# Intracellular lipid droplet targeting by apolipoprotein A-V requires the carboxyl-terminal segment

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**Abstract The expression of apolipoprotein A-V (apoA-V) in hepatoma cells results in homing of this protein to intracellular lipid droplets. When hepatoma cells transfected with a full-length apoA-V-green fluorescent protein fusion protein were cultured in medium that was not supplemented with oleic acid (OA), intracellular lipid droplet size and number were reduced compared with those of cells supplemented with OA. Confocal microscopy studies revealed that apoA-V associates with lipid droplets under both conditions. To define the structural requirements for apoA-V lipid droplet association, hepatoma cells were transfected with a series of C-terminal truncated apoA-V variants. Confocal microscopy analysis revealed that, in a manner similar to mature full-length apoA-V (343 amino acids), truncation variants apoA-V(1-292), apoA-V(1-237), and apoA-V(1-191) associated with lipid droplets, while apoA-V(1-146) did not. Western blot analysis of the relative abundance of apoA-V in cell lysates versus conditioned medium indicated that apoA-V variants associated with lipid droplets were poorly secreted while apoA-V(1-146) was efficiently secreted. Ultracentrifugation of conditioned medium revealed that, unlike full-length apoA-V, which associates with lipoproteins, apoA-V(1-146) was present solely in the lipoprotein-deficient fraction. Deletion of the N-terminal signal peptide from apoA-V resulted in an inability of the protein to be secreted into the medium, although it associated with lipid droplets. Taken together, these data suggest that the C terminus of apoA-V is essential for lipid droplet association in transfected hepatoma cells and lipoprotein association in conditioned medium while the signal peptide is required for extracellular trafficking of this protein.—**Shu, X., R. O. Ryan, and T. M. Forte. **Intracellular lipid droplet targeting by apolipoprotein A-V requires the carboxyl-terminal segment. *J. Lipid Res.* 2008.** 49: **1670–1676.**

**Supplementary key words** McA-RH7777 cells • confocal fluorescence microscopy • apolipoprotein A-V carboxyl-terminal truncation variants • lipoprotein association • apolipoprotein A-V-deficient high density lipoprotein • adipocyte differentiation-related protein • signal peptide

Apolipoprotein A-V (apoA-V), first described in 2001 (1, 2), has emerged as an important modulator of triglyceride (TG) metabolism (3). The protein is synthesized solely by the liver and is found in plasma associated with HDL and VLDL. Newly synthesized apoA-V consists of 366 amino acids, where the first 23 residues form the cleavable signal peptide. The mature, secreted form of apoA-V consists of 343 residues (39 kDa). The protein is extremely hydrophobic and not readily soluble in aqueous medium; however, once bound to lipid, it is soluble and stable (4). We recently reported that apoA-V, overexpressed in hepatoma cell lines, associates with cytoplasmic lipid droplets despite being synthesized with a signal peptide (5). Although the protein was poorly secreted from hepatoma cells, it was found in association with lipoproteins in conditioned medium. In hepatocytes, lipid droplets are a storage form of neutral lipids (particularly TG) that may be used for production of VLDL. Since it is conceivable that apoA-V lipid droplet association may be relevant to plasma TG homeostasis, we explored these observations further to determine structural elements within apoA-V that are involved in lipid droplet association and apoA-V secretion.

It was previously proposed that the C-terminal segment of apoA-V is required for lipid binding (6), and this proposal is supported by the in vitro observation that the apoA-V(1-292) variant had lower lipid binding ability than the full-length protein (7). In human subjects with a mutant form of apoA-V, Q139X, in which putative hydrophobic, amphipathic helices of the C terminus are deleted, association with lipoproteins in plasma did not occur (8). These observations strongly suggest a role for the C terminus in lipid binding. In the present study, several C-terminal apoA-V truncation variants were expressed in hepatoma cells to evaluate how much of the C-terminal region is required for homing to lipid droplets and/or lipoprotein association. In keeping with the results obtained in

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Abbreviations: ADRP, adipocyte differentiation-related protein; apoA-V, apolipoprotein A-V; DAPI, 4′,6-diamino-phenylindole; GFP, green fluorescent protein; McA-RH, McA-RH7777; OA, oleic acid; TG, triglyceride.

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human subjects carrying the Q139X apoA-V variant, apoA-V(1-146) described in this study did not associate with lipoproteins in conditioned medium. The latter variant was also unable to associate with lipid droplets, thus suggesting that the hydrophobic C-terminal region of this protein is required for lipid binding.

## MATERIALS AND METHODS

### Materials

Oleic acid (OA) and albumin were from Sigma. DMEM, horse serum, G418, and trypsin-EDTA were purchased from Invitrogen. FBS was from HyClone. Nile Red and 4′,6-diaminophenylindole (DAPI) were from Molecular Probes. Polyclonal rabbit anti-human apoA-V antibody was a kind gift from Dr. Robert J. Konrad (Eli Lilly and Co.). Monoclonal mouse anti-adipocyte differentiation-related protein (ADRP) antibody was from Progen Biotechnik.

### Cell culture

Rat hepatoma McA-RH7777-A18 (McA-RH) cells stably transfected with human apoB-100 (kindly provided by Dr. Zemin Yao, University of Ottawa) were cultured in DMEM containing 10% FBS, 10% horse serum, and 200 μg/ml G418. Cells were passaged every 4 days. Cells were transfected using Lipofectamine 2000 (Invitrogen).

### Construction of plasmids

DNA amplification was carried out using a plasmid template harboring the entire apoA-V coding region (a gift from Dr. Len Pennacchio, Lawrence Berkeley National Laboratory). Primers were designed to amplify full-length apoA-V and indicated truncations (**Fig. 1**). The amplification products were cloned into pcDNA3.1(+) (Invitrogen) or pEGFP-N1 (Clontech) in the case of expression of green fluorescent protein (GFP) fusions. The GFP tag was appended to the C terminus of the protein.

### Confocal microscopy

For fluorescence microscopy, McA-RH cells were grown on poly-L-lysine coverslips (BD Biosciences). After transfection with apoA-V or apoA-V-GFP constructs, cells were transferred to growth medium supplemented with 0.8 mM OA for 6 h. For immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS and processed as described (9). Cells were washed with PBS, and lipid droplets were stained with Nile Red as described (10); nuclei were stained with DAPI. To visualize ADRP, fixed cells were permeabilized and subsequently incubated with mouse anti-ADRP (1:10 dilution) followed by incubation with goat anti-mouse Alexa 594 secondary antibody. Images were captured with a LSM 510 Meta UV/Vis confocal microscope.

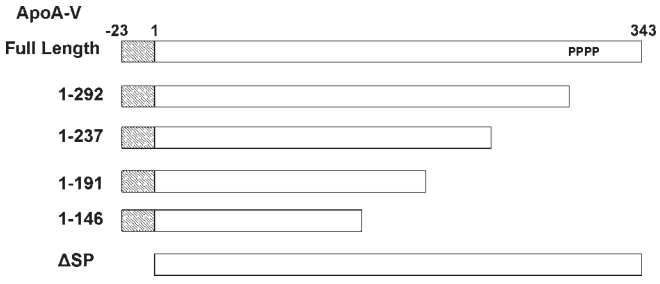


**Fig. 1.** Diagram depicting apolipoprotein A-V (apoA-V) truncation variants generated in this study. The full-length apoA-V schematically shows the signal peptide (SP) region (hatched box) as −23 to 1 and the mature secreted protein as region 1–343. The tetra-proline (PPPP) sequence beginning at residue 293 of the mature protein is indicated. Constructs of C-terminal truncation variants used in the present study included the signal peptide together with the region of the mature protein as shown. The deletion of the signal peptide is termed ΔSP.

### Cell/medium distribution of apoA-V and apoA-V variants

McA-RH cells were grown on poly-L-lysine-coated six-well plates (BD Biosciences). Twenty-four hours after transfection, medium was replaced with DMEM plus 10% FBS and 0.8 mM OA. After 16 h, cells were washed with PBS and incubated with serum-free medium for 24 h. Subsequently, conditioned medium was collected, cell monolayer was rinsed with PBS, and the cells were lysed with Cell Lysis Buffer (Cell Signaling Technology).

### Lipoprotein association of apoA-V and apoA-V variants

To determine whether full-length apoA-V or apoA-V(1-146) associated with lipoproteins upon secretion into the cell medium, conditioned medium was adjusted to density 1.21 g/ml by the addition of solid NaBr. The sample was then centrifuged at 100,000 *g* for 5.5 h in a Beckman TL100 centrifuge. The lipoprotein-rich $d \leqslant 1.21$ g/ml fraction and the $d > 1.21$ g/ml lipoprotein-poor fraction were harvested by aspiration as described by Lindgren, Jensen, and Hatch (11).

## RESULTS

### Effect of OA treatment on apoA-V lipid droplet association

We previously reported that, in OA-supplemented cultured hepatoma cells, ectopically expressed full-length apoA-V homes to lipid droplets coating their surfaces (5). Since OA supplementation drives intracellular lipid accumulation and new lipid droplet formation, we sought to determine whether OA supplementation is required for the lipid droplet association of transfected apoA-V. To examine this question, an apoA-V-GFP fusion protein was expressed in McA-RH cells cultured with or without OA supplementation. Lipid droplet association was then examined by confocal microscopy. As seen in **Fig. 2**, in the absence of OA, lipid droplets were fewer in number and smaller in size. Despite this, however, transfected apoA-V-GFP localized to lipid droplets in a manner similar to that seen in cells supplemented with OA.

### C-terminal sequence requirements for apoA-V lipid droplet association

Recently, Beckstead et al. (7) reported that the apoA-V (1-292) truncation variant (at the site of a unique tetra-proline sequence) can bind dimyristoylphosphatidylcholine vesicles but with lower binding efficiency than full-length apoA-V. These authors also showed that a 51 amino acid peptide encompassing residues 293–343 (the deleted portion of the protein) was highly efficient in binding to dimyristoylphosphatidylcholine. Taken together, these re-

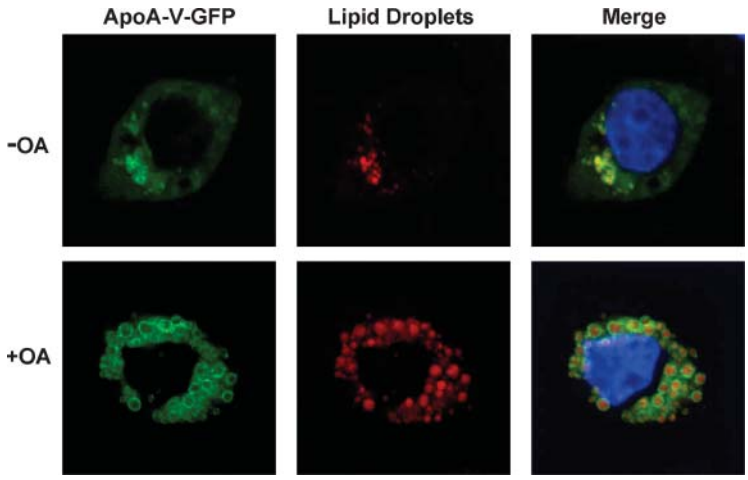


**Fig. 2.** Effect of oleic acid (OA) supplementation on apoA-V association with lipid droplets. Confocal fluorescence microscopy images show cultured McA-RH7777 (McA-RH) cells transfected with an apoA-V-green fluorescent protein (GFP) fusion construct (green fluorescence). Lipid droplets were stained with Nile Red (red fluorescence). Nuclei were stained with 4′,6-diamino-phenylindole (DAPI; blue fluorescence). Merged images are shown on the right. Top panel, no OA supplementation; bottom panel, supplementation with OA (0.8 mM for 6 h).

sults suggested that this region of apoA-V is important in lipid binding. To test whether deletion of these residues, as well as other more extensive C-terminal truncations, alters apoA-V association with lipid droplets, plasmid vectors encoding truncated apoA-V-GFP variants were generated (Fig. 1). The latter approach provides new insights into how much of the C-terminal domain is required for lipid droplet and lipoprotein association. Following transfection into McA-RH cells, the extent of association with lipid droplets was determined by confocal microscopy (**Fig. 3A**). Consistent with our earlier report (5), full-length apoA-V associates with lipid droplets in the cell (Fig. 3A, top panel). Homing of apoA-V to lipid droplets is not the result of the GFP fusion protein, since we previously showed (5) that without this fluorescent tag the protein still associated with lipid droplets. Upon deletion of 51 amino acids from the C terminus of apoA-V to generate apoA-V(1-292)-GFP, lipid droplet association persisted. Three additional apoA-V-GFP truncation variants were constructed, transfected into McA-RH cells, and examined for their ability to associate with lipid droplets. The apoA-V(1-237) variant (Fig. 3A) and the apoA-V(1-191) variant (data not shown) associated with intracellular lipid droplets, while apoA-V(1-146) did not. In the latter case (Fig. 3A), a diffuse fluorescent protein distribution pattern was observed and lipid droplets were smaller in size and dispersed throughout the cell.

### Effect of signal peptide deletion on apoA-V lipid droplet association

The recovery of apoA-V in association with cytosolic lipid droplets raises the question of whether the N-terminal signal peptide (SP) is required to direct the protein to cytosolic lipid droplets. To examine whether this 23 amino acid N-terminal sequence (12) plays a role in apoA-V post-translational fate, a plasmid construct encoding apoA-V-GFP that lacks a signal sequence, ΔSP-apoA-V-GFP, was transfected into McA-RH cells and the intracellular localization of the protein was assessed by confocal microscopy (Fig. 3A, bottom panel). Interestingly, compared with cells transfected with full-length apoA-V, "lipid droplets" present in the vicinity of ΔSP-apoA-V-GFP fluorescence intensity were amorphous entities.

ADRP, also known as adipophilin, is the major lipid droplet-associated protein in hepatocytes (13). Thus, we asked whether apoA-V and ADRP colocalize on lipid droplets and whether the ΔSP-apoA-V variant can potentially disrupt this association. Figure 3B (top panel) clearly demonstrates that full-length apoA-V and ADRP colocalize on the surface of lipid droplets in McA-RH cells. Control studies with cells incubated in the absence of apoA-V (Fig. 3B, middle panel) suggest that overexpression of apoA-V does not alter ADRP trafficking to lipid droplets. Expression of the ΔSP-apoA-V variant still results in its colocalization with ADRP (Fig. 3B, bottom panel), even though lipid droplets are amorphous (compare with Fig. 3A, bottom panel) suggesting that loss of the signal peptide did not misdirect ΔSP-apoA-V to another intracellular compartment.

### Effect of C-terminal sequence elements on the secretion efficiency of apoA-V

In cultured hepatoma cells, it is known that apoA-V is not as efficiently secreted as other apolipoproteins, such as apoA-I or apoB (5). The overall hydrophobicity and potent lipid binding ability of apoA-V may contribute to this phenomenon. The fact that transfected apoA-V(1-146) does not associate with lipid droplets suggests that this variant is less hydrophobic and has a lower lipid surface-seeking activity. Based on this, it was hypothesized that apoA-V(1-146) will be more efficiently secreted from transfected hepatoma cells compared with full-length apoA-V or apoA-V(1-292). To test this, full-length apoA-V, apoA-V (1-292), and apoA-V(1-146) GFP fusion protein constructs were transfected into McA-RH cells. After 24 h of culturing in serum-free medium, conditioned medium was collected and cell lysates were prepared. Equivalent aliquots of cell lysate and concentrated conditioned medium were then subjected to SDS-PAGE to determine the distribution of apoA-V (**Fig. 4A**). Whereas >80% of full-length apoA-V-GFP and apoA-V(1-292)-GFP were recovered in the cell

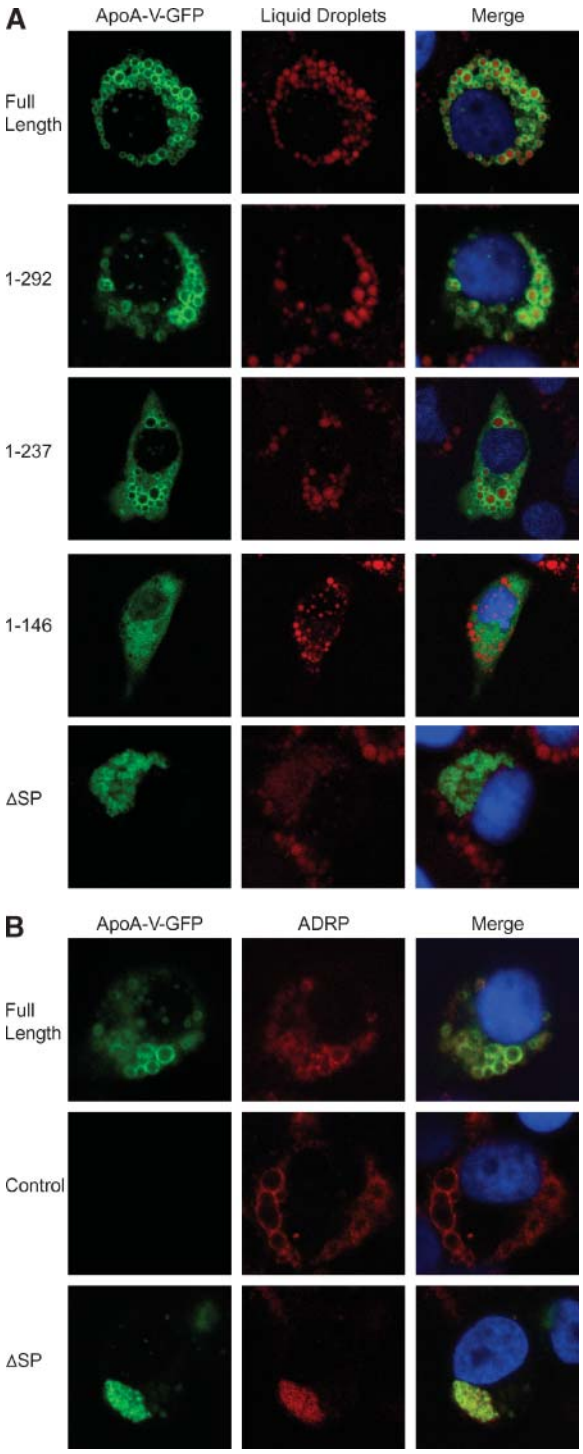


**Fig. 3.** Effect of C-terminal and signal peptide truncations on the lipid droplet association properties of apoA-V. Confocal fluorescence microscopy images show cultured McA-RH cells transfected with apoA-V-GFP fusion constructs (green fluorescence). McA-RH cells were incubated in the presence of 0.8 mM OA for 6 h and then fixed. Lipid droplets were visualized with Nile Red (red fluorescence), and nuclei were visualized with DAPI (blue fluorescence). A: Intracellular localization of full-length apoA-V, C-terminal truncation variants, signal peptide variant (ΔSP), and lipid droplets. Merged images are shown on the right. B: Effect of full-length apoA-V and ΔSP-apoA-V deletion on adipocyte differentiation-related protein (ADRP) distribution. Control cells (middle panel) were cells grown in the absence of transfected apoA-V-GFP. ADRP was visualized with goat anti-mouse Alexa 594 secondary antibody (red fluorescence).

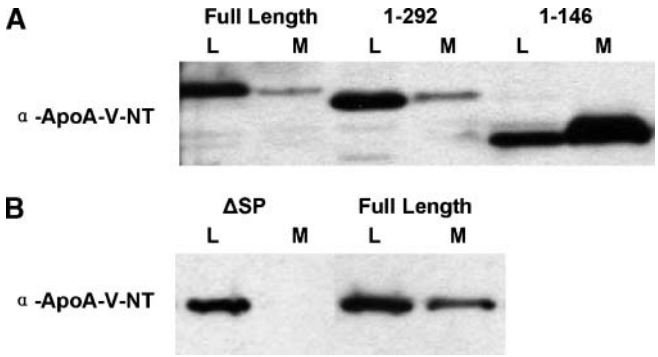


**Fig. 4.** Effect of C-terminal truncation of apoA-V on the secretion efficiency of apoA-V from transfected McA-RH cells. A: Cells were transfected with apoA-V-GFP fusion protein constructs including full-length apoA-V, apoA-V(1-292), and apoA-V(1-146). After 24 h of incubation of cells in serum-free medium, cell lysates (L) and conditioned medium (M) were obtained and equivalent aliquots were applied to gels. B: Comparison of the ΔSP-apoA-V deletion variant with full-length apoA-V, both transfected into cells without the GFP construct. Western blotting was carried out using a polyclonal anti-human apoA-V antibody (α-ApoA-V-NT).

lysate fraction, a much larger proportion of apoA-V(1-146)-GFP was recovered in the medium. ApoA-V(1-146) without the GFP tag showed a similar distribution (data not shown). Unlike the C-terminal truncations, 100% of ΔSP-apoA-V was recovered in the cell lysate (Fig. 4B). In this case, since a GFP tag was not employed, full-length apoA-V lacking a GFP tag was run for comparison and demonstrated that the ratio of protein in cell lysate versus medium was similar to that seen with apoA-V-GFP.

### Association of full-length and truncated apoA-V with lipoproteins in medium

Since transfected apoA-V(1-146) does not associate with intracellular lipid droplets, we sought to determine whether the secreted protein associates with lipoproteins in conditioned medium. Conditioned medium was collected and the $d \leqslant 1.21$ g/ml (lipoprotein-rich) and $d > 1.21$ g/ml (lipoprotein-poor) fractions were harvested. Whereas control full-length apoA-V was recovered in association with lipoproteins, apoA-V(1-146) was exclusively recovered in the lipoprotein-poor fraction (**Fig. 5**). The observation that a portion of the full-length apoA-V expressed in transfected hepatoma cells was recovered in the lipoprotein-poor fraction of conditioned medium seemingly contradicts previous observations that apoA-V was only found associated with lipoproteins in human (14) and mouse (15) plasma and that recombinant apoA-V is not soluble at physiological pH in a lipid-free form (4). In the present case, it is conceivable that apoA-V recovered in the $d > 1.21$ g/ml fraction may contain a small amount of lipid. It is also possible that, owing to the limited lipoprotein secretion efficiency of these cells combined with overexpression of apoA-V, there is insufficient lipid surface area for all of the apoA-V to bind to lipoproteins. To address whether the apoA-V recovered in the $d > 1.21$ g/ml fraction is capable of binding lipoproteins, the apoA-V-containing

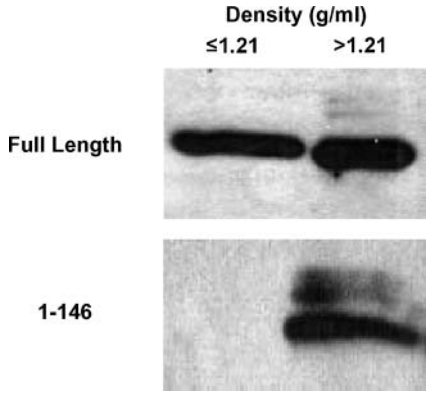


**Fig. 5.** Effect of C-terminal truncation on lipoprotein association in conditioned medium. McA-RH cells were transfected with full-length apoA-V and apoA-V(1-146). After incubation of the cells for 24 h in serum-free medium, conditioned medium was isolated and the lipoprotein-rich ($d \leq 1.21$ g/ml) and lipoprotein-poor ($d > 1.21$ g/ml) fractions were isolated and subjected to SDS-PAGE. Western blotting was carried out using a polyclonal anti-human apoA-V antibody.

lipoprotein-deficient fraction from conditioned medium was incubated with the HDL fraction obtained from *apoa5* knockout mice. Subsequent density ultracentrifugation showed that apoA-V transferred to the HDL particles. When the same experiment was performed with conditioned medium containing apoA-V(1-146), binding to HDL was not observed (data not shown).

## DISCUSSION

Lipid droplets are composed primarily of neutral lipids (TG or cholesteryl ester) and function as a dynamic lipid storage depot (16, 17). The surface coat of lipid droplets is composed of a phospholipid monolayer together with several associated proteins. Whereas lipid droplets are most abundant in adipocytes, they can be found in nearly all cell types, including hepatocytes. Research on the major lipid droplet-associated proteins perilipin, ADRP, and TIP47 (collectively referred to as "PAT" family proteins) has revealed structural similarities with the class of amphipathic apolipoproteins (18). Structural analysis of TIP47 provided evidence for a two-domain structure, and X-ray crystallography of its C-terminal domain revealed a helix bundle molecular architecture similar to the N-terminal four-helix bundle of apoE. Thus, although lipid droplets are of larger diameter (<1 μm in most cells but up to 50 μm in adipocytes) than lipoproteins, both possess a roughly spherical shape, a hydrophobic core, and a surface monolayer of phospholipid. Based on this, it is intriguing to consider that the binding interaction of PAT family proteins with lipid droplets may be analogous to that between apolipoproteins and lipoprotein particles. The helix bundle motifs of apoE and apoA-I are postulated to "open" upon lipid interaction, thereby exposing hydrophobic lipid binding sites in the bundle interior (19, 20). Given the comparable structures of the apoE N-terminal domain and the TIP47 C-terminal domain, a similar conformational adaptation may facilitate its association with the surface of lipid droplets. Apolipoproteins do not normally serve a dual function as lipid droplet- and lipoprotein-associated proteins and vice versa. An exception may be apoO, a recently identified apolipoprotein component of HDL, which is reported to associate with lipid droplets in cardiomyocytes (21). Based on this, it is not unreasonable that apoA-V can associate with cellular lipid droplets.

The observation that apoA-V associates with cytosolic lipid droplets is seemingly contradictory to its expression as a preprotein containing a 23 amino acid signal peptide. Classically, this N-terminal sequence will direct the newly synthesized protein to the endoplasmic reticulum (ER) for transit through the secretory pathway and, ultimately, export from the cell. While apparently not unprecedented (21), little is known about how proteins directed to a secretory pathway might escape this fate. In this case, examination of the mechanism of lipid droplet formation may provide a clue. Lipid droplets form rapidly in response to elevated fatty acid. Droplet formation occurs in discrete regions of the ER, with neutral lipid accumulating between leaflets of the bilayer. Mature lipid droplets are thought to bud from the ER membrane, forming an independent "organelle." Given the proximity of this process to the ER lumen, it is conceivable that lipid surface-seeking proteins (e.g., apoA-V) within this compartment may associate with nascent lipid droplets and migrate with them as they bud from the membrane toward the cytosol. While no definitive connection exists, it is interesting that partial hepatectomy in rats induces lipid droplet formation (22) as well as upregulation of apoA-V mRNA (2). Perhaps apoA-V facilitates lipid droplet formation or metabolism.

In the present study, we show that the lipid droplet association of apoA-V is not contingent upon OA supplementation of hepatocytes but does require the C-terminal portion of the protein. Not only full-length apoA-V but also C-terminal truncation variants, including apoA-V(1-292), apoA-V(1-237), and apoA-V(1-191), retained their ability to associate with lipid droplets following transfection into hepatoma cells. Indeed, loss of lipid droplet homing ability occurred only when the C terminus was truncated to residue 146 (out of 343 amino acids in full-length apoA-V). Furthermore, the similarity of lipid droplet morphology between full-length apoA-V and the apoA-V(1-292) variant suggests that, in the latter case, despite decreased lipid binding activity (7), sufficient amphipathic helix elements remain to effect binding of the protein to lipid droplets. On the other hand, the apoA-V(1-146) variant, in which a putative highly hydrophobic region (residues 171–241) has been deleted, did not associate with intracellular lipid droplets. Although apoA-V(1-146) was efficiently secreted by the cells, it did not associate with lipoproteins, consistent with its inability to associate with lipid droplets. The behavior of this variant is similar to that of a Q139X apoA-V mutant found in humans (8), in which the latter protein was found solely in the lipoprotein-deficient fraction of plasma. Thus, results obtained from studies of apoA-V(1-146)

provide direct evidence that the C-terminal region of apoA-V is necessary for lipoprotein association. At the same time, transfection studies indicate that the absence of the C-terminal sequence does not prevent secretion, again explaining the observation of circulating truncated apoA-V in subjects expressing Q139X apoA-V.

The significant differences in lipid droplet morphology between cells transfected with apoA-V with or without signal peptide suggest that this N-terminal sequence may be critical for normal lipid droplet association. The possibility that the signal peptide on apoA-V is defective can be eliminated on the basis of data with apoA-V(1-146), which contains a signal peptide identical to that present on full-length apoA-V. Results show that the signal peptide functions normally to direct this protein to a secretory pathway. The factors that regulate the cytosolic destination of a portion of the full-length apoA-V in these cells are not known but may be related to the overall hydrophobicity of the protein. In considering the molecular basis for this phenomenon, it is conceivable that the signal peptide-mediated transit of newly synthesized apoA-V to the ER compartment may result in localization of the protein to the site of lipid droplet genesis. Given that lipid droplet assembly occurs in the ER membrane, ultimately budding into the cytosol and pinching off to create a discrete droplet, apoA-V transiting the ER could make contact with the membrane site of droplet formation. In the absence of a signal peptide, apoA-V appears to alter lipid droplet morphology where the latter appears dispersed. Although speculative, owing to its high lipid binding affinity, ΔSP-apoA-V may disrupt the integrity of the lipid droplets. The ΔSP-apoA-V, however, does not appear to be mistargeted to another cell compartment, since its colocalization with ADRP, a major lipid droplet-associated protein in hepatocytes, is unaltered.

In humans, several common *APOA5* polymorphisms are known to be associated with reduced plasma apoA-V and increased plasma TG levels (23). One such polymorphism, cSNP 56C→G (S19W), which is found in the signal peptide domain of the protein, was shown to interfere with the translocation of apoA-V protein (24). In the latter study, substitution of serine-19 by tryptophan reduced the amount of fusion protein secreted into cell culture medium. In the present study, deletion of the signal peptide resulted in an inability of apoA-V to be secreted, suggesting that the signal peptide is required to direct extracellular trafficking of the protein.

In conclusion, the data presented provide indirect evidence for an intracellular mode of action of apoA-V. These findings are in keeping with the very low plasma concentration of this protein, its relatively poor secretion efficiency, and the observation that apoA-V mRNA is upregulated in response to partial hepatectomy. Furthermore, the observation that the C-terminal sequence is necessary for lipid droplet association, as well as lipoprotein association, suggests that this region of the protein may be essential for the manifestation of TG modulation by this protein. Future in vivo studies with truncated apoA-V variants will permit this hypothesis to be tested.

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