Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes

E. Joan Blanchette-Mackie,[1,*] Nancy K. Dwyer,[*] Therese Barber,[*] Robert A. Coxey,[*] Takehiko Takeda,[†] Cristina M. Rondinone,[†] Janice L. Theodorakis,[†] Andrew S. Greenberg,[†] and Constantine Londos[†]

Lipid Cell Biology Section,[*] Laboratory of Cell Biochemistry and Biology and Membrane Regulation Section,[†] Laboratory of Cellular and Developmental Biology, National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-0850

Abstract Immunocytochemistry was used to determine the intracellular location of perilipins in adipocytes and the occurrence of these proteins in tissues involved in triacylglycerol metabolism. Confocal microscopy and 3-dimensional analysis of 3T3-L1 adipocytes showed that perilipin immunofluorescence, present on the surfaces of all sized lipid droplets, appeared unevenly dispersed on the surfaces of many large lipid droplets. Electron microscopy revealed that immunogold staining for perilipin was located directly on the surface layer apposed to and surrounding the core triacylglycerol of intracellular lipid droplets of adipocytes in culture or from white and brown adipose tissue. Freeze-fracture electron microscopy indicated that the hydrophobic face of this surface monolayer contained particles identical in size and distribution to intramembranous particles (IMPs), which are unique structural features of the hydrophobic faces of bilayered membranes. Also, freeze-fracture replicas revealed areas of continuity between the surface layer of lipid droplets and the membrane leaflets of endoplasmic reticulum, suggesting that the droplet monolayer surface is an area of endoplasmic reticulum membrane leaflet modified by its unique content of perilipin. Microperoxisomes, identified by immunostaining for catalase, were found closely associated with lipid droplets, but external to and not in contact with the lipid droplet surface layer. Vimentin, identified by immunofluorescence, was present around the periphery of most lipid droplets in 3T3-L1 cells during early stages of adipocyte development but, in contrast to perilipins, vimentin was not around the periphery of many large lipid droplets in mature cells. Although perilipin was at the surface of lipid droplets in adipocytes of lactating mammary gland, none was found to be associated with the milk lipid droplets in alveolar epithelial cells, nor was the protein found on the surfaces of lipid droplets in hepatocytes. Studies in mammary gland show that perilipin immunostaining will be a valuable tool for the identification of tissue adipocytes severely depleted of their triacylglycerol stores and thus without their characteristic spherical shape. Perilipin's singular location on the surface monolayer of intracellular lipid droplets supports an intimate role for the protein in the triacylglycerol metabolic functions of adipocytes.—**Blanchette-Mackie, E. J., N. K. Dwyer, T. Barber, R. A. Coxey, T. Takeda, C. M. Rondinone, J. L. Theodorakis, A. S. Greenberg, and C. Londos.** Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *J. Lipid Res.* 1995. **36:** 1211–1226.

Supplementary key words triacylglycerol • endoplasmic reticulum • lipid droplet surface layer • peroxisomes • mammary gland • freeze-fracture • confocal microscopy

Perilipin, a major phosphoprotein of adipocytes, was discovered in the isolated fat layer from homogenized rat primary adipocytes (1, 2). This protein is the most abundantly radiolabeled phosphoprotein after lipolytic stimulation of adipocytes, and was identified initially as a 65/67 kDa doublet in SDS-PAGE gels (1). Perilipin has been shown to be associated with the periphery of intracellular lipid droplets in cultured murine 3T3-L1 adipocytes by light microscopic immunofluorescence with the use of rabbit antisera raised against rat perilipin (2). Recently, cloning of perilipin cDNA from a rat adipocyte expression library revealed two forms of the protein, the most abundant being one of 56 kDa, termed perilipin A, which, when phosphorylated migrates as an 65 kDa protein by SDS-PAGE; the second, less abundant, is 47 kDa, perilipin B (3). The two isoforms, which arise by alternative RNA splicing, are identical through their first 406 N-terminal amino acids but contain unrelated C-termini. The perilipins appear to be unique proteins as sequence analysis revealed no significant extended identity with any known proteins. Although the function of perilipin is unknown, several features suggest that it might have a role in lipid metabolism. These include the findings that perilipin is expressed in cells in which hormonal stimulation induces lipolysis, that it is located at the periphery of

Abbreviations: IMPs, intramembranous particles; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

[1]To whom correspondence should be addressed.

lipid storage droplets, and that lipolytic stimulation leads to polyphosphorylation of the protein in concert with the lipolytic response (1, 2).

The importance of perilipins to cellular lipid mobilization could be better evaluated by knowing their subcellular location in adipocytes and whether they are present in cells that synthesize but do not hydrolyze intracellular stores of triacylglycerol. Thus, murine 3T3-L1 adipocytes, isolated primary rat epididymal adipocytes, white and brown adipose tissue, mammary gland from lactating rats, and mouse liver were tested with immunofluorescence as well as with immunogold staining of cryosections by electron microscopy for the presence of perilipins. Our results in adipocytes showed that perilipin is located solely on the surface monolayer surrounding intracellular lipid droplets, and freeze-fracture electron microscopy showed that this perilipin-containing surface of intracellular lipid droplets contained intramembranous particles (IMPs). We also resolved the spatial relationship among endoplasmic reticulum, peroxisomes, vimentin, and lipid droplets in adipocytes. Perilipin was not detected in hepatocytes or mammary alveolar epithelial cells which also contain intracellular stores of triacylglycerol.

MATERIALS AND METHODS

Cell cultures

3T3-L1 fibroblasts were seeded in two-well plastic chambers (Lab-Tek plastic microscope culture wells from Miles Scientific, Naperville, IL) or 10-cm glass dishes for light and electron microscopy and grown in DMEM containing 10% fetal bovine serum. At confluence, the cells were washed extensively with phosphate-buffered saline (PBS) and the cell culture was continued in a chemically defined medium containing DMEM–Ham F-12 medium (1:1) with 30 μm biotin, 10 μg/ml transferrin, 1 mg/1ml fetuin, and 10 units/ml penicillin. Two days later (day 0), differentiation was initiated with 0.5 mM 3-isobutyl-1-methylxanthine and 0.1 μM dexamethasone for 3 days. Subsequently, the cells were incubated for an additional 3 days with 1 μM insulin, and thereafter maintained without hormones with a change in medium every other day.

For light microscopy, 3T3 L1 cells were fixed in 3% (w/v) paraformaldehyde in PBS for 30 min. Cells in chamber wells were immunolabeled for perilipin, catalase, or vimentin, stained with Nile Red for fluorescent observation of lipid droplets, or stained with $DiOC_6$ (Molecular Probes, Eugene, OR) for fluorescent observation of endoplasmic reticulum. For electron microscopy, 3T3-L1 cells were processed for cryosectioning, resin-embeding, or freeze-fracture analysis. For cryosectioning, 3T3-L1 cells grown in glass culture dishes (100 mm) were fixed in 3% paraformaldehyde and 0.2% glutaraldehyde in PBS at pH 7.4 for 40 min. After washing in PBS, a solution of 10% gelatin at 37°C was added to the dish for 5 min. The cells were scraped off the glass dish into a microfuge tube containing warm gelatin and centrifuged at 15,000 *g* for 1 min to form a pellet of cells in gelatin. The pellet was solidified at 4°C, refixed for 45 min, washed in PBS, removed from the tube, and cut into 1-mm cubes. Cubes were infiltrated with 2 M sucrose for 1.5 h at room temperature and frozen on cryopins in liquid N_2 for cryosectioning. For resin embeding, 3T3-L1 cells grown on plastic chamber slides were fixed in 0.5% potassium permanganate in PBS at pH 7.4 for 1 h, dehydrated in graded alcohols, and embedded in LX112 resin. After hardening in a 60°C oven overnight, the resin with embedded cells was easily separated from the plastic slide and sectioned. For freeze-fracture, 3T3-L1 cells in glass culture dishes were fixed in Karnovsky's fixative (4) for 30 min, scraped from the dishes, pelleted in fresh fixative in microfuge tubes (1 min at 15,000 *g*), cryoprotected in 30% glycerol in 0.2 M sodium cacodylate, pH 7.4, and immediately frozen in Freon cooled with liquid nitrogen. Freeze-fracture replicas of cell pellets were prepared in a Balzers model BAF 301 freeze-etch device.

Isolation of cells and tissues

Epididymal fat pads were obtained from Sprague-Dawley rats, mammary glands were from 6-day lactating rats, and brown fat pads and livers were from 2-day-old mice. Adipocytes were isolated from epididymal fat pads according to the method of Rodbell (5). For light microscopy, isolated adipocytes and tissues were fixed in 3% (w/v) paraformaldehyde in PBS at pH 7.4 for 30 min, washed in PBS, and prepared for cryosectioning in the same manner as 3T3-L1 cells. For electron microscopy, isolated adipocytes and tissues were fixed in 3% (w/v) paraformaldehyde and 0.2% glutaraldehyde in PBS, pH 7.4, for 30 min before preparation for cryosectioning. Cells and tissues were fixed in Karnovsky fixative (4), post-fixed in 2% OsO_4, resin-embedded, and sectioned for electron microscopy.

Cryosectioning

Thin cryosections (1.0 μm for light microscopy) and ultrathin cryosections (less than 0.1 μm for electron microscopy) were cut at −80 to −85°C on a Reichert Ultracut E microtome equipped with an FC4 attachment. Sections were transferred on drops of sucrose-gelatin to either slides or formvar-coated and carbon-coated 300-mesh gold grids by the method of Tokuyasu (6).

Immunolabeling

3T3-L1 cells in culture and cryosections on slides or grids were stained for perilipin, catalase, or vimentin with an indirect immunolabeling procedure. Primary antibodies used were affinity-purified rabbit anti-rat perilipin (2), rabbit anti-mouse vimentin antisera from R. M. Evans,

University of Colorado, (Denver, CO), and rabbit anti-bovine catalase from P. Lazarow, Mount Sinai Medical School (New York). All incubations (quench, antibody, and washes) were performed in PBS, at pH 7.4, containing saponin (0.1 mg/ml) and goat IgG (2.5 mg/ml). Cells and cryosections were incubated in PBS containing 0.2 M glycine for 45 min to quench aldehyde groups. Cells for vimentin immunostaining were fixed for 10 min in cold methanol instead of aldehyde. Cells were incubated with primary antibodies overnight at 4°C, washed (3 × 10 min) at room temperature, incubated with rhodamine-labeled secondary antibodies (1:50 dilution) for 1 h, and washed (3 × 10 min). For immunocytochemical controls, cells were incubated in rabbit preimmune serum or rabbit IgG at the same dilution as the immune antiserum and processed in an identical manner. For light microscopy, cells and cryosections were mounted in *p*-phenylenediamine glycerol (7) and rhodamine fluorescence was viewed with a Leitz fluorescence microscope with excitation filter, BP-530-560. Confocal microscopy was performed on a Nikon Optiphot microscope equipped with a Bio-Rad MRC-6000 confocal imaging system, a krypton argon laser, and dual channel filter set. For detecting rhodamine fluorescence, we used a 568 DF10 excitation filter, double dichroic, a 560 DR, LP dichroic reflector, and a 585 EF, LP emission filter. Ultrathin cryosections on grids were negatively stained with uranyl acetate and embedded in methyl cellulose by the method of Tokuyasu (6). Grids were viewed with a Phillips 400 transmission electron microscope.

Fluorescent staining

Lipid droplets in 3T3-L1 cells were stained for 10 min with 0.01% Nile Red in PBS (8). Cells were washed and mounted in glycerol for fluorescence microscopy with excitation filter BP-530-560. Endoplasmic reticulum in 3T3-L1 cells was stained for 30 sec with 0.005% D-273 3, 3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (9). Cells were viewed with a Leitz fluorescence microscope with excitation filter BP-450-490.

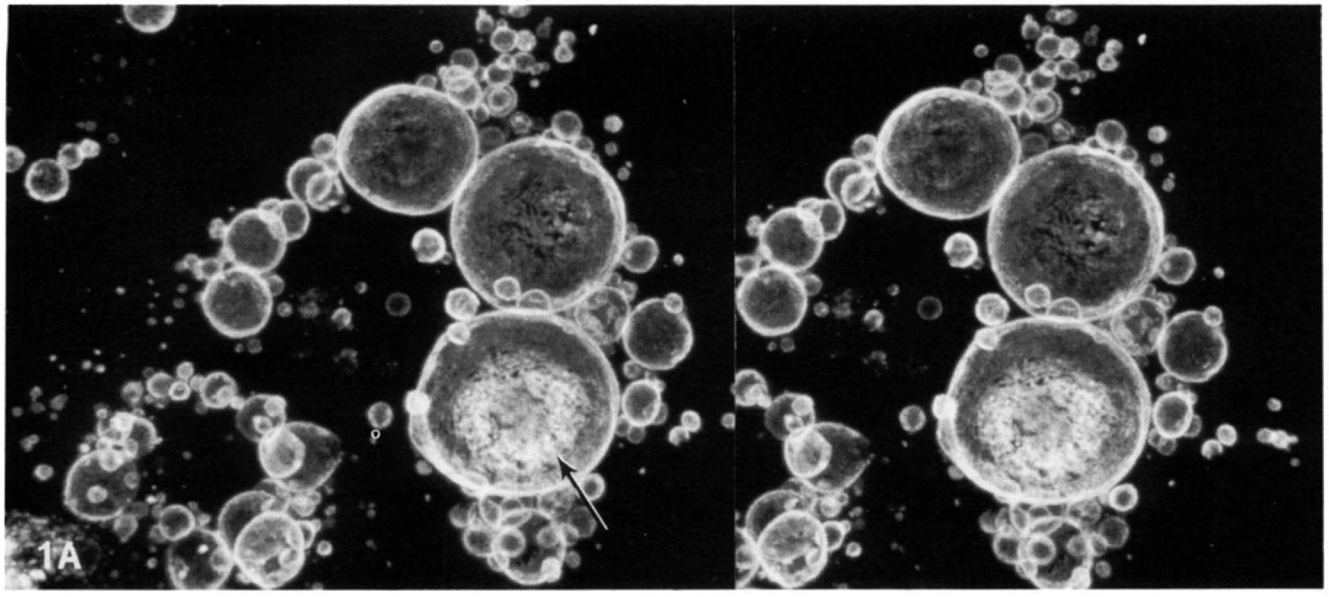
RESULTS

3T3-L1 adipocytes

Perilipin, lipid droplets and endoplasmic reticulum. With fluorescence microscopy we previously demonstrated perilipin immunostaining at the periphery of intracellular lipid droplets in murine 3T3-L1 adipocytes (2) that were identified by phase optics and Nile red staining. Optical sectioning through intracellular lipid droplets with confocal microscopy allowed greater resolution of the lipid droplet-perilipin association. Three-dimensional imaging of perilipin immunofluorescence showed that lipid droplets were present in clusters throughout adipocytes with small and large lipid droplets in contact (**Fig. 1A**). Perilipin covered the surfaces of lipid droplets of all sizes, although the surfaces of most larger droplets had an uneven distribution of perilipin (Figs. 1A, 1B); that is, there were surface areas of bright immunofluorescence and other sites with little or no immunostaining (Fig. 1B).

The relationship between perilipin and intracellular structures, including endoplasmic reticulum, lipid droplets, peroxisomes, and the intermediate filament protein, vimentin, was examined in 3T3-L1 cells with electron microscopic techniques. 3T3-L1 adipocytes were fixed with potassium permanganate, a selective fixative that preserves amphipathic molecules in cellular membranes, including endoplasmic reticulum, but does not preserve the nonpolar triacylglycerol of intracellular lipid droplets (10, 11). The surface layer of lipid droplets was also well preserved with this fixative and appeared in cross section as a single electron opaque line (**Figs. 2B, 2C** and **Fig. 3A**) and in grazing section as a moderately electron opaque layer adjacent to the lucent lipid droplet core (Fig. 2C). Staining of 3T3-L1 adipocytes with the fluorescent dye, $DiOC_6$, showed that elements of endoplasmic reticulum could be resolved as crescentric or ring-shaped structures at the periphery of lipid droplets (Fig. 2A). Electron microscopy showed that many tubular and vesicular elements of endoplasmic reticulum were in proximity to lipid droplets (Figs. 2C and 3A) and that such elements had areas of continuity with surface layers of lipid droplets (inset to Fig. 2C, at arrow). Some cisternae of endoplasmic reticulum either completely encircled lipid droplets (Fig. 2B) or followed the circular contours of the lipid droplet surface for extended distances (Fig. 3A). Immunogold electron microscopy of ultrathin cryosections of 3T3-L1 adipocytes revealed that perilipin was present only on the surface layer immediately adjacent to the lucent lipid droplet core (Fig. 3B); none was found on profiles of endoplasmic reticulum intimately associated with the lipid droplet surface nor elsewhere in cells. Small electron lucent, circular structures, 0.1 to 0.3 μm in diameter, that were in close association with cisternae of endoplasmic reticulum (**Fig. 4A**) could be identified as sections through lipid droplets by virtue of immunogold staining for perilipin. Both cross sections (Figs. 3B and 4A) and grazing sections (Fig. 4B) through lipid droplets showed an uneven, patchy distribution of immunogold on the surface layers of droplets. No regularly patterned arrays of immunogold were observed.

Further analysis of the perilipin-containing lipid droplet surface layer and its relationship to endoplasmic reticulum was made with freeze-fracture electron microscopy. Freeze-fracture replicas of 3T3-L1 adipocytes revealed that the surface layer of the lipid droplet contained intramembranous particles (IMPs) of approximately 75 Å located on the face of the surface layer in contact with the triacylglycerol core (**Fig. 5A,** stereo



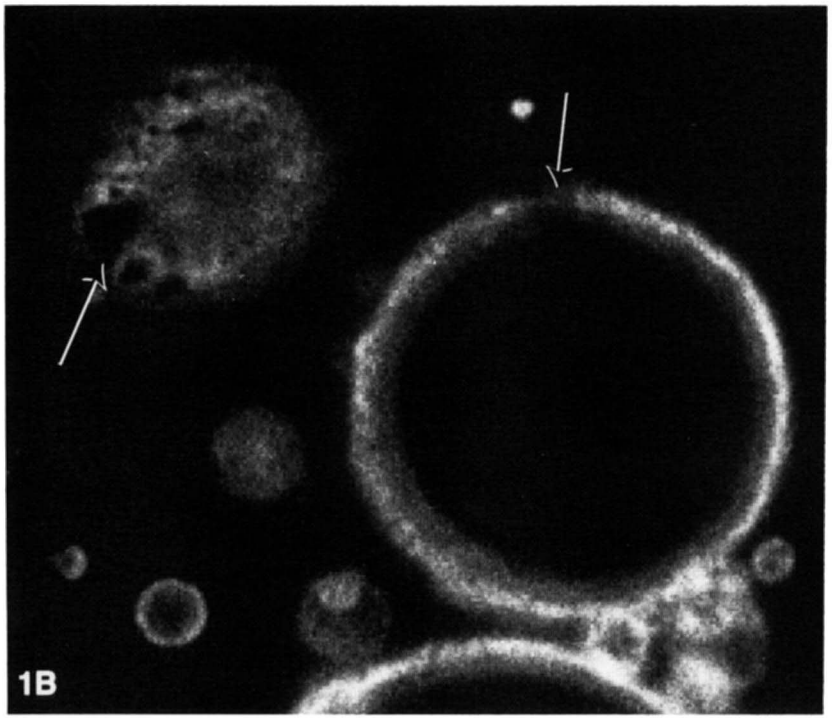


Fig. 1A. Stereo-paired confocal fluorescence images of lipid droplets in 3T3-L1 adipocytes immunostained for perilipin. Fluorescence immunostaining of some of the lipid droplet surface is more intense (arrow) than that at other sites, indicating perilipin is not homogeneously distributed on the surface. Note the small lipid droplets in contact with the surface of the large droplets. × 1,400. 1B: Confocal image of fluorescence immunostaining for perilipin at the surface of lipid droplets in 3T3-L1 cells. Note the discontinuous fluorescence at the surfaces of optically sectioned lipid droplets (arrows). × 4,000.

pair). Also, sites of continuity between the lipid droplet surface layer and membrane leaflets of endoplasmic reticulum were observed in freeze-fracture replicas (Fig. 5B at arrow).

Lipid droplets and peroxisomes. Peroxisomes in adipocytes are associated with the periphery of lipid droplets (11) and do not have a characteristic morphology, such as the core uricase crystals, that makes them easily recognizable in hepatocytes and other cells. Thus, we identified peroxisomes in adipocytes by immunogold staining for catalase. In 3T3-L1 adipocytes, many small characteristically dumbbell-shaped membranous structures were present at the periphery of lipid droplets and often between two or more lipid droplets (**Figs. 6A and 6C**). Immunogold staining of cryosections of 3T3-L1 cells for catalase showed that dumbbell-shaped as well as other elongate membranous structures associated with elements of endoplasmic reticulum surrounding lipid droplets were peroxisomes (Figs. 6B, 6D, and 6E).

Lipid droplets and vimentin. Studies indicate that as

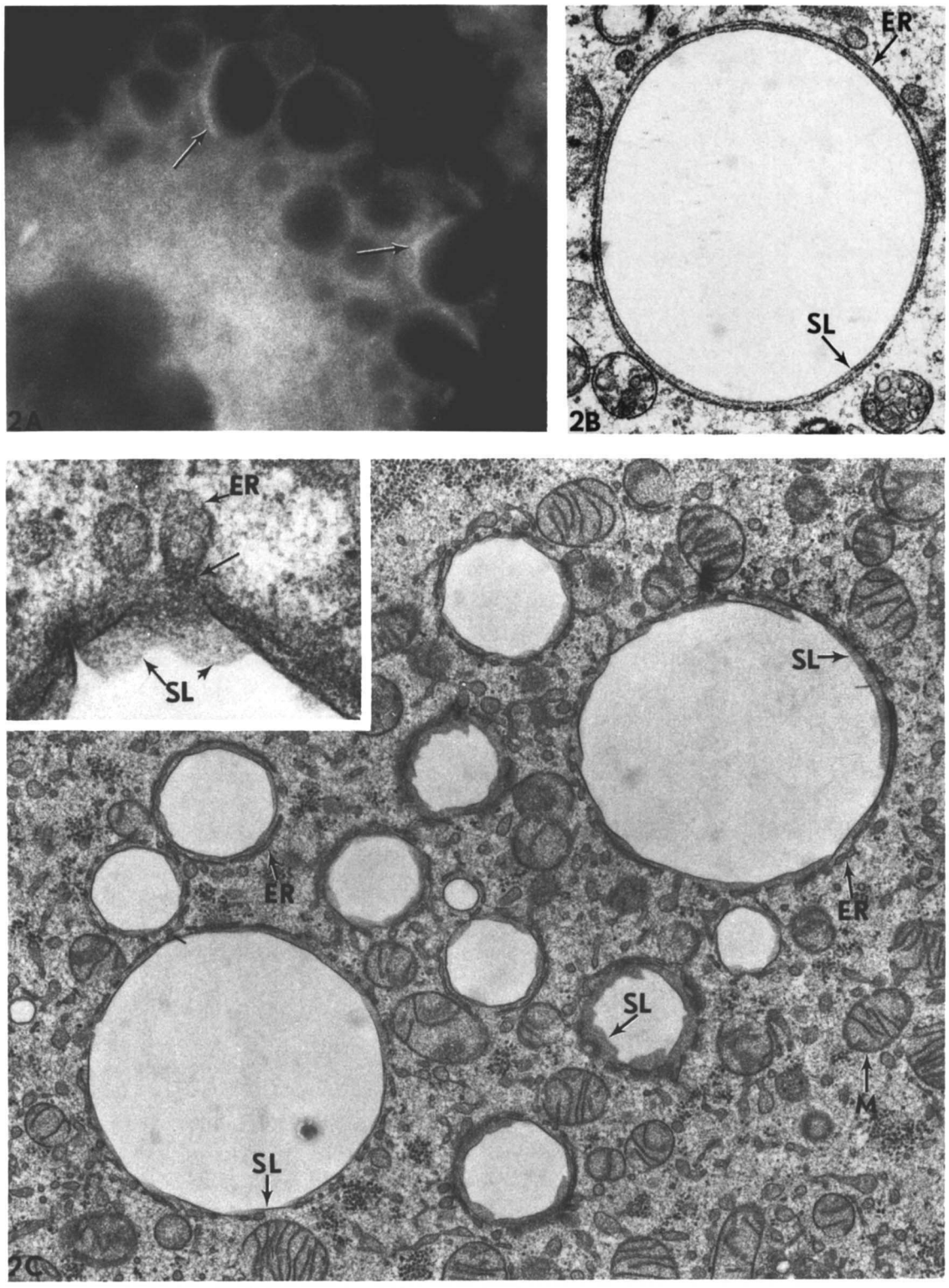
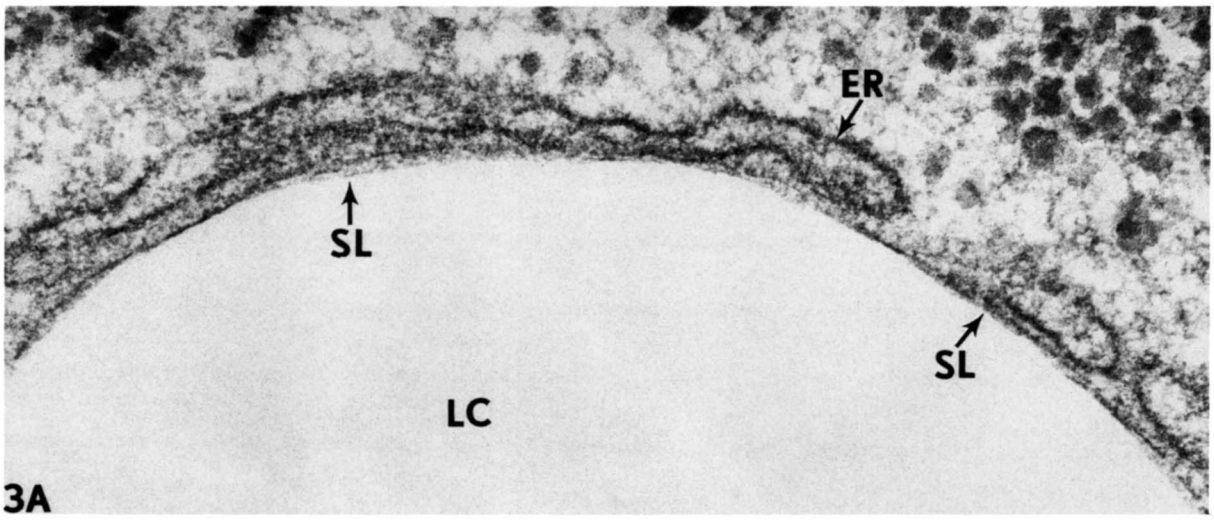


Fig. 2A. Light micrograph of 3T3-L1 adipocytes fluorescently stained with $DiOC_6(3)$ for endoplasmic reticulum (ER). Fluorescence staining is present in the central portion of the cell and in discrete crescentic shaped patterns (arrows) associated with the periphery of lipid droplets (LD). × 850. 2B and 2C: Electron micrographs of sections of 3T3-L1 adipocytes fixed with potassium permanganate. The lipid droplet triacylglycerol core (LC) is not preserved with this fixation and appears electron lucent while the surface layer (SL) of lipid droplets and endoplasmic reticulum (ER) are well preserved. 2B: A profile of endoplasmic reticulum (ER) appears to completely surround the lipid droplet periphery. × 41,000. 2C: The surface layer (SL) of lipid droplets is seen in both grazing sections and cross sections through droplets. Lipid droplets are encircled by endoplasmic reticulum which appears in section as cisternal, tubular and vesicular profiles. M, mitochondria. × 19,500. Inset shows area of continuity (at arrow) between membranous profile, presumably of endoplasmic reticulum (ER), and the "en face" sectioned surface layer (SL) of a lipid droplet. × 64,000.



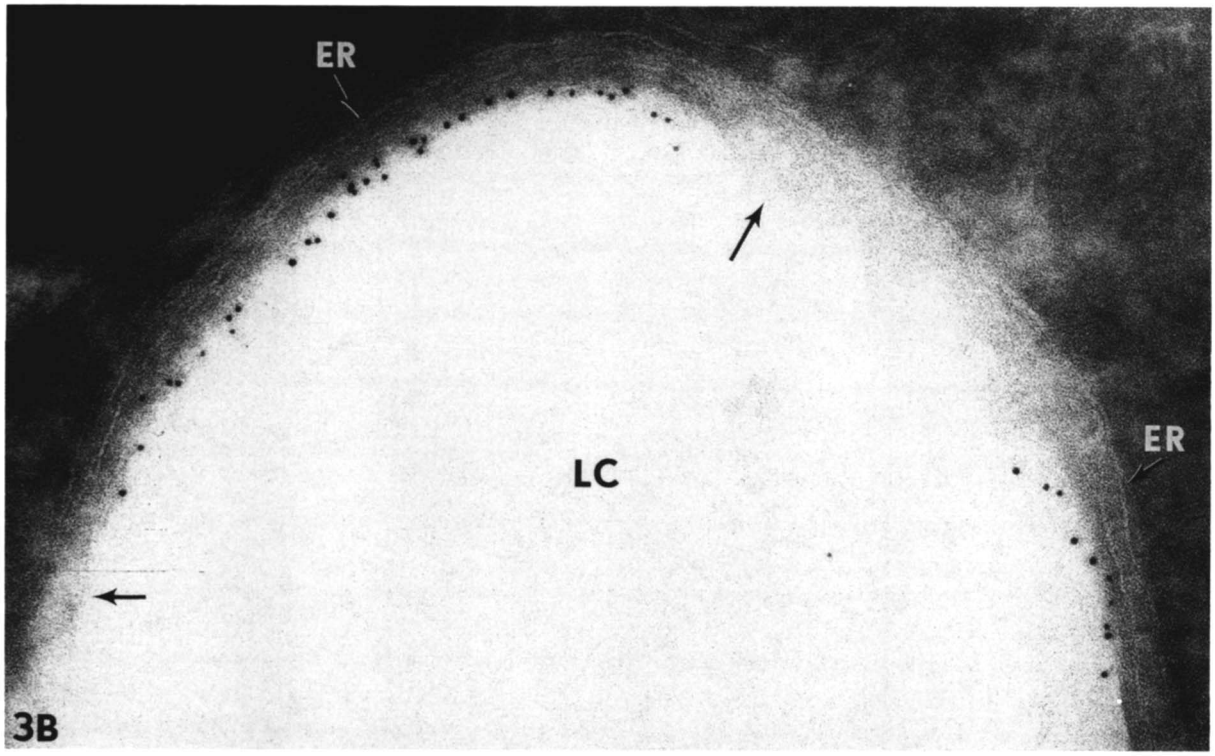
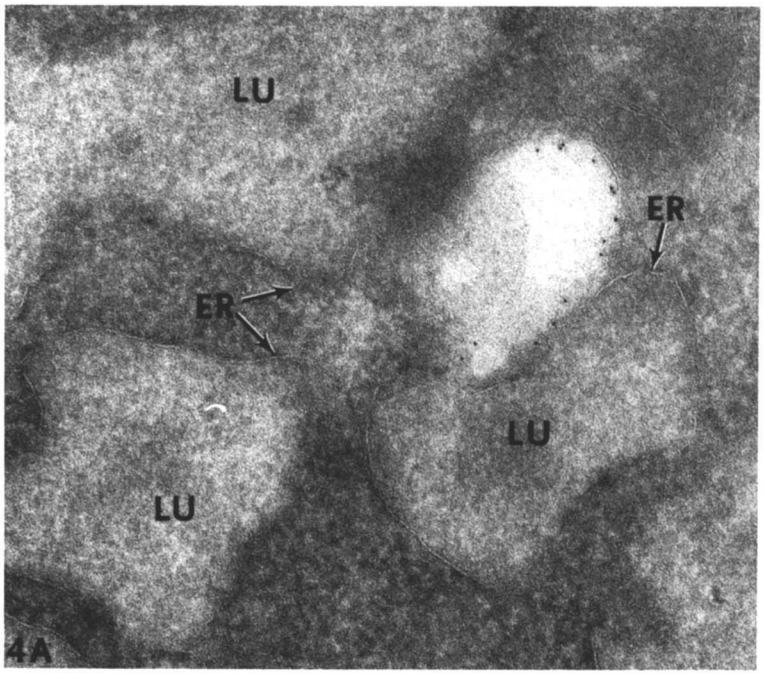
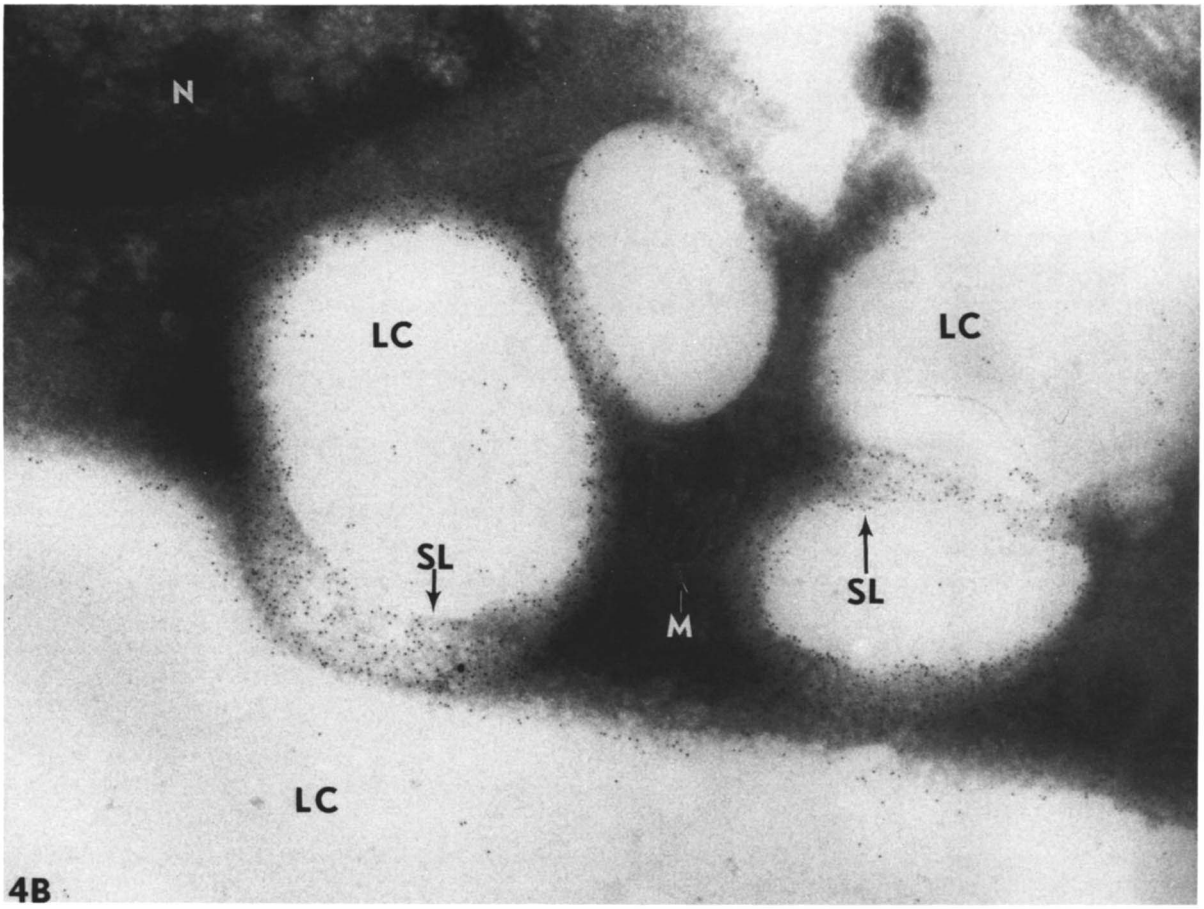


Fig. 3A. Electron micrograph of section through a lipid droplet in 3T3-L1 adipocyte fixed with potassium permanganate. The lipid droplet core (LC) is electron lucent, the surface layer (SL) appears as a single line in cross section and the endoplasmic reticulum (ER) is in close proximity to the lipid droplet periphery. × 70,000. 3B: Electron micrograph of a cryosection of 3T3-L1 adipocyte immunogold stained with antibodies to perilipin. Gold particles (5 nm) are present only at the lipid droplet surface. Note areas of the lipid droplet surface that do not immunostain with gold particles (arrows). Endoplasmic reticulum (ER) in close proximity to the lipid droplet periphery does not immunostain for perilipin. Lipid droplet core, LC. × 117,000.

differentiating 3T3-L1 fibroblastic-shaped cells accumulate lipid and develop into rounded mature adipocytes, some reorganization of cytoskeletal elements occurs (12). Aggregation of vimentin filaments around forming lipid droplets in 3T3-L1 adipocytes was shown with fluorescence microscopy (13). We identified vimentin in cells, with immunostaining, from day 3 to day 7 during differentiation of 3T3-L1 adipocytes. Optical sectioning with confocal microscopy enabled us to discern whether vimentin immunofluorescence occurred at the periphery of individual lipid droplets. At early stages of adipocyte differentiation, vimentin filaments were present not only





Figs. 4A and 4B. Cryosection of 3T3-L1 adipocytes immunogold stained with antibodies to perilipin. 4A: Immunogold particles (arrows) are present at the surface of a small (0.3 μ) electron lucent sphere in contact with endoplasmic reticulum (ER). Lu, lumen of endoplasmic reticulum × 58,000. 4B: This cryosection grazes the surfaces of intracellular lipid droplets to expose the surface layers (SL) adjacent to electron lucent cores (LC). The presence of gold particles indicates the location of perilipin on the surface layer of lipid droplets. N, nucleus; M, mitochondrion. × 39,000.

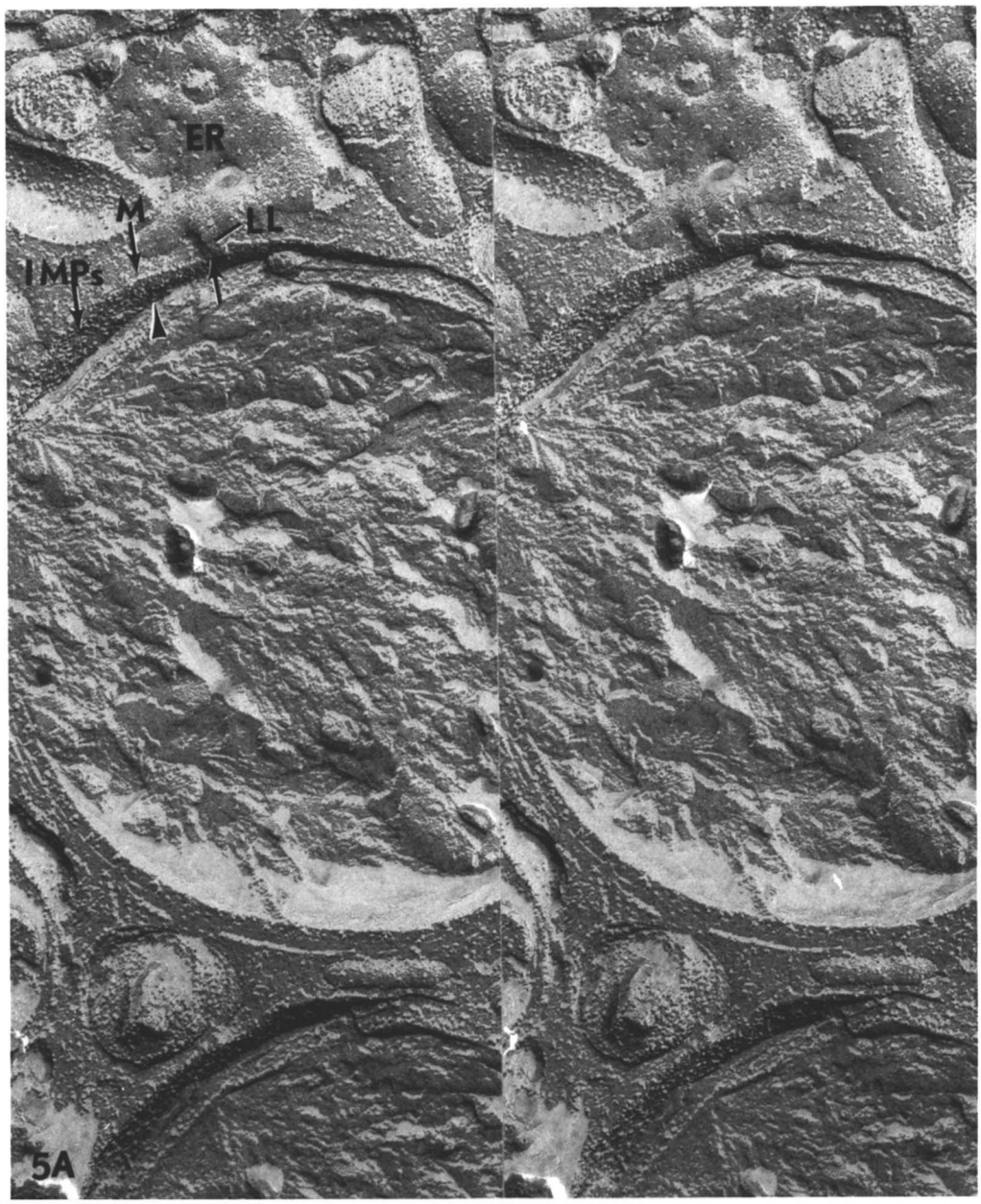


Fig. 5A. Stereo-paired electron micrographs of a replica of freeze-fractured lipid droplets in 3T3-L1 adipocyte. A membrane monolayer (M) containing intramembranous particles (IMPs) is in direct contact (at arrowhead) with the lipid droplet core. The membrane monolayer lies beneath the lumenal leaflet (LL) of the endoplasmic reticulum (arrow) and is continuous with the cytoplasmic leaflet of the endoplasmic reticulum (ER). × 69,000.

at the periphery of many small and medium sized lipid droplets but also surrounding nuclei and dispersed in the cytoplasm (**Fig. 7A**). By day 5, when most cells contained larger lipid droplets, vimentin filaments were not present around all lipid droplets (Fig. 7B). Most of the large lipid droplets had little or no vimentin staining (Fig. 7B). Vimentin was present at the cell periphery beneath the plasma membrane of adipocytes during all stages of differentiation (Figs. 7A and 7B).

Epididymal adipocytes, and suprascapular brown fat cells

The location of perilipin in isolated white adipocytes and adipocytes within intact white and brown adipose tissue was similar to that of cultured 3T3-L1 adipocytes. Immunofluorescence staining of cryosections of epididymal adipocytes showed that perilipin was present at the periphery of the large intracellular lipid droplet (**Fig. 8A**)

Fig. 5B. Higher magnification of area of continuity (at arrow) between the IMP containing membrane monolayer (M) and leaflet (LL) of endoplasmic reticulum (ER) shown in stereo in 5A. Note (at arrow) there is no fracture step between the lipid droplet monolayer and cytoplasmic leaflet (CL) of the endoplasmic reticulum. × 172,000.

while immunogold staining on ultrathin cryosections showed perilipin on the surface layers of small peripheral intracellular droplets as well as the large central lipid droplet (Fig. 8C). Peroxisomes, identified by immunostaining for catalase were present at the periphery of lipid droplets (Fig. 8B), similar to their location in 3T3-L1 cells. Immunofluorescence staining of cryosections of suprascapular brown fat and cultured brown fat cells revealed that perilipin was present at the periphery of all of the numerous intracellular lipid droplets (data not shown).

Lactating rat mammary gland and newborn mouse liver

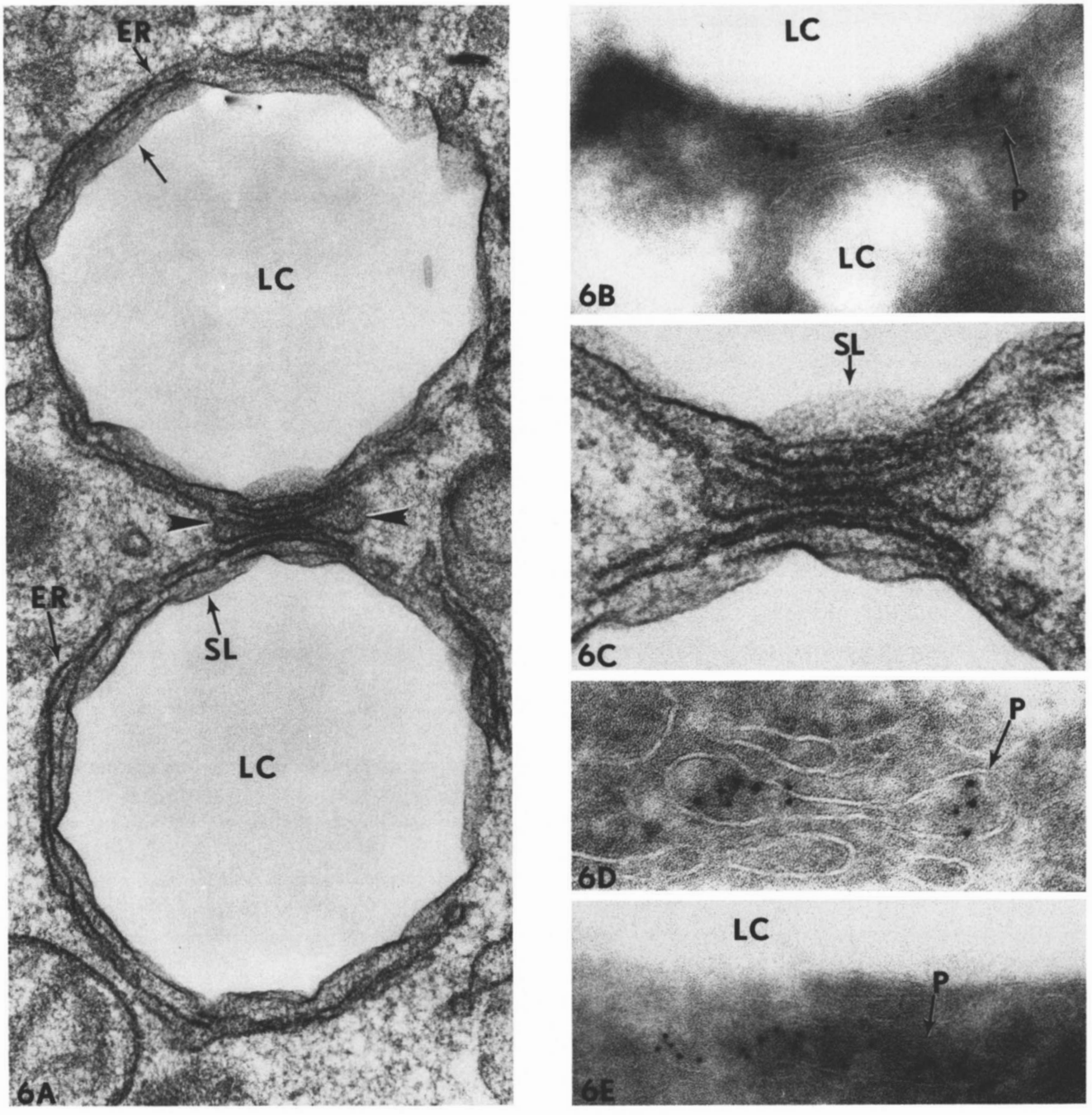
We investigated the location of perilipin in cells from lactating rat mammary gland, which contains both mammary alveolar epthelial cells and connective tissue adipocytes actively involved in triacylglycerol metabolism. Immunofluorescence staining of cryosections showed perilipin to be present in cells that were distributed randomly through the connective tissue or lining the basal surfaces of alveoli (Figs. 9A and 9B). By virtue of their intracellular immunofluorescence, which highlighted cell contours, they appeared elongate with attenuated cell bodies several microns in length (Fig. 9B). Electron microscopy of ultrathin sections determined that the elongated cells were adipocytes that contained many small lipid droplets extending through their cell bodies (Fig. 9C) and that surfaces of these small droplets immunolabeled for perilipin. We did not find perilipin immunofluorescence staining in mammary alveolar epithelial cells (Fig. 9A) although milk triacylglycerol-containing lipid droplets were abundant intracellularly and milk lipid droplets were present in alveolar lumena.

Hepatocytes from newborn mouse liver contained intracellular, Nile Red-positive lipid droplets but showed no immunostaining for perilipin. Sinusoidal cells and lipid-containing Ito cells also did not immunostain for perilipin (data not shown).

DISCUSSION

The major pool of triacylglycerol in mammals is the lipid storage droplets in adipocytes. There is a dearth of information on the biochemical and structural components of the lipid droplet surface, and it is not known if any surface components participate in lipogenesis or lipolysis. To date, the only proteins known to associate exclusively with intracellular lipid storage droplets are perilipins, named for their location at the periphery of the droplets as determined by immunocytochemistry with light microscopy (2). Other cellular components, such as endoplasmic reticulum, vimentin, peroxisomes, and mitochondria localized near lipid droplets are sometimes arranged in a pattern that encircles the peripheries of lipid droplets (13, 14). Thus, a goal of the present study was to define more precisely the location of perilipin as compared to other lipid droplet-associated structures and proteins.

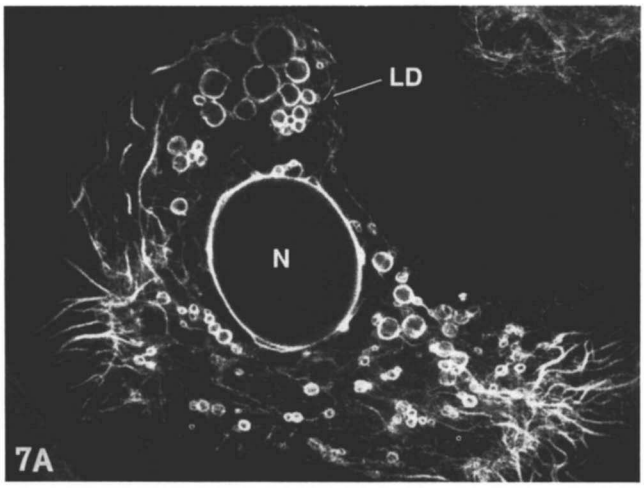
Perilipin is present at the surface of lipid droplets of all sizes in differentiating 3T3-L1 adipocytes. Confocal immunofluorescence reveals a close association between small and large droplets as well as a clustering of droplets in the endoplasmic region of cultured adipocytes. Three-dimensional constructs of optical slices through lipid droplets as well as electron microscopic studies reveal a patchy and uneven distribution of perilipin, suggesting that the protein is not associated with any organized structures, such as filaments. Both immunofluorescence and electron microscopy show not only this random distribution of perilipin but also areas devoid of perilipin,

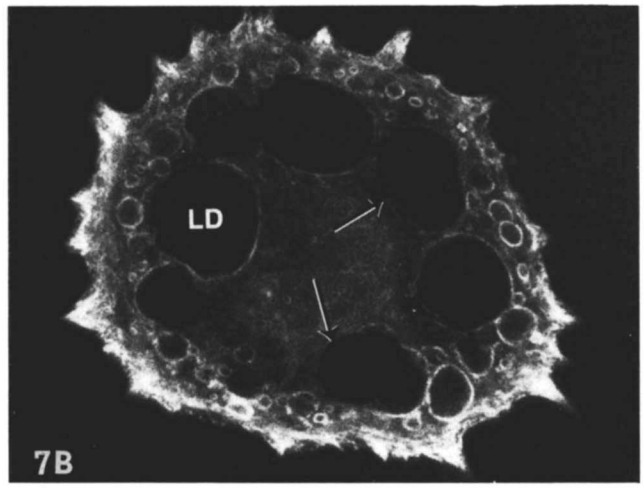


Figs. 6A and 6C. Electron micrographs of a section of a 3T3-L1 adipocyte fixed with potassium permanganate. Two lipid droplets of similar size have electron lucent cores (LC) and obliquely sectioned surface layers (SL) which collapsed during processing. Endoplasmic reticulum (ER) partially surrounds the lipid droplet periphery. A dumbbell-shaped structure is present between the two lipid droplets (at arrowheads and presented at higher magnification in 6C). 6A: × 52,000; 6C: × 81,000. 6B, 6D, and 6E: Cryosections of 3T3-L1 adipocytes which have been immunogold stained for catalase. Gold particles (5 nm), located in dumbbell (6B and 6D) and elongate shaped (6E) membrane-bounded structures present between lipid droplets (6B) show they are peroxisomes (P). LC, Lipid droplet core; SL, lipid droplet surface layer; ER, endoplasmic reticulum. 6B: × 108,000; 6D: × 124,000; 6E: × 108,000.

which may represent sites of contact between lipid droplets and other intracellular structures.

With electron microscopy, immunogold staining of cryosections of adipocytes shows gold particles randomly arranged on the layer immediately adjacent to the core of intracellular lipid droplets. This surface layer covering the core lipid can be seen easily in ultrathin cryosections of adipocytes fixed with aldehyde as the unfixed core triacyl-

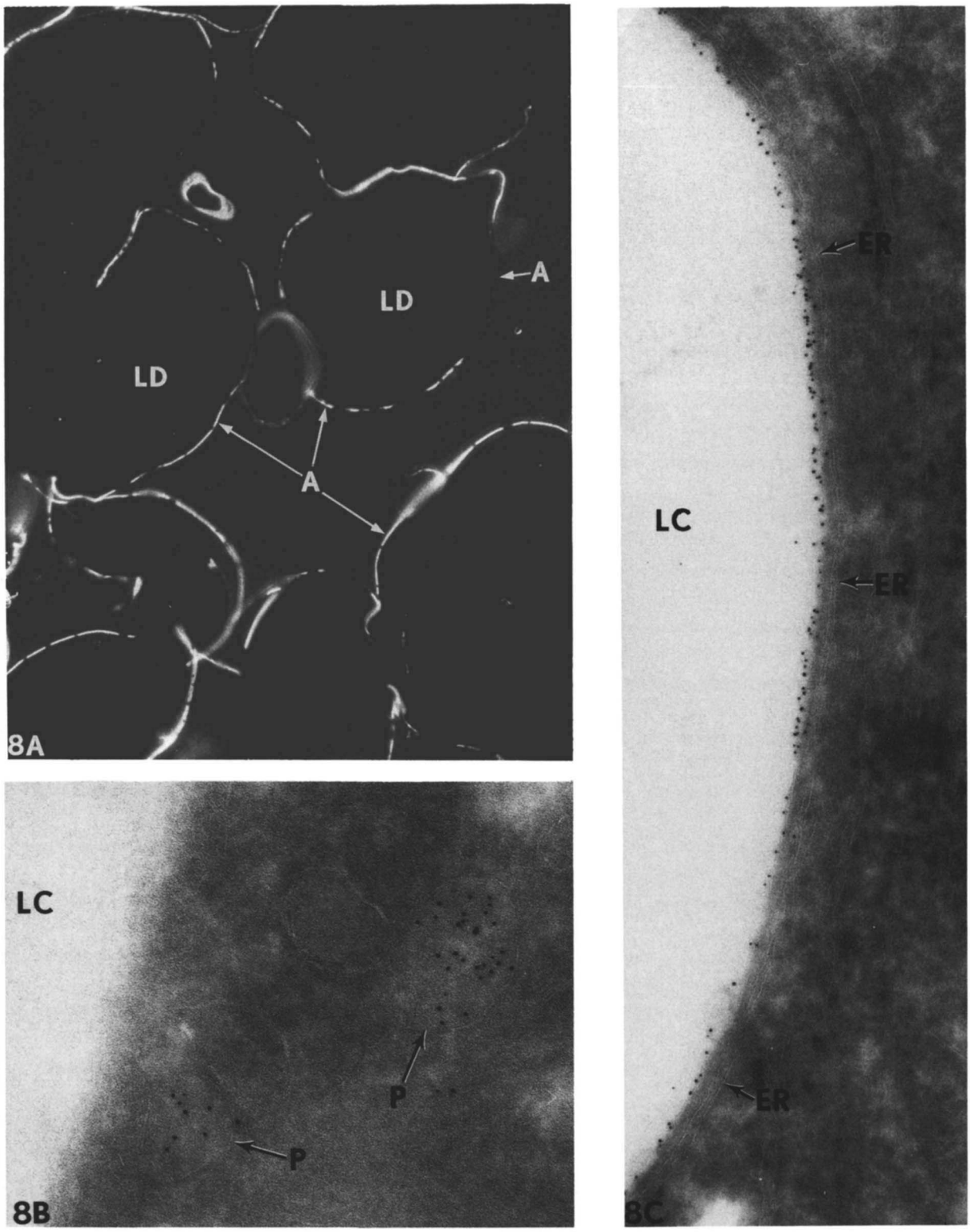




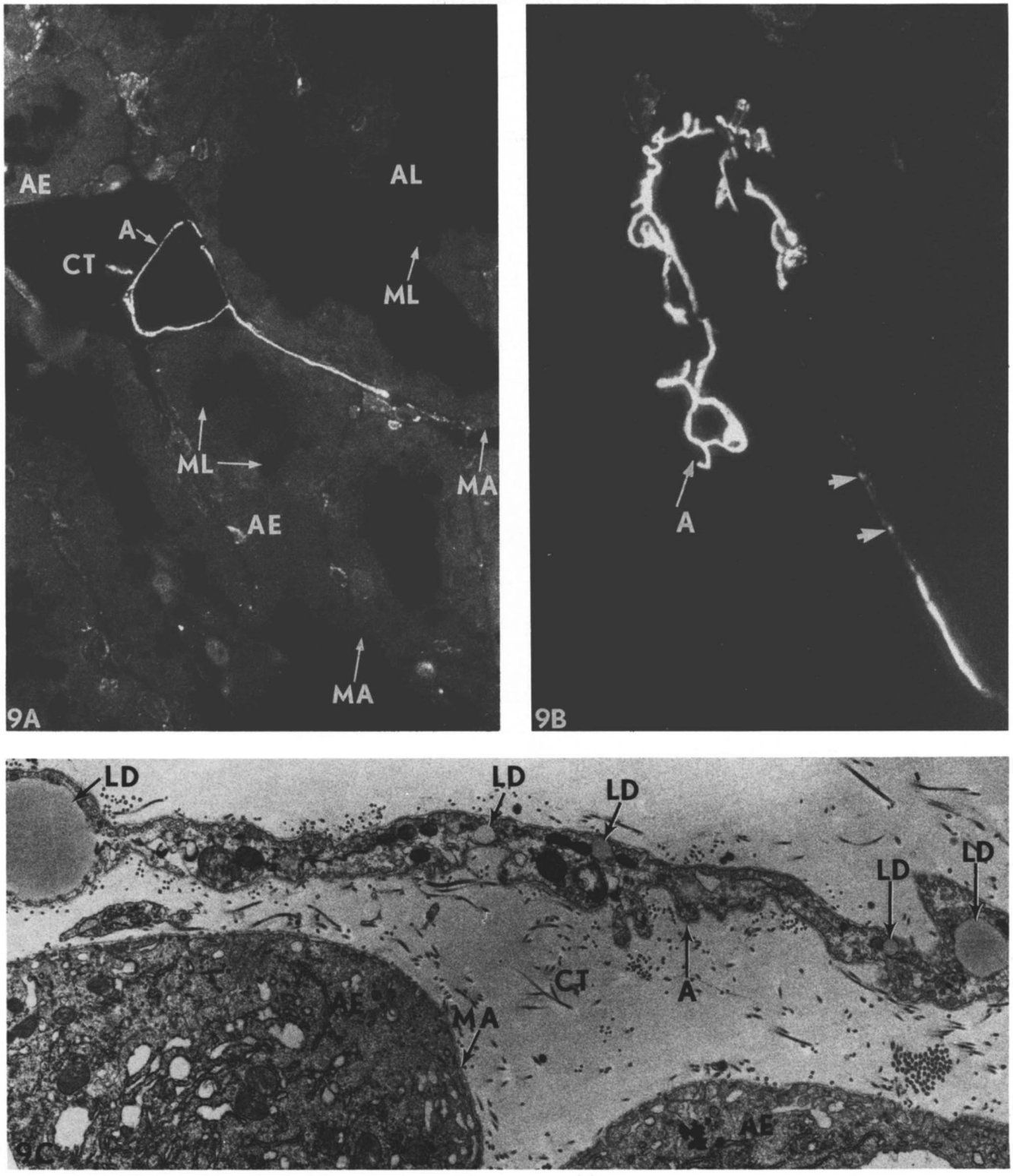
Figs. 7A and 7B. Confocal image of immunofluorescence staining of vimentin in 3T3-L1 adipocytes. 7A: In adipocytes at early stages of development (day 3) vimentin filaments are present around the periphery of small and medium size lipid droplets (LD), surrounding the nucleus (N) and dispersed in the cytoplasm. × 850; 7B: In adipocytes at late stages of development (day 5 and later) vimentin filaments are present at the periphery of some but not most of the lipid droplets. In particular, larger lipid droplets appear to have discontinuous or no peripheral vimentin staining (arrows). Vimentin is present also at the periphery of the cell beneath the cell surface (S). × 720.

glycerol of lipid droplets has little intrinsic electron opacity and also partially disperses from the section during subsequent aqueous procedures. In such preparations the lipid core appears mostly electron lucent and the surface layer, when cut obliquely in a grazing section, is moderately electron opaque. Immunogold labeling for perilipin was found exclusively on the surface layers of all sized droplets in cultured 3T3-L1 adipocytes and adipocytes from intact brown and white fat pads. Thus, perilipin becomes the first protein to be identified as an occupant of the limiting surface layer of adipocyte lipid droplets, an ideal location to play an important role in triacylglycerol metabolism.

In the present study we have used morphological techniques to assess the relationship of the perilipin-containing surface of lipid droplets to cellular organelles and cytoskeletal intermediate filaments. The results show a clear structural relationship between the endoplasmic reticulum and the lipid droplet surface and provide new information on the association among lipid droplets, peroxisomes, and vimentin. In early differentiation of 3T3-L1 cells, neutral lipid fluorescently stained with either Oil Red or Nile Red is diffusely distributed within the cell (15) in a pattern similar to that of endoplasmic reticulum stained with $DiOC_6$ (9). As adipocytes mature, discrete lipid droplets appear, concomitant with a decrease in the diffuse pattern of neutral lipid intracellular staining (15). Thus, neutral lipid, at first diffusely associated with endoplasmic reticulum membranes, subsequently forms into discrete lipid droplets with perilipin on their surface monolayers. Electron microscopic results elucidated a structural relationship between endoplasmic reticulum and the lipid droplet surface. Permanganate fixation, which preserves ampipathic phospholipid of intracellular membranes but not neutral triacylglycerol in lipid droplet cores (10, 11), preserved also the lipid droplet surface layer which, in sections, appeared to be continuous with membranes of branching tubular and cisternal endoplasmic reticulum. With freeze-fracture, the fracture plane is known to be along the hydrophobic region of intracellular membrane bilayers (16) and would be expected to fracture along the plane of the hydrophobic interface between the ampipathic lipid droplet surface layer and its hydrophobic core lipid. When the fracture plane crossed the lipid core to expose the surface layer adjacent to the core, it was studded with 75 Å particles. These particles on the hydrophobic face of the lipid droplet surface layer had the size, shape, and random distribution characteristic of intramembranous particles (IMPs), a unique feature of the hydrophobic faces of leaflets of cellular membranes (16). Also, freeze-fracture images showed areas of continuity between IMP-studded hydrophobic faces of lipid droplet surface layers and endoplasmic reticulum membrane leaflets, providing further evidence that the surface layer of the lipid droplet is a specialized area of the endoplasmic reticulum membrane leaflet. This demonstration of structural similarity between hydrophobic interiors of lipid droplet surface layers and membrane leaflets give strong support to the concept that triacylglycerol accumulates and lipid droplets develop in association with the hydrophobic region of endoplasmic reticulum membrane bilayers, where triacylglycerol synthesis is



Figs. 8A, 8B, and 8C. Cryosections of rat epididymal adipocytes (A). 8A: Light micrograph of cryosection immunostained with antibodies to perilipin. Immunofluorescence is present at the periphery of intracellular lipid droplets (LD). × 500. 8B: Electron micrograph of cryosection immunogold stained with antibodies to catalase. Gold particles (5 nm) are present in circular, membrane-bounded peroxisomes (P) adjacent to the lipid droplet periphery (labeled LC for lipid droplet core). × 90,000. 8C: Electron micrograph of cryosection immunogold stained with antibodies to perilipin. Gold particles (5 nm) are present at the periphery of a lipid droplet (labeled LC for lipid droplet core). A cisterna of endoplasmic reticulum (ER) follows the contours of the lipid droplet. × 90,000.



Figs. 9A, 9B, and 9C. Cryosections of mammary glands, from lactating rats, immunostained for perilipin. 9A: Immunofluorescence is present in an irregularly shaped, attenuated adipocyte (A) at the basal surfaces of mammary gland alveoli (MA). AE, alveolar epithelial cell; AL, lumen of alveolus; ML, milk lipid droplet; CT, connective tissue. × 560. 9B: Immunofluorescence is present in an irregularly shaped adipocyte (A) in mammary gland connective tissue. Note the punctate appearance of the immunofluorescence at arrowhead. × 840. 9C: Electron micrograph of adipocyte (A) at the basal surface of a mammary gland alveolus (MA). Several small intracellular lipid droplets (LD) are present in this attenuated portion of the adipocyte cell 1body. CT, connective tissue; AE, alveolar epithelial cell. × 12,000.

thought to occur (17). Other observations, including those on lipid droplet formation in 3T3-L1 adipocytes (14), lipid droplet formation and regression in epididymal adipocytes (18–20), milk lipid accumulation in alveolar epithelium of lactating mammary gland (21, 22), and lipoprotein accumulation in liver and intestine (23, 24), also show a structural relationship between triacylglycerol-rich lipid droplets and leaflets of membrane bilayers of endoplasmic reticulum. In adipocytes, lipid droplets may accumulate or regress within the confines of endoplasmic reticulum membranes. Adipocyte hormone-sensitive lipase, the rate-limiting enzyme for triacylglycerol lipolysis, translocates to lipid droplets upon hormonal stimulation (25). The fact that perilipin is found only in association with lipid droplets suggests a correlation between expression of perilipin and triacylglycerol metabolism.

In adipocytes, other circular and elongate membranous structures at the periphery of the lipid droplet but external to the lipid droplet surface layer were identified as microperoxisomes by immunostaining for catalase. Adipocyte microperoxisomes have none of the internal core density or crystalline structure of liver peroxisomes (26, 27), that would identify them from sectioned profiles of endoplasmic reticulum. Close structural relationships also occur among peroxisomes, unknown smooth membranous structures, and smooth membranes identifiable as cisternal elements of endoplasmic reticulum. Apparent continuities between peroxisomes and endoplasmic reticulum noted in 3T3-L1 cells (14) and liver (28–30) suggested that peroxisomes were derived as macromolecular aggregates from endoplasmic reticulum membranes. Even after rigorous isolation procedures smooth endoplasmic reticulum membrane fragments have been shown, with cytochemistry, to remain attached to peroxisomes (30). As both peroxisomal membrane and matrix proteins are synthesized on polysomes (31, 32), it was concluded that peroxisomes grow by import of proteins into existing peroxisomes and new peroxisomes are formed by fission (32, 33). In 3T3-L1 adipocytes we found a preferential accumulation of dumbbell-shaped peroxisomes, a profile that suggests organellar fission (28, 32), within constellations of lipid droplets. As newly formed peroxisomal membrane phospholipids probably originate in the endoplasmic reticulum, one might expect their close structural association to reflect this biosynthetic dependence. In 3T3-L1 adipocytes, developmentally induced foci in endoplasmic reticulum for the biosynthesis of phospholipid and triacylglycerol may simultaneously fill the need of proliferating peroxisomes for membrane components as well as increase surface area of endoplasmic reticulum membranes to accomodate developing lipid droplets.

Peroxisomes participate in glycerolipid, cholesterol, and sterol metabolism (34–39). A quantitatively unknown fraction of cellular triacylglycerol and phosphatidylglycerol is formed via the acyl dihydroxyacetonephosphate (DHAP) pathway, dependent on the DHAP-acyl transferase that is localized mainly in peroxisomes (35). Thus it is possible that triacylglycerol generated by the peroxisomal pathway is segregated from that generated by the glycerol-3-phosphate pathway in microsomes thought to be derived from endoplasmic reticulum (17, 34). That perilipin is associated only with triacylglycerol accumulation in endoplasmic reticulum and is not found in peroxisomes suggests yet another difference in regulation of lipid metabolism between the two organelles.

We could find no evidence for an association between perilipin and vimentin. Previous immunofluorescence studies showed that lipid droplets in 3T3-L1 adipocytes are surrounded by vimentin intermediate filaments during initial stages of differentiation (13), and electron microscopic studies of cross-sectioned lipid droplets revealed filaments of various size (75–100 Å) juxtaposed to the droplets but always external to the surface layer (13, 14). Although the data indicate that perilipin is not associated with vimentin, there is a clear relationship between vimentin bundles and small to medium sized lipid droplets in 3T3-L1 cells. However, vimentin bundles also surround the nuclei and extend inward from the plasma membrane, following the contours of the cells highlighting their general role in stabilizing cell structure. Optical sectioning by confocal microscopy permitted visualization of fluorescence to within less than 0.5 μm thickness of a single lipid droplet, thus excluding fluorescence interference from other levels of the cell. This technique showed that vimentin was often sparse or undetectable at the surfaces of large lipid droplets, in contrast to perilipin which is present on the surface layer of droplets of all sizes. One might speculate that early in 3T3-L1 cell differentiation the "caging" of droplets by vimentin (13) might provide a means of separating droplets, perhaps to foster more efficient lipid storage. As 3T3-L1 cells differentiate into adipocytes a decrease in cytoskeletal protein expression occurs (40, 41) and the decrease in vimentin expression may facilitate droplet coalescence to form the larger lipid droplets characteristic of mature adipocytes. However, a recent report showed no alteration in lipid droplet formation and accumulation in cultured adipocytes derived from epididymal fat pads of mutant mice lacking vimentin (42).

We questioned whether perilipin is present in cells other than cultured and tissue adipocytes that contain intracellular storage depots of triacylglycerol and thus, examined mammary glands from lactating rats and livers of newborn mice for perilipin. Alveolar epithelial cells of lactating mammary gland synthesize triacylglycerol from exogeneous fatty acids supplied by hydrolysis of blood lipoproteins (43). Cellular lipid droplets form in association with endoplasmic reticulum, enlarge, and are secreted into the alveolar lumen as milk lipid. We found

no evidence of perilipin in alveolar epithelial cells although they contained cytoplasm filled with lipid droplets in preparation for milk lipid secretion. On the other hand, immunostaining of cryosections of mammary gland, showed elongate, complexly shaped cells distributed throughout the connective tissue that stained for perilipin. Electron microscopic analysis identified these cells as extremely attenuated adipocytes, depleted of central deposits of intracellular triacylglycerol lipid. Perilipin immunostaining at the surface of small lipid droplets remaining in these cells became a marker for adipocytes with atypical structure, providing a potentially useful tool to follow adipocyte differentiation in mammary gland during development and adipocyte depletion during lactation. Immunostaining of liver from newborn mice showed that although hepatocytes and Ito cells contained Nile red-positive intracellular lipid droplets they did not contain perilipin. Thus, we conclude that perilipin is located on the lipid droplet surface layer of both tissue and cultured adipocytes but perilipin is not universally present in all cells that have capacity for triacylglycerol synthesis and storage as intracellular lipid droplets.

The only cells other than adipocytes in which we have found perilipin are steroidogenic adrenal cortical and Leydig cells (44, 45). In these cells perilipin is located at the surface of cholesteryl ester droplets that contain the precursors for steroid hormone synthesis. The interesting link between adipocytes and steroidogenic cells is that the cholesteryl ester hydrolase that catalyzes the initial step in steroid hormone synthesis is highly related to the hormone-sensitive lipase of adipocytes. The parallel, cAMP-dependent hydrolytic processes, plus the polyphosphorylation of perilipin in response to lypolytic and steroidogenic stimuli, point strongly to a role for perilipin in lipid hydrolysis. However, a role for perilipin in lipid packaging cannot be excluded. That is, perilipin may serve to sequester lipids and protect against inappropriate hydrolysis in the absence of elevated cAMP. In this scenario, perilipin may be involved indirectly in lipid hydrolysis, in contrast to a more direct role where perilipin serves as a docking protein for the lipid hydrolases. The currently available data do not permit one to distinguish between these possibilities.

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