PAT family proteins pervade lipid droplet cores

Horst Robenek,[1] Mirko J. Robenek, and David Troyer

Institute for Arteriosclerosis Research, University of Münster, Münster, Germany

Abstract The PAT family proteins, named after perilipin, adipophilin, and the tail-interacting protein of 47 kDa (TIP47), are implicated in intracellular lipid metabolism. They associate with lipid droplets, but how is completely unclear. From immunofluorescence studies, they are reported to be restricted to the outer membrane monolayer enveloping the lipid droplet and not to enter the core. Recently, we found another kind of lipid droplet-associated protein, caveolin-1, inside lipid droplets. Using freeze-fracture immunocytochemistry and electron microscopy, we now describe the distributions of perilipin and caveolin-1 and of adipophilin and TIP47 in lipid droplets of adipocytes and macrophages. All of these lipid droplet-associated proteins pervade the lipid droplet core and hence are not restricted to the droplet surface. Moreover, lipid droplets are surprisingly heterogeneous with respect to their complements and their distribution of lipid droplet-associated proteins. Whereas caveolin-1 is synthesized in the endoplasmic reticulum and is transferred to the lipid droplet core by inundating lipids during droplet budding, the PAT proteins, which are synthesized on free ribosomes in the cytoplasm, evidently target to the lipid droplet after it has formed. How the polar lipid droplet-associated proteins are accommodated among the essentially hydrophobic neutral lipids of the lipid droplet core remains to be determined.—Robenek, H., M. J. Robenek, and D. Troyer. **PAT family proteins pervade lipid droplet cores. *J. Lipid Res.* 2005.** 46: **1331–1338.**

Supplementary key words perilipin • adipophilin • tail-interacting protein of 47 kDa • freeze-fracture labeling

The past few years have seen great advances in elucidating lipid droplet function and biogenesis (1–3). Rather than being mere storage depots for hydrophobic neutral lipids, lipid droplets are currently recognized as indispensable and metabolically active organelles that participate in cell signaling (4), intracellular vesicle trafficking (5), and, most obviously, lipid homeostasis (6). Lipid droplets also appear to be involved in several important human diseases (7–9).

Recent studies have highlighted the importance of specific proteins that associate with lipid droplets (3, 10–20). In animal cells, lipid droplet-associated proteins include the structurally related members of the so-called PAT family proteins, named after perilipin, adipophilin, and the tail-interacting protein of 47 kDa (TIP47) (12, 13, 21), and the caveolins (3, 17, 19, 22–24). Perilipin associates with lipid droplets in adipocytes and steroidogenic cells. Adipophilin is a ubiquitously expressed lipid droplet-associated protein of all mammalian cell types. It has been found only associated with lipid droplets and not in any other subcellular compartment. Two PAT proteins, adipophilin and perilipin, and the caveolins have been described as colocalizing at the surfaces of lipid droplets (5). Recently identified lipid droplet-associated proteins are TIP47 (13, 25), lipotransin (26), a 200 kDa capsular protein (27), and the hepatitis C virus core protein (28). All lipid droplet-associated proteins are highly polar.

Lipid droplets are formed as neutral lipids, mainly triacylglycerol and sterol esters, and inundate between the leaflets of the membrane of the endoplasmic reticulum (ER). Distension of the membrane and budding of the membrane leaflet facing the cytoplasm are thought to leave the droplet surrounded by an envelope consisting of the former cytoplasmic leaflet of the ER membrane. Except for caveolin-1 (3, 17, 22), all lipid droplet-associated proteins described to date are thought to be confined exclusively to the lipid droplet envelope. Lipid droplet-associated proteins are generally assumed to be transferred to the droplet surface along with the cytoplasmic leaflet of the ER membrane (18, 29) and thus not to gain access to the droplet core. Alternatively, some of these proteins are thought to be directed from cytosolic sites of synthesis to the envelopes of nascent lipid droplets (12).

While exploring lipid droplet formation and the relationship of caveolin to lipid droplet structure, we localized caveolin-1 in cellular membranes and in lipid droplets of cultured smooth muscle cells using freeze-fracture immunocytochemistry and electron microscopy. We discovered that caveolin-1 is present not only in the outer membrane monolayer surrounding the lipid droplet, as concluded from immunofluorescence studies, but also throughout the core of the droplet (3, 17), a localization also found by

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[1] To whom correspondence should be addressed.
e-mail: robenek@uni-muenster.de

Tauchi-Sato et al. (22). We challenged the current model of lipid droplet biogenesis and proposed that caveolin-1 is extracted from the endoplasmic leaflet of the ER membrane and transferred into the core by the inundating lipids themselves.

Here, we show that PAT family proteins are likewise not confined exclusively to the surface of the lipid droplet but completely pervade the lipid droplet core. This finding indicates that the polar PAT proteins do access the lipid droplet core and are accommodated there, along with the hydrophobic neutral lipids, in some as yet unidentified way.

METHODS

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. (30) and differentiated to adherent macrophages by adding 100 mM phorbol 12-myristate 13-acetate to the medium for 3 days. Because normally cultured macrophages contain few lipid droplets, the cells were induced to accumulate lipid droplets before use by the further addition of 50 μg/ml acetylated low density lipoproteins at day 2 for 2 days as described by Hara et al. (31) and Gaus et al. (32). Stimulation of lipid droplet biogenesis by the addition of 600 μM oleic acid complexed to fatty acid-free BSA, as described by Barbero et al. (33) and Wolins, Rubin, and Brasaemle (13), produced results like those obtained when the cells were incubated with acetylated low density lipoproteins as described.

3T3-L1 cells were maintained in DMEM supplemented with 10% bovine fetal calf serum (FCS), 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Differentiation of 3T3-L1 cells to adipocytes was accomplished by incubating confluent monolayers of cells in DMEM supplemented with 10% FCS, 0.5 mM isobutylmethylxanthine, 10 μg/ml insulin, and 10 μM dexamethasone with fresh medium changes every 24 h for 72 h, followed by DMEM supplemented with 10% FCS with medium changes every 24 h for an additional 72 h (34).

Both cell types were maintained at 37°C in a 5% CO_2 atmosphere.

Antibodies

Adipophilin was immunolabeled using a mouse monoclonal antibody to a synthetic peptide representing the N terminus (amino acids 5–27) of human adipophilin (AP125; Progen Biotechnik, Heidelberg, Germany). A polyclonal antibody raised in guinea pig against a synthetic polypeptide representing the N terminus (amino acids 1–16) of human TIP47 (GP30; Progen Biotechnik) was used to detect TIP47. The GP30 antibody does not cross-react with adipophilin and perilipin (Progen Biotechnik). A polyclonal antibody raised in guinea pig against a synthetic peptide corresponding to the N-terminal region of human perilipin A and B was used to detect perilipin (GP29; Progen Biotechnik).

Caveolin-1 was immunolabeled using a mouse monoclonal anti-caveolin-1 antibody (clone 2297; BD Transduction Laboratories, Lexington, KY).

Ultrathin cryosectioning

Macrophages and adipocytes were prefixed for 5 min by adding an equal volume of 4% paraformaldehyde in PBS, pH 7.4, to the culture medium. The cells were washed briefly in PBS and fixed further in 4% paraformaldehyde for 1 h followed by incubation in 4% paraformaldehyde with 0.9 M sucrose and 90% (w/v) polyvinylpyrrolidone 25 for cryoprotection buffered with 50 mM sodium carbonate, pH 7.0, also for 1 h. The cells were scraped from the culture vessels with a rubber policeman, concentrated by centrifugation, placed on pins in a small volume of cryoprotectant, and frozen in liquid nitrogen. Ultrathin cryosections were prepared as described previously (35).

Freeze-fracturing

Cells were scraped from the culture vessels, centrifuged to remove excess medium, and recentrifuged briefly (<2 min) in 30% glycerol, fixed in Freon 22 cooled with liquid nitrogen, and freeze-fractured in a BA310 freeze-fracture unit (Balzers AG, Liechtenstein) 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. The replicas were incubated overnight in 5% sodium dodecyl sulfate (36) to remove cellular material except for those molecules adhering directly to the replicas, washed in distilled water, and incubated briefly in 5% BSA before immunostaining.

Immunolabeling

Both cryosections and freeze-fracture replicas were immunolabeled with GP30 or GP29 followed by a donkey anti-guinea pig 12 or 18 nm gold conjugate and AP125 or caveolin-1 followed by a goat anti-mouse 12 or 18 nm gold conjugate. Double labeling of adipocytes was carried out with a mixture of GP30 and anti-caveolin-1 antibody followed by a mixture of donkey anti-guinea pig 12 nm and goat anti-mouse 18 nm gold conjugates (both conjugates from Jackson Immunoresearch, West Grove, PA). Double labeling of THP-1 macrophages was carried out with a mixture of GP29 and AP125 antibodies followed by a mixture of donkey anti-guinea pig 12 nm and goat anti-mouse 18 nm gold conjugates. Immunolabeling is indicated in the figure legends. Control specimens, prepared without the first antibody, were essentially free of gold particles. Irrelevant antibodies against human Lamp-1, scavenger receptor BI, and connexin 43 used with appropriate gold conjugates did not immunolabel lipid droplets.

RESULTS

Freeze-fracture nomenclature

When frozen cells are fractured, the fracture plane skips between the various membranes, organelles, and compartments of the cell. The fracture plane runs preferentially along the middle of the phospholipid bilayer and splits the bilayer membrane into two leaflets. An understanding of the topological relationship between the different membrane systems of the cell and the conventions used in describing freeze-fractured membranes is essential for interpreting the present results. One monolayer or leaflet of all membranes is always situated next to the cytoplasm, and depending on the origin of the membrane, the other leaflet is adjacent to the extracellular, exoplasmic, or endoplasmic space (37). The extracellular space is delineated by the plasma membrane. Exoplasmic space is the interior of endocytotic vesicles, secretory granules, and lysosomes. Endoplasmic space denotes lumens of the ER, Golgi apparatus, and nuclear envelope. The P-face is defined as the fracture face of the cytoplasmic leaflet of the membrane, and the E-face as the fracture face of the extracellular, exoplasmic, or endoplasmic leaflet. Correct

identification of the fracture faces of membranes relies on multiple clues, such as membrane curvature, specific structural features of the membrane type/fracture face, the direction of metal evaporation, and labeling specificity.

Standard freeze-fracture nomenclature cannot be applied to lipid droplets, as these organelles do not have a limiting membrane enclosing a lumen. Of the first two layers surrounding the lipid droplet, the outermost is thought to be derived from the cytoplasmic leaflet of the ER membrane. The second is probably the first of a series of tightly compressed, alternately oriented lipid monolayers that make up the lipid droplet core. The hydrophobic planes between these layers can be freeze-fractured to reveal aspects similar to those of normal membrane fracture faces at various depths through the droplet. However, these fracture faces should not be designated P-face or E-face; they are better described by their location and the type of fracture through the lipid droplet (i.e., convex, concave, or cross-fracture). Here, we denote the two aspects of the outermost two lipid layers of the lipid droplet as the P-face equivalent and E-face equivalent, in keeping with the analogous biogenetic relationships to cellular membranes.

Adipophilin and TIP47 in THP-1 macrophages

In cryosectioned THP-1 macrophages, lipid droplets appear as electron-lucent vesicles with little internal structure. Weak labeling of adipophilin is generally found in the lipid droplet near the surrounding membrane monolayer (**Fig. 1A**). In such sections, it is difficult to discern exactly which structures are labeled.

Freeze-fracture replicas of lipid droplets in THP-1 macrophages vary in appearance, depending on the fracture plane and on the structural heterogeneity of different lipid droplets (Fig. 1B–E). Figure 1B, E illustrates examples in which the lipid droplets have been cross-fractured. They appear relatively featureless. In Fig. 1C, the outermost monolayer of a concavely fractured lipid droplet (P-face equivalent) is seen. A similar lipid droplet in which the fracture has followed the outermost membrane monolayer of the lipid droplet and then penetrated interiorly to cross-fracture the core of the lipid droplet is shown in Fig. 1D.

After immunogold labeling of the freeze-fracture replicas with AP125 and immunogold, almost all lipid droplets show adipophilin labeling. Adipophilin is present in a variety of locations within the lipid droplets. Label is observed not only on the outermost monolayer of cross-fractured (Fig. 1B) and concavely fractured lipid droplets (P-face equivalent; Fig. 1C) but also throughout the interior of the lipid droplets (Fig. 1D, E).

Lipid droplets of THP-1 macrophages also contain TIP47 (**Fig. 2A–C**). TIP47 labeling occurs in the outermost monolayer of concavely fractured lipid droplets (Fig. 2A). Dou-

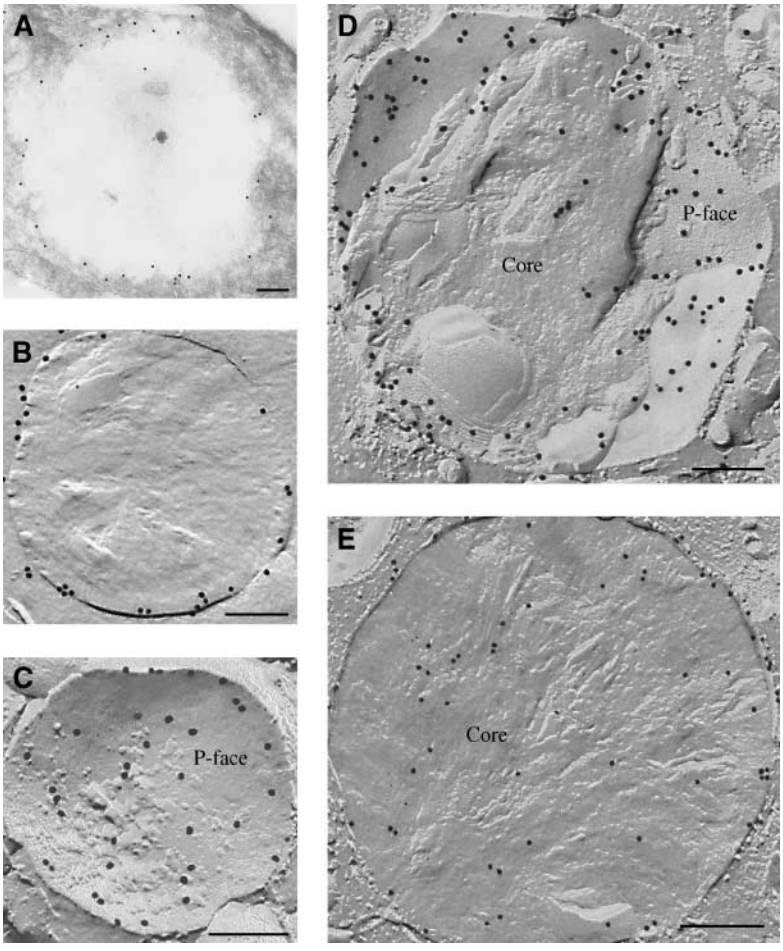

Fig. 1. Distribution of adipophilin in lipid droplets of THP-1 macrophages. A: Immunogold labeling of adipophilin (12 nm gold) in a cryosection. B–E: Immunogold labeling of adipophilin (18 nm gold) in freeze-fracture, showing the presence of adipophilin on the outermost monolayer (P-face equivalent) of cross-fractured (B) and concavely fractured droplets (C, D) and throughout the lipid droplet cores (D, E). Bars = 0.2 μm.

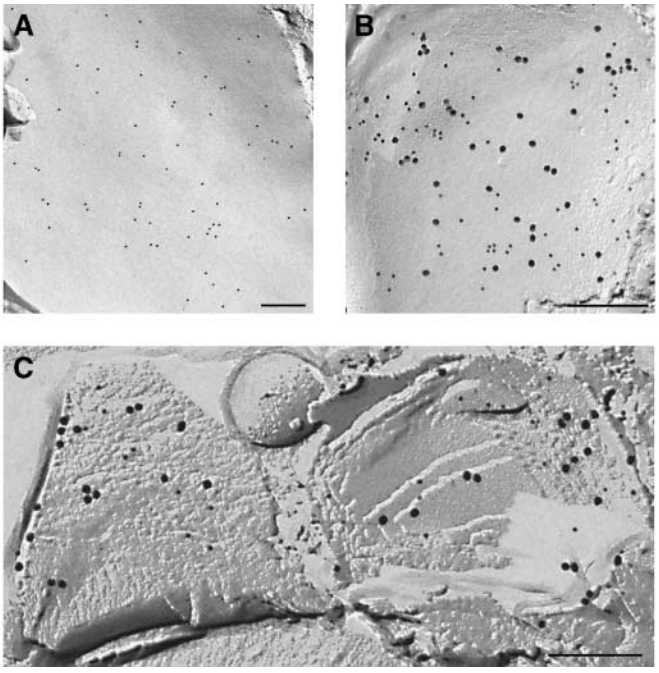

Fig. 2. Distribution of adipophilin and tail-interacting protein of 47 kDa (TIP47) in lipid droplets of THP-1 macrophages. Immunogold labeling of TIP47 (12 nm gold; A) and of TIP47 (12 nm gold) and adipophilin (18 nm gold) on the outermost monolayer (P-face equivalent) of a concavely fractured lipid droplet (B) and on cross-fractured lipid droplets (C). TIP47 and adipophilin are colocalized on the outermost monolayer (P-face equivalent) and in the droplet core. Bars = 0.2 μm.

ble labeling of TIP47 and adipophilin reveals codistribution of both lipid droplet-associated proteins on the outermost monolayer of concavely fractured lipid droplets (Fig. 2B) and in the lipid droplet cores (Fig. 2C).

Perilipin and caveolin-1 in adipocytes

Perilipin is found on the outermost monolayer of concavely fractured lipid droplets (P-face equivalent; **Fig. 3A**) and on the outermost monolayer of convexly fractured lipid droplets (E-face equivalent; Fig. 3B) in adipocytes. However, the concentration of perilipin label is much lower in the E-face equivalent than in the P-face equivalent.

Significant amounts of perilipin label are found on cross-fractured lipid droplets. The distribution of perilipin is highly variable, ranging from uniformly distributed across the droplet core to concentrated at specific locations within the droplet core. Examples are shown in **Fig. 4A–F**. In Fig. 4A, perilipin label is distributed uniformly over the entire droplet core. Figure 4B illustrates a lipid droplet in which the fracture has followed the outermost monolayer and penetrated interiorly to cross-fracture the lipid core. The concentration of perilipin label is noticeably lower in the lipid core than in the P-face equivalent of the outermost monolayer. Nonhomogeneous distribution of perilipin in lipid droplets is visible in Fig. 4C. This cross-fractured lipid droplet contains perilipin along part of the periphery, with an outpocketing of perilipin at high concentration extending to the very center of the core. Peripheral concentration of perilipin is shown in Fig. 4D, E. In Fig. 4D, a less regular peripheral distribution with weak labeling at the center of the core is demonstrated. No labeling in the core, but strong, sharply delineated labeling at part of the periphery, is seen in Fig. 4E. A similar but superficially fractured droplet with equivalent labeling appears in Fig. 4F. In this lipid droplet, the outermost monolayer (P-face equivalent) and the lipid layers of the peripheral core are strongly labeled, but the inner core is only weakly labeled.

Double labeling of perilipin and caveolin-1 reveals extreme heterogeneity with respect to the distribution of these two lipid droplet-associated proteins in lipid droplets of adipocytes (**Figs. 5**, **6**). Figure 5A illustrates one lipid droplet in which the fracture has exposed the outermost monolayer and another that has been cross-fractured. The outermost monolayer exhibits colocalization of perilipin and caveolin-1, but the core of the other droplet contains almost exclusively caveolin-1. In Fig. 5B, two cross-fractured droplets containing perilipin and caveolin-1 label in almost equal amounts are shown. In another droplet (Fig. 5C), in which the fracture has exposed the outermost monolayer and the core, perilipin and caveolin-1 are colocalized in the outermost layer, whereas caveolin-1 appears almost exclusively in the core. In Fig. 6, three droplet cores have been exposed, the first of which is strongly and exclusively labeled with caveolin-1, the second

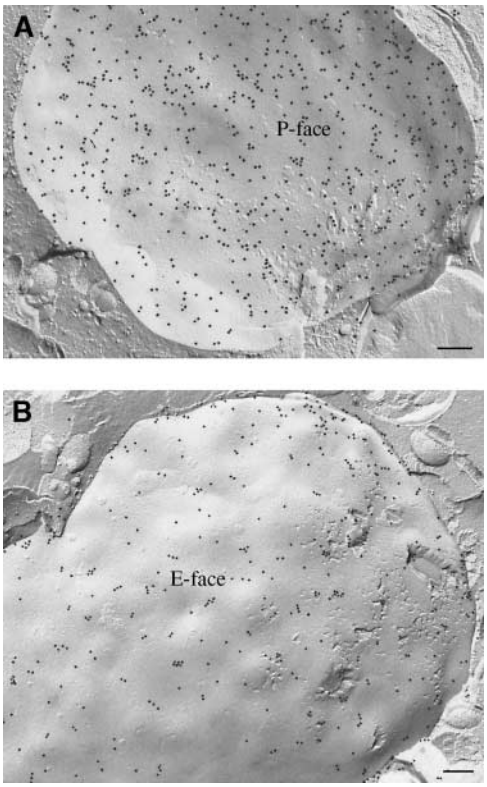

Fig. 3. Distribution of perilipin on the lipid droplet surface in adipocytes. Immunogold labeling of perilipin (18 nm gold) is found on the outermost monolayer of concavely fractured (P-face equivalent) lipid droplets (A) and of convexly fractured (E-face equivalent) lipid droplets (B). Bars = 0.2 μm.

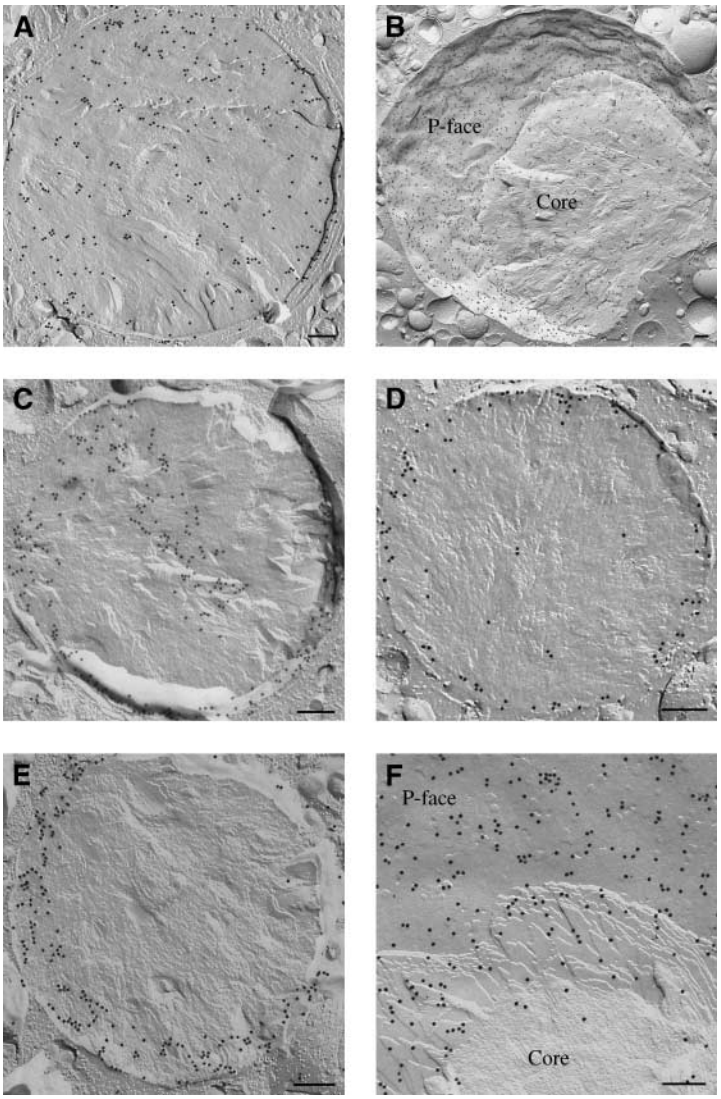

Fig. 4. Distribution of perilipin on cross-fractured lipid droplets of adipocytes. Perilipin label (18 nm gold) is localized randomly over the entire droplet core (A), on the outermost monolayer (P-face equivalent) and the core (B), at part of the periphery and in an outpocketing extending to the very center of the core (C), less regularly over the peripheral region with weak labeling of the center of the core (D), on a sharply delineated peripheral region with no labeling of the core (E), and on the outermost monolayer (P-face equivalent) and on lipid layers of the peripheral core (F), whereas the inner core is only weakly labeled. Bars = 0.2 μm.

with perilipin and caveolin-1 in almost equal amounts, and the third with only traces of perilipin and caveolin-1.

DISCUSSION

The key findings of the present study are that in mature lipid droplets, the PAT proteins perilipin, adipophilin, and TIP-47 and caveolin-1 occur *1*) on both the P-face and E-face equivalents of the outer droplet lamella and *2*) throughout the core and *3*) that lipid droplets are surprisingly heterogeneous with respect to their complements of lipid droplet-associated proteins. These findings are at variance with reports of the localization of these proteins exclusively at the surfaces of lipid droplets.

Lipid droplet-associated proteins clearly gain access to the successive interior lamellae and the amorphous interior of the lipid droplets, as indicated by the presence of label in these zones. Labeling of the lipid droplet-associated proteins in lipid droplets is far from being confined to the fracture face of the outermost monolayer (P-face equivalent). These proteins are frequently seen on the fracture face of the inner monolayer of the outermost bilayer, a position corresponding to that of the E-face of the ER, suggesting a location of the proteins in the E-face equivalent. This is in keeping with a mechanism of lipid droplet formation in which the E-face of the ER membrane is incorporated into the lipid droplet, not solely its cytoplasmic leaflet, a possibility we have discussed elsewhere (3).

Current hypotheses of lipid droplet biogenesis, however, propose that lipids initially accumulate between the endoplasmic leaflet (E-face) and the cytoplasmic leaflet (P-face) of ER membranes (2, 22–24, 29). As the lipid pool enlarges, it pinches off the ER, generating a droplet enveloped in a phospholipid monolayer derived from the cytoplasmic leaflet of the ER membrane. At present, lipid droplet proteins are thought to gain access to the lipid droplet surface by lateral diffusion along this outer monolayer. A major implication of this hypothesis is that protein transfer from the ER to the lipid droplet occurs within the plane of the cytoplasmic leaflet of the ER membrane constituting the droplet envelope; proteins located in the endoplasmic leaflet of the ER membrane are not transferred to the lipid droplet. Our recent finding of caveolin-1 in

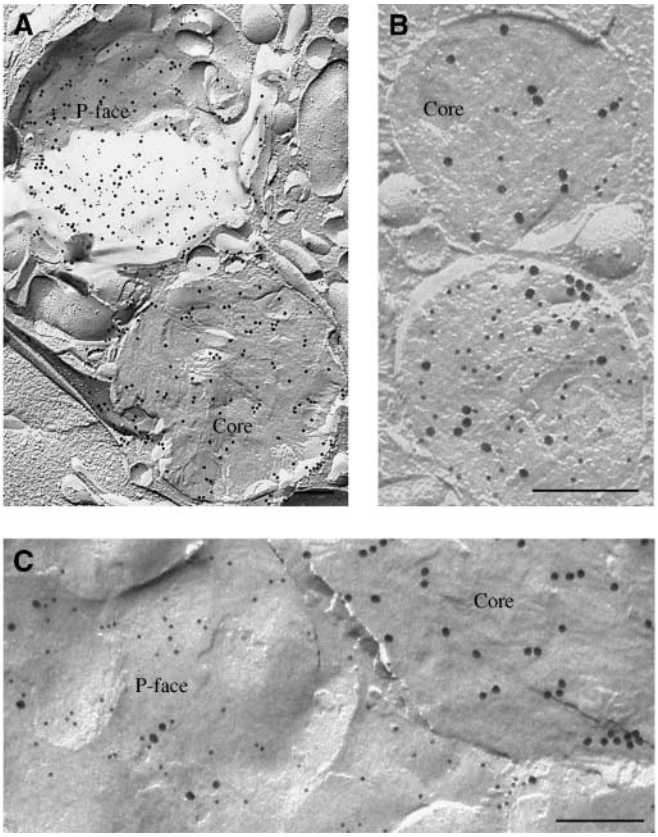

Fig. 5. Distribution of perilipin and caveolin-1 in lipid droplets of adipocytes. A: Immunogold labeling of perilipin (12 nm gold) and caveolin-1 (18 nm gold) on two lipid droplets in the same cell. One droplet shows colocalization of both proteins on the outermost monolayer (P-face equivalent) in almost equal amounts, whereas the other is cross-fractured and the core contains almost exclusively caveolin-1 label. B: The cores of these two cross-fractured lipid droplets contain both perilipin and caveolin-1 in almost equal amounts. C: Perilipin and caveolin-1 are colocalized on the outermost monolayer (P-face equivalent) of this lipid droplet, whereas the core of the same droplet contains almost exclusively caveolin-1 label. Bars = 0.2 μm.

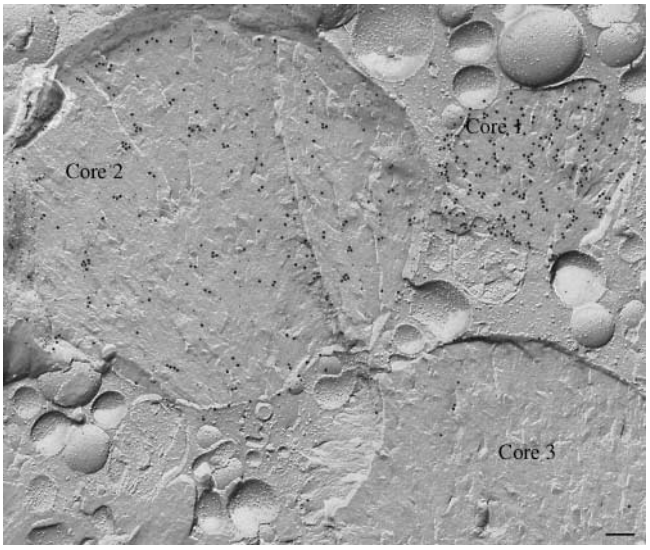

Fig. 6. Distribution of perilipin and caveolin-1 in three lipid droplet cores of an adipocyte. Core 1 is strongly and exclusively labeled with caveolin-1, core 2 with perilipin and caveolin-1 in almost equal amounts, and core 3 contains only traces of perilipin and caveolin-1 label. Bars = 0.2 μm.

the E-face of the ER membrane and within lipid droplet cores is thus incompatible with this hypothetical mechanism (3). We surmised that the known high affinity of lipids for caveolin-1 (38) causes lipids to carry caveolin-1 into the droplet core during droplet formation (3, 17).

Unfortunately, the source of perilipin and adipophilin in the lipid droplet core is difficult to deduce at present. Perilipin and adipophilin have not been detected in sedimentable membranes (10, 11), suggesting that they are not present on membranes of the ER in which neutral lipids are synthesized (39–41). Londos et al. (12) and Brasaemle et al. (11) concluded that perilipins and adipophilin are produced on free and not membrane-bound ribosomes. Thus, the presence of these proteins in the core, together with the currently available information on sites of perilipin and adipophilin synthesis, imply that PAT proteins do not gain access to the lipid droplet by lateral diffusion along the ER outer monolayer but are initially directed toward the lipid droplet from a cytosolic site of synthesis after the droplet has already formed. Gao and Serrero (42) described the translocation of adipophilin to the plasma membrane in COS-7 cells during differentiation. We also have preliminary data that the PAT family proteins are components of the cytoplasmic leaflet of the plasma membrane of macrophages and adipocytes (data not shown). Stimulation of lipid droplet formation by incubation of the cells with acetylated low density lipoprotein leads to clustering of the PAT family proteins in the plasma membrane and close apposition of the lipid droplets to these clusters. We speculate that whereas caveolin-1 is synthesized in the ER and transferred from the endoplasmic leaflet of the ER membrane to the lipid droplet by inundating lipids, the PAT family proteins, which are synthesized on free ribosomes in the cytoplasm, are first inserted in the cytoplasmic leaflet of the plasma membrane and from there to closely juxtaposed lipid droplets. How the polar PAT proteins could be integrated into the core along with the hydrophobic neutral lipids is enigmatic. Nevertheless, at least adipophilin has been characterized as a high-affinity lipid binding protein (43), and lipids are known to exhibit high affinity for caveolin-1 (38), itself polar and a resident of the core.

Finally, lipid droplet cores do indeed contain other polar molecules such as cyclooxygenase (44).

We believe that previous failures to detect lipid droplet-associated proteins in lipid droplet cores depend less on the absence of these proteins there than on the lack of suitability of the methods used to detect them. Traditionally, immunofluorescence microscopic techniques and analyses of subcellular isolates have been used to study lipid droplet-associated proteins and lipid droplet structure. It is now recognized that the fixatives and lipid solvents that are indispensable for immunofluorescence

studies alter the size and shape of lipid droplets and the distribution of lipid droplet-associated proteins (34, 45). In some cases, insufficient antibody penetration may be responsible for the enhanced staining at the lipid droplet margins typically seen in immunofluorescence microscopy. Therefore, methods to achieve higher inherent spatial resolution than that attainable with immunofluorescence microscopy and to avoid chemical fixation and permeabilization are mandatory. Cryosectioning techniques yield high resolution but are inadequate for demonstrating lipid droplet-associated proteins, because fixation is required and because the contents of the lipid droplets leach out during the many washing steps before immunolabeling is complete. Visualization of lipid droplet-associated proteins in lipid droplets by freeze-fracture immunocytochemistry is inherently more reliable than conventional immunofluorescence microscopic and cryosectioning methods, because fixation, lipid solvents, and permeabilization are avoided. Although the effects of freezing on the internal structure of lipid droplets have not been determined (22), possible artifactual redistribution of droplet surface proteins to the core during snap-freezing as an explanation for immunolabeling in the droplet core seems highly unlikely. Differences in labeling intensities and in the labeling patterns for individual lipid droplet-associated proteins in adjacent lipid droplets of the same cell and after double labeling procedures, as well as nonlabeling of lipid droplets by irrelevant antibodies, support this view entirely. We see no a priori reason for anticipating labeling artifacts from the freeze-fracture, replica, and immunocytochemistry procedures, and certainly all the less than for previously used methods of visualization.

The demonstration of PAT proteins inside lipid droplet cores undermines the validity of their being unique to the droplet envelope and makes current hypotheses of lipid droplet formation untenable. On the other hand, it is difficult to understand how these highly polar proteins could be integrated into the core of the droplet among the hydrophobic lipids. New explanations for these processes must be sought. Fusion between smaller lipid droplets, progressive accretion of lipids and lipid droplet-associated proteins onto the surface of the lipid droplet directly from the ER membrane or from the cytosol, and movement of proteins across lipid bilayers are among the crucial aspects that require reevaluation in formulating new models. Other fundamental questions are related to the mechanisms by which nascent lipid droplets bud off ER membranes, which proteins are involved, and how PAT proteins enter lipid droplets. Determining how lipid droplet-associated proteins are packaged among the lipids of the lipid droplet core constitutes a formidable future challenge.

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