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Spatial Integration of TIP47 and Adipophilin in Macrophage Lipid Bodies*

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We studied the distribution of the PAT family proteins TIP47 and adipophilin in lipid bodies of THP-1 cell-derived macrophages using freeze-fracture immunolabeling and other techniques. Lipid bodies in macrophages comprise lipid droplets and extensive, previously scantily characterized sheet-like organelles, which we descriptively call "lipid sails." TIP47 and adipophilin are components of many, but not all, the lipid droplets. Both proteins are not confined to the surface of lipid droplets, as supposed, but are also inside lipid droplet cores. They are not codistributed stoichiometrically in lipid droplets. How TIP47 and adipophilin, which are polar proteins, enter the lipid droplets and are packaged among the hydrophobic neutral lipids of the core is unclear. However, in the lipid layers of the core, these proteins are directed sometimes inward and sometimes outward. Because TIP47 and adipophilin also localize to lipid sails, lipid sails are intimately involved in intracellular lipid metabolism.

An entirely new view of the functions and composition of lipid bodies is currently emerging from studies on proteins associated with these organelles. Lipid bodies are clearly more than mere storage depots for superfluous neutral lipids in times of metabolic stress (1). Aside from their obvious involvement in lipid homeostasis, they appear also to partake exquisitely in cell signaling (2, 3), intracellular vesicle trafficking (3, 4), and disease (5, 6). Lipid bodies are metabolically active organelles in cells of virtually all animals, many plants, and even some prokaryotes (7, 8).

Several proteins with similar amino-terminal amino acid sequences and hence common structural motifs, the PAT (perilipin, adipophilin, tail-interacting protein of 47 kDa) family proteins, interact with lipid bodies in as yet unknown ways. PAT family proteins are believed to localize exclusively to the surface of lipid bodies and to be evolutionarily conserved (9–12). Similarities in the structure of PAT family proteins and their targeting to and localization in lipid bodies are suggestive of a common underlying function in lipid homeostasis and/or lipid body biogenesis.

Among the members of the PAT family proteins, the tail-interacting protein of 47 kDa (TIP47) is still of uncertain status. TIP47 seems to be involved in delivering mannose-6-phosphate receptors from endosomes to the trans-Golgi network (13, 14) and, at the same time, to be a component of the surface of lipid bodies (10, 15). These two cellular compartments, lipid bodies and the late endosome sorting compartment, are currently thought to be completely divorced.

Several controversial reports on the putative association of TIP47 with lipid droplets, the most common form of lipid body in eukaryotic cells, have appeared recently. Wolins *et al.* (15) described TIP47 in lipid droplets, but Barbero *et al.* (16) explicitly refuted this result by demonstrating that the antibody used by Wolins *et al.* (15) cross-reacts with a confirmed lipid droplet protein, adipophilin. Miura *et al.* (10) subsequently studied green fluorescence protein (GFP)[1]-tagged TIP47 in Chinese hamster ovary cells transfected with a vector containing the coding sequence for murine TIP47. GFP-TIP47 appeared to be at the lipid droplet surface, despite the fact that TIP47 was also strongly expressed throughout the cytoplasm of the cells. Nevertheless, the authors concluded that TIP47 is indeed a component of lipid droplets because the fluorescence patterns from isolated lipid droplets from GFP-TIP47-, GFP-adipophilin-, and GFP-perilipin-expressing cells were indistinguishable. Until now, TIP47 has been directly visualized in lipid droplets only by immunofluorescence microscopy. Whether TIP47 is truly a component of lipid droplets and, if so, the exact spatial distribution of TIP47 (and adipophilin) inside lipid droplets still lack independent clarification, ideally using methods with higher inherent spatial resolution than that attainable by fluorescence microscopy.

We studied the distribution of TIP47 and adipophilin in lipid bodies of THP-1 cell-derived macrophages at high resolution using freeze-fracture immunogold labeling and electron microscopy. We found that lipid bodies in macrophages consist of normal lipid droplets and membranous organelles, which we call "lipid sails." We describe the relationship of lipid sails to lipid droplets and to the PAT family proteins for the first time. Our studies show that TIP47 and adipophilin are integral components of both lipid droplets and lipid sails and that lipid sails are organelles of intracellular lipid metabolism in macrophages.

EXPERIMENTAL PROCEDURES

Antibodies—A polyclonal antibody raised in guinea pig against a synthetic polypeptide representing the amino terminus (amino acids 1–16) of human TIP47 (GP30; Progen Biotechnik, Heidelberg, Germany) was used to detect TIP47. Adipophilin was immunolabeled using a mouse monoclonal antibody to a synthetic peptide representing the amino terminus (amino acids 5–27) of human adipophilin (AP 125; Progen Biotechnik).

Cell Culture—Human THP-1 monocytes from the American Type Culture Collection (Manassas, VA) were cultured in suspension in RPMI 1640 medium containing the supplements recommended by Iwashima *et al.* (17) and differentiated to adherent macrophages by adding 100 mM phorbol 12-myristate 13-acetate to the medium for 3

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[1] The abbreviations used are: GFP, green fluorescence protein; PBS, phosphate-buffered saline; acLDL, acetylated low density lipoprotein; ER, endoplasmic reticulum.

days. Because normally cultured macrophages contain few lipid bodies, the cells were lipid-loaded before use by the further addition of 50 μg/ml acetylated low density lipoprotein at day 2 for 8 h to 2 days as described by Hara *et al.* (18) or by the addition of 600 μmol of oleic acid complexed to bovine serum albumin at a ratio of 6 mol of oleic acid per mole of albumin (15, 16).

Western Blotting—To test whether GP30 (TIP-47) cross-reacts with adipophilin, extracts from macrophages were probed with GP30 and AP 125. After removal of the medium and rinsing with phosphate-buffered saline (PBS), the cells were extracted with radioimmune precipitation assay buffer. Proteins in equal amounts of lysate were separated by polyacrylamide gel electrophoresis on 9% gels and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Bedford, MA). After incubation in PBS containing 5% skim milk for 1 h to block nonspecific binding, the membranes were reacted with GP30 (1:1500) or AP 125 (1:20) for 1 h at 37 °C, washed in PBS containing 0.5% skim milk and 0.05% Tween 20 for 1 h, incubated with peroxidase-conjugated secondary antibodies (DAKO, Hamburg, Germany) for 1 h at 37 °C, and treated with ECL reagents (Amersham Biosciences). Detection of both TIP47 and adipophilin was performed by successively incubating the membrane with GP30 followed by AP 125 (or vice versa). Chemiluminescence signals from bound antibodies were recorded on Hyperfilm (Amersham Biosciences).

Immunofluorescence Staining—Cultured THP-1 cells were differentiated and lipid-loaded in chamber slides. They were rinsed with PBS and fixed in 4% paraformaldehyde in PBS at room temperature for 30 min. After extensive washing, they were incubated for 1 h in PBS containing 1% bovine serum albumin to block nonspecific binding and 0.05% Tween 20 for permeabilization. They were immunolabeled with GP30 (1:500) and AP 125 (1:5) for 1 h, followed by washing and incubation with anti-guinea pig cy3-conjugated and anti-mouse cy2-conjugated secondary antibodies (Dianova, Hamburg, Germany). To visualize TIP47 or adipophilin and neutral lipids at the same time, the fluorophore BODIPY 493/503 (Molecular Probes), which specifically stains neutral lipids (19), was dissolved in ethanol at 1 mg/ml and added to the secondary antibody solutions to a final concentration of 20 μg/ml. Nuclei were stained with Hoechst 33258 dye (Sigma). The preparations were mounted in fluorescent mounting medium (DAKO Cytomation, Hamburg, Germany) and examined in a fluorescence microscope or a confocal laser scanning microscope (Zeiss, Jena, Germany).

Thin Sectioning—Lipid-laden macrophages were grown in flasks, fixed with 2% glutaraldehyde and 0.5% osmium tetroxide in PBS, and dehydrated with ethanol using standard procedures. A few milliliters of propylene oxide were added to the flasks, and the cells were removed by gently swirling and embedded in Epon as usual. Thin sections were cut in an ultramicrotome, contrasted with uranyl acetate and lead citrate, and photographed in the electron microscope.

Ultracryo-sectioning—Lipid-laden macrophages were fixed in 1% paraformaldehyde in PBS, scraped from the culture vessels and prepared further for cryoimmunoelectron microscopy basically as outlined by Tokuyasu (20), immunostained for TIP47 as described below, and examined in an electron microscope.

Freeze-fracture Immunogold Labeling—To survey for TIP47 and adipophilin in lipid bodies in the electron microscope, macrophages were lipid-laden for 2 days and freeze-fractured and replicated using metal evaporation and immunogold labeling as outlined in detail elsewhere (21–23). Briefly, chemically unfixed macrophages were scraped from the culture flasks, incubated in 30% glycerol (for a maximum of 2 min), collected by mild centrifugation, frozen in Freon at −200 °C, and fractured in a vacuum coating unit (BA310, Balzers, Liechtenstein). The fractured cell surfaces were coated with 2 nm of platinum-carbon at an angle of 38° followed by pure carbon. The replicas were incubated overnight in 5% SDS to remove cellular material except for molecules adhering directly to the replicas, rinsed, and immunolabeled. Immunolabeling was performed with GP30 (5 μg/ml) followed by a donkey anti-guinea pig 12-nm gold conjugate, AP 125 (5 μg/ml), followed by a goat anti-mouse 18-nm gold conjugate or a mixture of GP30 and AP 125 followed by a mixture of donkey anti-guinea pig 12-nm and goat anti-mouse 18-nm gold conjugates (both conjugates were from Jackson ImmunoResearch, West Grove, PA) as indicated in the figure legends. The interpretation of immunogold-labeled replicas of freeze-fractured cells is explained elsewhere (22, 23).

RESULTS

Anti-TIP47 and Anti-adipophilin Antibodies React Specifically—The amino-terminal domains of TIP47 and adipophilin are known to have a high degree of homology, and one anti-

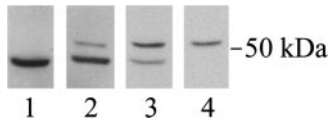

FIG. 1. **GP30 does not cross-react with adipophilin; AP 125 does not cross-react with TIP47.** Cell proteins from extracts of THP-1 cell-derived macrophages lipid-laden for 24 h with acLDL were separated electrophoretically, blotted to filters, and probed with GP30 (TIP47; *lane 1*), AP 125 followed by GP30 (adipophilin + TIP47; *lane 2*), GP30 followed by AP 125 (TIP47 + adipophilin; *lane 3*), or AP 125 (adipophilin; *lane 4*).

serum to TIP47 is known to cross-react with adipophilin as already mentioned. Therefore, we tested the specificity of the antibodies by Western blotting. THP-1 cell extracts reacted with GP30 (TIP47) and AP 125 (adipophilin) yielded unique specific bands of 47 kDa for TIP47 and 52 kDa for adipophilin (Fig. 1). No cross-reactivity was observed.

Lipid Bodies in Macrophages Consist of Lipid Droplets and Lipid Sails—Conventionally thin-sectioned THP-1 cell-derived macrophages contain solitary lipid droplets, clusters of lipid droplets, and sheet-like structures (Figs. 2 and 3). Solitary lipid droplets usually appear structureless. Clustered lipid droplets show evidence of internal structure and are often associated with the cytocenter. As we will show, the sheet-like structures are also lipid bodies, and we descriptively call them lipid sails. Lipid sails in cells exposed to acetylated low density lipoproteins (acLDLs) for 12 h are made up of individual sheets or whorls of membrane-like structures, each consisting of two surfaces separated by a lumen-like space (Fig. 3). One surface only of each lipid sail is decorated conspicuously with glycogen particles. After moderate lipid loading (12 h), lipid sails are often accompanied by solitary lipid droplets. Under prolonged lipid loading (48 h), the glycogen layer is absent (Fig. 2). Both lipid droplets and lipid sails are visible in the light microscope in phase contrast (Fig. 4*A*).

TIP47 and Adipophilin Localize to Lipid Droplets—Macrophages immunostained with GP30 and AP 125 contain both TIP47 and adipophilin, which clearly colocalize in acLDL- and oleate-laden macrophages. In cryosectioned macrophages, lipid droplets appear as electron lucent vesicles with little internal structure. Labeling of adipophilin is generally found in the lipid droplet and the surrounding membrane monolayer. In such sections, it is difficult to determine exactly which structures are labeled (Fig. 4*B*). High levels of TIP47 immunofluorescence occur in lipid droplets in cells lipid-laden before use by acLDL (Fig. 4, *C* and *E*) or oleate (Fig. 4*D*) and, to a lesser extent, in punctate structures throughout the entire cell (Fig. 4, *C* and *D*). Similarly, AP 125 (adipophilin) prominently and specifically stains lipid droplets of varying sizes (Fig. 4, *C* and *D*). Whereas small lipid droplets are usually solitary, large lipid droplets are often clustered at specific regions in the cell. Adipophilin- and TIP47-stained lipid droplets appear as rings of quite substantial but inconstant thickness, making them easy to identify (Fig. 4*E*). Furthermore, cells lipid-loaded with acLDL and doubly stained for TIP47 or adipophilin and for neutral lipids using BODIPY 493/503 reveal that the ring structures are indeed inside the lipid droplets because immunostaining and staining of neutral lipids colocalize; lipid droplets show a distinct ring of TIP47 (Fig. 5*A*) or adipophilin (Fig. 5*B*) staining surrounding the neutral lipid core. Cytoplasmic adipophilin is found in some cells in the form of punctate staining not associated with lipid droplets, and TIP47 shows a punctate distribution throughout the cytoplasm in some cells (data not shown). Lipid sails in macrophages cannot be identified by TIP47 or adipophilin immunofluorescence.

Freeze-fracture immunogold labeling also reveals that both TIP47 and adipophilin are associated with lipid droplets. After

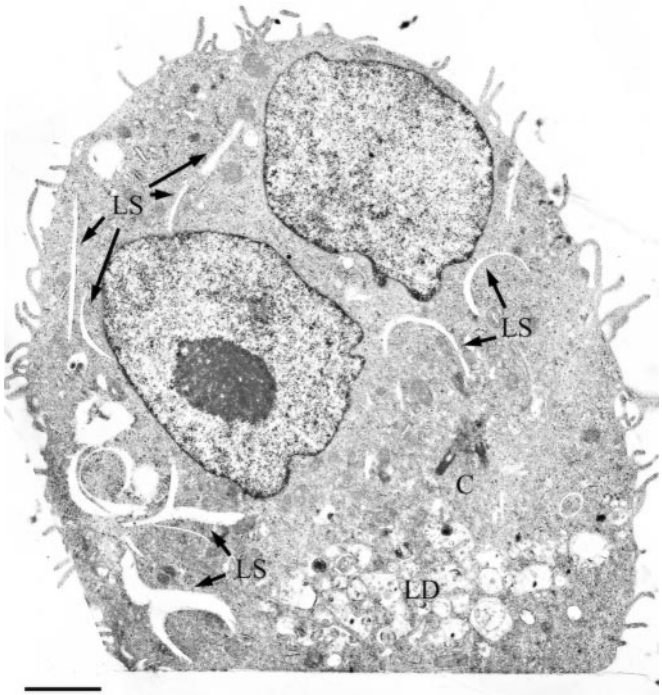

FIG. 2. **Lipid bodies of THP-1 cell-derived macrophages consist of lipid droplets and lipid sails.** Overview. Macrophages were lipid-laden for 2 days by incubation with acLDL. Most of the lipid droplets (*LD*) are seen coalesced at the cytocenter (*C*). Large membrane-like sheets, which we call lipid sails (*LS*), are visible throughout the cell. Conventional thin section. *Bar*, 2 μm.

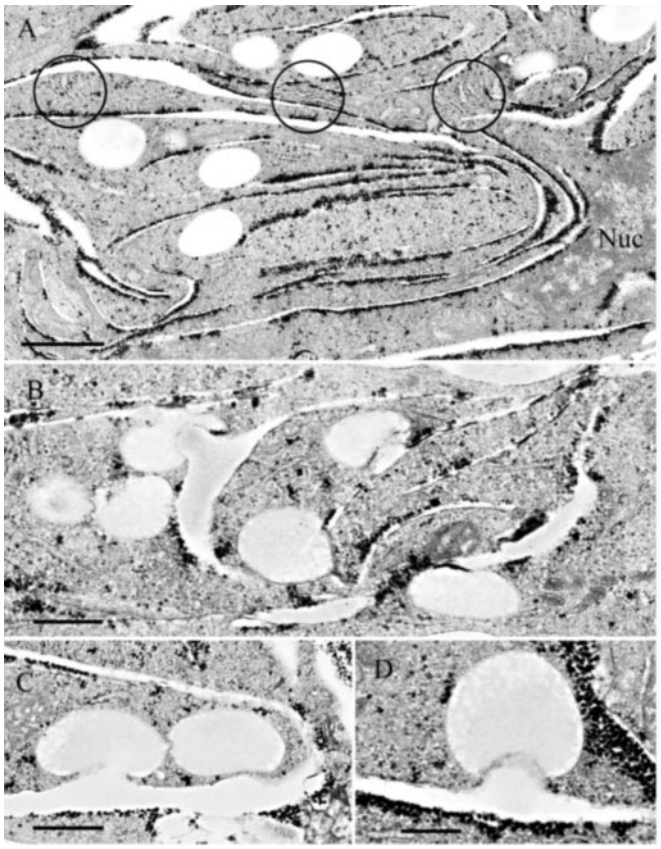

FIG. 3. **Lipid droplets seem to bud off lipid sails.** *A*, after lipid loading for 12 h with acLDL, macrophages contain many solitary lipid droplets associated with lipid sails. Lipid sails bear a unilateral coat of glycogen. Lipid sails appear obviously different than membranes of rough ER (*circles*). *B*–*D*, lipid droplets seem to bud off lipid sails. Thin sections. *Nuc*, nucleus. *Bars*: *A*, 1 μm; *B* and *C*, 0.5 μm; and *D*, 0.3 μm.

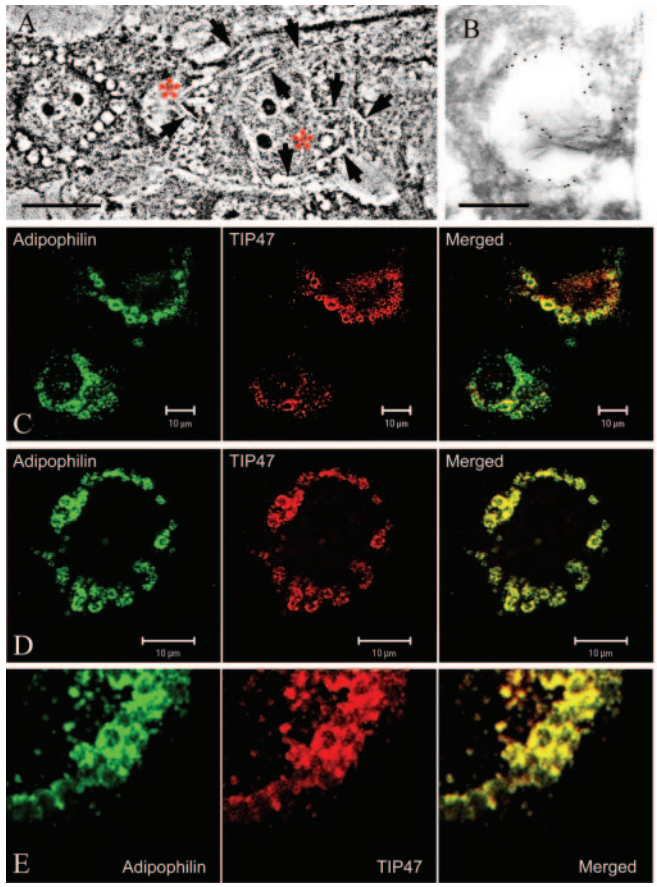

FIG. 4. **Macrophage lipid droplets contain adipophilin and TIP47.** Macrophages were lipid-loaded for 12 or 24 h using acLDL (*A*–*C* and *E*) or oleic acid complexed to bovine serum albumin (*D*). *A*, cells incubated with acLDL for 12 h contain lipid droplets (*lucent round profiles*) and lipid sails (*arrows*), and lipid droplets are frequently associated with the lipid sails (to the *right* of *asterisks*). Phase contrast. *B*, structured lipid droplets labeled for adipophilin with AP 125-immunogold in macrophages treated with acLDL for 12 h. Grazing ultra-cryosection. *C*, views of macrophages incubated with acLDL for 24 h. Solitary lipid droplets contain adipophilin (*green*) as well as TIP47 (*redl*). Colocalization of adipophilin and TIP47 appears *yellow* in the merged figure. In addition, fluorescence for adipophilin and TIP47 appears with punctate distribution in the cytoplasm. Confocal microscopy. *D*, views of macrophages incubated with oleic acid complexed to bovine serum albumin for 24 h. The distribution of adipophilin and TIP47 in lipid droplets in oleate-loaded cells is similar to that in acLDL-treated cells. TIP47 staining in the cytoplasm is weaker in oleate-loaded cells. Confocal microscopy. *E*, macrophages incubated with acLDL for 24 h. At high magnification both adipophilin (*green*) and TIP47 (*red*) are seen at the periphery of lipid droplets. Staining around the droplets varies in thickness, and several lipid droplets show staining for adipophilin in the core. Confocal microscopy.

labeling with AP 125, most adipophilin is visualized at the surface of lipid droplets (Figs. 6, 7, *B* and *C*, and 8, *A* and *B*). In addition, adipophilin is frequently found inside the lipid droplet core, where it is commonly confined to the outermost lipid layers (Figs. 7*B* and 8, *A* and *B*). Single labeling with GP30 and double labeling with GP30 and AP 125 reveal a similar distribution for TIP47. TIP47 is likewise present primarily at the lipid droplet surface as well as on the layers of the core near the lipid droplet surface (Figs. 7, *A*–*C*, and 8, *A* and *B*). However, both antigens are usually absent deeper in lipid droplet cores (Fig. 7, *B* and *C*). Surprisingly, the antibodies, which are directed exclusively toward the amino termini of their respective antigens, label both the concave and convex surfaces of lipid layers in the lipid droplet cores (Fig. 7, *A* and *C*). Although TIP47 and adipophilin are colocalized in lipid droplets, they are present in widely varying proportions (Fig. 8, *A* and *B*).

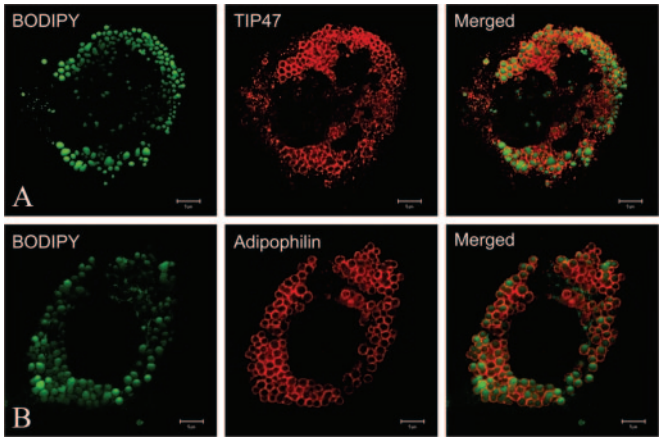

FIG. 5. **TIP47 and adiopophilin are associated with droplets containing neutral lipids.** Macrophages were lipid-loaded for 24 h using acLDL. Solitary droplets containing neutral lipids (BODIPY; *green*; *left panels*) also contain (*A*) TIP47 (*red*; *middle panel*) and (*B*) adipophilin (*red*; *middle panel*). The merged figures (*right panels*) reveal distinct rings of TIP47 (*A*) and adipophilin (*B*) surrounding the neutral lipid cores. Confocal microscopy.

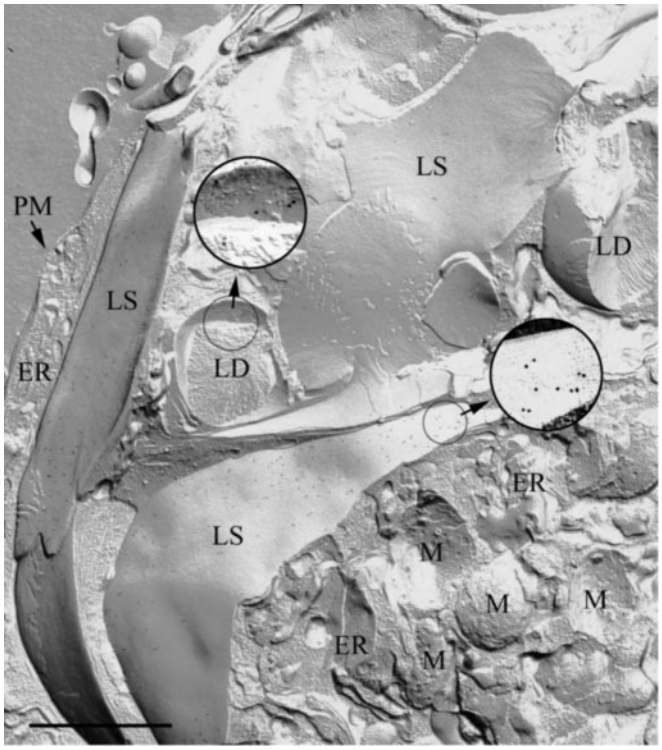

FIG. 6. **Adipophilin in lipid droplets and lipid sails of macrophages.** Cells were freeze-fractured and immunogold-labeled for adipophilin. Adipophilin is localized in lipid droplets (*LD*) and in lipid sails (*LS*; *insets*). No labeling is found in ER or mitochondria (*M*). Macrophages were incubated with acLDL for 12 h. *PM*, plasma membrane. *Bar*, 1 μm.

TIP47 and Adipophilin Are Components of Lipid Sails—TIP47 and adipophilin are also components of lipid sails. In freeze-fractured cells, the exposed surfaces of lipid sails are characteristically smooth and featureless (Figs. 6, 9, and 10), compared with the membranes of mitochondria (Fig. 6) and the plasma membrane (Fig. 9), which contain many intramembrane particles. Immunogold labeling shows that TIP47 (Figs. 6 and 9) and adipophilin (Figs. 6 and 10) are associated with most surfaces of lipid sails (Figs. 6 and 9). However, TIP47 is conspicuously absent on some surfaces as well as in the intervening spaces (Fig. 9). Double labeling with GP30 and AP 125

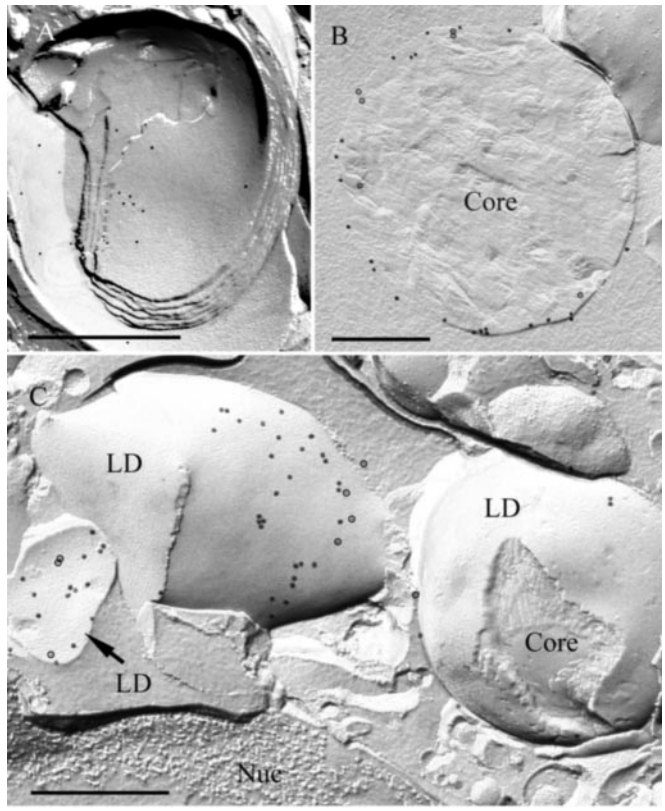

FIG. 7. **TIP47 and adipophilin are components of the envelope and core of lipid droplets.** *A*, immunogold localization of TIP47 in a concavely fractured lipid droplet. TIP47 labeling is on the outer surfaces of several layers inside the droplet core. *B*, cross-fractured lipid droplet double labeled for TIP47 (*small gold particles in circles*) and adipophilin (*large gold particles*). TIP47 and adipophilin labeling is generally near the surface of the droplet, but both the envelope and the core are marked. *C*, double-labeled freeze-fractured lipid droplets (*LD*) showing colocalization of TIP47 (*small gold particles in circles*) and adipophilin (*large gold particles*) on the inside surface of the outer layer of the core (*left lipid droplet*) and on the outside surface of a lipid droplet envelope (*arrow*). Deeper regions of the core of the lipid droplet at the *right* are unlabeled. Macrophages were incubated with acLDL for 12 h. *Nuc*, nucleus. *Bars*: *A*–*C*, 0.5 μm.

indicates that TIP47 and adipophilin are colocalized in lipid sails (Fig. 10).

No immunogold labeling of TIP47 or adipophilin is found on mitochondria, endoplasmic reticulum (ER), Golgi, or nuclear membranes or in the cytoplasm. However, both antigens are present in the plasma membrane in highly specific configurations relative to lipid droplets (data not shown).

DISCUSSION

We have studied the distribution of the PAT family proteins TIP47 and adipophilin in macrophages. We also provide new data on the spatial distribution of TIP47 and adipophilin in lipid droplets. We show that these lipid body proteins are not confined to the surface phospholipid monolayer of the lipid droplet envelope, as often tendered (1, 7), but are an integral component of lipid droplet cores; TIP47 and adipophilin do not merely coat lipid droplets. Macrophages doubly stained with antibodies to TIP47 or adipophilin and for neutral lipids using BODIPY 493/503 reveal distinct rings of TIP47 or adipophilin staining surrounding the neutral lipid cores of lipid droplets. The subcellular structures detected by anti-TIP47 or anti-adipophilin and BODIPY 493/503 are clearly lipid droplets because the two stains colocalize. These results demonstrate unequivocally that TIP47 and adipophilin are components of lipid droplets. The association of TIP47 with lipid droplets is not

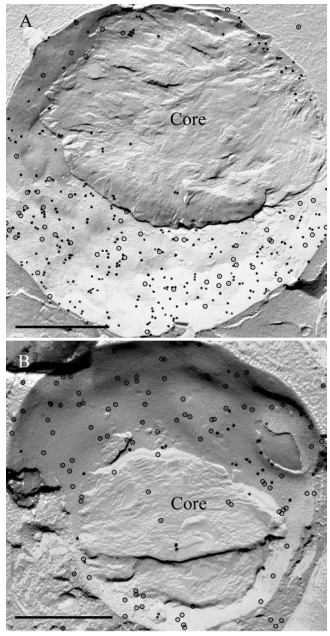

FIG. 8. **TIP47 and adipophilin are codistributed at the periphery of lipid droplets, but in varying amounts.** Concavely fractured lipid droplets showing colabeling of TIP47 (*small gold particles in circles*) and adipophilin on the outer surface of two lipid droplet envelopes. Both antigens are found in superficially exposed regions of the cores. In *A*, the ratio of TIP47 to adipophilin labeling is ~4:1, in *B*, it is 1:4. Macrophages were incubated with acLDL for 12 h. *Bars*: *A* and *B*, 0.5 μm.

FIG. 9. **Distribution of TIP47 in lipid sails.** Immunogold labeling of TIP47 is seen on most, but not all (*arrows*), surfaces of lipid sails. Macrophages were incubated with acLDL for 12 h. *LD*, lipid droplet; *PM*, plasma membrane. *Bar*, 0.5 μm.

unique to macrophages because Wolins *et al.* (15) described lipid droplets in other cells including primary mouse fibroblasts, human melanocytes, human HeLa cells, and 3T3-L1

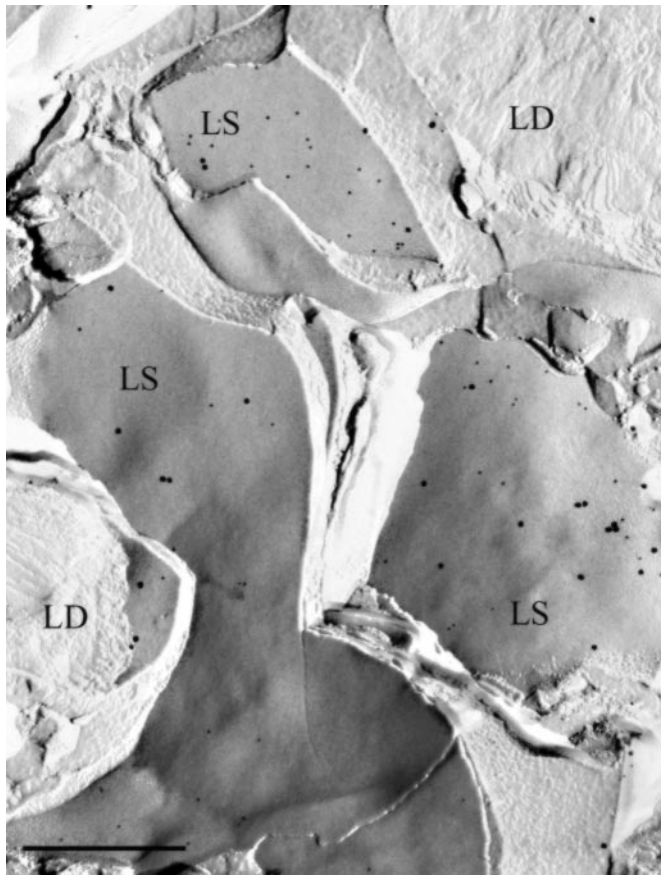

FIG. 10. **Lipid sails contain TIP47 and adipophilin.** TIP47 (*small gold particles*) and adipophilin (*large gold particles*) are colocalized in lipid sails (*LS*). Macrophages were incubated with acLDL for 12 h. *LD*, lipid droplet. *Bar*, 0.5 μm.

adipocytes with surface immunostaining for TIP47, an observation that we confirmed in HeLa cells and 3T3-L1 adipocytes (data not shown). Using freeze-fracture, we found no differences in the labeling pattern and intensities of TIP47 and adipophilin between cells loaded with acLDL and cells loaded with oleic acid. In our images and those in the literature, focused adipophilin-stained lipid droplets appear in the fluorescence microscope as rings, but the staining pattern is of irregular and substantial thickness. This appearance indicates that adipophilin is indeed predominantly concentrated at the lipid droplet surface, but the fluorescence signal, and hence adipophilin, is not restricted to the phospholipid monolayer enveloping the lipid droplets. We also note that the distribution of lipid droplet proteins inside macrophage lipid droplet cores is no exception because we have found traces of TIP47 in lipid droplets of adipocytes and very substantial amounts of adipophilin, perilipin, and another lipid droplet protein, caveolin-1, not only at the surface but also completely pervading the cores of lipid droplets of adipocytes (data not shown), milk fat globules (data not shown), and lipid droplets of smooth muscle cells (23), respectively. Finally, lipid droplet cores are known to contain other polar molecules such as cyclooxygenase (24), flotillins (25), and caveolin-2 (26).

The finding that TIP47 and adipophilin are not merely associated with lipid droplets but are actually components of the cores of lipid droplets raises at least four difficult questions. Where do these proteins come from, how do they target to lipid droplets, how can they get into the lipid droplet core, and, in particular, how are these polar proteins packaged among the essentially hydrophobic neutral lipids in the lipid droplet core?

These questions can be only incompletely addressed at the

present time. First, whether TIP47 and adipophilin arise at the ER, as caveolin-1 (a lipid droplet protein we localized in ER membranes) does (22, 23), is unclear. One early report describing a form of adipophilin in ER membranes from homogenates of lactating mammary gland (27) has not been confirmed. In the present study, we found no trace whatsoever of gold labeling for TIP47 or adipophilin on ER membranes. Diaz and Pfeffer (28) described TIP47 as a cytosolic protein, Brasaemle *et al.* (29) reported a lack of sedimentable membranes containing adipophilin, and Londos *et al.* (1) discussed the synthesis of adipophilin and perilipin, another PAT protein, on free ribosomes. Synthesis of TIP47 on free ribosomes would explain the high background levels of TIP47 in the cytoplasm of macrophages and other cells (10). Immunofluorescence from cytoplasmic TIP47 would tend to mask the signal from TIP47 in lipid droplets, and, not being membrane-associated, cytoplasmic free-ribosomal TIP47 would go undetected by freeze-fracture immunogold labeling as we observed. All in all, it is unlikely that these proteins arise in the ER. A cytosolic origin of TIP47 and adipophilin appears more probable.

Second, TIP47 and adipophilin are thought to be essentially soluble proteins, so initial binding of TIP47 and adipophilin to the lipid droplet envelope might take place via fatty acid groups, if these proteins are indeed acetylated, and once bound to lipid droplets, TIP47 and adipophilin should potentially form hairpin structures in the lipid droplet envelope (7). Caveolin-1, another lipid droplet protein, is known to be hairpin-shaped, with its hydrophilic ends directed to the cytosol.

The answers to the third (how TIP47 and adipophilin could be transported through the lipid droplet envelope and gain access to the deeper lipid layers of the core) and fourth questions (how TIP47 and adipophilin could be orderly packaged among the predominantly hydrophobic neutral lipids in the core) are simply matters for conjecture at the moment. With regard to the packaging of TIP47 and adipophilin, we found labeling of these proteins on both concave and convex fractures of the core layers, *i.e.* on both outward- and inward-facing surfaces of the core layers. Because GP30 and AP 125 specifically recognize the amino termini of their antigens, the amino-terminals of the molecules are arranged sometimes inward and sometimes outward in the layers of the lipid droplet core. It may be possible to gain more information on the orientation of these molecules in the core using appropriate truncated proteins and antibodies raised against the carboxyl-terminals of TIP47 and adipophilin.

In the context of atherogenesis, macrophages are germane for coping with superfluous lipids in the vessel wall. Shiratori *et al.* (30) demonstrated lipid sails in acLDL-laden J774 macrophages treated with the ACAT inhibitor 58035. Because we now know that both TIP47 and adipophilin are present in lipid sails, lipid sails evidently represent a kind of lipid body in macrophages. These organelles are also present in native peritoneal macrophages,[2] but to our knowledge, they are not present in other cells, so these organelles may be unique to macrophages. The smooth appearance of the surfaces of lipid sails exposed by freeze-fracturing compared with the surfaces of intracellular membranes suggests a high lipid content in lipid sails, and this supposition is in agreement with early findings of Shio *et al.* (31). The association of lipid sails with solitary lipid droplets and the distended appearance of the lumens of lipid sails at moderate durations of lipid loading indicate that they might be sites of lipid droplet formation. Our images seem to depict budding of lipid droplets from lipid sails. The membranous appearance of lipid sails suggests they could be specialized extensions of the ER. Nevertheless, their content of TIP47 and adipophilin, which are completely lacking in the ER, and their unusual unilateral coats of glycogen make a direct relationship with the ER questionable. At the very least, it is clear that lipid sails are organelles of lipid metabolism in macrophages.

TIP47 and adipophilin and other lipid droplet proteins are clearly components of both the envelope and core of lipid droplets and of macrophage lipid sails. Explaining their presence in these situations and unraveling their functions there is going to be a challenging task.

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[2] H. Robenek, S. Lorkowski, M. Schnoor, and D. Troyer, unpublished data.

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