnight in 3 μ M MG132. Consistent with the findings of Xu et al. (Xu et al., 2005), we found that MG132 treatment induced formation of CLDs coated with ADPH[fl]-VSV (Fig. 3Ai). These structures were verified to be CLDs by Nile Red staining and sensitivity to triacsin C (supplementary material Figs S2 and S3). MG132 treatment, however, did not induce formation of CLDs coated with TIP47 or $\Delta 2,3$ ADPH-VSV (Fig. 3Ag,h). Incubation in medium containing both MG132 and OA did not result in additional CLD accumulation in parental 293 cells or either cell line (Fig. 3Aj–l). Together, these results suggest that preventing the degradation of full-length ADPH promotes CLD accumulation, but inhibiting proteasomal degradation does not independently promote the accumulation of CLDs or enhance the abilities of TIP47 or $\Delta 2,3$ ADPH-VSV to induce their formation.

The N-terminal region of ADPH influences access of TIP47 to CLDs

Previous studies have suggested that the presence of ADPH limits access of TIP47 to the CLD surface (Sztalryd et al., 2006; Listenberger et al., 2007; Russell et al., 2008). Consistent with this hypothesis, we found that incubation with OA led to translocation of TIP47 from its punctate cytosolic localization to CLDs in parental 293 cells but not in cells expressing ADPH[fl]-VSV, in which CLDs are coated with ADPH[fl]-VSV (Fig. 3Aa,c,d,f). Time-course experiments demonstrated that TIP47 retained its punctate cytosolic distribution at all times between 4 hours (the earliest time CLDs are detected) and 96 hours after OA addition in the ADPH[fl]-VSV-expressing cells (data not shown). By contrast, incubating $\Delta 2,3$ ADPH-VSV-expressing cells with either OA alone or with OA plus MG132 resulted in translocation of TIP47 to CLDs, where it coexisted with $\Delta 2,3$ ADPH-VSV (Fig. 3Ab,e,k). In many cases, CLDs in these cells exhibited yellow immunofluorescence, suggesting that $\Delta 2,3$ ADPH-VSV and endogenous TIP47 were in close proximity on the CLD surface. To address the possibility that this co-existence is due to permeabilization/solvent effects, we repeated this experiment in cells permeabilized with 0.1% saponin and obtained similar results (supplementary material Fig. S4). Additionally, we found that differences in the localization of TIP47 in parental 293 cells and in cells expressing

ADPH[fl]-VSV or $\Delta 2,3$ ADPH-VSV were not due to different levels of TIP47, as all three lines had similar levels of TIP47 mRNA and protein (data not shown).

ADPH-TIP47 chimeras confirm the functional importance of their N-terminal regions

To confirm that the differences in the properties of ADPH and TIP47 are specified by their N-terminal regions, we generated stable cell lines expressing chimeras of TIP47 and ADPH. We reasoned that if the N-terminal region of ADPH controlled stability and CLD access, chimeric molecules composed of the N-terminal region of ADPH and the C-terminal region of TIP47 should behave like ADPH, whereas those composed of the N-terminal region of TIP47 and the C-terminal region of ADPH should behave like TIP47. The chimeric proteins were constructed by swapping the N- and C-terminal halves of mouse ADPH and TIP47 and placing a VSV epitope tag at the C-terminal end of each molecule for ease of detection (Fig. 4A,B). In immunoblot analyses of the chimera-expressing cell lines (Fig. 4C), we were unable to detect the ADPH-TIP47-VSV chimera unless the cultures were treated with OA or MG132 (Fig. 4C,E). The levels of this chimera in cultures treated with OA were not significantly different from those treated with MG132 ($P < 0.25$). Incubation with both OA and MG132 increased the levels of the ADPH-TIP47-VSV chimera over the levels of OA alone by ~50% ($P < 0.04$) and over MG132 alone by ~70% ($P < 0.002$). By contrast, the TIP47-ADPH-VSV chimera was detected in untreated cultures and its levels were not significantly altered by incubation in OA ($P < 0.08$), MG132 ($P < 0.13$), or both agents together ($P < 0.12$). These findings support the conclusion that the N-terminal region of ADPH is responsible for targeting it for degradation by the proteasomal pathway. In addition, the data suggest that differences in the N-terminal regions of ADPH and TIP47 are responsible for differences in their stabilities under fatty acid-deficient conditions, as well as for differences in their susceptibility to proteasomal degradation.

Both chimeric proteins localized to CLDs (Fig. 5). Consistent with the immunoblot data, the ADPH-TIP47-VSV chimera was not detected by immunofluorescence unless cells were incubated in culture media supplemented with OA and/or MG132 (Fig. 5A–D). Like ADPH[fl]-VSV, the ADPH-TIP47-VSV chimera localized to CLDs in cells incubated with OA, MG132 or these agents together (Fig. 5B–D). Conversely, like endogenous TIP47, the corresponding TIP47-ADPH-VSV chimera was detected in the cytoplasm of cells cultured in the absence of added OA, where it had a punctate distribution (Fig. 5E). Incubation with OA, or MG132 in combination with OA, resulted in translocation of this chimera to CLDs (Fig. 5F,H); however, it did not localize to CLDs when incubated with MG132 alone (Fig. 5G). In all cases, CLD localization of chimeric proteins was verified by the ability of 5 μ M triacsin C to inhibit the formation of these structures (data not shown).

Lastly, the chimeric proteins were used to test the hypothesis that the N-terminal region of ADPH is responsible for limiting access of TIP47 to CLD, as suggested by the results shown in Fig. 3. In order to carry out this experiment, it was first necessary to establish the specificity of our antibodies. Immunoblot analyses (Fig. 6A) showed that anti-human TIP47 detects a ~47 kDa band corresponding to endogenous TIP47 in both chimera-expressing cell lines, as expected because the parental 293 cell line is human in origin. However, this antibody did not detect either the TIP-ADPH-VSV chimera, which runs at ~52 kDa, or the ADPH-TIP-VSV chimera, which runs at ~49 kDa. By contrast, antibodies to the N-terminus of mouse TIP47 or VSV specifically detected the 52 kDa TIP47-ADPH-VSV chimera, and antibodies to the N-terminus of mouse ADPH or VSV specifically detected the 49 kDa ADPH-TIP47-VSV chimera.

Fig. 6B shows the effects of ADPH-TIP47-VSV and TIP47-ADPH-VSV chimeric proteins on the ability of endogenous TIP47 (green fluorescence, Fig. 6Ba–d) to localize to CLDs after incubation in OA for 24 hours. In cells expressing ADPH-TIP47-VSV (red

fluorescence), the chimeric protein localized to CLDs following incubation with OA (Fig. 6Bb), but was not detected in control cells (Fig. 6Ba). Endogenous TIP47 in these cells retained its punctate distribution in both the presence and absence of OA. In cells expressing TIP47-ADPH-VSV (Fig. 6Bc,d), the chimeric protein (red fluorescence) and endogenous TIP47 were diffusely distributed in the cytoplasm under control conditions; however, following OA addition, both proteins localized to CLDs (Fig. 6Bd). Here again, we observed that many CLDs in these cells exhibited yellow immunofluorescence, suggesting close proximity of the TIP47-ADPH-VSV chimera and endogenous TIP47 proteins.

Discussion

The sequences of the N-terminal ~100 amino acids (PAT-1 domain) of perilipin, adipophilin and TIP47 are highly related (Lu et al., 2001). Until now, however, the functional importance of this domain has been obscure. We have used cell lines expressing full-length or N-terminally modified forms of ADPH, as well as ADPH-TIP47 chimeras, to demonstrate that sequences in the PAT-1 domain of ADPH encode multiple functions, including regulating proteasomal degradation, CLD stabilization and controlling access of TIP47 to the CLD surface. Our data further suggest that the N-terminal regions of ADPH and TIP47 contribute to the differences in their properties.

Studies by Xu et al. (Xu et al., 2005) convincingly demonstrated that proteasomal degradation is responsible for ADPH turnover in the absence of triglycerides; however, the region of ADPH that mediates this process was not identified. Our study provides several lines of evidence that the N-terminal region of ADPH plays a major role in regulating its degradation. First, modification of the N-terminal region, either by addition of a GFP tag or by truncation, prevents the rapid loss of ADPH in the absence of TAG synthesis. Second, MG132 inhibition of proteasomal degradation has comparatively small effects on the levels of N-terminally modified forms of ADPH. Third, TIP47 is normally stable in the absence of added OA; however, replacing the N-terminal half of TIP47 with that of ADPH generates a chimeric protein, the stability of which, like that of full-length ADPH, is dependent on either inhibiting proteasomal degradation or stimulating triglyceride synthesis. Conversely, replacing the N-terminal half of ADPH with that of TIP47 generates a stable chimeric protein, the levels of which, like those of endogenous TIP47, are relatively unaffected by inhibiting proteasomal degradation.

Proteasomal degradation appears to be the primary mechanism for degrading full-length forms of ADPH in our cultures, whereas lysosomal processes do not normally appear to contribute to this process. Our observation that MG132 has a relatively modest effect on Δ 2,3 ADPH-VSV levels suggests that the region between amino acids 1 and 75 plays an important role in regulating rapid proteasomal degradation of full-length ADPH. At present, we do not know if this region is directly ubiquitinated or whether it indirectly serves a permissive function in ADPH degradation. Mouse ADPH contains four lysine residues within this region that are possible sites of ubiquitylation, and studies are in progress to determine whether these lysines undergo ubiquitylation and to what extent they might contribute to proteasomal degradation of ADPH. Furthermore, we cannot dismiss the possibility that other regions of ADPH also contribute to its degradation by the proteasomal pathway. Indeed, the observation that MG132 treatment produces modest increases in the levels of Δ 2,3 ADPH-VSV suggests that sequences distal to amino acid 75 might also contribute to the proteasomal degradation of ADPH, albeit to a lesser extent than the N-terminal sequences.

Evidence from Xu et al. (Xu et al., 2005) suggests that inhibiting ADPH breakdown promotes TAG synthesis and CLD formation. Our data are consistent with their observations

and further suggest that the region deleted in Δ 2,3 ADPH-VSV might contribute to these functions. Since Δ 2,3 ADPH-VSV binds CLDs, our data further suggest that sequences responsible for targeting of ADPH to CLDs are distinct from those involved in CLD stabilization.

The finding that MG132 slightly elevated TIP47 levels also suggests that degradation by the proteasomal pathway might contribute to the processes that regulate its steady-state levels. In addition, leupeptin and chloroquine increased TIP47 levels, and the magnitude of their effects were similar to that of MG132. Thus, both lysosomal and proteasomal processes are likely to contribute to the regulation of TIP47 levels. The observation that leupeptin and chloroquine also produced modest increases in the levels of Δ 2,3 ADPH-VSV, but did not affect the levels of ADPH[fl]-VSV, suggests that the removal of sequences targeting ADPH to proteasomal degradation converts it into protein that is degraded by mechanisms similar to those that degrade TIP47. In summary, our inhibitor studies suggest that both proteasomal and lysosomal processes contribute to the degradation of ADPH and TIP47, and that sequences within the first 75 amino acids of ADPH are responsible for targeting it for rapid proteasomal degradation.

Recent studies demonstrating that TIP47 localizes to CLDs in cells deficient in ADPH suggests that ADPH might restrict access of TIP47 to the CLD surface (Xu et al., 2005; Russell et al., 2008). Consistent with this possibility, Listenberger et al. (Listenberger et al., 2007) recently demonstrated that exogenous expression of ADPH displaces TIP47 from CLDs in 293 cells. In agreement with these results, we found that CLDs in 293 cells that contain undetectable amounts of endogenous ADPH by immunoblot, are coated by TIP47, whereas in cells expressing ADPH[fl]-VSV, CLDs are coated with ADPH but lack TIP47. By contrast, we found both the N-terminally truncated ADPH and endogenous TIP47 on the same CLDs in cells expressing Δ 2,3 ADPH-VSV. Thus, our data support previous conclusions that ADPH impairs the association of TIP47 with CLDs (Xu et al., 2005; Russell et al., 2008) and suggest that sequences within the PAT-1 domain of ADPH are responsible for this function. In support of this, we found that replacing the N-terminal half of TIP47 with that of ADPH generated a chimeric protein that prevented endogenous TIP47 from localizing to CLDs, whereas replacing the N-terminal half of ADPH with that of TIP47 generated a chimeric protein that allowed endogenous TIP47 to co-exist with it on CLDs. These chimeric protein data further suggest that the exclusion of TIP47 from CLDs is due to a specific function of the N-terminal region of ADPH, and not to simple mass action effects.

Despite their sequence and structural similarities, ADPH and TIP47 appear to possess distinct biological properties. Based on differences in their stability when not bound to CLDs, Wolins et al. (Wolins et al., 2006) have classified ADPH as a constitutively TAG-associated PAT protein (CPAT), whereas TIP47 is described as an exchangeable TAG-associated PAT protein (EPAT). Our data suggest that the N-terminal regions of ADPH and TIP47 are primarily responsible for these classifications. Indeed, removal of the N-terminal region of ADPH appears to convert it from a CPAT protein that is unstable when not bound to CLDs, to an EPAT-like protein that is found in a stable pre-existing cytosolic pool that translocates to CLDs in response to added OA. Our chimera data further support this notion by showing that the C-terminal regions of ADPH and TIP47 do not appear to influence their stabilities when not bound to CLDs. Specifically, the ADPH/TIP47 chimera is unstable and appears to exist exclusively in association with CLDs, like a CPAT protein, whereas the TIP47/ADPH chimera is stable and capable of transferring from the cytosol to CLDs following OA addition, like an EPAT protein.

At present, we know little about the molecular, structural and/or conformational determinants that account for the differences in the functional properties of ADPH and

TIP47. Hickenbottom et al. (Hickenbottom et al., 2004) have identified regions of sequence and structural similarity within the N- and C-terminal portions of PAT proteins, respectively referred to as PAT-N and PAT-C regions. The PAT-N region is predicted to be composed of a series of 11mer repeats distal to the PAT-1 sequence identified by Lu et al. (Lu et al., 2001). The PAT-C region is an independently folding unit comprising a 4-helix bundle flanked by α/β domains. It is interesting that the region in ADPH that we have identified as mediating differences in the properties of ADPH and TIP47 lies within the PAT-1 sequence, before the predicted 11mer repeat region. Thus, it is unlikely that the 11mer repeats contribute to the observed differences between ADPH and TIP47, suggesting the presence of at least one additional functional determinant in the PAT-N region.

In summary, our results provide evidence that the N-terminal PAT-1 domain of ADPH participates in multiple cellular functions. Additional studies will be required to determine whether these functions involve direct and/or indirect actions of this region, as well as to identify the precise structural features responsible for a particular function. Our conclusion that these functions are encoded by a limited region within the N-terminus of ADPH provides the basic framework needed for defining the mechanisms and molecular determinants that mediate the functions of its PAT-1 domain.

Materials and Methods

Antibodies

Antibodies to the N-terminal 25-amino-acid sequence of murine ADPH were generated in rabbits then affinity purified (Affinity Bioreagents, Golden, CO) or were obtained commercially as guinea pig antibodies to murine ADPH (Research Diagnostics Incorporated/Fitzgerald Industries International, Flanders NJ). Rabbit antibodies to the C-terminal sequence (amino acids 152–434) of human TIP47 were kindly provided by Dr Suzanne Pfeffer (Stanford University, Palo Alto, CA). Chicken antibodies to human TIP47 were obtained commercially (GenWay, San Diego, CA). Mouse monoclonal antibody to the VSV-G, 11-amino-acid epitope was purchased from Roche (Indianapolis, IN; clone P5D4).

Plasmids

Construction of pcDNA3 plasmids containing cDNAs encoding native full-length mouse ADPH (ADPH[fl]) and C-terminal VSV-tagged mouse ADPH (ADPH[fl]-VSV) have been described (McManaman et al., 2003). The VSV-G epitope tag (referred to as VSV) is encoded by the amino acid sequence YTDIEMNRLGK (Roche Diagnostics, Indianapolis, IN). To make the N-terminal GFP-tagged, C-terminal VSV-tagged mouse ADPH (GFP-ADPH[fl]-VSV) plasmid, the ADPH[fl]-VSV-encoding portion of the ADPH[fl]-VSV plasmid was excised and ligated into the pEGFP-C3 vector. To make the N-terminal GFP-tagged mouse TIP47 (GFP-TIP47), mouse *Tip47* was PCR amplified and ligated into the pEGFP-C3 vector. To make the C-terminal VSV-tagged mouse ADPH (1–204)-TIP47 (225–437) (ADPH-TIP-VSV) plasmid, the sequence encoding mouse ADPH (1–204) was PCR amplified and inserted into pcDNA3; the sequence encoding mouse TIP47 (225–437) was amplified and inserted into the same plasmid just downstream of the ADPH (1–204); and the VSV tag sequence was PCR amplified and inserted just downstream of the TIP47 (225–437) sequence. This resulted in a chimeric molecule with the sequence GGTACC (*KpnI* site; encoding Gly-Thr) in between sequences encoding ADPH (1–204) and TIP47 (225–437). To make the C-terminal VSV-tagged mouse TIP47 (1–224)-ADPH (205–425) (TIP47-ADPH-VSV) plasmid, the sequence encoding mouse TIP47 (1–224) was PCR amplified and inserted into pcDNA3; the sequence encoding mouse ADPH (205–425) was amplified and inserted into the same plasmid just downstream of the TIP47 (1–224); and the VSV tag sequence was PCR amplified and inserted just downstream of the ADPH (205–425).

sequence. This resulted in a chimeric molecule with the sequence GGTACC (*KpnI* site; encoding Gly-Thr) in between sequences encoding TIP47 (1–224) and ADPH (205–425). All constructs were verified by sequencing. Construction of the $\Delta 2,3$ ADPH-VSV-encoding plasmid has been described (Russell et al., 2008).

Cell lines

HEK 293 (293) cells were obtained from ATCC (Manassas, VA) and used as the parental cell line from which all the other cell lines were derived. Parental 293 cells, and all stably transfected cell lines derived from them, were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose (25 mM) and supplemented with 6% bovine calf serum. The ADPH[fl], ADPH[fl]-VSV, GFP-ADPH[fl]-VSV, GFP-TIP47, ADPH-TIP-VSV, TIP47-ADPH-VSV and $\Delta 2,3$ ADPH-VSV plasmids were transfected into 293 cells by the calcium phosphate method (Russell et al., 2008). Cells were selected with G418 and clones were isolated by limiting dilution, selected by immunohistochemical and immunoblot analysis and recloned to ensure purity. Stock stably transfected cell lines were maintained in G418; however, all experiments were conducted in culture media in its absence. Both non-cloned cell lines (transfected and cultured for at least 2 weeks in G418) and cloned lines were used in experiments and in all cases produced the same results. Only data from twice-cloned lines are presented. Experiments on non-cloned cell lines were performed to ensure that the cloned cell lines accurately reflected the phenotype of the population.

Immunoblot analysis

Following removal of cells from the culture dishes with 0.5 mM EDTA in PBS, cell proteins were separated on 10% polyacrylamide gels, transferred to nitrocellulose membranes and probed with primary antibodies at the following dilutions: guinea pig anti-ADPH, 1:1000; rabbit anti-ADPH, 1:5000; chicken anti-TIP47, 1:2000; and mouse anti-VSV, 1:1000. Corresponding horseradish peroxidase-conjugated secondary antibodies (Sigma, St Louis, MO) were used according to manufacturer's specifications. Western Lightening Chemiluminescence Reagent (Perkin Elmer, Boston, MA) was used to detect bands, and band intensities were quantified by densitometry (ChemiDoc System, Bio-Rad, Hercules, CA). Triplicate dishes were assayed for each data point and all experiments were performed at least twice. Band intensities were normalized to that of β -actin on stripped and reprobed blots. Statistical analysis was performed using the Student's *t*-test. Preliminary studies were performed to verify that the responses were in the linear range.

Immunofluorescence microscopy

Cultured cells were grown on glass coverslips in culture dishes for at least 3 days prior to experiments. Following experimental treatments, media were removed and immediately replaced with 3.7% formaldehyde for 10 minutes to fix the cells, then with 50% ethanol for 4 minutes to permeabilize the cells, and finally the cells were washed with PBS for 30 minutes. Blocking solution (2% BSA in PBS) was then applied for 20 minutes, followed by the antibodies to ADPH, TIP47 or VSV (all diluted with blocking buffer) for 25 minutes. Coverslips were washed in PBS, reblocked for 5 minutes and then incubated with Alexa Fluor 594- or 488-conjugated donkey anti-rabbit, anti-guinea pig, or anti-mouse IgG (Invitrogen/Molecular Probes, Carlsbad, CA) for 25 minutes. The coverslips were washed again in PBS, incubated with DAPI (0.05 μ g/ml) for 20 minutes, washed and finally mounted on glass slides with Aquamount (ScyTek, Logan, UT). CLDs coated with ADPH variants or TIP47 were confirmed to contain neutral lipid by Nile Red colocalization experiments as described previously (McManaman et al., 2003). Multiple methods of permeabilization, including the use of Triton X-100, saponin and different ethanol concentrations, were investigated to optimize staining of PAT proteins and CLDs in our

cells. In our hands, all methods give similar results in terms of PAT protein localization, but 50% ethanol give the best image quality.

Image analysis and CLD quantitation

Immunofluorescence images were captured at room temperature on a Nikon Diaphot fluorescence microscope equipped with a Cooke SensiCam CCD camera (Tonawand, NY) using Slidebook software (Intelligent Imaging Innovations, Denver, CO) as previously described (McManaman et al., 2003). All images were digitally deconvolved using the No Neighbors algorithm (Slidebook), converted to TIFF files and processed by Photoshop (Adobe Systems, Mountain View, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Brasaemle DL, Barber T, Wolins NE, Serrero G, Blanchette-Mackie EJ, Londos C. Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J Lipid Res.* 1997; 38:2249–2263. [PubMed: 9392423]
- Gao J, Ye H, Serrero G. Stimulation of adipose differentiation related protein (ADRP) expression in adipocyte precursors by long-chain fatty acids. *J Cell Physiol.* 2000; 182:297–302. [PubMed: 10623894]
- Garcia A, Sekowski A, Subramanian V, Brasaemle DL. The central domain is required to target and anchor perilipin A to lipid droplets. *J Biol Chem.* 2003; 278:625–635. [PubMed: 12407111]
- Garcia A, Subramanian V, Sekowski A, Bhattacharyya S, Love MW, Brasaemle DL. The amino and carboxyl termini of perilipin a facilitate the storage of triacylglycerols. *J Biol Chem.* 2004; 279:8409–8416. [PubMed: 14610073]
- Greenberg AS, Egan JJ, Wek SA, Garty NB, Blanchette-Mackie EJ, Londos C. Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J Biol Chem.* 1991; 266:11341–11346. [PubMed: 2040638]
- Heid HW, Schnolzer M, Keenan TW. Adipocyte differentiation-related protein is secreted into milk as a constituent of milk lipid globule membrane. *Biochem J.* 1996; 320:1025–1030. [PubMed: 9003395]
- Heid HW, Moll R, Schwetlick I, Rackwitz HR, Keenan TW. Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. *Cell Tissue Res.* 1998; 294:309–321. [PubMed: 9799447]
- Hickenbottom SJ, Kimmel AR, Londos C, Hurley JH. Structure of a lipid droplet protein; the PAT family member TIP47. *Structure.* 2004; 12:1199–1207. [PubMed: 15242596]
- Igal RA, Wang P, Coleman RA. Triacsin C blocks de novo synthesis of glycerolipids and cholesterol esters but not recycling of fatty acid into phospholipid: evidence for functionally separate pools of acyl-CoA. *Biochem J.* 1997; 324:529–534. [PubMed: 9182714]
- Knott TJ, Pease RJ, Powell LM, Wallis SC, Rall SC Jr, Innerarity TL, Blackhart B, Taylor WH, Marcel Y, Milne R, et al. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature.* 1986; 323:734–738. [PubMed: 3773997]

- Listenberger LL, Ostermeyer-Fay AG, Goldberg EB, Brown WJ, Brown DA. Adipocyte differentiation-related protein reduces lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. *J Lipid Res.* 2007; 12:2751–2761. [PubMed: 17872589]
- Lu X, Gruia-Gray J, Copeland NG, Gilbert DJ, Jenkins NA, Londos C, Kimmel AR. 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.* 2001; 12:741–749. [PubMed: 11641724]
- McManaman JL, Palmer CA, Wright RM, Neville MC. Functional regulation of xanthine oxidoreductase expression and localization in the mouse mammary gland: evidence of a role in lipid secretion. *J Physiol.* 2002; 545:567–579. [PubMed: 12456835]
- McManaman JL, Zabaronick W, Schaack J, Orlicky DJ. Lipid droplet targeting domains of adipophilin. *J Lipid Res.* 2003; 44:668–673. [PubMed: 12562852]
- McManaman JL, Russell TD, Schaack J, Orlicky DJ, Robenek H. Molecular determinants of milk lipid secretion. *J Mammary Gland Biol Neoplasia.* 2007; 12:259–268. [PubMed: 17999166]
- Miura S, Gan JW, Brzostowski J, Parisi MJ, Schultz CJ, Londos C, Oliver B, Kimmel AR. Functional conservation for lipid storage droplet association among Perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, *Drosophila*, and *Dictyostelium*. *J Biol Chem.* 2002; 277:32253–32257. [PubMed: 12077142]
- Murphy DJ. The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Prog Lipid Res.* 2001; 40:325–438. [PubMed: 11470496]
- Nielsen RL, Andersen MH, Mabhout P, Berglund L, Petersen TE, Rasmussen JT. Isolation of adipophilin and butyrophilin from bovine milk and characterization of a cDNA encoding adipophilin. *J Dairy Sci.* 1999; 82:2543–2549. [PubMed: 10629799]
- Ostermeyer AG, Ramcharan LT, Zeng Y, Lublin DM, Brown DA. Role of the hydrophobic domain in targeting caveolin-1 to lipid droplets. *J Cell Biol.* 2004; 164:69–78. [PubMed: 14709541]
- Russell TD, Palmer CA, Orlicky DJ, Fischer A, Rudolph MC, Neville MC, McManaman JL. Cytoplasmic lipid droplet accumulation in developing mammary epithelial cells: roles of adipophilin and lipid metabolism. *J Lipid Res.* 2007; 48:1463–1475. [PubMed: 17452747]
- Russell TD, Palmer CA, Orlicky DJ, Bales ES, Chang BH, Chan L, McManaman JL. Mammary glands of adipophilin-null mice produce an amino-terminally truncated form of adipophilin that mediates milk lipid droplet formation and secretion. *J Lipid Res.* 2008; 49:206–216. [PubMed: 17921437]
- Schlegel A, Lisanti MP. A molecular dissection of caveolin-1 membrane attachment and oligomerization. Two separate regions of the caveolin-1 C-terminal domain mediate membrane binding and oligomer/oligomer interactions in vivo. *J Biol Chem.* 2000; 275:21605–21617. [PubMed: 10801850]
- Sztalryd C, Bell M, Lu X, Mertz P, Hickenbottom S, Chang BH, Chan L, Kimmel AR, Londos C. Functional compensation for adipose differentiation-related protein (ADFP) by TIP47 in an adfp null embryonic cell line. *J Biol Chem.* 2006; 281:34341–34348. [PubMed: 16968708]
- Targett-Adams P, Chambers D, Gledhill S, Hope RG, Coy JF, Girod A, McLauchlan J. Live cell analysis and targeting of the lipid droplet-binding adipocyte differentiation-related protein. *J Biol Chem.* 2003; 278:15998–16007. [PubMed: 12591929]
- Tauchi-Sato K, Ozeki S, Houjou T, Taguchi R, Fujimoto T. The surface of lipid droplets is a phospholipid monolayer with a unique Fatty Acid composition. *J Biol Chem.* 2002; 277:44507–44512. [PubMed: 12221100]
- Wolins NE, Quaynor BK, Skinner JR, Schoenfish MJ, Tzekov A, Bickel PE. S3-12, Adipophilin, and TIP47 package lipid in adipocytes. *J Biol Chem.* 2005; 280:19146–19155. [PubMed: 15731108]
- Wolins NE, Brasaemle DL, Bickel PE. A proposed model of fat packaging by exchangeable lipid droplet proteins. *FEBS Lett.* 2006; 580:5484–5491. [PubMed: 16962104]
- Wu CC, Howell KE, Neville MC, Yates JR 3rd, McManaman JL. Proteomics reveal a link between the endoplasmic reticulum and lipid secretory mechanisms in mammary epithelial cells. *Electrophoresis.* 2000; 21:3470–3482. [PubMed: 11079566]

Xu G, Sztalryd C, Lu X, Tansey JT, Gan J, Dorward H, Kimmel AR, Londos C. Post-translational regulation of adipose differentiation-related protein by the ubiquitin/proteasome pathway. *J Biol Chem*. 2005; 280:42841–42847. [PubMed: 16115879]

Fig. 1.

Effects of oleic acid on the stability of ADPH variants in stably transfected HEK 293 cells. (A) Immunoblot analysis of parental 293 cultures and cultures stably expressing ADPH variants following a 20-hour incubation in the absence (C) or presence of 300 μM oleic acid (OA). Parental 293 culture extracts were immunoblotted with chicken anti-human TIP47, extracts of cultures expressing ADPH were immunoblotted with guinea pig anti-mouse ADPH, and extracts of cultures expressing VSV-tagged forms of ADPH were immunoblotted with mouse anti-VSV. Immunoblots of whole gels are shown to demonstrate specificity and the absence of breakdown products. The boxed regions beneath show the results of immunoblots that were stripped and re-probed for β-actin to control for sample loading. (B) Representative anti-VSV immunoblots of cultures expressing ADPH[fl]-VSV or Δ2,3 ADPH-VSV following a 20-hour incubation in the indicated concentrations of OA. Anti-β-actin immunoblots are shown beneath the anti-VSV immunoblots. (C) Quantitation of the immunoblots in B. The data show relative steady-state levels of ADPH[fl]-VSV (white bars) and Δ2,3 ADPH-VSV (black bars) normalized to β-actin. The values are means ± s.d. for triplicate cultures. (D) Effects of OA and/or triacsin C on ADPH[fl]-VSV and Δ2,3 ADPH-VSV levels. Representative anti-VSV immunoblots of extracts of ADPH[fl]-VSV or Δ2,3 ADPH-VSV cell lines following a 20-hour incubation in unsupplemented basal culture media (Cont), or in basal media supplemented with 300 μM OA, 5 μM triacsin C (Triac-C), or with 300 μM OA plus 5 μM triacsin C (Triac-C + OA). β-actin loading controls for each sample are shown beneath the anti-VSV blots. Representative immunoblots are from a single well of triplicate cultures treated as indicated. All experiments were repeated at least twice, with similar results.

Fig. 2.

N-terminally modified ADPH is resistant to proteolytic degradation. (A) Effects of MG132 concentration on steady-state levels of ADPH variants and TIP47. Representative immunoblots of parental 293 cultures and ADPH variant cultures following a 20-hour incubation in unsupplemented basal culture media containing increasing concentrations of MG132. Anti-β-actin immunoblots are shown beneath the anti-VSV immunoblots. The relative effects of increasing MG132 concentrations on steady-state levels of each ADPH variant and endogenous TIP47 are shown alongside each immunoblot. The values are averages ± s.d. for six samples, each normalized to β-actin. (B) Effects of MG132 and OA on steady-state levels of ADPH[f]-VSV, Δ2,3 ADPH-VSV and TIP47. Representative

immunoblots of parental 293 cultures and ADPH[fl]-VSV and $\Delta 2,3$ ADPH-VSV cultures following a 20-hour incubation in basal culture media (control), or in basal culture media supplemented with 3 μ M MG132 (MG), 300 μ M OA, or 3 μ M MG132 plus 300 μ M OA (MG/OA). The relative effects of these treatments on steady-state levels of each protein are shown alongside each immunoblot. The values are averages $\pm$ s.d. for six samples, each normalized to β -actin. (C) Effects of lysosomal protease inhibitors on steady-state levels of ADPH[fl]-VSV, $\Delta 2,3$ ADPH-VSV and endogenous TIP47. Representative immunoblots of parental 293 cultures and ADPH[fl]-VSV and $\Delta 2,3$ ADPH-VSV cultures after a 20-hour incubation with the indicated inhibitors of lysosomal proteases. The relative effects of these inhibitors on steady-state cellular levels of each protein are shown alongside each blot. The values are averages $\pm$ s.d. for six samples, each normalized to β -actin. All experiments were repeated twice, with similar results. In-depth statistical analyses of these results are given in the text.

Fig. 3.

Immunolocalization of endogenous TIP47, ADPH[fl]-VSV and $\Delta 2,3$ ADPH-VSV. (A) Immunostaining of endogenous TIP47 (green) and ADPH[fl]-VSV (red) or $\Delta 2,3$ ADPH-VSV (red) following a 24-hour incubation of 293 cells (a,d,g,j), $\Delta 2,3$ ADPH-VSV cells (b,e,h,k) and ADPH[fl]-VSV cells (c,f,i,l) in basal media (Cont, a–c), 300 μ M OA (d–f), 5 μ M MG132 (MG, g–i) or 300 μ M OA plus 5 μ M MG132 (OA + MG, j–l). Panels a,d,g,j were immunostained with chicken anti-human TIP47; b,e,h,k and c,f,i,l were immunostained with mouse anti-VSV and chicken anti-human TIP47. (B) Enlargement of regions in Ad-f are shown (a–c) to better illustrate CLD staining properties of endogenous TIP47, $\Delta 2,3$ ADPH-VSV and ADPH[fl]-VSV. Nuclei were stained with DAPI (blue). All experiments were performed at least three times, with similar results. Multiple images were captured in each experiment; representative images are shown. Images were originally taken at 600 $\times$ magnification. Scale bar: 20 μ m.

Fig. 4. Specificity of N-terminal control of ADPH stability. (A,B) Schematics of ADPH-TIP47-VSV and TIP47-ADPH-VSV chimeras. (C,D) Representative immunoblots of ADPH-TIP47-VSV (C) and TIP47-ADPH-VSV (D) chimeras following a 20-hour incubation of their respective cultures in basal culture media (control) or in basal culture media supplemented with 3 μ M MG132 (MG), 300 μ M OA, or 3 μ M MG132 plus 300 μ M OA (MG/OA). Chimeras were detected by probing with mouse anti-VSV antibodies; blots were stripped and reprobed for β -actin to control for loading. (E,F) Quantitation of the respective immunoblots in C and D. The values are averages $\pm$ s.d. for three samples, each normalized to β -actin. The experiment was repeated twice, with similar results. Statistical analyses of these results are given in the text.

Fig. 5.

Immunolocalization of chimeric proteins. Cultures stably expressing ADPH-TIP47-VSV (A-D) or TIP47-ADPH-VSV (E-H) were incubated for 20 hours in unsupplemented basal media (Cont; A,E), or in basal media supplemented with 300 μ M OA (B,F), 3 μ M MG132 (MG, C,G), or 3 μ M MG132 plus 300 μ M OA (MG+OA, D,H). Chimeric proteins were localized in cells by staining with mouse anti-VSV (green); nuclei were stained with DAPI (blue). All experiments were performed at least three times, with similar results. Multiple images were captured in each experiment; representative images are shown. Images were originally taken at 600 $\times$ magnification. Scale bar: 20 μ m.

Fig. 6.

Specificity of N-terminal control of TIP47 binding to CLDs. (A) Specificity of TIP47 antibodies. Extracts of cultures stably expressing the TIP47-ADPH-VSV or ADPH-TIP47-VSV chimeric protein were immunoblotted with chicken anti-human TIP47 antibody (α hTIP47), rabbit antibodies to the N-terminus of mouse TIP47 (α N-term mTIP47) or mouse anti-VSV antibody (α VSV). α hTIP47 detected a single species at ~47 kDa in cultures of both chimeric cell lines (calculated molecular weight of hTIP47=47032.88; ProSite, <http://ca.expasy.org/tools/dna.html>). α N-term mTIP47 and α VSV antibodies detected a single species of ~52 kDa in TIP-ADPH-VSV cultures (calculated molecular weight=50907.48) and a single species of ~49 kDa (calculated molecular weight=48019.52) in ADPH-TIP47-VSV cultures. (B) Immunolocalization of endogenous TIP47 (green, a–d) and chimeric proteins in ADPH-TIP-VSV (red, a,b) and TIP-ADPH-VSV (red, c,d) in cells after a 20-hour incubation in the absence (Cont, a,c) or presence of 300 μ M OA (b,d). TIP47 was localized by staining with α hTIP47; chimeric proteins were localized by staining with mouse anti-VSV; nuclei were stained with DAPI (blue). All experiments were performed at least three times, with similar results. Multiple images were captured in each experiment; representative images are shown. Images were originally taken at 600 $\times$ magnification. Scale bar: 20 μ m.