TIP47, a Lipid Cargo Protein Involved in Macrophage Triglyceride Metabolism

Insa Buers, Horst Robenek, Stefan Lorkowski, Yvonne Nitschke, Nicholas J. Severs, Oliver Hofnagel

Objective—Uptake of lipids by macrophages (MΦ) leads to lipid droplet accumulation and foam cell formation. The PAT family proteins are implicated in lipid droplet formation, but the precise function of the 47-kDa tail interacting protein (TIP47), a member of this family, is poorly defined. The present study was performed to determine the function of TIP47 in MΦ lipid metabolism.

Methods and Results—Freeze-fracture cytochemistry demonstrates that TIP47 is present in the plasma membrane of MΦ and is aggregated into clusters when the cells are incubated with oleate. Suppression of adipophilin levels using siRNA knockdown leads to migration of TIP47 from a cytoplasmic pool to the lipid droplet. Further, reduction of TIP47 decreases triglyceride levels, whereas raising TIP47 levels by expression of EGFP-TIP47 shows the opposite effect.

Conclusion—Our results show that the TIP47 protein levels directly correlate with triglyceride levels. We propose that TIP47 may act as a carrier protein for free fatty acids and in this way participates in conversion of MΦ into foam cells. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: TIP47 ■ triglyceride metabolism ■ macrophages ■ PAT proteins ■ lipid droplets

The uptake of lipids by macrophages (MΦ) resulting in their conversion to foam cells is a hallmark of atherosclerosis. The accumulation of cholesterol esters in MΦ can be induced in vitro by incubation of the cells with modified low density lipoprotein (LDL), eg, acetylated LDL (acLDL), which is taken up by scavenger receptors. The accumulation of triglycerides in MΦ can be mediated in vitro by incubation with free fatty acids (eg, oleate) or very low density lipoprotein (VLDL). The lipid loading of MΦ with cholesterol esters and triglycerides results in the formation of lipid droplets (LD), a process critical not only to atherosclerosis, but also to diabetes and obesity.1–5 The molecular mechanisms regulating the formation and metabolism of LD are not well understood. Structurally, LD consist of a lipid core surrounded by a phospholipid monolayer, which contains various proteins involved in lipid metabolic processes such as lipolysis or lipogenesis.6–8

The most abundant LD-associated proteins are those of the PAT family (the collective term for perilipin, adipophilin and the tail interacting protein of 47 kDa, TIP47). Perilipin expression is limited to adipocytes, steroirogenic cells, and MΦ9–11; whereas adipophilin and TIP47 are more widely expressed.12,13

The most prominent LD-associated protein in MΦ is adipophilin, found both at the surface of LD and also within the core.14,15 Although adipophilin is widely held not to be present in any other subcellular compartments, our recent studies using high-resolution freeze-fracture electron microscopy combined with immunogold labeling demonstrate that this protein is also expressed in the plasma membrane and endoplasmic reticulum.16,17 Sequence analysis and functional studies reveal that adipophilin is able to bind free fatty acids18 and cholesterol.19

TIP47 was originally described as a cytosolic protein which binds to the cytoplasmic domains of the cation-dependent and cation-independent mannose 6-phosphate receptors.20 Subsequent studies reported TIP47 at the LD surface of HeLa cells21 and other cell types.14,15,22 Our recent studies revealed that TIP47 is not restricted to cytosolic compartments but is also localized in the LD core and in the plasma membrane of THP-1 MΦ in a similar manner to that found with adipophilin.14–16 The highly conserved C-terminal PAT domain of TIP47 and adipophilin suggests that both proteins share a common lipid binding site.23 This lipid binding site consists of a 4 helix bundle which forms, together with αβ sheets, a hydrophobic cleft that is suggested to bind fatty acids.

The function of adipophilin and TIP47 in foam cell formation is only poorly understood. Lipid loading of human monocytes24 or MΦ25 results in an increased adipophilin expression. Overexpression of adipophilin increased lipid accumulation5; conversely, adipophilin knockdown de-
creased the lipid content of THP-1 MΦ.5 Paul et al.26 revealed that MΦ of ApoE−/−/adipophilin−/− mice exhibit an impaired ability to accumulate intracellular LD and that the lack of adipophilin in these mice protects against atherosclerosis.

In contrast to adipophilin, little is known regarding TIP47 in MΦ lipid metabolism and foam cell formation. The present study therefore set out to investigate the function of TIP47 on THP-1 MΦ lipid metabolism.

Methods

Cell Culture

Human monocyteic THP-1 cells were cultured in RPMI 1640 medium containing the supplements recommended by Schnoor et al.27 THP-1 monocytes differentiated to adherent MΦ by addition of 100 μmol/L phorbol 12-myristate 13-acetate (Sigma-Aldrich) to the medium for 72 hours.

Preparation of Lipoproteins

VLDL (d<1.006 g/mL) was isolated from human plasma of individual normolipemic volunteers by sequential ultracentrifugation in a Beckmann L7-65 ultracentrifuge using a 70 Ti rotor operated at 59 000 rpm at 4°C for 24 hours. The top fraction was dialyzed at 4°C for 72 hours in 0.15 mol/L NaCl containing 0.3 mmol/L EDTA (pH 7.4). Oleate and acLDL was prepared as described previously.15

Antibodies

Antibodies were purchased from Progen Biotechnik, abcam, Dianova, and Jackson Immunoresearch. For details, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde, blocked, permeabilized, fluorescently labeled with specific antibodies, and examined using a laser-scanning confocal microscope LSM510 (Zeiss). For details please see the supplemental materials.

Freeze-Fracture Replication

Freeze-fracture replication, nomenclature, and interpretation of freeze-fracture replica immunolabeling data were as described by Robenek et al.14–17 For details please see the supplemental materials.

Plasmid Generation and siRNA Construction

Full-length TIP47 sequence and 4 truncation mutations were cloned into pEGFP-C1 expression vector (Clontech Laboratories). TIP47 specific siRNA and the negative control were cloned into pEGFP-C1 expression vector (Clontech Laboratories). For details please see the supplemental materials.

Transfection

THP-1 MΦ were transfected using the nucleofection method of amaxa. For details please see the supplemental materials.

Real-Time RT-PCR

Isolation of RNA and synthesis of cDNA were performed as described by Schnoor et al.27 We performed real-time RT-PCR using the ABI PRISM 7900HT sequence detection system (Applied Biosystems) and the QuantiTect SYBR Green PCR kit (Qiagen). For details please see the supplemental materials.

LD Isolation

Transfected THP-1 MΦ were scraped in 40% iodixanol buffer (Progen Biotechnik). Cells were homogenized by sonication and then transferred to the bottom of a centrifuge tube and overlaid with 30% and 20% iodixanol buffer. LD were isolated by using density gradient centrifugation. For details please see the supplemental materials.

Figure 1. Immunolabeled freeze-fraction replica demonstrating endogenous TIP47 in the P-face (PF) of the plasma membrane (PM) of THP-1 MΦ. TIP47 clusters after oleate incubation on elevated membrane domains. Fractures that penetrate beneath these domains demonstrate that LD lie immediately below. Bar: 0.5 μm.

SDS-PAGE and Western Blot Analysis

Transected THP-1 MΦ were scraped in HIDE buffer. 10 μg of total protein per lane was loaded on 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore). Membranes were labeled with specific primary and secondary antibodies. For details please see the supplemental materials.

Measurement of Cholesterol and Triglyceride Content

Triglyceride and total cholesterol content were measured using the GPO/PAP and CHOD/PAP method (ProDiagnostica) according to the manufacturer’s instructions. Protein content of the lysate was measured by using the Bradford protein assay (Bio-Rad). For details please see the supplemental materials.

Results

TIP47 Clusters in Specific Plasma Membrane Domains After Oleate Incubation

Freeze-fracture replica immunogold labeling demonstrated that TIP47 is present in the plasma membrane of THP-1 MΦ (Figure 1). The TIP47 label was widely dispersed in the plasma membrane under normal culture conditions (data not shown), but lipid loading with oleate for 24 hours resulted in its clustering in discrete raised plasma membrane domains. Fractures penetrating beneath the plasma membrane demonstrate that LD are closely apposed to the raised TIP47 protein-enriched domains (Figure 1).

TIP47 Localizes to LD After Adipophilin Knockdown

The close association between the TIP47 plasma membrane clusters and underlying LD suggests a specialized function...
for these membrane domains in MΦ lipid metabolism. To explore this possibility we applied siRNA-induced knockdown of TIP47 and adipophilin. Transient transfection of TIP47 siRNA resulted in a 60% reduction of TIP47 protein expression with no significant alteration in the level of adipophilin (supplemental Figure IA). The TIP47 mRNA level was correspondingly reduced by 80% and the adipophilin mRNA level unchanged (supplemental Figure IB). Transfection of adipophilin siRNA knocked down adipophilin protein concentration by 55% and transcript levels by 80% without affecting TIP47 protein or transcript concentrations (supplemental Figure IA and IB).

We then investigated the PAT protein distribution pattern of MΦ after transfection of adipophilin and TIP47 siRNA. Forty-eight hours after transfection with adipophilin siRNA, THP-1 MΦ were incubated for 24 hours with acLDL or oleate. Immunofluorescence microscopy revealed that perilipin was undetectable at the LD surface in the knockdown cells. TIP47 labeling in untransfected control cells was mostly in the cytoplasm and adipophilin exclusively at the LD surface (Figure 2A through 2C). After acLDL incubation of adipophilin suppressed cells, however, a remarkable redistribution of TIP47 to the LD was apparent (Figure 2D through 2F, arrows).

As with the nontransfected acLDL loaded cells, nontransfected cells incubated with oleate showed adipophilin prominently at the LD and TIP47 diffusely localized in the cytoplasm with only occasional instances of colocalization of the proteins at the LD surface (Figure 2G through 2I, arrows). Oleate incubation of adipophilin suppressed cells led to a marked redistribution of TIP47 to the LD surface (Figure 2J through 2L, arrows) similar to but more pronounced than that observed in the corresponding acLDL adipophilin knockdown cells.

To verify these results, we isolated LD from control and siRNA transfected cells after incubation with acLDL, VLDL, and oleate. In accordance with the immunofluorescence findings, the TIP47 protein level was slightly increased in
suppressed samples, but the magnitude of the reduction after TIP47 suppression was not altered by triacsin C (Figure 3A).

AcLDL incubation resulted in strongly increased cholesterol levels compared to unloaded cells. The cholesterol level of adiopophilin knockdown cells was significantly decreased in unloaded and lipid loaded cells, whereas that of TIP47 knockdown cells was unaffected in acLDL treated cells (Figure 3B). Incubation with VLDL or oleate had no influence on the cholesterol content compared to control cells. Triacsin C had no effect on the cholesterol level.

Suppression of TIP47 Does Not Influence aP2 or mal1

The altered triglyceride content after TIP47 or adiopophilin suppression suggests that TIP47 is able to transport fatty acids to LD. To exclude the possibility that the altered triglyceride content could be attributable to regulation of other fatty acid binding proteins (FABP) such as aP2 or mal1, we investigated the protein level of both these proteins after suppression of TIP47 or adiopophilin. Western blot analysis showed that alterations in TIP47 or adiopophilin protein levels do not influence aP2 or mal1 in the presence or absence of oleate or VLDL (supplemental Figure IV).

Effects of Enhancing TIP47 Levels Using Full-Length and Truncated EGFP-TIP47 Vectors

To verify further the relationship between TIP47 expression and triglyceride levels as deduced from the siRNA knockdown, we determined the effects of expression of full-length and truncated fragments of TIP47 (Figure 4A) in MΦ. Western blot analysis (supplemental Figure III) demonstrated expression of EGFP-TIP47 and the truncated fragments of

Figure 3. Effect of adiopophilin and TIP47 knockdown on (A) triglyceride and (B) cholesterol levels. Transfected THP-1 MΦ were incubated with acLDL, VLDL, and oleate in the presence or absence of triacsin C. Values for each bar are means ± SEM, n=4; *P<0.05 vs control siRNA cells.

The altered localization of TIP47 observed after adiopophilin knockdown or lipid loading raises the possibility that TIP47 plays a role in lipid storage in MΦ. We therefore analyzed the triglyceride and cholesterol content of unloaded and lipid-loaded control and siRNA transfected cells (Figure 3). Knockdown of adiopophilin and TIP47 in unloaded cells significantly decreased triglyceride content (Figure 3A). Incubation with acLDL did not influence the triglyceride content of control cells, but suppression of TIP47 or adiopophilin expression slightly reduced the triglyceride content. Supplementation of acLDL with triacsin C, an inhibitor of the acyl-coenzyme A synthetase which esterifies free fatty acids with coenzyme A, did not enhance this effect. Both VLDL and oleate incubation resulted in strongly increased triglyceride levels compared to unloaded cells. Triglyceride levels in adiopophilin-suppressed cells were enhanced after VLDL and oleate incubation. Knockdown of TIP47 resulted in a significantly lower triglyceride content after VLDL or oleate incubation. Incubation with triacsin C abrogated partly the VLDL or oleate-induced triglyceride increase. When triacsin C was added to VLDL or oleate incubated cells, the overall levels of triglyceride were reduced in controls and TIP47-

Figure 4. Expression of EGFP-TIP47 constructs in THP-1 MΦ. A, Diagram of the structure of the constructs. B, Triglyceride content of cells expressing EGFP-TIP47, EGFP-TIP47-aa1-416, or EGFP-TIP47-aa198-434 is enhanced compared to control cells. Values for each bar are means ± SEM, n=4; *P<0.05 vs control cells.
TIP47. The additional expression of EGFP-TIP47, EGFP-TIP47-aa1-416, or EGFP-TIP47-aa198-434 had the opposite effect to that obtained by siRNA knockdown of TIP47, ie, triglyceride levels were elevated rather than depressed. This effect was found in both VLDL and oleate incubated cells (Figure 4B). No effect was found after acLDL incubation (data not shown). Triacsin C supplementation resulted in decreased triglyceride levels of all treatments. EGFP-TIP47, EGFP-TIP47-aa1-416, or EGFP-TIP47-aa198-434 expression led to enhanced triglyceride levels after VLDL and oleate incubation, even in the presence of triacsin C compared to the control (Figure 4B). In contrast to this, the expression of EGFP-TIP47-aa1-198 and EGFP-TIP47-aa1-248 had no effect on the triglyceride level compared to EGFP-control cells. Similar to the siRNA knockdown experiments, the cholesterol content was not altered when overall levels of TIP47 were increased by expression of EGFP-TIP47 and the truncated TIP47 fragments.

TIP47 Colocalizes With DGAT at the LD Surface
If TIP47 is able to transport fatty acids to lipid droplets, then these fatty acids would be incorporated into triglycerides for storage. We therefore investigated whether acyl-coenzyme A:diacylglycerol acyl-transferase (DGAT), the enzyme which catalyzes the terminal step in triglyceride synthesis, is localized at the LD surface. Immunofluorescence microscopy showed that, after adipophilin suppression, DGAT is indeed localized at the LD surface as is TIP47 (Figure 5A through 5C). Freeze-fracture replica immunogold labeling confirmed the colocalization of TIP47 and the DGAT at the LD monolayer (Figure 5D and 5E).

Discussion
A novel key finding of the present study is that TIP47 is actively redistributed from the cytosolic compartment to the LD to replace adipophilin when the latter is removed by siRNA knockdown. This is linked to an enhanced cellular triglyceride level after oleate and VLDL incubation. When TIP47 expression is suppressed, triglyceride levels decrease; conversely, when TIP47 levels are increased by expression of EGFP-TIP47 alongside endogenous TIP47, the triglyceride content is increased. These findings shed new light on the relevance of TIP47 in MΦ lipid accumulation, in particular by pointing to a role for this protein in triglyceride metabolism.

Decreased expression of one PAT family protein can in some instances lead to increased expression and functional substitution by another, as reported with adipophilin and perilipin. It might, therefore, be predicted that TIP47 expression would increase in adipophilin knockdown cells. Recent studies have indeed reported increased TIP47 expres-
sion in fibroblasts of adipophilin knockout mice and that TIP47 may substitute for adipophilin at the LD surface of these cells.28 However, peritoneal ΜΦ of these mice show no alteration in TIP47 mRNA and protein expression, raising the possibility that other LD binding proteins may compensate for the lack of adipophilin.26

Our findings show that when adipophilin is lacking, despite no change in TIP47 level, a major redistribution of TIP47 occurs to the normal site of adipophilin, the LD surface. The fact that TIP47 mRNA and protein level did not increase in ΜΦ after adipophilin suppression may be explained by the ability of TIP47, unlike other ΕΑΤ family proteins, to exist in a soluble cytosolic form.26,29,30 The ability to bind to the LD surface arises from the crystalline structure of TIP47 which reveals 4 amphipathic helices23 that form a hydrophilic bundle similar to that of lipid-free apolipoprotein E. During lipid binding, the bundle of lipid-free apolipoprotein E opens to interact with the surface phospholipid monolayer of lipoproteins,31,32 and similar properties may permit TIP47 to bind to the LD monolayer.30 New synthesis of TIP47 in response to reduced adipophilin may not be necessary in view of the substantial cytosolic pool of TIP47 that is available immediately for recruitment to the LD. The dramatic nature of such recruitment in response to the reduction of adipophilin expression is clearly demonstrated by our immunofluorescence results and confirmed by Western blot of isolated LD.

From our localization findings, it might be predicted that TIP47 compensates functionally for adipophilin in ΜΦ cholesterol or triglyceride metabolism. To investigate this possibility, we examined total cholesterol and triglyceride contents in cells with suppressed adipophilin and TIP47 levels. Larigauderie and coworkers5 have previously shown that overexpression of adipophilin in the presence of acLDL leads to a higher cholesterol level in THP-1 ΜΦ and that suppression of adipophilin in the presence of acLDL conversely decreased the cholesterol content. We analyzed the influence of cholesterol ester-ricer acLDL, triglyceride-rich VLDL, and oleate incubation on THP-1 ΜΦ with suppressed adipophilin and TIP47 levels. In accord with Larigauderie et al,5 our data show that adipophilin suppression decreases the total cholesterol content. Thus, the presence of LD TIP47 fails to prevent the effect arising from lack of adipophilin. Our data show that neither loss nor enhancement of TIP47 alters the cholesterol content. Thus, TIP47 is unable to compensate functionally for adipophilin in THP-1 ΜΦ.

Previous studies have reported that oleate incubation increases TIP47 protein concentration in HeLa cells.13 Others showed that TIP47 is rapidly recruited from the cytosol to nascent LD when cells are provided with fatty acid,30 again raising the possibility that TIP47 might influence the triglyceride metabolism of ΜΦ. In contrast to the cholesterol level, our experiments showed that the triglyceride level was clearly influenced by TIP47 in THP-1 ΜΦ. Our findings show that translocation of TIP47 from the cytosol to LD in adipophilin-suppressed cells treated with oleate or VLDL is also associated with markedly enhanced triglyceride levels. This positive correlation between TIP47 and triglyceride levels was apparent irrespective of whether TIP47 expression was suppressed or enhanced. This finding stands in contrast to that of Larigauderie et al,25 who reported that adipophilin suppression results in decreased triglyceride levels after VLDL incubation. The reasons for this discrepancy are unclear, but one possibility is that it might be related to the use of different transfection methods. Whereas Larigauderie et al used a standard liposome-based transfection method, we applied the nucleofection method with a protocol that gives much higher transfection efficiency and excellent cell survival compared with established techniques, which we developed specifically for use with THP-1 cells.

To exclude that changes in triglyceride content arise from the regulation of other FABPs in adipophilin or TIP47 knockdown cells, we examined aP2 and mal1 content in the THP1 ΜΦ. These two proteins are known to be involved in ΜΦ foam cell formation and atherogenesis.33 Our findings showed that neither adipophilin suppression nor suppression of TIP47 influence the levels of aP2 and mal1. We thus conclude that neither aP2 nor mal1 are responsible for the observed differences in the triglyceride content after TIP47 or adipophilin suppression.

Because previous studies showed that adipophilin is able to bind free fatty acids18 and TIP47 exhibits high sequence homology to adipophilin, we examined which part of TIP47 is able to bind fatty acids. Our results showed that, apart from full length TIP47, only the TIP47 fragments containing the 4 helix domain were able to enhance the triglyceride content. This is in accordance with analysis of the crystal structure of TIP47 which showed that the α/β-sheets and the 4 amphipathic helices of TIP47 build a hydrophobic cleft which is able to bind monomers. This hydrophobic cleft appears to facilitate the binding of free fatty acids.

Having shown that the TIP47 redistribution in response to loss of adipophilin is implicated in triglyceride rather than cholesterol metabolism, our next step was to explore the possible mechanism by applying triacsin C to inhibit acyl-coenzyme A synthetase, a key enzyme of triglyceride synthesis. This enzyme esterifies long chain fatty acids with coenzyme A as they are incorporated into triglycerides. Our findings showed that TIP47 suppression results in decreased triglyceride levels. The magnitude of this effect was not enhanced by addition of triacsin C, indicating that TIP47 influences the triglyceride level at the step of acyl-coenzyme A synthetase-mediated triglyceride synthesis. Expression of full length-TIP47 as well as TIP47 fragments containing the 4 helix domain leads to an enhanced triglyceride level. This increase was not influenced by triacsin C treatment. Our findings are consistent with the idea that TIP47 may transport free fatty acids activated by acyl-coenzyme A synthetase from the cytosolic side of the plasma membrane or from the cytoplasm to LD.

If TIP47 is able to transport free fatty acids to LD, the fatty acids would have to be incorporated into triglycerides for long-term storage. This would imply that the LD monolayer contains enzymes for triglyceride metabolism. Our results revealed that the DGAT, which catalyzes the terminal step in triglyceride synthesis, colocalizes with TIP47 in the LD monolayer. Our data are thus consistent with the notion that TIP47 may transport free fatty acids to LD, and at the LD surface the activated free fatty acids are incorporated into triglycerides by DGAT.

Finally, given our conclusion that TIP47 may substitute for the lack of adipophilin in triglyceride metabolism, what is the significance of the altered distribution of this protein found in the plasma membrane of ΜΦ on oleate incubation? We
propose that the clustering of TIP47 in the plasma membrane may enhance the direct transport of activated free fatty acids from the plasma membrane to LD. We speculate that TIP47 is able to bind activated free fatty acids at the cytoplasmic monolayer of the plasma membrane or in the cytoplasm and transports them to LD. Opening of the 4 helix bundles may facilitate TIP47 binding at the LD surface. The integration of TIP47 in the LD monolayer may promote the transfer of free fatty acids and their incorporation into triglycerides by DGAT. After releasing the lipids, TIP47 may dissociate from the LD and become available for new lipid binding. We thus envisage TIP47 as stabilizing the triglyceride content of MΦ by acting as a lipid cargo protein. It is in this manner that we suggest that TIP47 may promote MΦ-derived foam cell formation and act as a player in the pathogenesis of atherosclerosis.

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Disclosures

None.

References

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Supplement material

TIP47, a lipid cargo protein involved in macrophage triglyceride metabolism

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Methods

Antibodies

A polyclonal antibody raised in guinea pig against a synthetic polypeptide corresponding to the N-terminus (amino acids 1-16) of human TIP47 (GP30; Progen Biotechnik) was used to detect TIP47. Adipophilin was detected using a mouse monoclonal antibody against a synthetic peptide representing the N-terminus (amino acids 5-27) of human adipophilin (AP125; Progen Biotechnik). A polyclonal antibody raised in guinea pig against a synthetic peptide corresponding to the N-terminal region of human perilipin, which is shared by all known perilipin isoforms, was used to detect “pan”-perilipin (GP29, Progen Biotechnik). Acyl-Coenzyme A:diacylglycerol acyl-transferase (DGAT) was detected using a goat polyclonal antibody against a synthetic polypeptide corresponding to the internal sequence (amino acids 305-317) of human DGAT (ab59034; abcam). EGFP was immunolabeled using a rabbit polyclonal antibody raised against the entire sequence of GFP (ab290, abcam). A polyclonal antibody raised in rabbit was used to detect full human full length aP2 (ab37267, abcam). Human mal1 was detected using a mouse monoclonal antibody against the amino acids 1-133 (ab54617, abcam). For fluorescence microscopy anti-guinea pig, anti-mouse or
anti-goat Cy3-conjugated or Cy2-conjugated secondary antibodies were used (Dianova). The secondary antibodies used for electron microscopy were donkey anti-guinea pig or donkey anti-goat coupled to 12 nm or 18 nm colloidal gold (Jackson Immunoresearch). For Western blot analysis peroxidase-conjugated anti-guinea pig, anti-mouse or anti-rabbit antibodies (Dianova) were used.

**Immunofluorescence microscopy**

THP-1 MΦ transfected with EGFP-TIP47 vector, TIP47 siRNA or adipophilin siRNA and incubated with 50 µg/ml acLDL or 150 mM oleate/BSA were washed twice with PBS and fixed with 4% paraformaldehyde. After extensive washing the cells were incubated for 1 h in PBS containing 1% BSA to block nonspecific binding, and 0.05% Tween 20 for permeabilization. Cells were labeled using guinea pig anti-TIP47 (Progen Biotechnik), guinea pig anti-perilipin (Progen Biotechnik) and mouse anti-adipophilin (Progen Biotechnik) antibodies for 1 h, followed by incubation with anti-guinea pig Cy3-conjugated or anti-mouse Cy2-conjugated secondary antibodies for 1 h. The preparations were mounted in fluorescence mounting medium (Dako) and examined in a confocal laser-scanning microscope LSM510 (Zeiss).

**Freeze-fracture replication**

THP-1 MΦ incubated with 150 mM oleic acid or transfected with adipophilin siRNA were scraped from the culture vessels, centrifuged to remove excess medium, re-centrifuged briefly in 30% glycerol for 30 sec, frozen in Freon 22 cooled with liquid nitrogen, and freeze-fractured in a BA310 freeze-fracture unit (BAL-TEC AG) at -100°C. Replicas of the freshly fractured cells were immediately made by electron beam evaporation of platinum-carbon and carbon at angles of 38° and 90° and to thicknesses of 2 and 20 nm, respectively. Replicas were incubated overnight in 5% SDS to remove cellular material except for those molecules
adhering directly to the replicas. Replicas were then washed in distilled water and incubated briefly in 5% BSA before immunolabeling.

*Freeze-fracture nomenclature and interpretation of freeze-fracture replica immunolabeling data*

When frozen membranes are fractured, the fracture preferentially splits membranes into their two constituent half-membrane leaflets along a plane between the hydrophobic tails of the phospholipids in the bilayer. In the case of the plasma membrane, one leaflet remains attached to the extracellular space (E-half), whereas the other leaflet remains attached to the cytoplasm or protoplasm (P-half). The view of the E-half revealed by freeze-fracturing is termed the E-face, and that of the P-half is termed the P-face. A corresponding nomenclature is applied to intracellular membranes. The fracture faces of the cytoplasmic leaflets of the ER and nuclear membranes are designated as P-face views, and those of the endoplasmic leaflets adjacent to the ER lumen and perinuclear space are the complementary E-face views. In this way, a consistent terminology describes structurally and functionally equivalent portions of the different membrane systems of the cell. The envelope surrounding the lipid droplets presents a special case, because it is not part of a classic bilayer, but a phospholipid monolayer apposed against the lipids of the core. The fracture plane often exposes the interface between the hydrophobic aspect of the monolayer and the core. The fracture face of the monolayer revealed in concavely fractured lipid droplets is considered to be the P-face view, and the complementary aspect seen in convexly fractured lipid droplets (which actually represents a view of the outermost layer of the neutral lipid core) is the E-face equivalent. Correct identification of the fracture faces of membranes relies on multiple cues, such as membrane curvature, specific structural features of the membrane type or fracture face, the distribution of metal evaporation, and labeling specificity. Rather than removing all cellular material from the replicas with strong oxidants (the standard procedure in freeze-fracture replica
preparation), replicas for immunolabeling are washed with SDS. This preserves molecules adhering directly to the replicas, while the remaining cellular material is flushed away. Integral membrane proteins may than be labeled using immunocytochemical techniques. Labeling of the SDS-treated freeze-fracture replicas was carried out using the primary antibodies against TIP47 and DGAT described above followed by matching secondary antibodies coupled to 12 nm or 18 nm colloidal gold. Viewed in the electron microscope, the electron-dense gold particles clearly mark the positions of these proteins, superimposed upon the en face views of the membranes or lipid droplet phospholipid monolayers.

Plasmid generation and siRNA construction

Human TIP47 ORF cDNA was amplified by polymerase chain reaction from THP-1 MΦ using primers corresponding to the 5’ and 3’ ends of TIP47 ORF to which XhoI (5’-end) and BamHI (3’-end) cutting sites were attached. The amplified TIP47 sequence was cloned into the pEGFP-C1 expression vector (Clontech Laboratories). Four additional truncation mutations of TIP47 (EGFP-TIP47-aa1-198, EGFP-TIP47-aa1-248, EGFP-TIP47-aa1-416, EGFP-TIP47-aa198-434) were prepared as well as human full length TIP47. Primer sequences used for TIP47 cDNA amplification are described in table 1. The sequences of the inserts were verified using 5’-TGCATTCATTTTATGTTTCAGGTTCA-3’ and 5’-CGATCACATGGTCCTGCTGG-3’ sequencing primers and the BD Terminator Cycle Sequencing kit 3.1 (Applied Biosystems). The sequences were determined with a capillary electrophoresis sequencer (Applied Biosystems) and then analyzed with SeqMan from the DNASTAR software package (DNASTAR).

The sequence-specific siRNA directed against human TIP47 was designed by Qiagen. Human adipophilin-specific siRNA was obtained from Invitrogen. Among three siRNAs for target genes TIP47 or adipophilin, the sequences specific for adipophilin (sense: 5’-
CCUACCUGAGUCUGUGUGAGAU-3' and antisense: 5'-
AUCUCACACACAGACUUCAGGUAGG-3') and TIP47 (sense: 5'-
GUUCUCCCCUGCAGAAUUU-3' and antisense: 5'-AAAUCUGCAGGGGAAC-3')
were selected based upon their efficiency to specifically inhibit target gene expression. An unmodified siRNA pre-designed by Qiagen with the following sequence (sense: 5'-
UUCUCCGAACGUGUCACGUdTDdT -3' and antisense: 5'-
ACGUGACACGUUCCGGAGAAdTdT -3') was used as a negative control.

Transfection

Two days before transfection, cells were plated in 75 cm² culture flasks at a density of 1.5×10⁷ cells. Detached THP-1 MФ were transfected using the nucleofection method of Amaxa. The transfection efficiency using 0.5 µg of vector DNA or 1 µg siRNA was approximately 50% or 90%, respectively. LD formation in THP-1 MФ was induced by incubation with 50 µg/ml acLDL, 50 µg/ml VLDL or 150 mM oleate complexed to bovine serum albumin (BSA) for 24 h.

Real-time RT-PCR

Isolation of RNA and synthesis of cDNA were performed as described by Schnoor et al.. We performed real-time RT-PCR using the ABI PRISM 7900HT sequence detection system (Applied Biosystems) and the QuantiTect SYBR Green PCR kit (Qiagen). Primers were obtained from Invitrogen and the primer sequences were as follows: TIP47 forward, 5'-GGCCCTAAGCCTGATGGAAA-3'; TIP47 reverse, 5'-CTGGCCTTCCACCAGCTTCT-3'; adipophilin forward, 5'-CTGATGAGTCCCACTGTGCTGA-3'; adipophilin reverse, 5'-TGTGGCACGTGGTCTGGAG-3'; SRP14 forward, 5'-AGCACTGTGGTGAGCTCCAAG-3'; SRP14 reverse, 5'-TCAGCCCATCCATGTAGCTCTA-3'; GAPDH forward, 5'-
CAACAGCGACACCCACTCCT-3’; GAPDH reverse, 5’-CACCCTGGTGCTGTAAGCCAAA-3’. Cycling parameters and analyses were performed as described previously.1

**Lipid droplet isolation**

THP-1 MΦ transfected with TIP47 siRNA or adipophilin siRNA were washed twice with PBS and scraped into 40% iodixanol buffer (Progen Biotechnik) containing 30 mM Tricine. Cells were homogenized by sonication and then transferred to the bottom of a centrifuge tube and overlaid with 30% and 20% iodixanol buffer. After centrifugation at 10,000 x g for 60 min the LD fraction was transferred to acetone (1:6) for protein precipitation. The precipitate of the LD was centrifuged for 10 min at 10,000 x g and dissolved in HIDE lysis buffer (0.5% Nonidet P40, 50 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 mM EDTA, and 50 mM NaF) containing protease inhibitor cocktail from Boehringer. Lysates were used for Western blot analysis.

**SDS-PAGE and Western blot analysis**

THP-1 MΦ transfected with TIP47 or adipophilin siRNA or transfected with full length EGFP-TIP47 or truncated mutations of EGFP-TIP47 were washed twice with PBS and lysed in HIDE buffer. 10 µg of total protein per lane was loaded on 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore). Membranes were incubated with guinea pig anti-human TIP47 (Progen Biotechnik) or mouse anti-human adipophilin (Progen Biotechnik) antibodies followed by incubation with peroxidase conjugated anti-guinea pig or anti-mouse antibodies (Dianova). Densitometric analysis was performed to measure the amount of TIP47 and adipophilin protein. The TIP47 and adipophilin protein per lane was normalized to the total protein amount determined by Ponceau S staining.
Measurement of cholesterol and triglyceride content

48 h after transfection 2.5 µM triacsin C (Biomol) was added to the medium 4 h before lipid loading. MΦ transfected with TIP47 and adipophilin siRNA as well as MΦ transfected with EGFP-TIP47 or truncated mutations of EGFP-TIP47 were incubated with 50 µg/ml acLDL, 50 µg/ml VLDL or 150 mM oleate. The cells were washed twice with PBS and scraped in 400 µl 0.9% NaCl. After sonication the protein content of the lysate was measured by using the Bradford protein assay (Bio-Rad). Triglyceride and total cholesterol content were measured using the GPO/PAP and CHOD/PAP method (ProDiagnostica) according to the manufacturer’s instructions.
Results

Figure I

**Figure I.** Expression of adipophilin and TIP47 protein and mRNA in siRNA transfected cells. THP-1 МΦ were transfected with adipophilin- or TIP47-specific siRNA. 72h after transfection, total protein and RNA were isolated. A) Western blot analysis of adipophilin and TIP47 in THP-1 МΦ. Densitometric analysis showed that knockdown of TIP47 or adipophilin results in 60% decreased TIP47 or 55% decreased adipophilin protein level (n=5, p < 0.01). B) RNA levels of adipophilin and TIP47 mRNA were quantified using real-time RT-PCR. The differences between adipophilin or TIP47 knockdown cells and control cells was significant at p < 0.01, n=6.
Figure II

A. Western blot analysis showing the expression of TIP47 and adipophilin proteins. The gel is loaded with unloaded, acLDL, VLDL, and oleate samples, with control and adipophilin siRNA treatments.

B. Bar graph showing the TIP47 protein concentration as a percentage of control for unloaded, acLDL, VLDL, and oleate samples with control siRNA, adipophilin siRNA, and TIP47 siRNA treatments.

C. Bar graph showing the adipophilin protein concentration as a percentage of control for unloaded, acLDL, VLDL, and oleate samples with control siRNA, adipophilin siRNA, and TIP47 siRNA treatments.
**Figure II.** TIP47 and adipophilin protein expression in TIP47 or adipophilin suppressed THP-1 MΦ after incubation with acLDL, VLDL, or oleate for 24 hours. Suppression of adipophilin leads to enhanced TIP47 protein levels after VLDL and oleate incubation (A, B). Unloaded cells or treatment with acLDL had no effect on TIP47 protein levels after adipophilin knock down (A, B). Suppression of TIP47 did not influence the adipophilin protein expression (A, C). Suppression of TIP47 in the presence of oleate and VLDL is lower compared to unloaded or acLDL loaded cells (A). This might be due to a higher affinity of TIP47 to triglyceride rich lipid droplets and a subsequent reduced turn-over rate of TIP47 protein. Values for each bar are means SEM from 3 experiments (*P_0.05 versus control).

**Figure III**

![Western blot analysis using a rabbit anti-GFP antibody showed the expression of full length and truncated EGFP-TIP47 proteins.](image)

**Figure III.** Western blot analysis using a rabbit anti-GFP antibody showed the expression of full length and truncated EGFP-TIP47 proteins.
**Figure IV.** Expression of aP2 and mal1 protein in TIP47 or adipophilin siRNA transfected THP-1 cells. Suppression of TIP47 or adipophilin has no influence on the protein level of aP2 or mal1 in the presence or absence of oleate or VLDL.

**Table 1.** Primers used for cloning full length and truncated TIP47. XhoI and BamHI cloning sites are underlied.

<table>
<thead>
<tr>
<th>Primer specificity</th>
<th>Sequence 5’ → 3’ forward</th>
<th>Sequence 5’ → 3’ reverse</th>
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<tr>
<td>TIP47 full length</td>
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<td>CTCTCGGATCCCCATCTTCTCTCCTCCG</td>
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References: