Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein

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Abstract The adipose differentiation-related protein (ADRP) was first characterized as a mRNA induced early during adipocyte differentiation (Jiang, H. P., and G. Serrero. 1992. *Proc. Natl. Acad. Sci. USA.* **89:**7856–7860). The present study demonstrates that ADRP mRNA is expressed in a variety of tissues and cultured cell lines. Immunocytochemical examination revealed that ADRP localizes to neutral lipid storage droplets in cultured murine 3T3-L1 adipocytes, murine MA-10 Leydig cells, Chinese hamster ovary (CHO) fibroblasts, and human HepG2 hepatoma cells; the association of ADRP with lipid droplets was confirmed by subcellular fractionation of MA-10 Leydig cells. In addition to ADRP, steroidogenic cells and adipocytes express the perilipins, a family of lipid droplet-associated proteins that share a highly related sequence domain with ADRP. ADRP and perilipins co-localize on lipid droplets in MA-10 Leydig cells. While ADRP was found on small lipid droplets in 3T3-L1 preadipocytes and early differentiated adipocytes, it was absent in maturing adipocytes. In contrast, perilipins were absent early during differentiation, but were found on small and large lipid droplets at later stages. The transition in surface protein composition of adipocyte lipid droplets from ADRP to perilipins occurred 3 days after the initiation of differentiation when cells displayed co-localization of both proteins on the same lipid droplets. The specific localization of adipose differentiation-related protein to lipid droplets in a wide variety of cells suggests that ADRP plays a role in management of neutral lipid stores.—**Brasaemle, D. L., T. Barber, N. E. Wolins, G. Serrero, E. J. Blanchette-Mackie, and C. Londos.** Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J. Lipid Res.* 1997. **38:** 2249–2263.

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In many different types of mammalian cells, intracellular neutral lipids are stored in discrete lipid storage droplets composed of a core of triacylglycerol and cholesteryl esters surrounded by a limiting osmophilic boundary generally thought to be a phospholipid monolayer. In adipocytes, large triacylglycerol-rich lipid storage droplets hold the body's major energy reserves. Smaller cholesteryl ester-rich lipid storage droplets in steroidogenic cells of adrenal cortex, testes, and ovary provide a source of cholesterol that can be used as a substrate for steroid hormone synthesis (1–3) and membrane biogenesis (4). Intracellular lipid storage droplets occur in a wide variety of other cells from liver, muscle, heart, kidney, intestine, and mammary gland (5–7) and in cultured fibroblasts and macrophages (8, 9). Most cultured cells can rapidly take up and esterify extracellular fatty acids and the resulting lipid storage droplets are rapidly depleted after the withdrawal of substrate (8). Thus, the ability to package neutral lipids into discrete lipid storage droplets is a general property of most cells.

Little is known about the composition of the proteins at the surfaces of mammalian intracellular lipid storage droplets. By contrast, circulating lipoproteins and milk lipid globules are similar secreted structures that have been characterized in detail. The first described intrinsic lipid droplet-associated proteins were the perilipins which localize to the periphery of the intracellular neutral lipid storage droplets in adipocytes (10–12) and steroidogenic cells of adrenal cortex, testes, and ovary (T.

Abbreviations: ADRP, adipose differentiation-related protein; ALBP, adipose lipid binding protein; CHO, Chinese hamster ovary.

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Barber, N. K. Dwyer, J. Wolff, D. Servetnick, D. L. Brasaemle, C. Londos, and E. J. Blanchette-Mackie, unpublished results) (13). To date, we have detected the perilipins in no other types of cells. While the perilipins exhibit no extended sequence similarity to other proteins in the current databases, they share a limited sequence similarity with the adipose differentiation-related protein (ADRP). The amino terminal 105 amino acid region of the perilipins is 32% identical and 67% similar to the amino terminus of ADRP.

ADRP was first identified during a search for genes expressed early during the differentiation of murine 1246 adipocytes in culture (14). Initial scans of total RNA from various tissues indicated that ADRP mRNA is expressed at high levels in adipose tissue, but not in liver, kidney, heart, or brain of lean mice (15). Neither subcellular fractionation nor immunofluorescence studies suggested the association of ADRP with lipid storage droplets in murine 1246 adipocytes 2 to 4 days after the initiation of differentiation (15).

The current study addresses the tissue distribution and subcellular localization of ADRP. As ADRP shares limited sequence homology with the perilipins, we hypothesized that ADRP may be a lipid storage droplet-associated protein. Furthermore, as we had recently found the perilipins in low abundance in steroidogenic cells (13) in addition to adipocytes, we examined steroidogenic cells and a number of tissues and cell lines for the expression of ADRP mRNA. And finally, we compared the time-course of expression and the subcellular localization of ADRP to that of the perilipins during the differentiation of cultured 3T3-L1 adipocytes.

MATERIALS AND METHODS

Materials

Nile Red was obtained from Molecular Probes, Inc. (Eugene, OR). Lissamine rhodamine- or fluorescein-conjugated secondary antibodies raised against rabbit or guinea pig IgGs were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). TRIzol and powdered Dulbecco's Modified Eagle's medium, Waymouth's MB 752/1 medium, Ham's F-10 medium, Ham's F-12 medium, and Minimum Essential Medium were purchased from Life Technologies, Inc (Grand Island, NY). 3-Isobutyl-1-methylxanthine, insulin, and dexamethasone were obtained from Sigma (St. Louis, MO). Horse serum and fetal bovine serum were obtained from Summit Biotechnology (Ft. Collins, CO). Rat R2C Leydig cells, Chinese hamster ovary (CHO) fibroblasts, human HepG2 hepatoma cells, and murine 3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, MD). MA-10 Leydig cells were generously provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA). A blot containing poly $(A)^+$ RNA from various tissues was purchased from Clontech Laboratories, Inc. (Palo Alto, CA).

Cell culture

MA-10 Leydig cells (16) were maintained in Waymouth's MB 752/1 medium supplemented with 15% horse serum. R2C Leydig cells were maintained in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. CHO fibroblasts were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum. HepG2 hepatoma cells were maintained in Minimum Essential Medium supplemented with 10% fetal bovine serum. 3T3-L1 cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum. The media for all cells were supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, and all cells were maintained in a 5% CO_2 atmosphere at 37°C. Cells were maintained in culture flasks and dishes from Corning/Costar, except for the use of Lab-Tek Chamber Slides from Nunc for microscopy experiments. In one experiment, intracellular neutral lipid storage was increased by the addition of oleic acid coupled to bovine serum albumin (17) to the cell culture medium.

The differentiation of 3T3-L1 cells into adipocytes was accomplished by incubating confluent monolayers of cells in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum and 10^{-5} M dexamethasone, 0.5 mM isobutylmethylxanthine, and 10 μg/ml insulin for 72 h with a change to fresh medium every 24 h. After 72 h, the differentiation reagent mixture was withdrawn and the medium was changed every 24 h to Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum.

Immunofluorescence microscopy

Cells were prepared for immunofluorescence microscopy and stained as described previously (12). Immunostaining was accomplished with the following polyclonal antibodies used singly or in combination: *1*) affinity-purified rabbit immunoglobulins raised against a recombinant fusion protein of glutathione S-transferase with ADRP (15); *2*) rabbit antiserum raised against a recombinant 6-histidine-tagged amino terminal peptide (105 amino acids) of perilipin; and *3*) affinity-purified guinea pig immunoglobulins raised against a recombinant 6-histidine-tagged carboxyl terminal peptide (96 amino acids) of perilipin A. Antibodies *1*) and *2*), above, did not cross-react on immunoblots or in im-

munofluorescence studies in cultured cells. Control experiments indicated no specific staining pattern in cells incubated with the fluorescent secondary antibody alone, or with pre-immune antisera followed by a fluorescent secondary antibody. Neutral lipids were visualized by staining 3% paraformaldehyde-fixed cells with 0.01% Nile Red in phosphate-buffered saline for 10 min. Cells were viewed with a Leitz Diaplan immunofluorescence microscope or a Nikon Optiphot microscope equipped with a Bio-Rad MRC-1024 confocal imaging system.

Subcellular fractionation

MA-10 Leydig cells were disrupted by incubation in a hypotonic lysis medium containing 10 mM Tris, pH 7.4, 1 mM EDTA, 10 mM sodium fluoride, 20 μg/ml leupeptin, 1 mM benzamidine, and 100 μM [4-(2-aminoethyl)-benzenesulfonylfluoride] hydrochloride for 10 min at 4°C followed by 10 strokes in a Teflon/glass homogenizer. The homogenate was centrifuged at 500 *g* for 5 min and the supernatant was adjusted to 30% sucrose, layered beneath a 0–30% sucrose gradient and centrifuged for 4 h at 280,000 *g* at 4°C. Harvested fractions were assayed for NADH cytochrome c reductase activity (18) to identify fractions containing endoplasmic reticulum and prepared for SDS-PAGE on 10% polyacrylamide gels by the addition of Laemmli sample buffer (19).

Immunoblots

Proteins from subcellular fractions or whole cell homogenates were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose for immunoblotting. Immunoblots were probed with either affinity-purified anti-ADRP immnuoglobulin or antisera against the amino terminus of perilipin as described above and as identified in the figure legends. Blots were incubated with secondary antibodies conjugated with horseradish peroxidase; an enhanced chemiluminescent substrate (Pierce, Rockford, IL) was used for signal detection.

Northern blot analysis

RNA was extracted from cells using TRIzol according to the protocol of the manufacturer. Total RNA was electrophoresed on 1% agarose gels containing formaldehyde; the RNA was transferred to Hybond N+ (Amersham) charged nylon membranes. Northern blots were hybridized with ^{32}P-labeled cDNA probes corresponding to the full coding sequence of perilipin A or 0.8 kb of the 3′ end of ADRP at 37°C in 50% formamide, 0.5 M sodium chloride, 10 mM EDTA, 1% sodium dodecyl sulfate, 10 μg/ml salmon sperm DNA, 50 mM sodium citrate, 120 mM sodium phosphate, pH 6.8.

RESULTS

ADRP mRNA is expressed in various tissues and cultured cell lines

Based on the finding of the perilipins in steroidogenic cells (13) in addition to adipocytes, and given the structural similarities between the amino termini of ADRP and the perilipins, we reasoned that the expression of ADRP may not be limited to adipocytes. Previous analysis of total RNA from mouse tissues indicated a high level of expression of ADRP mRNA in adipose tissue (15). To increase the sensitivity of detection, poly $(A)^+$ RNA from various other tissues was probed with a radiolabeled ADRP cDNA probe (**Fig. 1a**). An approximate 1.9 kb ADRP mRNA was detected in all tissues examined, although the relative abundance varied. Lung expressed the highest levels of ADRP mRNA followed by liver, testes, spleen, brain, heart, skeletal muscle, and kidney, in order of decreasing abundance; mRNA from

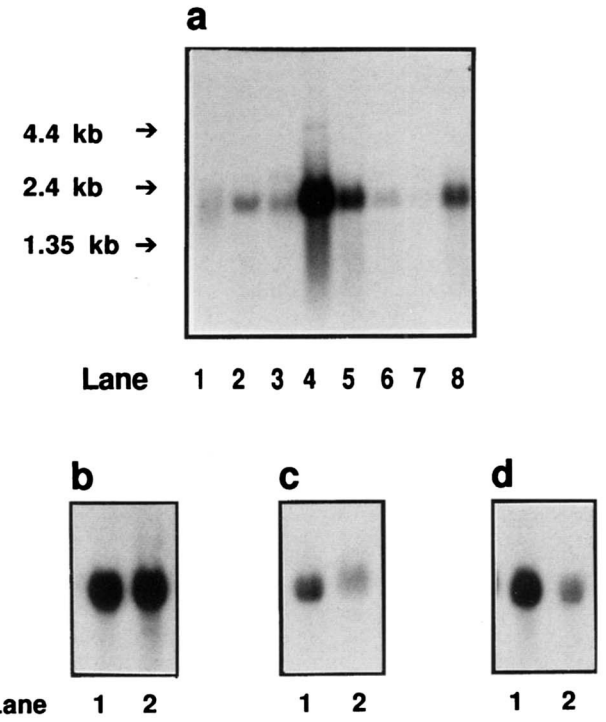

Fig. 1. Northern blot analyses of RNA from various tissues and cultured cell lines probed with radiolabeled ADRP cDNA probes. a) Northern blot of poly $(A)^+$ RNA (2 μg/lane) from murine heart (lane 1), brain (lane 2), spleen (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and testes (lane 8). b) Northern blot of total RNA from murine 3T3-L1 adipocytes, 7 days after the initiation of differentiation (15 μg; lane 1) and murine MA-10 Leydig cells (15 μg; lane 2). c) Northern blot of total RNA from MA-10 Leydig cells (15 μg; lane 1) and human HepG2 hepatoma cells (30 μg; lane 2). Slight differences in mobility are due to inconsistencies in the gel and not due to a difference in mRNA size. d) Northern blot of total RNA from MA-10 Leydig cells (20 μg; lane 1) and CHO fibroblasts (20 μg/lane 2).

adipose tissue was not included on this blot. To address the possibility of contamination of the poly $(A)^+$ RNA preparations with RNA from small amounts of fat contaminating the dissected tissues, the blots were reprobed for perilipins and adipose lipid binding protein (ALBP; data not shown). No perilipin or ALBP mRNA was detected in lung, heart, kidney, or skeletal muscle. Surprisingly, testes showed a moderately strong hybridization to ALBP mRNA; however, a relatively strong signal for perilipin C was accompanied by a relatively weak signal for perilipin A. This pattern of perilipin expression is unlike that of adipose tissue (11) and more similar to that of steroidogenic cells (13). In brain, a relatively strong ADRP hybridization was accompanied by relatively weak signals for ALBP and perilipins. The only samples for which contamination by adipose tissue might explain the presence of ADRP mRNA were liver and spleen, as each contained both ALBP and perilipin mRNAs. Northern blot analysis of total RNA extracted from cultured cell lines identified ADRP mRNA in murine MA-10 Leydig cells at approximately equal abundance to that in differentiated murine 3T3-L1 adipocytes (Fig. 1b). Human HepG2 hepatoma cells (Fig. 1c), CHO fibroblasts (Fig. 1d), and 3T3-L1 pre-adipocytes (see Fig. 6a) all expressed similar amounts of ADRP mRNA and less than that of MA-10 Leydig cells. By contrast, among these cultured cell lines, only differentiated 3T3-L1 adipocytes and MA-10 Leydig cells express perilipin mRNAs (data not shown for MA-10 Leydig cells; see Fig. 6b for 3T3-L1 adipocytes).

ADRP is a lipid droplet-associated protein

To determine the subcellular location of ADRP, a number of cell lines were examined by immunofluorescence microscopy using an ADRP-specific polyclonal

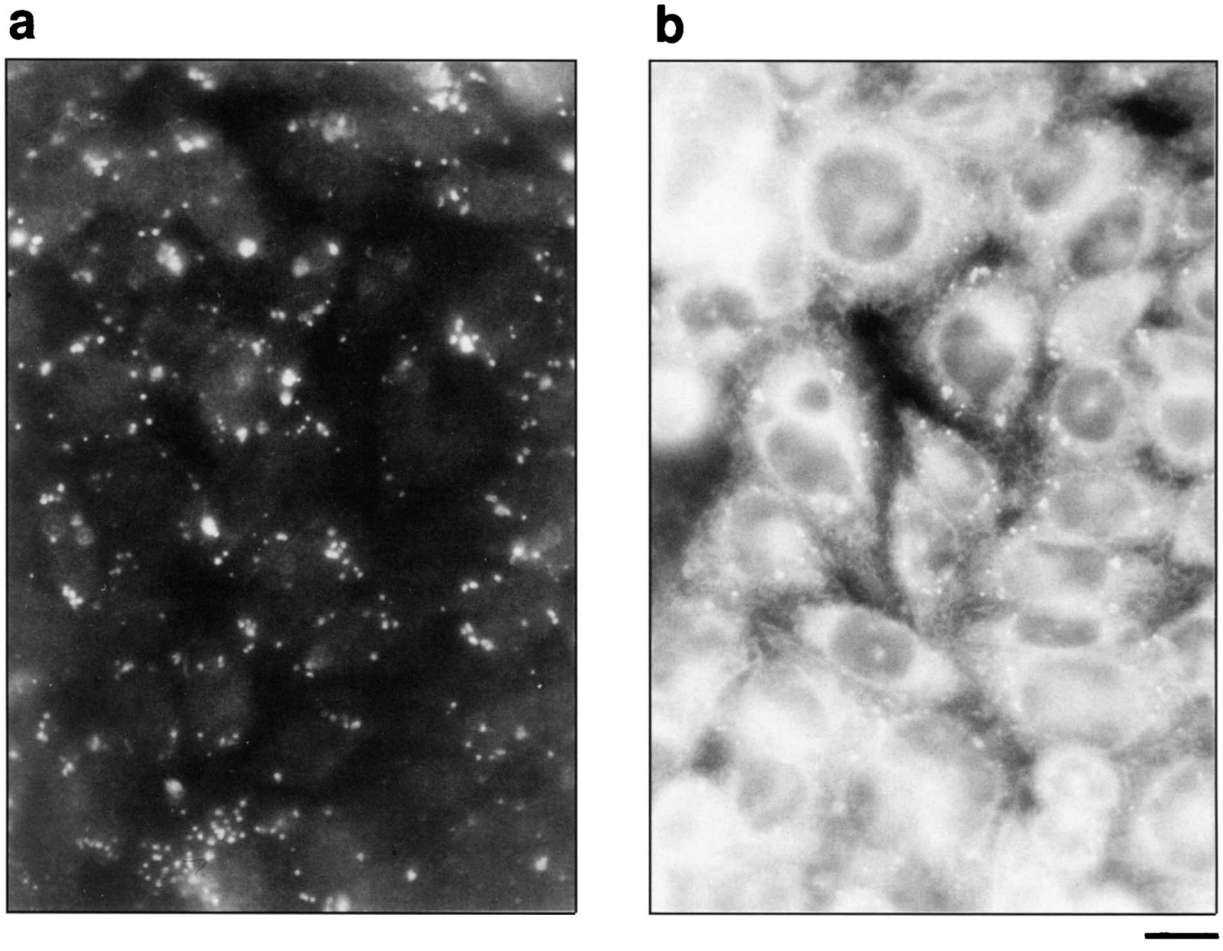

Fig. 2. ADRP is a lipid droplet-associated protein in CHO fibroblasts. a) Fluorescence of CHO fibroblasts stained with anti-ADRP antibodies. b) Fluorescence of a separate group of CHO fibroblasts stained with Nile Red to detect neutral lipids. Diffuse bright areas within cells in 2b likely represent staining of neutral lipid distributed throughout reticular regions of the cells. Lipid storage droplets in 2b are indicated by bright punctate structures arrayed singly and in small clusters. ADRP staining in 2a identifies similar bright punctate structures arrayed singly and in small irregularly shaped clusters. Bar = 12 microns.

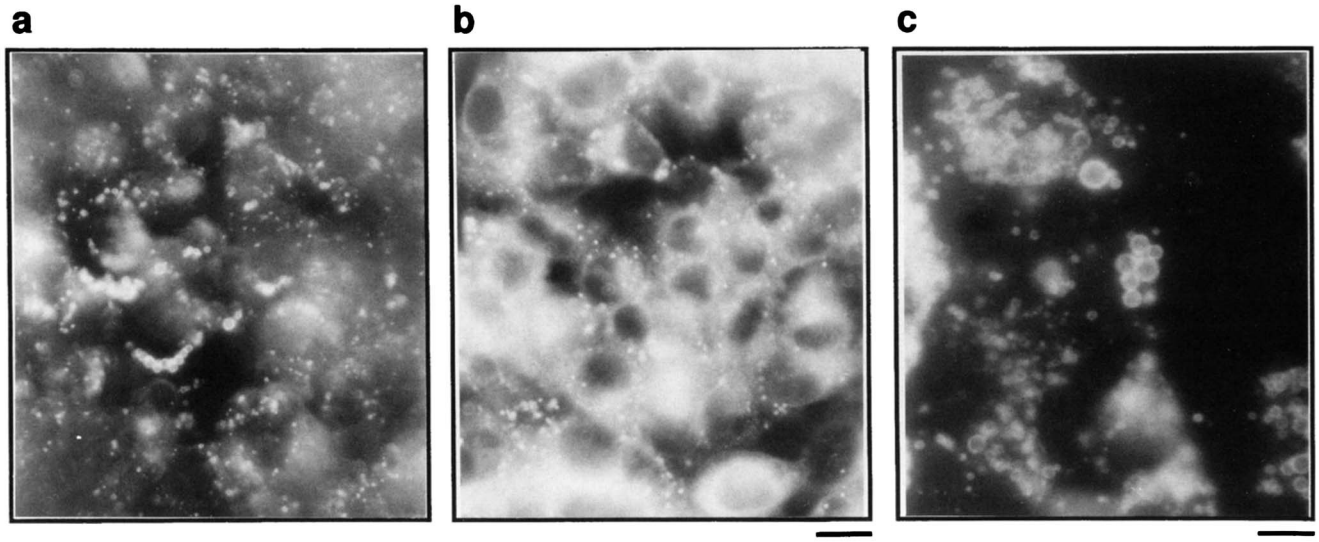

Fig. 3. ADRP is a lipid droplet-associated protein in human HepG2 hepatoma cells. a) and c) Fluorescence of HepG2 hepatoma cells stained with anti-ADRP antibodies. b) Fluorescence of a separate group of HepG2 hepatoma cells stained with Nile Red to detect neutral lipids. Lipid storage droplets in 3b are indicated by brightly stained punctate structures arrayed singly and in small clusters. ADRP staining in 3a identifies similar brightly stained punctate structures arrayed singly and in small irregularly shaped clusters, as well as some ringed structures indicating larger lipid storage droplets. Bar below 3b = 12 microns and applies to both 3a and b. c) shows ADRP staining of HepG2 hepatoma cells incubated with 400 μM oleic acid coupled to bovine serum albumin for 24 h to increase storage of neutral lipids at higher magnification; distinct rings are visible. Bar = 7 microns.

antibody. The lipid storage droplets of CHO fibroblasts (**Fig. 2**) and HepG2 hepatoma cells (**Fig. 3**) are very small and displayed a punctate pattern when stained for neutral lipids. ADRP staining of both of these types of cells (Fig. 2a and 3a) was largely indistinguishable from the neutral lipid staining pattern (Figs. 2b and 3b). Supplementation of the culture medium of HepG2 cells with fatty acids increased the number of lipid storage droplets per cell and, concomitantly, the amount of staining for ADRP (HepG2 cells, Fig. 3c). At high magnification, ADRP staining of fatty acid-supplemented HepG2 cells (Fig. 3c) revealed bright rings indicating the localization of ADRP at the surfaces of lipid storage droplets. Similar observations were made for fatty acid-supplemented CHO cells (data not shown). Additional cell lines displaying an ADRP-specific staining pattern comparable to that of neutral lipids were rat R2C Leydig cells and murine Y-1 adrenal cortical cells (data not shown).

Subcellular fractionation of MA-10 Leydig cells confirms a lipid storage droplet localization of ADRP

Intracellular localization of ADRP was addressed further by subcellular fractionation of MA-10 Leydig cells. Centrifugation of homogenates immediately after hypotonic lysis of cells resulted in the co-flotation of a large portion of ADRP with lipid storage droplets; however, a significant portion of ADRP was spread throughout the supernatant and membrane fractions (data not shown). For improved resolution, the components of post-nuclear supernatants were separated on a 0–30% sucrose gradient (**Fig. 4**). The vast majority of ADRP fractionated with lipid storage droplets (Fig. 4a; fraction 11). While a small portion of ADRP banded lower in the gradient, it was found in fractions more buoyant than those containing NADH-cytochrome c reductase activity (Fig. 4b; fractions 3–4), a marker for endoplasmic reticulum (18). These results were obtained from cells grown in normal culture conditions and from cells supplemented with fatty acids to increase neutral lipid synthesis and storage. Hence, ADRP is found in low density fractions that do not co-fractionate with microsomes, indicating that ADRP is a component of a compartment highly enriched with neutral lipids.

ADRP and perilipins co-localize to the lipid storage droplets of MA-10 Leydig cells

MA-10 Leydig cells are unique among the cells examined in displaying constitutive expression of both ADRP and the perilipins. Thus, we asked whether ADRP and the perilipins are on the same lipid storage droplets or segregated on separate structures. In contrast to adipocytes (see below), it was not feasible to test for co-localization in MA-10 Leydig cells by double staining cells with anti-ADRP and anti-perilipin antibodies. MA-10 cells express substantial amounts of perilipin C; the only available antibody that recognizes this isoform was raised in rabbits, as was the anti-ADRP antibody. This

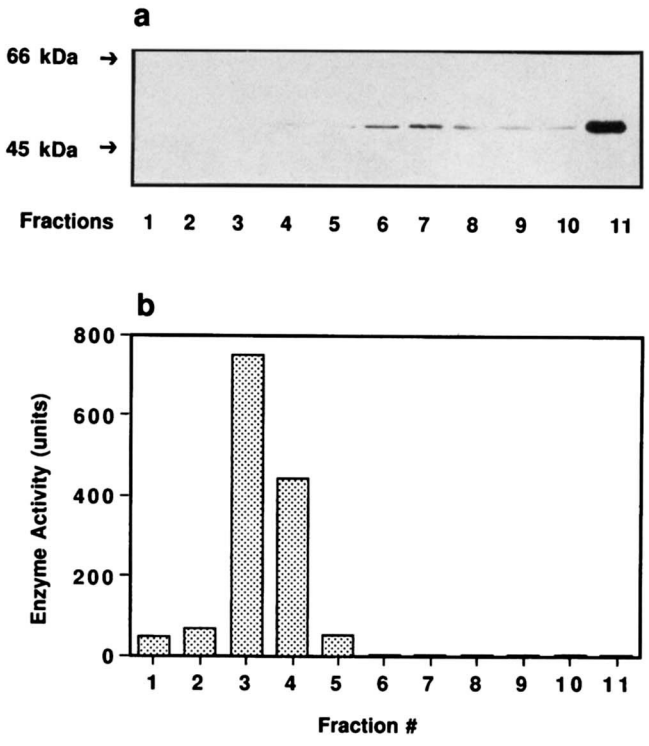

Fig. 4. Subcellular fractionation of MA-10 Leydig cells shows that the majority of ADRP is found in fractions more buoyant than microsomes. a) Immunoblot probed with polyclonal ADRP antibodies showing fractions of a 0 to 30% sucrose gradient of post-nuclear supernatant from MA-10 cell homogenates. Fraction 1 corresponds to the bottom of the gradient. The majority of ADRP (50 kDa) was found in the most buoyant fraction (#11), with an additional small peak in fractions 6 and 7. b) Histogram of NADH cytochrome c reductase activity (in relative units) of gradient fractions showing a peak of activity in fractions 3 and 4.

problem was circumvented by the following approach. Neutral lipid staining of MA-10 Leydig cells (**Fig. 5C**) showed that lipid storage droplets are visible as phase dense structures (Fig. 5F). All visible phase dense structures (Fig. 5D and 5E) stained positively for ADRP (Fig. 5A) and perilipin (Fig. 5B). ADRP and perilipin immunostaining of the largest of these structures produced bright rings around the periphery of the droplets; some of the small structures displayed a punctate staining pattern. These observations establish that ADRP and the perilipins co-localize to the same lipid storage droplets.

Differentiation of 3T3-L1 adipocytes is accompanied by an increase in levels of ADRP mRNA but a decrease in levels of ADRP protein

ADRP mRNA levels increase during the differentiation of murine 1246 adipocytes and primary adipocytes (H. Ye and G. Serrero, unpublished results) (14, 15). In 3T3-L1 cells, there was a reproducible initial decrease in ADRP mRNA levels followed by a large increase as differentiation proceeded (**Fig. 6a**). While ADRP mRNA was present at low levels in 3T3-L1 cells before the initiation of differentiation, perilipin mRNA was absent and not detectable until 3 days after the initiation of differentiation, and increased thereafter (Fig. 6b).

Cellular protein levels of ADRP and perilipin were also assayed throughout the differentiation of 3T3-L1 cells. Immunoblots of whole cell homogenates revealed that ADRP was present before the initiation of differentiation; its level doubled by 1 day after the initiation of differentiation, remained constant through day 3, dropped by day 4, and was barely detectable by day 8 (**Fig. 7a**). This temporal profile of ADRP protein was surprising as it showed the opposite trend when compared to that of ADRP mRNA (Fig. 6a); ADRP protein levels had decreased by day 4 as ADRP mRNAs were showing the largest increases. By contrast, perilipin A was detectable by day 3 and levels continued to rise during adipocyte differentiation (Fig. 7b); perilipin B was detectable by 8 days. Overall, levels of perilipin protein increased in parallel with perilipin mRNA (Fig. 6b).

Transition of lipid droplet surface protein composition from ADRP to the perilipins during the differentiation of 3T3-L1 adipocytes

Immunofluorescence microscopy was used to determine the cellular locations of ADRP and the perilipins throughout the differentiation of 3T3-L1 adipocytes. Before the induction of differentiation, pre-confluent, actively dividing (data not shown) and confluent, growth arrested 3T3-L1 cells (**Fig. 8A**) showed a punctate staining pattern for ADRP comparable to that obtained in cells stained for neutral lipids (data not shown); hence, tiny lipid storage droplets containing ADRP were present before the initiation of differentiation. No perilipin was detectable (Fig. 8I). One day after the induction of differentiation, there was a modest increase in specific staining for ADRP in punctate pattern (Fig. 8B) similar to that of cells stained for neutral lipids (data not shown); again, no perilipin was detectable (Fig. 8J). Specific staining for perilipins was detected by 3 days after the initiation of differentiation (Fig. 8K) and appeared both as a punctate staining pattern and as bright rings surrounding growing lipid storage droplets. The pattern of staining for ADRP was similarly punctate with some distinct rings (Fig. 8C). At day 3, cells that contained large lipid storage droplets exhibited perilipin staining more frequently than ADRP staining; ADRP staining was more prevalent among cells containing very small lipid droplets. This pattern became even more striking 5 days into the differentiation program when cells stained for the perilipins showed bright rings surrounding growing lipid storage droplets as well as some punctate staining (Fig. 8L). By contrast, few

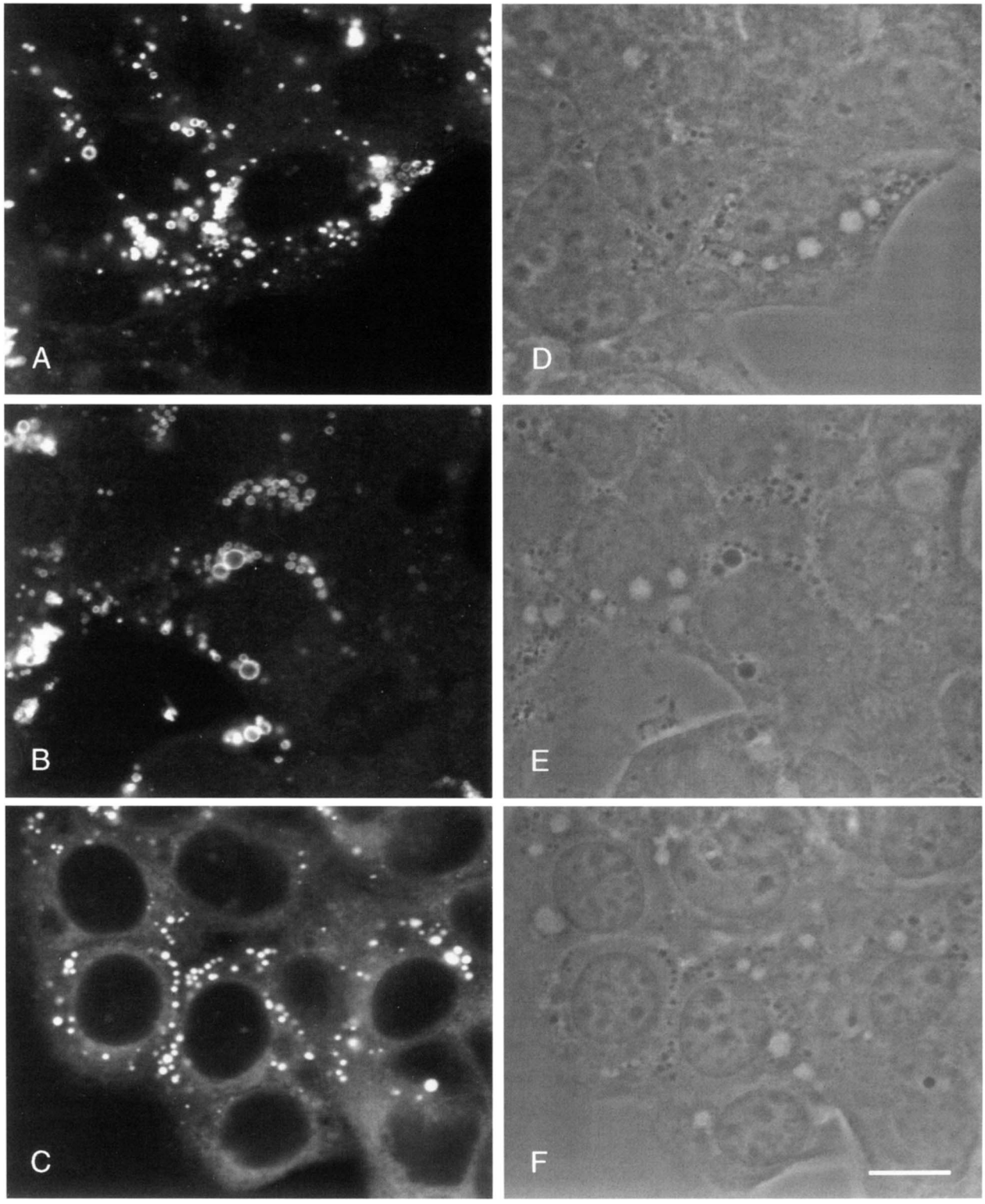

Fig. 5. ADRP is a lipid droplet-associated protein in MA-10 Leydig cells and shows the same localization pattern as the perilipins. D), E), and F) are phase images of cells in A), B) and C), respectively. A) Confocal image of immunofluorescence of MA-10 Leydig cells stained with anti-ADRP antibodies showing punctate staining and small rings corresponding to phase dense structures in 5D. B) Immunofluorescence of MA-10 Leydig cells stained with polyclonal antibodies raised against a recombinant amino terminal peptide of perilipin showing punctate staining and small rings corresponding to phase dense structures in 5E. C) Fluorescence of MA-10 cells stained with Nile Red to detect neutral lipid. Neutral lipid staining in 5C identifies small lipid storage droplets that appear phase dense in the corresponding image 5F. Bar = 6 microns.

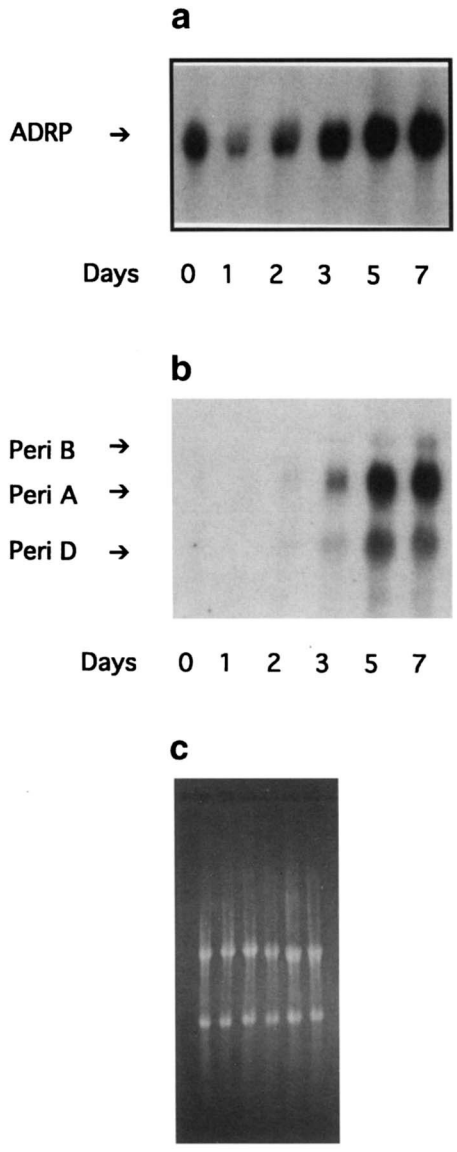

Fig. 6. Northern blot analysis of RNA from differentiating 3T3-L1 adipocytes shows increasing ADRP and perilipin mRNA levels during adipose differentiation. 3T3-L1 cells were grown to confluence (day 0); dexamethasone, insulin and isobutylmethylxanthine were added for 72 h to induce adipocyte differentiation. Days 1 through 7 designate time after the induction of differentiation. Blots of total RNA (15 μg/lane) from cells extracted throughout the time-course of differentiation were probed with radiolabeled cDNA probes for ADRP (a) or perilipin (b). Perilipins A, B, and D are alternative splice variants from a single gene; perilipin A and B mRNA yield unique protein products; 6c shows the ethidium bromide-stained gel used to prepare the blot in 6a and 6b.

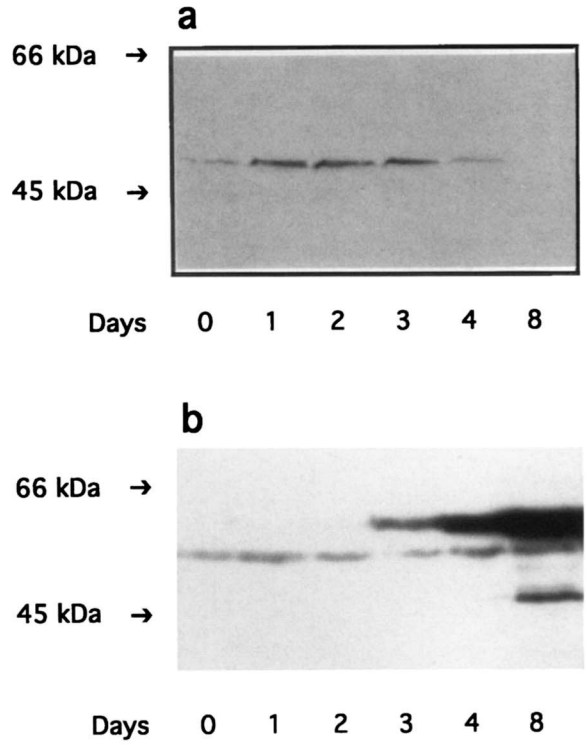

Fig. 7. Immunoblots of homogenates from 3T3-L1 adipocytes reveal a decrease in ADRP but an increase in perilipins during differentiation. Cells were induced to differentiate as described in the legend to Fig. 6. Days 1 to 8 designate the time after the initiation of differentiation. Whole cell homogenates were normalized on a per cell basis by loading SDS-PAGE gels with homogenate equivalent to 15 μg DNA/lane; this mass of cellular DNA corresponds to approximately 150 μg protein for undifferentiated cells and 375 μg protein for differentiated adipocytes. Proteins were separated by SDS-PAGE and transferred to nitrocellulose; immunoblots were probed with anti-ADRP antibodies (a) or anti-perilipin antibodies (b). The upper band in 7b at approximately 60 kDa is perilipin A, while the lower band at approximately 47 kDa (Day 8) is perilipin B; the faint band in all lanes at approximately 55 kDa is a non-specific band. Cellular ADRP levels increase after the initiation of differentiation but then decrease by day 4 and thereafter, while levels of perilipins increase from day 3.

cells showed a specific staining pattern for ADRP (Fig. 8D); these cells displayed primarily punctate staining and lacked the larger lipid storage droplets characteristic of well differentiated adipocytes, and hence, were probably lagging in the differentiation program.

Co-localization of ADRP and the perilipins on the lipid storage droplets of 3T3-L1 adipocytes 3 days after the initiation of differentiation

The transition from ADRP to the perilipins at the surfaces of the lipid storage droplets of 3T3-L1 adipocytes occurred 3 days into the differentiation program. To address whether the two proteins localize transiently on the same lipid storage droplets, cells were doubly stained with polyclonal antibodies raised in rabbits against ADRP and antibodies raised in guinea pigs against perilipin. Lipid droplets that co-stained for both ADRP and perilipin were evident in many cells (**Fig. 9D** and **9E**) indicating that the two proteins transiently occupied the same lipid storage droplets. Other cells in the population exhibited only ADRP staining (Fig. 9A)

or only perilipin staining (Fig. 9B). As the cells do not differentiate synchronously, it was impossible to determine whether or not all lipid storage droplets pass through a stage on day 3 when both proteins are present.

DISCUSSION

Few mammalian proteins intrinsic to intracellular lipid storage droplets have been identified. We now report that ADRP is a lipid droplet-associated protein expressed in a wide variety of cultured cell lines including adipocytes, steroidogenic Leydig cells, fibroblasts, and hepatoma cells. Immunocytochemical studies revealed a bright staining pattern specific to the surfaces of lipid storage droplets in all cells tested with no evidence for association with any other cellular compartment. Furthermore, in sucrose gradients containing MA-10 Leydig cell homogenates, the vast majority of ADRP was found in low density fractions well separated from fractions containing an enzyme marker for endoplasmic reticulum. Two additional studies highlight the relationship between ADRP and stored neutral lipids. Treatment of rats with etomoxir, an inhibitor of carnitine palmitoyltransferase I, induces ADRP expression in liver concomitant with the accumulation of neutral lipids (20). The authors speculate that the induction of ADRP expression may be related to the increased lipid deposition; however, subcellular association of ADRP with neutral lipid was not investigated. A second study found that ADRP is a component of secreted milk lipid globule membranes in cow, human, and rat milk (21); fractionation of bovine mammary tissue identified ADRP in lipid droplet and endoplasmic reticulum fractions. Thus, ADRP is a component of both stored neutral lipid droplets and structurally similar secreted droplets in milk.

ADRP mRNA is expressed in all tissues that were examined (Fig. 1). This observation suggests that most organs contain cells that have intracellular neutral lipid storage droplets. While adipose tissue expressed the most ADRP mRNA (15) when total RNA was assayed from a number of tissues, examination of poly $(A)^+$ RNA from other tissues revealed that moderate levels of ADRP mRNA are observed in lung, liver, and testes; specific cell types within all three of these organs have cytoplasmic lipid droplets. Neutral lipid droplets are prevalent in interstitial fibroblasts within the walls of alveoli in the lungs of fetal, neonatal, and young rats (22–24), in both hepatocytes and Ito cells in liver (6), and in Leydig and Sertoli cells in testes (T. Barber, N. K. Dwyer, J. Wolff, D. Servetnick, D. L. Brasaemle, C. Londos, and E. J. Blanchette-Mackie, unpublished results) (6, 7). We suggest that the lower levels of ADRP mRNA observed in spleen, brain, heart, skeletal muscle and kidney may reflect a reduced requirement for stored neutral lipids in these tissues.

The amino terminus of ADRP is highly similar to the amino termini of the perilipins, a family of lipid storage droplet-associated proteins. The role of this common domain is unknown; it may play a functional role or contain targeting signals that direct the association of the proteins with lipid storage droplets. Whereas ADRP has been found in all cells examined, the distribution of the perilipins is limited to adipocytes and steroidogenic cells. While steroidogenic MA-10 Leydig cells express both proteins simultaneously and display them together on the same lipid storage droplets, differentiating 3T3-L1 adipocytes show a temporal transition from the early appearance of ADRP protein to a later display of the perilipins on growing lipid storage droplets. In 3T3-L1 pre-adipocytes and early during differentiation, ADRP associates with minute cytoplasmic lipid droplets. Perilipin expression initiates 3 days into the differentiation program (this study and C. R. Rondinone, T. Takeda, J. Theodorakis, T. Barber, E. J. Blanchette-Mackie, R. Pointer, A. R. Kimmel, A. S. Greenberg, and C. Londos, unpublished results) when individual lipid storage droplets can contain both ADRP and the perilipins. By 5 days, most cells show exclusively perilipin staining

Fig. 8. Lipid storage droplet content of ADRP and the perilipins switches during 3T3-L1 cell differentiation; ADRP coats minute lipid storage droplets early in differentiation while the perilipins coat growing lipid storage droplets. 3T3-L1 cells were grown to confluence (8A, E, I, M); and then stimulated to differentiate. Cells in 8B, F, J, and N were fixed and stained 1 day after the initiation of differentiation; cells in 8C, G, K, and O were fixed and stained 3 days after the initiation of differentiation; cells in 8D, H, L, and P were fixed and stained 5 days after the initiation of differentiation. 8A), B), C), and D) show confocal images of cells stained with anti-ADRP antibodies. 8E), F), G), and H) are phase images of the cells in A, B, C, and D, respectively. 8I), J), K), and L) show confocal images of cells stained with anti-perilipin antibodies. 8M), N), O), and P) are phase images of cells in 8I, J, K, and L, respectively. Immunostaining for ADRP (8A, B, C, and D) shows bright punctate staining and some small rings throughout the course of adipocyte differentiation; however, few cells showed a specific staining pattern by 5 days (8D). Specific staining for ADRP was particularly lacking in well differentiated cells where growing lipid storage droplets are indicated by arrowheads. Immunostaining for perilipins (8I, J, K, and L) shows the absence of specific staining in cells at confluence (8I) and 1 day after the initiation of differentiation (8J), with bright staining visible in cells at 3 and 5 days after the initiation of differentiation (8K and L) in a punctate pattern as well as in rings around developing lipid storage droplets. Bar in 8H applies to 8A and E as 23 microns and 8B, C, D, F, G, and H as 16 microns. Bar in 8P applies to 8I through 8P as 21 microns.

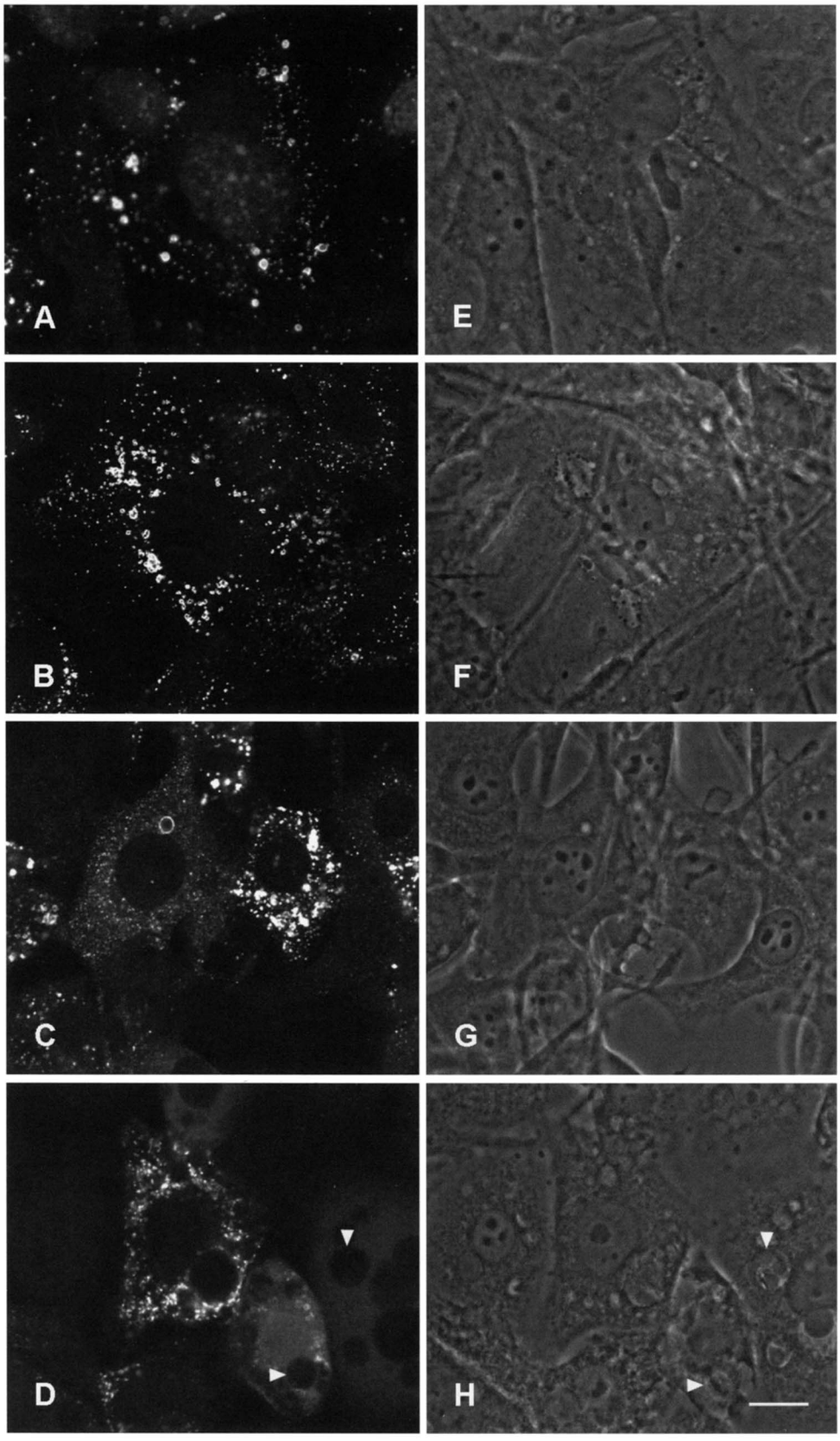

Fig. 8.

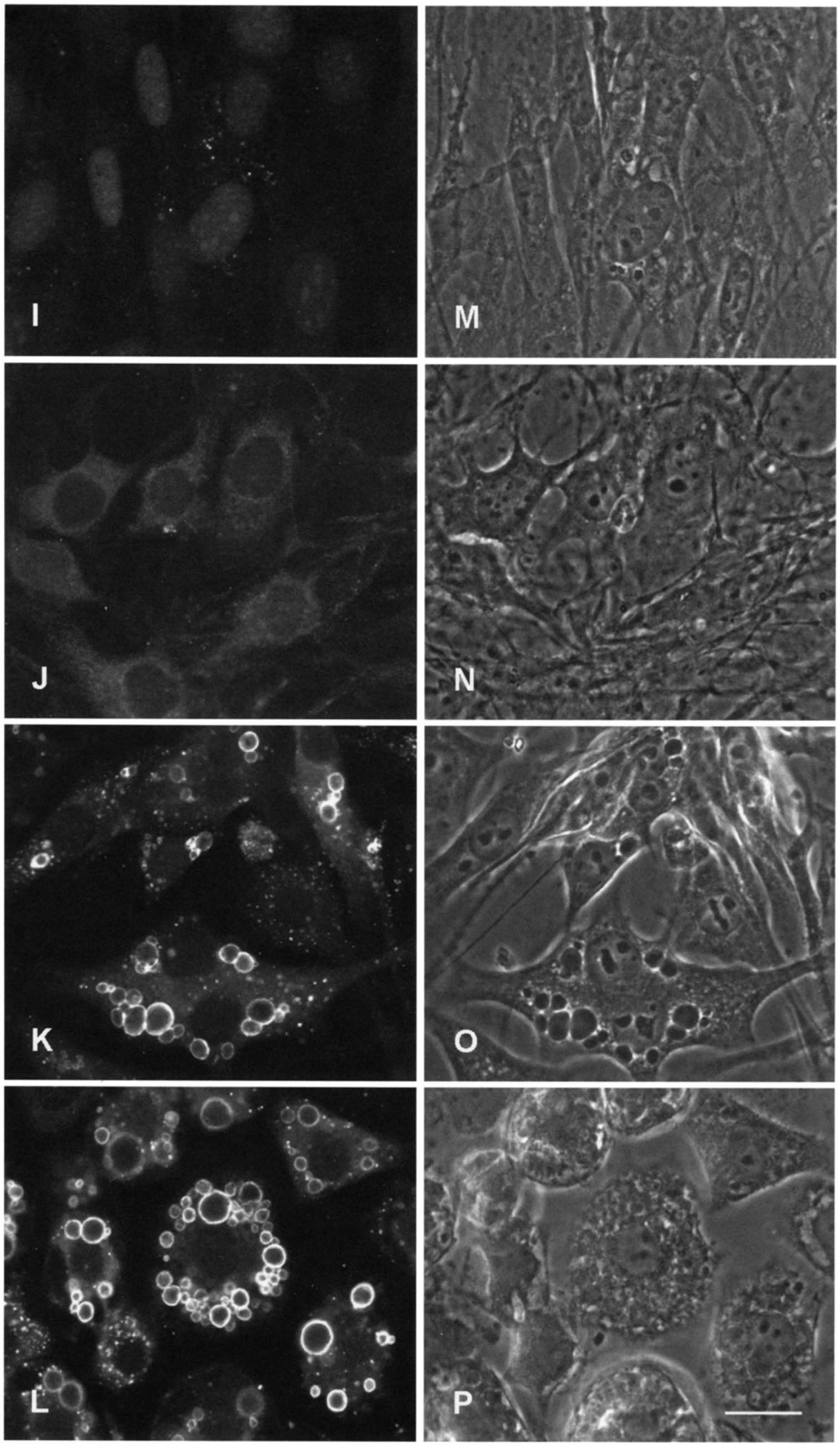

Fig. 8.

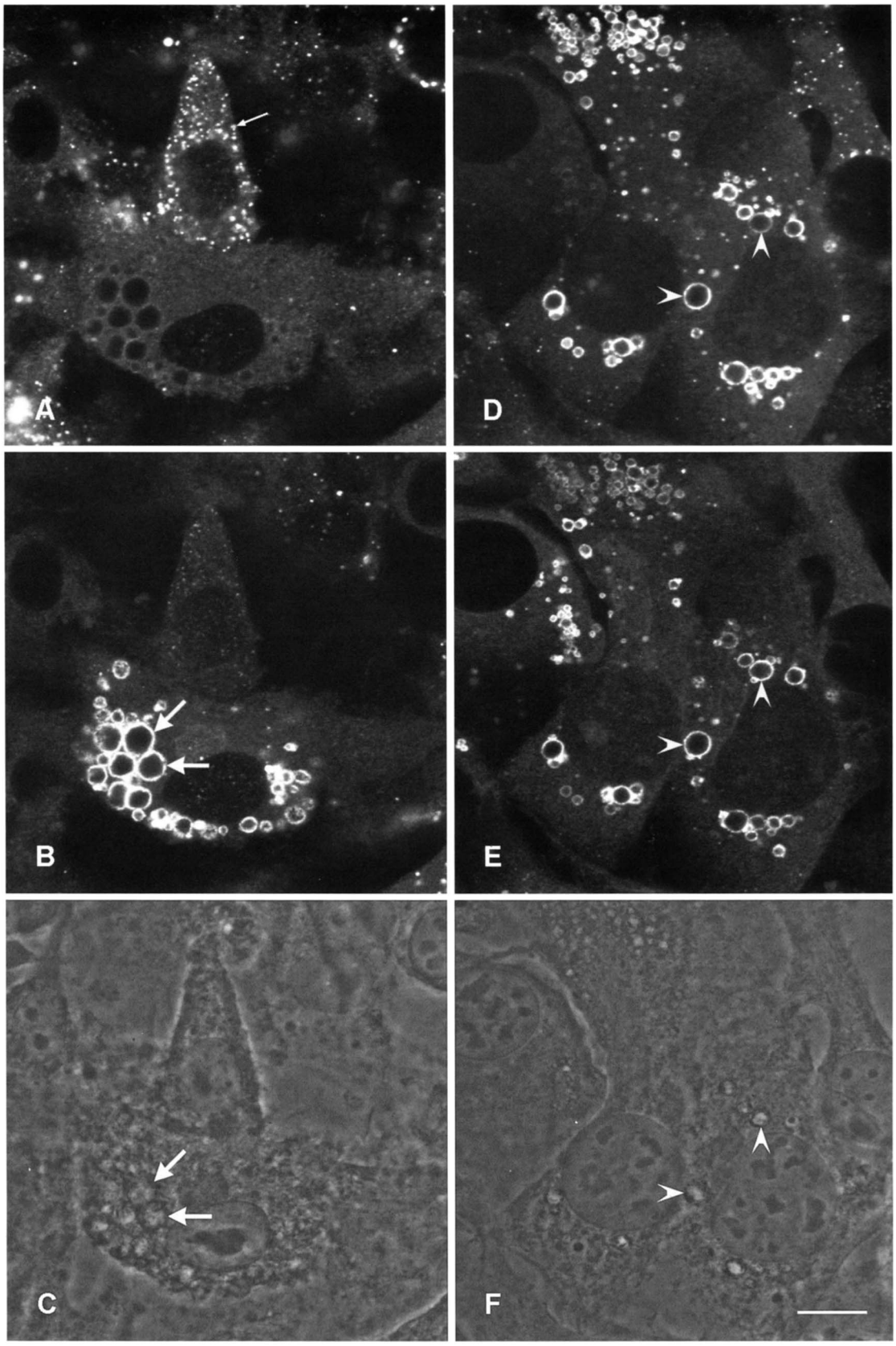

Fig. 9.

around all sizes of lipid droplets; only a few cells show ADRP staining which is limited to minute droplets. Thus, the surface protein transition from ADRP to the perilipins occurs at a time when large increases in triacylglycerol storage are observed.

Is the onset of perilipin expression responsible for the loss of ADRP from adipocyte lipid droplets? ADRP protein levels are regulated during the differentiation of 3T3-L1 adipocytes by a post-transcriptional mechanism; ADRP protein declines while ADRP mRNA increases. Thus, the reduction in ADRP protein may result from decreased translation of ADRP mRNA, or ADRP may be precluded from associating with growing lipid storage droplets, resulting in destabilization and degradation of the protein. We have shown that association with lipid storage droplets is required for stabilization of perilipins (17). The stabilization of ADRP may also require an accessible droplet surface. If ADRP and the perilipins compete for a common binding site on the surfaces of growing lipid storage droplets, then the induction of perilipin expression and subsequent association of perilipins with droplets may exclude ADRP from the droplet surface, resulting in degradation of ADRP. Nonetheless, ADRP and perilipins coexist on lipid droplets in MA-10 Leydig cells as well as on adipocyte droplets 3 days into the differentiation program. It remains to be determined whether these examples of co-occupancy result from an excess of ADRP relative to perilipin, or whether they signify a lack of direct competition between ADRP and the perilipins for binding sites on lipid droplets. We have no explanation for the persistence of high ADRP mRNA levels in differentiated 3T3-L1 adipocytes; it will be interesting to determine whether changes in lipid metabolism can induce the reduction of perilipin levels and the reappearance of ADRP on lipid droplets.

The transition of lipid droplet protein composition from ADRP to the perilipins during the differentiation of 3T3-L1 adipocytes appears not to be a direct function of the growing size of the lipid storage droplets; ADRP is not always associated with small structures and the perilipins are not found solely on larger structures. Indeed, extremely small structures containing the perilipins are observed in 3T3-L1 adipocytes from 3 days into the differentiation program onward, suggesting that nascent droplets formed later during differentiation contain perilipins and not ADRP.

The finding that ADRP is a widely distributed, probably ubiquitous, lipid storage droplet protein implies that it plays an essential role in lipid metabolism. The appearance of ADRP around minute lipid droplets suggests a role in the nucleation of neutral lipid packaging. The mechanisms of lipid droplet formation are unknown; however, the early stages of droplet formation likely occur in or near the endoplasmic reticulum where the activities of diacylglycerol acyltransferase (25 and references contained within, 26) and acyl-CoA cholesterol acyltransferase (27 and references contained within), the final and committed steps of triacylglycerol synthesis and cholesterol esterification, are located. We have found no evidence for the association of ADRP with the endoplasmic reticulum. Furthermore, ADRP is synthesized on free polysomes (C. J. Schultz, N. E. Wolins, and C. Londos, unpublished observations) and contains no previously characterized signal sequences; hence, ADRP may be recruited to lipid storage droplets from the cytosol and not from within the endoplasmic reticulum. Thus, a role for ADRP in lipid packaging would be manifested after the initial deposition of neutral lipids into a nascent droplet and may include stabilization of droplet structure or regulation of lipid hydrolysis.

The switch from ADRP to the perilipins at lipid droplet surfaces in differentiating 3T3-L1 adipocytes implies that the perilipins may fulfill additional functions that ADRP cannot provide. Additional investigation is needed to determine whether or not the perilipins are required for the coalescence of droplets to produce the characteristic large lipid storage droplets found in mature adipocytes. Additionally, the placement of perilipins on mature droplets may facilitate neutral lipid catabolism via mechanisms that are specific to mature adipocytes, and not pre-adipocytes. Perilipins, but not ADRP, contain consensus sequences for phosphorylation by cAMP-dependent protein kinase (10, 11). These sites are phosphorylated in response to lipolytic stimuli, suggesting that the perilipins may have a role in lipid hydrolysis (28, 29).

Lipid storage droplets are a dynamic subcellular compartment found in a broad spectrum of organisms ranging from yeast and plants to vertebrates. These organ-

Fig. 9. Co-localization of ADRP and the perilipins on the lipid storage droplets of some 3T3-L1 adipocytes at 3 days. Three days into the differentiation program, 3T3-L1 adipocytes were double stained with anti-ADRP antibodies and anti-perilipin antibodies. A), B), and C) are images of the same cells; D), E), and F) are images of the same cells. 9A and D show ADRP immunostaining; 9B, and E show perilipin immunostaining; 9C shows a phase image of cells in 9A and B; 9F shows a phase image of cells in 9D and E. Double immunostaining for ADRP and perilipins shows that both proteins can be found on some lipid storage droplets (arrowheads), while other lipid storage droplets show immunostaining for perilipin only (heavy arrows), or ADRP only (small arrow). Phase images of large lipid storage droplets appear phase lucent in 9C and F (arrowheads and heavy arrows), while tiny lipid droplets appear as phase dense granules in 9C. Bars = 6 microns.

elles have been best characterized in plant seeds, where they provide a source of energy and building materials for germination and early development. There is a burgeoning literature on the oleosins, proteins intrinsic to plant lipid droplets (reviewed in 30 and 31); proteins associated with yeast lipid droplets are also being identified (32). Perilipins were the first described proteins intrinsic to mammalian intracellular lipid droplets and have only been found in adipocytes (10–12) and steroidogenic cells (T. Barber, N. K. Dwyer, J. Wolff, D. Servetnick, D. L. Brasaemle, C. Londos, and E. J. Blanchette-Mackie, unpublished results) (13). The present study establishes that ADRP also associates with lipid storage droplets but appears to be expressed ubiquitously. The identification of lipid droplet-specific proteins has begun to provide definition of this organelle in mammalian cells. Interestingly, there is no apparent structural similarity between the perilipins or ADRP and the oleosins, potentially analogous plant lipid droplet proteins. Identification of additional components will begin to unravel the complex biology regulating lipid storage and release from neutral lipid storage droplets.

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