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Reorganization of a Novel Vimentin-Associated Protein in 3T3-L1 Cells During Adipose Conversion

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Abstract We have found that the antibody A2, a marker for the capsule of steroidogenic lipid droplets, reacts with an intermediate filament-associated protein, P_{200}, in 3T3-L1 preadipocytes. Supporting evidence came from the colocalization pattern of P_{200} with vimentin in double label experiments. The association of P_{200} with vimentin was further confirmed by its copurification with vimentin after high salt extraction and colocalization of these two proteins in high salt-extracted and vinblastine-treated cells. In preadipocytes this protein was distributed on the vimentin filament network. At the early stage of adipose conversion, this protein was found to encircle nascent lipid droplets ranging from 0.1 to 0.2 μm, accompanied with a decreased distribution on the vimentin filament system. This infers a possible translocation of P_{200} from the vimentin filaments to the droplet surface. Meanwhile, the vimentin filaments remained in a normal distribution in the cytoplasm and were apparently not associated with the nascent droplet. The association of vimentin filaments to droplet surfaces became prominent in lipid droplets larger than 0.2 μm, forming a typical vimentin cage. Immunogold staining also confirmed the translocation of P_{200} immunoreactivity from the droplet surface to the vimentin cage. The relocation of P_{200} from the cytoplasmic vimentin filaments to the droplet surface prior to the formation of the vimentin cage, as well as the reorganization of this protein in the vimentin cage, suggests a stabilizing role in the lipid droplet formation and an inducing function of this protein in the formation of the vimentin cage. J. Cell. Biochem. 67:84–91, 1997.

Key words: vimentin-associated protein; capsule of lipid droplet; vimentin cage; adipocyte

INTRODUCTION

Lipid droplets in mouse adrenal Y-1 and bovine adrenal fasciculata cells are surrounded by an electron-dense layer, the capsule [Almahbobi et al., 1992]. Recently, we had used a monoclonal antibody A2 to label the capsule of lipid droplets in cultured rat adrenal cells and hamster Leydig cells, in which lipid droplets are entangled in masses of irregularly arranged vimentin filaments [Wang and Fong, 1995; Fong et al., 1996]. The distribution of this 160-kD capsular protein on lipid droplets is dynamic; it detaches from lipid droplets in response to lipolytic stimulation and this process is regulated by protein kinase A system [Fong et al., 1996; Fong and Wang, 1997].

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3T3-L1 preadipocytes can be converted into adipocytes by conditioned induction [Green and Menth, 1974]. The expression of vimentin, actin, and tubulin is maintained at decreased levels during adipose conversion [Sidhu, 1979; Spiegelman and Farmer, 1982]. The arrangement of vimentin filaments, however, changes dramatically; they are reorganized from a cytoplasmic network pattern to a regular cage structure around lipid droplets of varying sizes in fully differentiated 3T3-L1 cultures [Franke et al., 1987]. What causes the translocation of the vimentin filaments to the lipid globules is still unknown. On the basis of the property of tenacious binding of lipids to vimentin during biochemical isolation [Traub et al., 1985], the vimentin cage appears to have a role in stabilizing lipid globule formation and preventing direct hydrophobic association of lipid globules with various membranous organelles [Franke et al., 1987].

The purposes of this study were to use mAb A2 to examine the presence of the capsule of lipid droplets in 3T3 adipocytes and to deter-

mine its dynamic distribution in the formation of the vimentin cage by immunofluorescence and immunoelectron microscopy. We will also address the role of this capsular protein in vimentin cage formation.

MATERIALS AND METHODS

Antibodies

The characterization of the mAb A2 was reported by Wang et al. [1997]. The rabbit antivimentin polyclonal antibody used in this study was a generous gift from Dr. Eugenia Wang (Lady Davis Institute, Montreal, Canada). For double immunofluorescence staining with the two antibodies mentioned above, a combination of biotinylated horse antimouse IgG whole molecule (Vector, Burlingame, CA) plus Texas red-conjugated avidin D (Vector) and fluorescein-isothiocyanate (FITC)-labeled goat antirabbit IgG whole molecule (Sigma, St. Louis, MO) was used. Ten nanometer gold-conjugated goat antimouse IgG whole molecule (Sigma) was used as secondary antibody in immunoelectron microscopic localization.

Cell Culture

Mouse embryo 3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Long Island, NY) supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). Cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. The medium was changed at 48 h intervals. To induce adipose conversion, near-confluent 3T3-L1 cells were treated with 0.25 µM dexamethasone and 0.5 mM 1-methyl-3-isobutylxanthine (Sigma) for 2 days. The culture medium was then removed and the cells were incubated in fresh medium for several days. Differentiation of preadipocytes was examined by phase contrast microscopy. To achieve better visualization of individual cells, the cells were replated on coverslips and incubated for another 24 h, according to the method of Franke et al. [1987].

Treatments

Cells were incubated at 4°C for 90 min, followed by addition of 15 µM colchicine for another 2 h at 37°C to depolymerize the microtubules. For high salt extraction, cells were extracted with 0.6 M KCl in PBS containing 1% Triton X-100, 10 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM ethylene-diaminotetraacetic acid (EDTA) for 1 min [Zackroff and Goldman, 1979] to remove actin filaments and microtubules.

Immunofluorescence

3T3-L1 cells attached to coverslips were rinsed in phosphate-buffered saline (PBS), pH 7.4. For double staining, the cells were fixed with 0.15% glutaraldehyde in PBS (containing 0.15% Triton X-100) for 5 min, followed by incubation with $NaBH_4$ (1 mg/ml) for 15 min twice to reduce free aldehyde groups, which can nonspecifically bind antibodies. Each coverslip was overlaid with primary antibodies (mAb A2 and rabbit anti-vimentin polyclonal antibody) and incubated in a humidified chamber for 2 h at room temperature. After washing with PBS, biotinylated horse antimouse IgG (H + L) antibody (1:100 dilution) was applied, followed by 2-h incubation at room temperature. After rinsing in PBS, cells were incubated with Texas red-conjugated avidin D (1:100 dilution) and FITC-labeled goat antirabbit IgG whole molecule antibody (1:50 dilution) for 2 h. Cells were then washed with PBS, mounted on glass slides and viewed with a Reichert Polyvar 2 microscope (Leica, Wien, Austria) equipped with epifluorescence illumination.

Immunoelectronic Microscopy

3T3-L1 cells grown in 35-mm plastic dishes were rinsed in phosphate buffer (PB), pH 7.4 and pretreated with 0.2% Triton X-100 for 5 min. Cells were then fixed in 0.2% glutaraldehyde for 10 min at 4°C and treated with 1 mg/ml $NaBH_4$ for 15 min twice to block nonspecific binding. Incubation with mouse mAb A2 was carried out at 4°C overnight, followed by three washes in PB. Subsequently, cells were incubated with 10 nm gold-conjugated goat antimouse IgG (H + L) (1:10 dilution) for 2 h at room temperature. The samples were rinsed twice with PB and postfixed, first in 2% glutaraldehyde, and then in 1% osmic acid in PB. Samples were then dehydrated in a series of alcohols and embedded in Epon 812 by standard procedures. Thin sections were prepared and stained with both uranyl acetate and lead citrate.

Gel Electrophoresis and Western Blotting

Confluent 3T3-L1 cells grown in 100-mm plastic dishes were rinsed in PBS, collected and directly dissolved in sample buffer by gentle homogenization and heating at 95°C for 5 min. Gel electrophoresis and immunoblotting were performed according to the protocols of Fritz et al. [1989] and Towbin et al. [1979], respectively. After electrophoresis one sample lane was sliced from the gel and stained with 0.1% Coomassie Blue R-250 for reference purposes. The unstained portion of the gel was electrotransfered onto nitrocellulose paper. Strips were cut from the nitrocellulose paper, blocked with 5% nonfat milk in TBS (150 mM NaCl in 50 mM Tris, pH 8.2), and incubated with primary antibody (mAb A2 or rabbit anti-vimentin antibody) at 4°C overnight. The strips were then washed with TBS-0.1% Tween 20 and incubated for 2 h at 37°C with alkaline phosphatase-conjugated secondary antibodies (1:7,500 dilution) (Sigma). After washing with TBS-0.1% Tween 20, the strips were incubated with a substrate solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as chromagen.

RESULTS

mAb A2 detected a 200-kD protein (P_{200}) in 3T3-L1 preadipocytes (Fig. 1A). Intermediate filaments harvested from cells after extraction with 0.6 M KCl were also found to contain this protein (Fig. 1B), in addition to vimentin (Fig. 1D). Furthermore, the A2 antibody also reacted with P_{200} in converted adipocytes (Fig. 1C). The relative amount of vimentin appeared to be less in mature adipocytes (Fig. 1F) compared with that in preadipocytes (Fig. 1D).

We performed double immunofluorescence staining to study the distribution of the P_{200} and its relationship to vimentin filaments in 3T3-L1 preadipocytes. Anti-vimentin Ab revealed a fine network of filaments in the cytoplasm (Fig. 2A), a pattern identical to that found with mAb A2 (Fig. 2B). When 3T3-L1 preadipocytes were extracted with 0.6 M KCl to remove most proteins, except the intermediate filaments and their associated proteins [Zacroff and Goldman, 1979], P_{200} remained codistributed with vimentin filaments (Fig. 2C,D). Furthermore, colchicine treatment induced vimentin filaments to form thick bundles that colocalized with P_{200} in the perinuclear region (Fig. 2E,F). As shown in Figure 3, immunoreactive-gold particles were discontinuously distributed along the 10-nm intermediate filaments in 3T3-L1 preadipocytes.

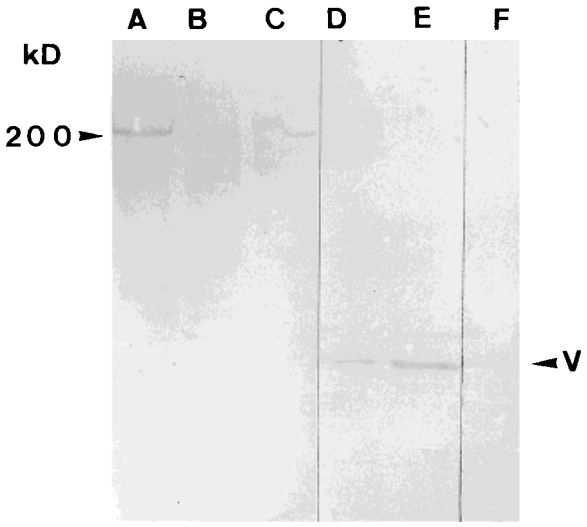

Fig. 1. Immunoblot analysis of mAb A2 and the anti-vimentin antibody in 3T3-L1 cells. **A,D:** Lysates of 3T3-L1 preadipocytes. **B,E:** Cell residues after KCl extraction. **C,F:** Lysates of 3T3-L1 adipocytes. A–C: immunoblots using mAb A2. **D–F:** Immunoblots with anti-vimentin antibody. P_{200} and vimentin (V) are identified in preadipocytes and adipocytes by antibody A2 and anti-vimentin antibody, respectively. P_{200} is copurified with vimentin in KCl-extracted residues of preadipocytes (B,E).

When 3T3-L1 preadipocytes underwent adipose conversion, small lipid droplets gradually accumulated in the cytoplasm. At an early stage, preadipocytes were flattened, polygonal and contained many vimentin filaments. Nascent lipid droplets with a diameter of 0.1–0.13 μm were enclosed by an A2-positive capsule (Fig. 4E) and negative for vimentin (Fig. 4A). To prevent steric hindrance between an IgM (A2) and IgG (anti-vimentin), we tried different sequences of primary antibody incubation and consistently obtained the same result (Fig. 4B,F). The specific capsular staining persisted in lipid droplets grown up to 0.3 μm (Fig. 4C,G). The association of P_{200} to the droplet surface was not disrupted by high salt or 0.15% Triton X-100 extraction (data not shown), indicating the presence of tight binding. The cells at the intermediate stage of adipose conversion became rounded, with decreased filamentous staining for P_{200} and vimentin (Fig. 4C,G). Meanwhile the capsule on these medium-size lipid droplets (>0.2 μm) was now positively stained with both antibodies. At a more advanced stage, large lipid droplets (0.8 μm) were encircled by a prominent rim with both antibodies (Fig. 4D,H).

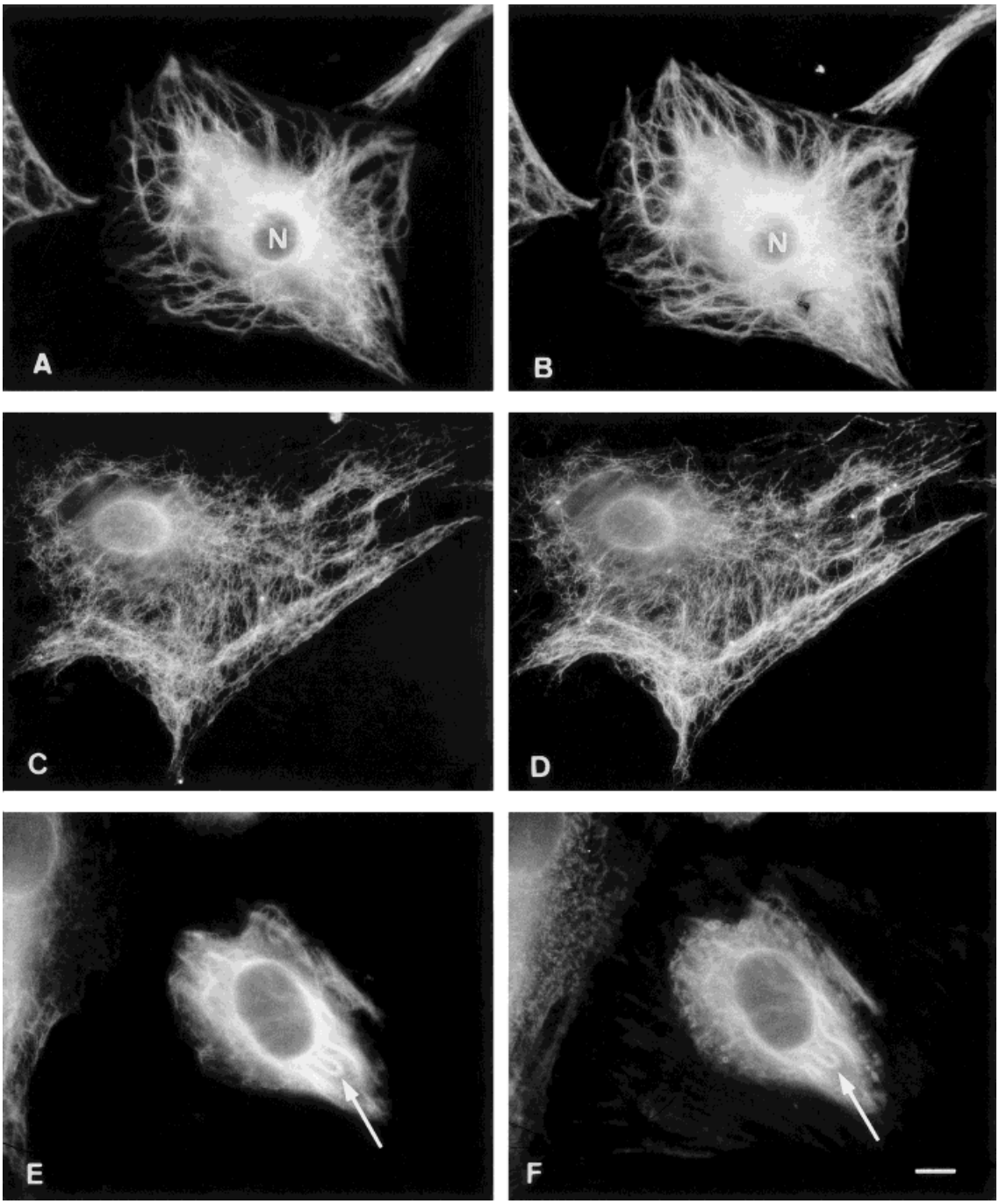

Fig. 2. Co-distribution of P_{200} and vimentin in early 3T3-L1 preadipocytes. Cells are doubly stained with anti-vimentin antibody **(A,C,E)** and mAb A2 **(B,D,F).** P_{200} is mostly colocalized with vimentin intermediate filaments in normal (A,B), KCl-extracted (C,D) and colchicine-treated cells (E,F). It is noted that the capsule of nascent lipid droplets is positive for P_{200} (**F**) but not for vimentin **(E).** Arrows, (E and F) indicate the coarse perinuclear vimentin bundles. N, nucleus. Bar = 10 μm.

Immunogold staining with mAb A2 provided more details of the distribution of P_{200} in vimentin-cage formation. The immunoreactivity of mAb A2 was located on vimentin filaments that were close to, but did not encircle, nascent lipid droplets (Fig. 5A). Some gold particles were directly situated on the surface of lipid droplets of different sizes (Fig. 5A–C). Occasionally, a few intermediate filaments with gold labeling were attached to the droplet surface (Fig. 5B).

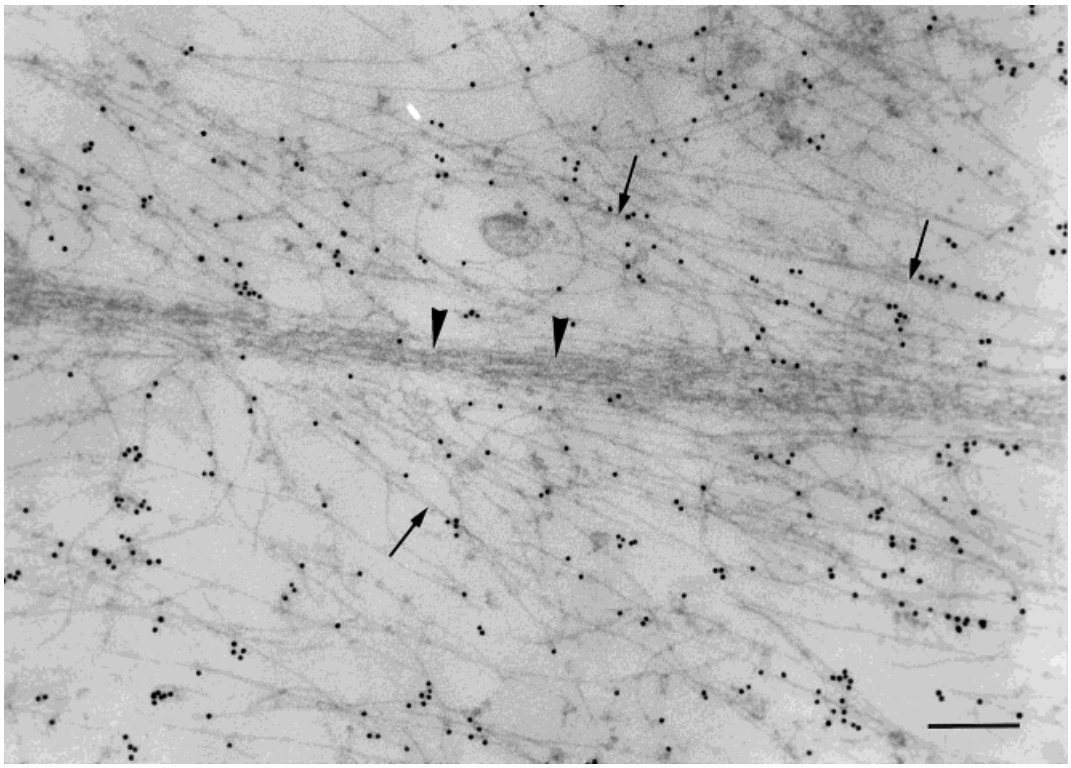

Fig. 3. Immunogold staining for P_{200} in 3T3-L1 preadipocytes. Colloidal gold particles are seen on interconnected networks of intermediate filaments (arrows). A bundle of microfilament filaments is devoid of gold particles (arrowheads). Bar = 0.2 µm.

In large lipid droplets (>1 µm), the droplet surfaces were often encased by groups of intermediate filaments decorated with gold particles when tangentially sectioned (Fig. 5C). In cross-sectioned lipid droplets, the peripheries of lipid droplets were directly associated with gold particle-labeled intermediate filaments (Fig. 5E).

DISCUSSION

We have used mAb A2 to identify the P_{200} in 3T3-L1 preadipocytes. This protein not only colocalizes with vimentin filaments, but also copurifies with vimentin after high salt extraction by immunoblot analysis. Moreover, when vimentin filaments were aggregated by vinblastine treatment, the staining for P_{200} persisted on these intermediate filament bundles. Immunoelectron microscopic observation also confirmed that P_{200} is sporadically distributed along intermediate filaments. Since 3T3-L1 preadipocytes contain only intermediate filaments of the vimentin type [Franke et al., 1978; Spiegelman and Farmer, 1982], we conclude that P_{200} is a vimentin-associated protein in 3T3-L1 cells.

Greenberg et al. [1991] found that perilipins were present at the periphery of lipid droplets in preadipocytes. In the present study, we found that the periphery of newly formed lipid droplets in preadipocytes also contained P_{200}. It appears that the binding of P_{200} to the droplet surface could stabilize lipid spheres up to 0.2 µm in diameter in the absence of the vimentin cage during adipose conversion. Concomitant with the new location of P_{200} on the capsule of lipid droplets, the amount of cytoplasmic vimentin filaments was significantly decreased, with a gradual loss of A2 immunoreactivity. The relocation of P_{200} from vimentin filaments to the capsule of lipid droplets was confirmed by immunogold staining, which demonstrated an intimate association between P_{200} and the lipid surface. No vimentin filaments were found associated with these nascent lipid droplets. Currently, we can not determine whether the capsular staining was due to the translocation of P_{200} from vimentin filaments, or to a redistribution of newly synthesized P_{200}. Biochemical modification of P_{200} protein may take place upon stimu-

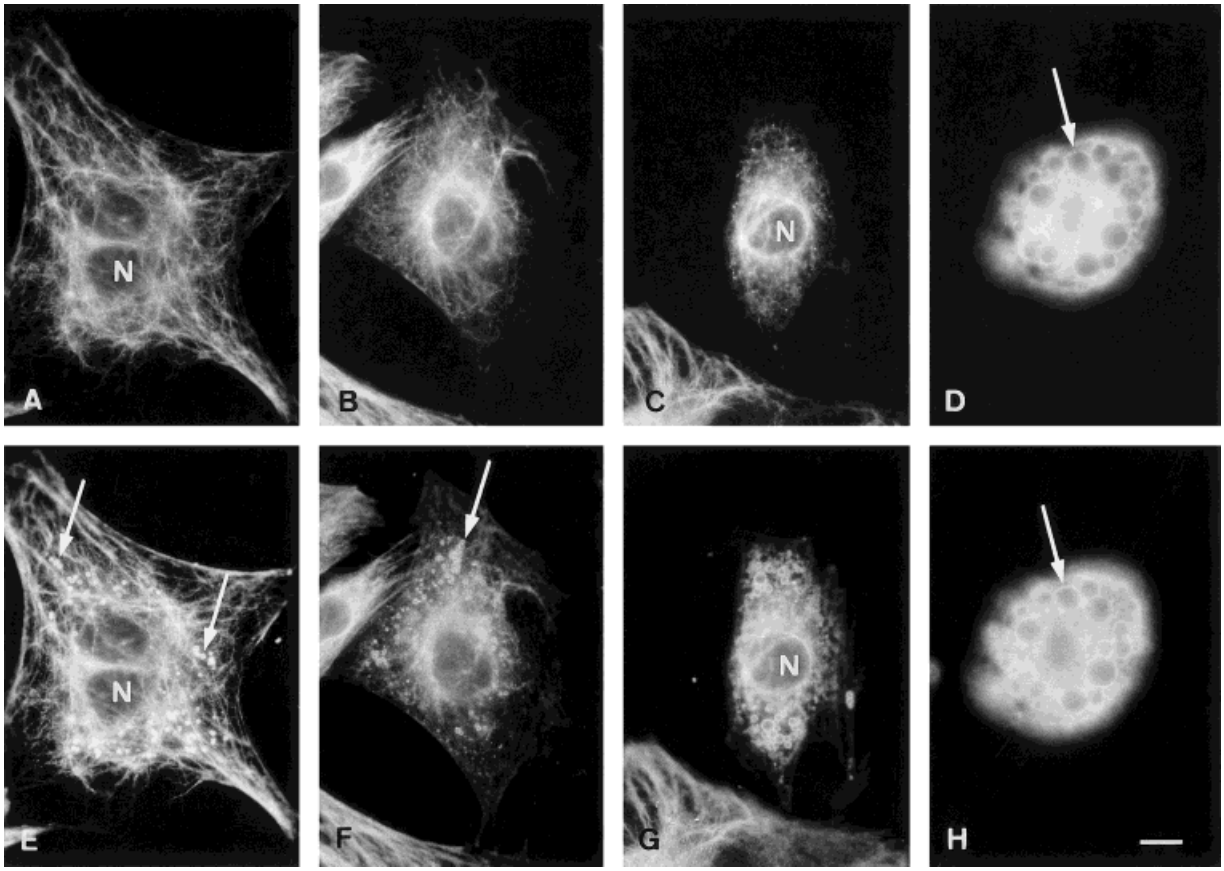

Fig. 4. Double immunofluorescence staining of the anti-vimentin antibody and mAb A2 in converting adipocytes. **A–D:** Anti-vimentin staining. **E–H:** mAb A2 staining. A and E are cells incubated with mAb A2 first and then anti-vimentin. Cytoplasmic intermediate filaments are positive for both P_{200} (E) and vimentin (A). The capsules of nascent lipid droplets are only stained with antibody A2 (arrows, **E,F**). **B–F,C–G,D–H:** Cells stained with anti-vimentin first and then mAb A2. **G:** Most of the mAb A2 immunoreactivity is distributed on the capsule of lipid droplets, while immunoreactivity on the filaments is weak. D and H represent terminally differentiated adipocytes. Specific staining for vimentin and P_{200} is located on the lipid droplet capsule (arrows). Bar = 10 μm.

lation of adipose conversion and result in its detachment from vimentin filaments.

Between the intermediate and final stages of adipose conversion, vimentin filaments were found to encircle the lipid droplets and form the vimentin cage, as described by Franke et al. [1987]. We detected P_{200} on the vimentin filaments at the surface of lipid droplet, indicating that an internal reorganization of vimentin filaments accompanies with the formation of the vimentin cage. This observation also suggests that during the formation of lipid droplets, P_{200} binds to the droplet surface prior to the vimentin filaments, confirming the role of this protein in protecting the nascent lipid globules and inducing the formation of the vimentin cage.

As a vimentin-associated protein, P_{200} may behave as a linker between lipids and vimentin filaments. In rat adrenal cells, mAb A2 only reacts with the 160-kD capsular proteins, not with any other vimentin-associated proteins [Wang and Fong, 1995; Fong et al., 1996]. This might explain the reason why the vimentin cage is not present in this particular cell type. The absence of the vimentin cage in rat adrenal cells and Leydig cells may account for the failure of the lipid droplets in these two cell types to grow up to large lipid globules. The differences in the mechanisms of lipid mobilization between lipid droplets with and without a vimentin cage deserve further study.

Perturbation of the organization of vimentin filaments by nocodozole, microinjection of anti-intermediate filament proteins, or transfection of a vimentin-mutant gene significantly reduces the formation of lipid droplets in preadipocytes, and this suggests that the presence of the vimentin cage stabilize the accumulation of

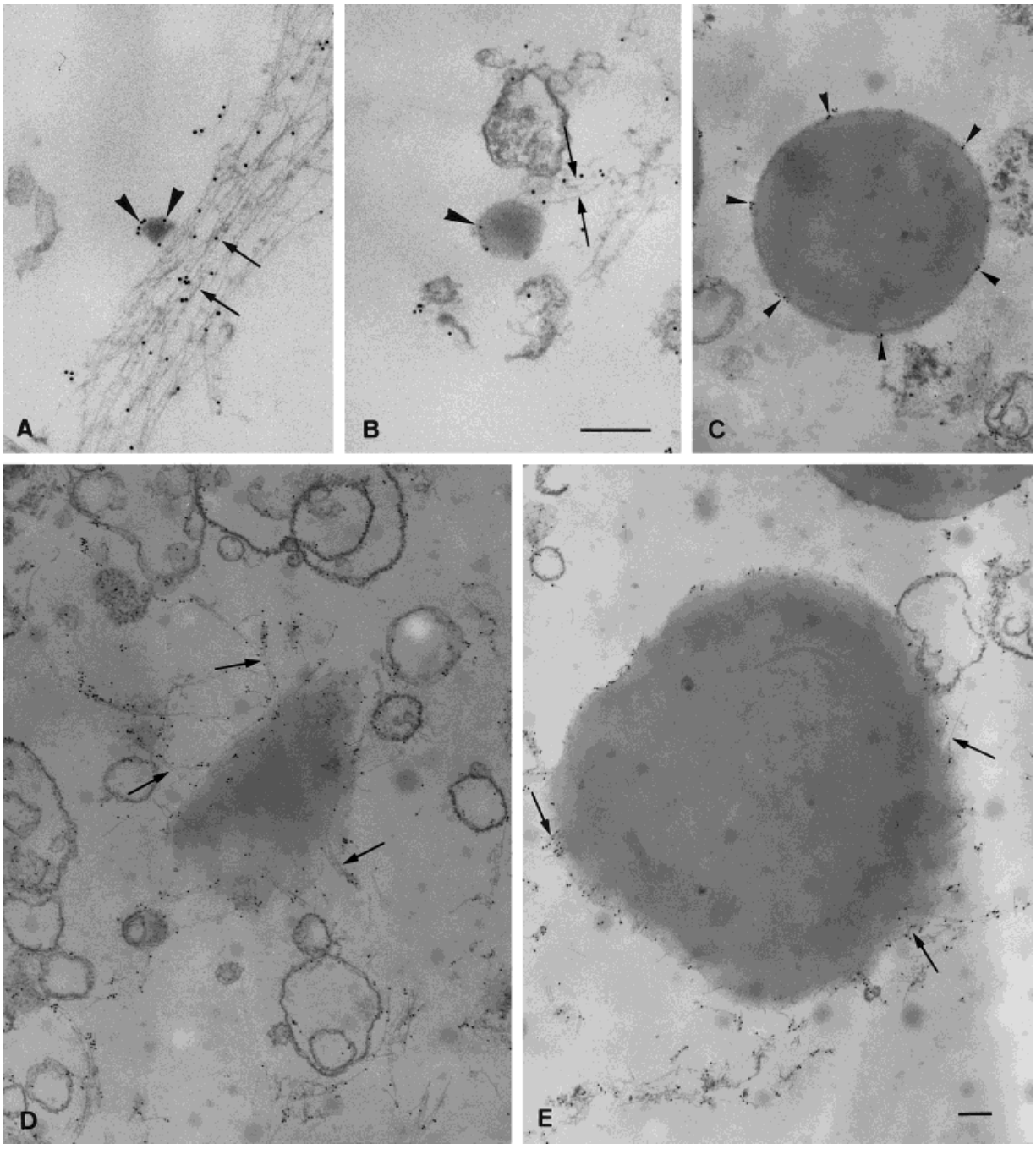

Fig. 5. Immunogold staining for P_{200} on growing lipid droplets. **A:** Gold labeling on a nascent lipid droplet. **B:** A few gold-labeled intermediate filaments are attached to the droplet surface. **C:** Gold labeling on a growing lipid droplet. **D:** Immunoreactivity is seen on the 10-nm filaments ensheathed in a tangentially sectioned lipid droplet. **E:** Gold particles in association with intermediate filaments are distributed on the surface of a large lipid droplet. Arrowheads, indicate gold particles on lipid droplet; arrows, indicate gold-labeled intermediate filaments. Bar = 0.2 μm.

triglyceride in lipid droplets [Liebert and Evans, 1996]. By contrast, it is intriguing that adipocytes from vimentin-null mice are able to form lipid droplets; Colucci-Guyon et al. [1994] proposed that there is some adaptive mechanism for stabilizing lipid droplets that compensates for the absence of vimentin filaments. Our discovery of the presence of P_{200} on the surface of nascent lipid droplets in adipocytes suggests that this might be the mechanism that provides

a stable environment for the growth of lipid droplets. It is possible that the treatments described by Liebert and Evans [1996] also detach P_{200} from the lipid droplets. Therefore, the absence of the vimentin cage may not be the only reason for the failure of lipid droplet formation. Further examination of the distribution of P_{200} under the same experimental manupulations is needed to explain this phenomenon.

REFERENCES

Almahbobi G, Williams LJ, Hall PF (1992): Attachment of steroidogenic lipid droplets to intermediate filaments in adernal cells. J Cell Sci 101:383–393.

Colucci-Guyon E, Portier MM, Dunia I, Paulin D, Pournin S, Babinet C (1994): Mice lacking vimentin develop and reproduce without an obvious phenotype. Cell 79:679–694.

Fong TH, Wang SM, Lin HS (1996): Immunocytochemical demonstration of a lipid droplet-specific capsule in cultured Leydig cells of the golden hamsters. J Cell Biochem 63:366–373.

Fong TH, Wang SM (1997): Dissection of the signaling mechanism for capsule detachment of lipid droplets in rat adrenocortical cells. J Cell Biochem 64:1–8.

Franke WW, Schmid E, Osborn M, Weber K (1978): Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc Natl Acad Sci USA 75: 5034–5038.

Franke WW, Hergt M, Grund C (1987): Rearrangement of the vimentin cytoskeleton during adipose conversion: Formation of an intermediate filament cage around lipid globules. Cell 49:131–141.

Fritz JD, Swartz DR, Greaser ML (1989): Factors affecting polyacrylamide gel electrophoresis and electroblotting of high molecular-weight myofibrillar proteins. Anal Biochem 180:205–210.

Green H, Menth M (1974): An established preadipose cell line and its differentiation in culture. Cell 3:127–133.

Greenberg AS, Egan JJ, Wek SA, Garty NB, Blanchette-Mackie EJ, Londos C (1991): Perilipin, a major hormonally regulated adipocytes-specific phosphoprotein associated with the periphery of lipid storage droplets. J Biol Chem 266:11341–11346.

Liebert JG, Evans RM (1996): Disruption of the vimentin intermediate filament system during adipose conversion of 3T3-L1 cells inhibits lipid droplet accumulation. J Cell Sci 109:3047–3058.

Sidhu RS (1979): Two-dimensional electrophoretic analyses of proteins synthesized during differentiation of 3T3-L1 preadipocytes. J Biol Chem 254:11111–11118.

Spiegelman BM, Farmer SR (1982): Decreases in tubulin and actin gene expression prior to morphological differentiation of 3T3 adipocytes. Cell 29:53–60.

Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Traub P, Perides G, Scherbarth A, Traub U (1985): Tenacious binding of lipids to vimentin during its isolation and purification from Ehrlich ascites tumor cells. FEBS 193:217–221.

Wang SM, Fong TH (1995): A lipid droplet-specific capsule is present in rat adrenal cells: Evidence from a monoclonal antibody. Biochem Biophys Res Commun 217:81–88.

Wang SM, Chen JS, Fong TH, Lim SS (1997): Characterization of a novel filament system in goldfish xanthophores. Cell Motil Cytoskel 36:216–227.

Yang HY, Lieska N, Goldman AE, Goldman RD (1985): A 300,000-mol-wt intermediate filament associated protein in baby hamster kidney (BHK-21) cells. J Cell Biol 100: 620–631.

Zackroff RV, Goldman RD (1979): In vitro assembly of intermediate filaments from baby hamster kidney (BHK-21) cells. Proc Natl Acad Sci USA 76:6226–6230.