Characterization of a Distinct Plasma Membrane Macromdomain in Differentiated Adipocytes*

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Robert G. Parton‡‡**, Juan Carlos Molero††††, Matthias Floetemeyer‡‡‡‡, Kathryn M. Green§§§§, and David E. James****

From the ‡Institute for Molecular Bioscience, §Centre for Microscopy and Microanalysis, and ¶School of Biomedical Sciences, University of Queensland, Queensland 4072, Australia

Caveolae are small invaginations of the cell surface that are abundant in mature adipocytes. A recent study (Kanzaki, M., and Pessin, J. E. (2002) J. Biol. Chem. 277, 25867–25869) described novel caveolin- and actin-containing structures associated with the adipocyte cell surface that contain specific signaling proteins. We have characterized these structures, here termed “caves,” using light and electron microscopy and observe that they represent surface-connected wide invaginations of the basal plasma membrane that are sometimes many micrometers in diameter. Rather than simply a caveolar domain, these structures contain all elements of the plasma membrane including clathrin-coated pits, lipid raft markers, and non-raft markers. GLUT4 is recruited to caves in response to insulin stimulation. Caves can occupy a significant proportion of the plasma membrane area and are surrounded by cortical actin. Caveolae density in caves is similar to that on the bulk plasma membrane, but because these structures protrude much deeper into the plane of focus of the light microscope molecules such as caveolin and other plasma membrane proteins appear more concentrated in caves. We conclude that the adipocyte surface membrane contains numerous wide invaginations that do not represent novel caveolar structures but rather large surface caves.

Adipocytes play a major role in whole body fuel homeostasis. They represent a major site of lipid storage and in turn a major energy source for other tissues. 3T3-L1 adipocytes have been widely used as a model system to study adipogenesis (1). Originally isolated from disaggregated mouse embryos by selecting clones that had the potential to differentiate into adipocytes, they share many of the properties of bona fide mature adipocytes in vivo (2–6). Shortly after the initiation of differentiation, the expression of a number of gene products that regulate adipogenesis and insulin action are markedly increased (7, 8). The cells then round up, presumably due to a rearrangement of the cytoskeleton, and electron-lucent lipid droplets can be seen scattered throughout the cell cytoplasm (9). If the cells are cultured for long periods of time, the lipid droplets tend to fuse to form one large droplet resembling mature white adipocytes.

The morphological changes that accompany adipocyte differentiation have been studied in detail (9, 10). Most notably, each rounded lipid droplet is surrounded by a single lipid layer, which is in turn ensheathed by endoplasmic reticulum. Another characteristic morphological feature of differentiating adipocytes is the increase in abundance of caveolae. Caveolae are evident as single pits at the cell surface as well as associated with surface-connected spherical structures, to form characteristic caveolae-studded features termed “rosettes” (9). Caveolar rosettes are found in many eukaryotic cells (11) but they are particularly abundant in adipocytes. The relationship between these organelles and adipogenesis is not clear, although it may be related to a role in cholesterol homeostasis and/or lipid transport between the lipid droplet and the cell surface.

One of the metabolic processes that is acquired during 3T3-L1 adipocyte differentiation is a highly insulin-responsive glucose-transport system (12). Insulin stimulates glucose transport in muscle and adipocytes in vivo via the translocation of a facilitative glucose transporter GLUT4 from intracellular tubulovesicular elements to the plasma membrane (13). It has been shown that two separate insulin-responsive signal-transduction pathways regulate cell surface levels of GLUT4 in an additive manner (reviewed in Ref. 14). The first pathway involves the lipid kinase, phosphatidylinositol 3-kinase, and the serine/threonine protein kinase Akt/PKB. The second pathway is independent of phosphatidylinositol 3-kinase and involves the recruitment of a multimeric complex that includes C-Clbl and CAP to a specialized microdomain of the plasma membrane (15, 16). This specialized domain has been characterized as a lipid raft domain that is highly enriched in the lipid raft marker proteins caveolin and flotillin. Very recently, these structures have been defined as a specialized form of caveolae associated with the filamentous actin cytoskeleton, referred to as Cav-actin (17). We have also observed the presence of large ring-like structures associated with the plasma membrane of 3T3-L1 adipocytes. In the present study, we have further characterized these rings using both light and electron microscopy. These structures are detected in differentiated adipocytes but not in preadipocytes. They represent large surface-connected invaginations of the plasma membrane and hence have been named caves. In contrast to previous studies (15–17), we show that although these structures are studded with caveolae, they simply represent an extension of the cell surface, also containing clathrin-coated pits and non-caveolar markers (raft and non-raft markers). By light microscopy we observe an enrich-
ment of actin staining around caves and by electron microscopy we observe a filamentous network associated with these structures resembling actin filaments. Thus, caves represent an adipocyte-specific specialization of the cell surface.

EXPERIMENTAL PROCEDURES

Cell Culture—3T3-L1 fibroblasts, from the American Type Culture Collection, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) \(^1\) supplemented with 10% (v/v) newborn calf serum (CSL Limited, Parkville, Victoria, Australia), 2 mM l-glutamine, 100 units/liter penicillin and 100 \(\mu\)g/liter streptomycin at 37°C in 10% CO\(_2\), and passaged at 70% confluence. Confluent fibroblasts were induced to differentiate 1 d after reaching confluence by the addition of DMEM containing 10% (v/v) heat-inactivated fetal calf serum (FCS; Invitrogen), 4 mg/ml insulin, 0.25 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 100 ng/ml d-biotin. After 72 h, the differentiation medium was replaced with fresh FCS/DMEM containing insulin. Adi-

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\({\text{DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SNAP, soluble NSF attachment protein; CT-B, cholera toxin binding subunit B; HRP, horseradish peroxidase.}}\)

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Fig. 1. Glut4 redistributes to surface rings in 3T3-L1 adipocytes. Presence of ring-like structures in 3T3-L1 adipocytes determined by immunofluorescence microscopy. A and B, plasma membrane lawns were obtained from insulin-stimulated 3T3-L1 adipocytes, as described under “Experimental Procedures,” and then fixed and immunolabeled with antibodies specific for either GLUT4 (A) or caveolin-1 (B). C and D, 3T3-L1 adipocytes were incubated in the absence (C) or presence of insulin (D) for 15 min at 37°C. Cells were fixed in acetone and immunolabeled with an anti-GLUT4 polyclonal antibody. Confocal laser-scanned sections were obtained from the base of the cells to the perinuclear region, which were then stacked. E, confocal image of microtubule and actin staining in 3T3-L1 adipocytes. Microtubules (shown in red) were visualized with an antibody specific for alpha-tubulin. Actin filaments (shown in green) were visualized in parallel by staining with phalloidin-FITC. Bar = 5 \(\mu\)m.

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Fig. 2. Caveolin-1 and cholera toxin labeling of 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with CT-B-FITC (CTx) for 30 min at 4°C. They were then labeled for caveolin-1 and examined by confocal microscopy. B, M, and T represent optical sections close to the base, middle, or top of the cell. The lower panels (B') show higher magnification views of the base of one of the cells showing colocalization of CT-B-FITC and caveolin-1 in ring-shaped structures (arrowheads). Bar = 5 \(\mu\)m.

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1 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SNAP, soluble NSF attachment protein; CT-B, cholera toxin binding subunit B; HRP, horseradish peroxidase.
Adipocytes were obtained as described under “Experimental Procedures” and then fixed and immunolabeled with a clathrin-specific antibody. Representative images of a plasma membrane lawn showing focal planes close to the substratum (A) in which the entire plasma membrane lawn is visible or above this plane (B) in which only the ring-shaped structures are prominent. B’ shows a higher magnification of B. C–G’ and C’–G’, plasma membrane lawns from insulin-treated (C and C’) or basal cells (D–G, D’–G’). Lawns were immunolabeled with specific antibodies to IRAP (C), transferrin receptor (D), GLUT1 (E), syntaxin 4 (F), or SNAP 23 (G). C–G’ show corresponding high magnification views of caves. Bar = 10 μm (A–G) or 1 μm (B’–G’).

Experimental Procedures—The insulin-regulated aminopeptidase (IRAP) polyclonal antibody was kindly provided by Dr. Susanna Keller. Clathrin monoclonal antibody X22 was a kind gift of Dr. Frances Brodsky. Phalloidin-FITC and all the fluorophore-tagged secondary antibodies were obtained from Molecular Probes (Eugene, Oregon).

Indirect Immunofluorescence—Cells cultured on coverslips in 6- or 12-well dishes were fixed for 2 h in DMEM/0.2% BSA or Krebs Ringer Phosphate buffer (KRP)/0.2% BSA buffers and incubated with the appropriate treatments. Cells were fixed in acetone for 5 min, washed with PBS, washed again with PBS/0.15 M glycine, and incubated with 1% BSA/PBS for 30 min. Cells were incubated in primary antibodies diluted in PBS/1% BSA for 1 h. Cells were then washed with PBS/0.1% BSA and incubated for 30 min with phallolidin-FITC or the corresponding FITC or Texas Red-conjugated secondary antibody diluted in PBS/1% BSA. Normal rabbit serum was used as a negative control. The coverslips were washed with PBS, mounted onto glass microscope slides, and viewed using a X63/1.4 Zeiss oil immersion objective on a Zeiss Axioplan fluorescence microscope equipped with a Bio-Rad MRC-600 laser confocal imaging system. In experiments designed for staining of cytoskeletal elements, cells were fixed and permeabilized simultaneously in cytokeleton-stabilizing buffer CBS (10 mM PIPES pH 6.9, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA) as described in Ref. 21.

Plasma Membrane Lawn Assay—Plasma membrane fragments were prepared from basal and insulin-stimulated adipocytes as described previously (22, 23). Briefly, adipocytes were grown on glass coverslips and sonicated using a probe sonicator (Kontes) to generate a lawn of plasma membrane fragments that remained attached to the glass. These fragments were then immunolabeled with the corresponding antibodies as indicated in the figure legends. Coverslips were visualized and imaged using a confocal laser scanning immunofluorescence microscope.

Electron Microscopy—3T3-L1 fibroblasts or differentiated adipocytes on 3-cm-diameter plastic dishes were incubated with 50 μg/ml CT-B-HRP (Sigma) for 30 min at 4 °C in DMEM containing 0.1% BSA as described previously (24). They were then washed, fixed, and processed for HRP detection. Alternatively, cells were fixed in 2.5% glutaraldehyde containing 1 mg/ml ruthenium red. The cell monolayer was embedded in a thin layer of Epon resin in situ. Sections were cut parallel to the culture substratum from the base of the cell. In some experiments, pieces of polymerized resin containing the cells were sandwiched together with fresh resin between them, and after polymerization vertical sections were prepared perpendicular to the culture substratum.

For analysis of plasma membrane lawns by electron microscopy, fibroblasts were cultured on carbon/Formvar-coated gold grids in a 6-well multidish. After differentiating for 10 days, grids were transferred to a new well of a 6-well multidish containing 10 ml of PBS. Grids were fixed with 15% methyl alcohol on the bottom of the well, and the grid was then sonicated for 1–3 s using a Sonifier 250 with a microtip (Branson Ultrasonic) 5 mm above the grid at output level 1 until the carbon/Formvar coating started to peel off. The plasma membrane lawns were then immunolabeled with polyclonal antibodies against caveolin-1 and protein A-gold 5 nm followed by fixation and staining as described elsewhere (25).

RESULTS AND DISCUSSION

Light Microscopic Characterization of Cell Surface Architecture in Adipocytes—We have previously described a technique that we refer to as the plasma membrane lawn assay for examining the insulin-dependent movement of GLUT4 to the plasma membrane (22). Using this technique, highly purified plasma membrane fragments, termed plasma membrane lawns, are generated by sonication of whole cells attached to adipocytes were re-fed with FCS/DMEM every 72 h and utilized for experiments at 8–10 days post differentiation.

Reagents and Antibodies—All tissue culture media were purchased from Invitrogen, except fetal calf serum, which was obtained from Trace Biosciences (Clayton, Australia). Insulin was obtained from Calbiochem. Bovine serum albumin (BSA) was purchased from ICN (Costa Mesa, CA). The GLUT4 polyclonal antibody (R017) was raised against a synthetic peptide corresponding to the COOH-terminal 20 amino acids of rat GLUT4. Monoclonal anti-tubulin antibody (DM10) was purchased from Sigma. Polyclonal anti-caveolin antibody was obtained from Zymed Laboratories Inc. Syntaxin4, GLUT1, and SNAP 23 polyclonal antibodies are described elsewhere (18). The insulin-regulated aminopeptidase (IRAP) polyclonal antibody was kindly provided by Dr. Susanna Keller. Clathrin monoclonal antibody X22 was a kind gift of Dr. Frances Brodsky. Phalloidin-FITC and all the fluorophore-tagged secondary antibodies were obtained from Molecular Probes (Eugene, Oregon).
glass coverslips. Labeling of these fragments with antibodies specific for GLUT4 reveals very little labeling in the absence of insulin, whereas with insulin there is a marked increase in labeling. The GLUT4 labeling appears quite uniform across the plane of the membrane but also, in many cells, strongly labels very large ring structures that we have in the past not been able to explain. These structures are shown in Fig. 1. Lawns from insulin-stimulated 3T3-L1 adipocytes were labeled with antibodies to GLUT4 (Fig. 1A) or caveolin-1 (Fig. 1B) and processed for immunofluorescence. Both proteins showed a strong labeling of the striking ring-like structures in many plasma membrane lawns, indicating that both raft-associated proteins such as caveolin and non-raft proteins such as GLUT4 (26) are present in these structures. Although caveolin and GLUT4 were both found distributed over the entire basal surface of the adipocyte, this labeling appeared weaker than that in rings and generally appeared in a more restricted focal plane (data not shown). We then examined the distribution of GLUT4 in intact cells (control or insulin-stimulated) using three-dimensional reconstruction of confocal sections taken through the basal half of the cell. In the absence of insulin, GLUT4 was predominantly found in intracellular perinuclear structures (Fig. 1C), but after insulin stimulation there was a dramatic increase in the general cell surface GLUT4 labeling and in labeling of the rings (Fig. 1D).

The observation that these ring-like structures remained attached to the plasma membrane during preparation of plasma membrane lawns suggested that they were surface-connected. To test this possibility, we labeled adipocytes with FITC-labeled cholera toxin B subunit at 4°C. Under these conditions, the probe labels the entire cell surface and is not internalized. As shown in Fig. 2, the ring-shaped structures
were clearly labeled by cholera toxin and colocalized with the caveolae marker, caveolin-1. We also examined the relationship of the rings to the cortical actin network. The ring-shaped structures were surrounded by FITC-phalloidin labeling indicative of f-actin (Fig. 1E).

We next investigated whether other plasma membrane markers are associated with the ring-shaped structures. Plasma membrane lawns were prepared from adipocytes and labeled for immunofluorescence. We first labeled with antibodies to the coated pit marker, clathrin. As shown in Fig. 3A, a focal plane close to the substratum shows clathrin labeling over the entire surface with a faint indication of ring-shaped labeling (arrowheads). However, a higher focal plane above the lawn (Fig. 3, B and B’) still reveals strong staining of the characteristic rings, now in the absence of general plasma membrane staining. Note that the limiting membrane of the cell is also evident in this plane, presumably representing part of the plasma membrane protruding above the substratum after sonication. We investigated whether other plasma membrane markers are present in the rings. As shown in Fig. 3, C–G and C’–G’, the insulin-regulated aminopeptidase, IRAP, was evident within the caves of insulin-treated cells, and the transferrin receptor, the glucose transporter GLUT1, and the SNARE proteins syntaxin 4 and SNAP23, were all clearly evident within the rings of basal cells. Thus, markers of caveolae, clathrin-coated pits (clathrin, transferrin receptor), and other surface markers (GM1, GLUT1, syntaxin 4, and SNAP23) are all associated with the rings.

Electron Microscopic Characterization of 3T3-L1 Surface Architecture—To further characterize the surface morphology of differentiated 3T3-L1 adipocytes, we used an HRP-labeled cholera toxin B subunit, CT-B-HRP, and electron microscopy. Cells were labeled with CT-B-HRP at 4 °C and then processed for HRP visualization and resin embedding in situ. Semi-thick sections were cut parallel to the culture substratum or samples were processed for vertical sections perpendicular to the culture plane. CT-B-HRP showed labeling of the entire surface and allowed good visualization of associated caveolae (Fig. 4A). As expected, CT-B-HRP labeled both single caveolae as well as the characteristic groups of caveolae around a spherical surface structure, termed rosettes, which have been well characterized in both 3T3-L1 cells (9) and other cell types (11). As in other cell types (11), clathrin-coated pits were also associated with these structures in adipocytes (data not shown), suggesting that they
are not simply an elaborate caveolar structure. Sections parallel to the substratum also revealed long thin invaginations of the surface connecting groups of caveolae to the cell surface (Fig. 4B). These surface-connected invaginations appear to account for the presence of small groups of caveolae deep within the cell interior. A striking example of this is shown in Fig. 4C, in which a caveolae group can be observed within an area of cytoplasm surrounded by the nucleus. Without a cell surface marker these structures could be assumed to be internal caveolae, endosomes, or caveosomes (27).

The most striking observation in these studies was the presence of huge CT-B-HRP-labeled structures within the cell interior. Although variable from cell to cell, these structures were readily visualized in sections cut parallel to the culture substratum after CT-B-HRP labeling (Figs. 5, 6, and 8). Because cells were labeled at 4 °C, these structures are clearly surface-connected and correlate with the ring-shaped structures labeled with CT-B-FITC and caveolin-1 seen by light microscopy. As is evident in Figs. 5–8, at low magnification these structures could easily be mistaken for lipid bodies owing to their large size, electron-lucent lumen, and position in the cell (Figs. 5–8). However, the labeling of these structures with CT-B-HRP as well as the presence of associated caveolae showed that these structures are surface-connected. We also used a different surface marker, the nonspecific plasma membrane marker ruthenium red (Fig. 7). Ruthenium red also showed striking labeling of the surface-connected “internal” structures in differentiated adipocytes. Neither ruthenium red nor CT-B-HRP labeled these structures in undifferentiated 3T3-L1 adipocytes (results not shown), showing that they are specific to the differentiated cells. We operationally

Fig. 6. Electron microscopic analysis of adipocyte caves. 3T3-L1 adipocytes were processed and sectioned as described in the legend to Fig. 4 (A and B) or were resectioned perpendicular to the culture substratum (C). The large caves (asterisks) are several micrometers in diameter and surround the nucleus. The caves possess numerous caveolae (arrows), as shown at higher magnification in B. The perpendicular section in C shows that there are large invaginations of the basal surface next to the culture substratum (arrowheads) caves. Bar = 600 nm.
termed these structures “adipocyte caves” to emphasize their presence in the differentiated cells and their unique surface architecture.

Despite their surface connection, the caves were often found extremely close to the nucleus, Golgi complex, and lipid bodies in the perinuclear area of the cell (Figs. 5–8). Close examination of the caves showed them to be composed of both invaginated caveolar membrane and intervening non-caveolar planar membrane (e.g., see Figs. 6B and 7C). The density of caveolae associated with the cave membrane was variable but in the same range as that observed at the peripheral plasma membrane. Clathrin-coated pits were also associated with the caves (Fig. 5A) as well as thin filaments which, based on the immunofluorescence data (Fig. 1), we assume represent actin filaments (Fig. 5, A and C). We used perpendicular sections to further examine the relationship of the caves to the basal plasma membrane. In some sections, we fortuitously observed large invaginations at the base of the cell that extended upward toward the interior (Fig. 6C), suggesting that the caves are huge pits in the basal plasma membrane. We also performed serial sectioning of CT-B-HRP-labeled cells, cutting a series of sections from the base of the cell (see Fig. 8 for representative sections). The first sections showed, as expected, that the entire basal cell surface was covered in densely packed CT-B-HRP-labeled caveolae (Fig. 8A). Large CT-B-HRP-labeled structures were clearly visible in these, and in
subsequent, serial sections (Fig. 8, B, C, and C'). Note that lipid bodies and nuclei were in the same plane as the caves, showing that the caves extend up from the base of the cell into the perinuclear area. Serial section analysis, combined with analysis of perpendicular sections (see above) showed that the caves are extremely large in the xy plane (up to several micrometers) but generally smaller in the z plane (one micrometer or less).

Plasma membrane lawns have been extensively characterized by light microscopy. To allow exact correlation of the labeling seen by light microscopy, we developed methods to analyze the ring-shaped structures seen in plasma membrane lawns at the ultrastructural level by immunogold electron microscopy. 3T3-L1 adipocytes were cultured on Formvar-coated grids and then sonicated to leave plasma membrane lawns attached to the grids. The grids were then immunogold labeled using antibodies to caveolin-1. As shown in Fig. 9, putative caves were readily visualized with this technique, appearing as circular or multilobed structures within the center of the lawns. Caveolae, labeled with anti-caveolin antibodies, and clathrin-coated pits (recognized by characteristic morphology) were very abundant around many of the caves (Fig. 9, B–E). Other putative caves appeared to have been sheared off close to the plane of the plasma membrane and showed no evident concentration of caveolae or coated pits (Fig. 9F). Taken together with observations of sectioned material, these observations suggest that concentration of markers within caves simply represents the amount of plasma membrane retained with the caves after sonication.

The abundance of the rings was variable among different cells in the field, and we were unable to find any significant correlation between their abundance and other parameters such as density of lipid droplets, cell size, and insulin sensitivity. Regardless, based on serial section analysis we have estimated that the contribution of these structures to the total plasma membrane area may be as high as 25% in certain cells and so these structures may play an important role in transmitting signals or nutrients across the cell surface.

This study represents the first ultrastructural description of a novel plasma membrane specialization in differentiated 3T3-L1 cells and has important implications. It has recently been suggested that adipocytes possess a novel signal-transduction pathway involved in insulin action that is localized to surface-connected caveolae (15–17). However, the structures in which caveolin and the signaling proteins colocalize are identical morphologically to those reported here. Because the ring-shaped caves are the prominent structures observed in plasma membrane lawns (because they have greater depth than the flat plasma membrane between), it can appear that proteins colocalize in these domains by light microscopy. Colocalization of proteins, such as flotillin, with caveolin in the ring-like structures has been interpreted as evidence of caveolae localization (15). However, in view of the results presented here it is
important to assess at the ultrastructural level whether such proteins are actually enriched in these domains as compared with the bulk plasma membrane. It is clear that higher resolution is required to show caveolae localization. Based on our data the rings, or caves as we call them, are not simply groups of caveolae but represent large plasma membrane domains with associated caveolae. GLUT4, a well characterized membrane protein that does not show raft association (26), is also clearly associated with this domain. These domains also appear to act as non-selective docking sites for exocytic vesicles. We have observed the presence of SNAREs in these structures (Fig. 3) and at times soon after insulin stimulation we have observed the simultaneous appearance of GLUT4 in both caves and the flat part of the membrane (data not shown). In addition, we have shown the association of clathrin-coated pits and transferrin receptors with caves. Analysis of plasma membrane lawns by electron microscopy, as described here, indicates that in some instances the caves, which extend away from the plane of the membrane, may remain associated with the lawns whereas in other cases they may be removed, presumably during the sonication process. This likely explains why on occasions we observe large holes in the lawns by immunofluorescence microscopy (data not shown). In cases where the membrane is retained, it may appear that marker molecules are relatively enriched in these structures compared with the flat plasma membrane by light microscopy due to their extended height. This is well illustrated for clathrin (Fig. 3), in which the relative abundance of this marker in the flat part of the membrane versus the caves depends on where the plane is taken. Thus, further work is required to establish caveolae, or raft localization, of specific signaling pathways.

The caves that we have described here appear to form during the process of adipocyte differentiation, because we were unable to detect them in 3T3-L1 preadipocytes. Notably, caves were not observed in all adipocytes in the culture suggesting that they may have a rather specialized role. Further study will be required to determine the function, biogenesis, and dynamics of these structures in adipocytes. The caves clearly represent areas of plasma membrane detachment from the substratum. Of note was the presence of filamentous material,
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we assume represents extracellular matrix, traversing the lumen of each cave (see Fig. 8 for examples). Whether a simple absence of adhesion molecules in certain areas of the plasma membrane, or masking of these molecules, could cause the generation of the caves is presently unclear. After trypsinization of adipocytes, cave-like structures could still be detected but only in a minority of cells (results not shown). It also remains to be established whether caves are dynamic structures that are constantly being remodeled. Images of actin at the neck of two connected structures (e.g. Fig. 5) would certainly be consistent with this view, although previous studies suggest that clustered caveolae are not significantly affected by actin disruption (17).

In conclusion, we have shown that in a model adipocyte system large “internal” vacuoles are connected to the “limiting” membrane. Rather than simply groups of caveolae, these membranes have the characteristics of a plasma membrane domain; they are composed of caveolae, coated pits, raft and non-raft membranes and have an associated cortical actin network.

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