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Association of Globular β-Actin with Intracellular Lipid Droplets in Rat Adrenocortical Cells and Adipocytes

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Proteins located on the surface of lipid droplets may mediate intracellular lipid metabolism. In the present study, immunofluorescent staining and polyacrylamide gel electrophoresis demonstrated that actin (43 kD) is associated with isolated intracellular lipid droplets of rat adrenocortical cells and adipocytes. Two-dimensional gel electrophoresis and immunoblot analysis further confirmed that the lipid droplet-associated actin is the beta isoform. In cultured adrenocortical cells, stress fibers and the surface of intracellular lipid droplets were labeled with anti-beta-actin monoclonal antibody, whereas FITC-phalloidin staining did not mark the rim of lipid droplets. The present results provide the first morphological evidence that globular beta-actin is associated with intracellular lipid droplets. This significant association of actin with the surface of lipid droplets suggests that beta-actin might be involved in the regulation of intracellular lipid metabolism, particularly providing insight into the important transport of lipid constituents. © 2001 Elsevier Science

Key Words: **β-actin; lipid droplet; adrenocortical cells; adipocytes.**

Actin, a cytoskeletal protein, exists in vertebrates as six different isoforms (1). β- and γ-isoforms of actin are abundant in many vertebrate nonmuscle cells (2, 3). Interactions between actin and lipids of cell membranes have been well documented to play important roles in many cell activities such as maintenance of cell shape, cell motility, and cell adhesion (4, 5). In general, actin is anchored to the membrane by actin-associated proteins such as α-actinin (6), vinculin (7), talin (8), etc. Purified actin can also interact directly with liposomes composed of pure lipids without the need of a linker protein (9, 10). That is, actin can bind directly or indirectly to lipids of plasma membranes. It is unclear whether actin is also associated with intracellular lipid droplets, which are constituted by lipids.

Several lipid droplet-associated proteins have been investigated. Perilipins, hormonally regulated phosphoproteins, have been found on the periphery of lipid droplets in adipocytes (11, 12), adrenocortical cells, and Leydig cells (13). In addition, recent studies reported that 'capsular proteins' are also located on the surface of lipid droplets of rat adrenocortical cells (14, 15), hamster Leydig cells (16), and 3T3-L1 adipocytes (17). As to their potential functions, these lipid droplet-associated proteins may be involved in mediating lipid metabolism such as lipid packaging or lipid hydrolysis in response to hormone stimulation. However, the molecular processes that govern either the deposition or catabolism of the lipid droplets are still a mystery (18). It is possible that some other lipid droplet-associated proteins have not been yet discovered.

In this study, we examine the proteins associated with intracellular triglyceride-rich lipid droplets of adipocytes and the cholesterol-rich lipid droplets of adrenocortical cells by immunofluorescence, polyacylamide gel electrophoresis, and immunoblotting methods. The present data demonstrate the binding of β-actin with intracellular lipid droplets. The significant roles of β-actin on intracellular lipid droplets are also discussed.

MATERIALS AND METHODS

Materials. Adult male Wistar rats (200–400 g) were housed in standard conditions with sufficient food and water. Type II collagenase, poly-L-lysine, rabbit polyclonal anti-actin antibodies, mouse monoclonal anti-β-actin antibody, Texas-Red conjugated anti-rabbit IgG antibody, FITC conjugated anti-mouse IgG antibody that is preabsorbed with rat serum, and FITC-phalloidin were all purchased from Sigma (St. Louis, MO). Biotin-conjugated anti-mouse IgG that is preabsorbed with rat serum and streptavidin conjugated with peroxidase was purchased from Vector (Burlingame, CA). DMEM/Ham's F-12 (1:1 v/v) medium, horse serum, fetal bovine serum, and

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FIG. 1. Immunofluorescent staining of isolated lipid droplets from rat adrenocortical cells (A and B) and adipocytes (C and D) by anti-actin polyclonal antibodies. A and C are the phase pair of B and D, respectively. The isolated droplet from adipocytes is larger than that from adrenocortical cells. Bright rims labeled with actin can be seen encircling the lipid droplets (arrowheads). Bar = 20 μm.

penicillin/streptomycin were obtained from Gibco (Grand Island, NY).

Isolation of intracellular lipid droplets. Rats were anesthetized with 7% chloral hydrate (4 ml/kg) by intraperitoneal injection. Adrenal glands and epididymal fat pads were isolated and rinsed with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4).

The intracellular lipid droplets of adrenocortical cells were purified as described in a previous report (19). Each adrenal gland was trimmed to remove fat and homogenized in 1 ml of a cold 0.25 M sucrose solution with a Teflon/glass homogenizer on ice. The lipid droplet fraction was isolated by discontinuous gradient centrifugation. Briefly, the homogenate was layered on top of 3 ml of a 0.5 M sucrose solution that was at the bottom of a centrifuge tube. After that, 1.2 ml of a 0.125 M sucrose solution was loaded on top of the homogenate. The gradient was centrifuged at 13,000 g for 3 h at 4 °C. The white floating lipid droplets were collected and stored at −20°C.

Isolation of adipocytes from epididymal fat pads has been previously reported (20). Adipocytes were then hypotonically lysed in lysing medium (containing 10 mM Tris buffer, pH 7.4, 1 mM EDTA, 1 mM NaN_3, and 1 mM PMSF) and then disrupted with 10 strokes in a Teflon/glass homogenizer on ice. After centrifugation at 27,000 rpm for 30 min at 4°C, the floating fat cake containing intracellular lipid droplets was collected and stored at −20°C as described before (13).

SDS-PAGE and immunoblotting. The lipid droplet preparations from adrenocortical cells or adipocytes were assayed for protein

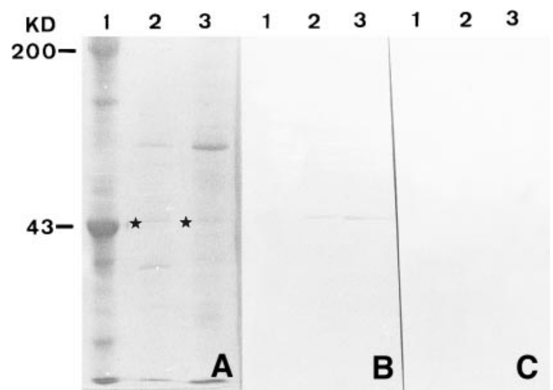

FIG. 2. SDS-PAGE and immunoblot analysis of lipid droplet preparations. A: Coomassie blue-stained gel. B: Corresponding immunoblot with beta-actin monoclonal antibody. C: Primary antibody blank control. Lane 1 contains myofibrillar proteins of rat soleus muscle. Lanes 2 and 3 contain proteins in lipid droplet preparations of adrenocortical cells and adipocytes, respectively. The myosin heavy chain (200 kD) and α-actin (43 kD) of myofibrils are abundant. β-actin (43 kD) (asterisks in A and visualized bands in B) is identified in both lipid droplet preparations but not in myofibrils or the blank control.

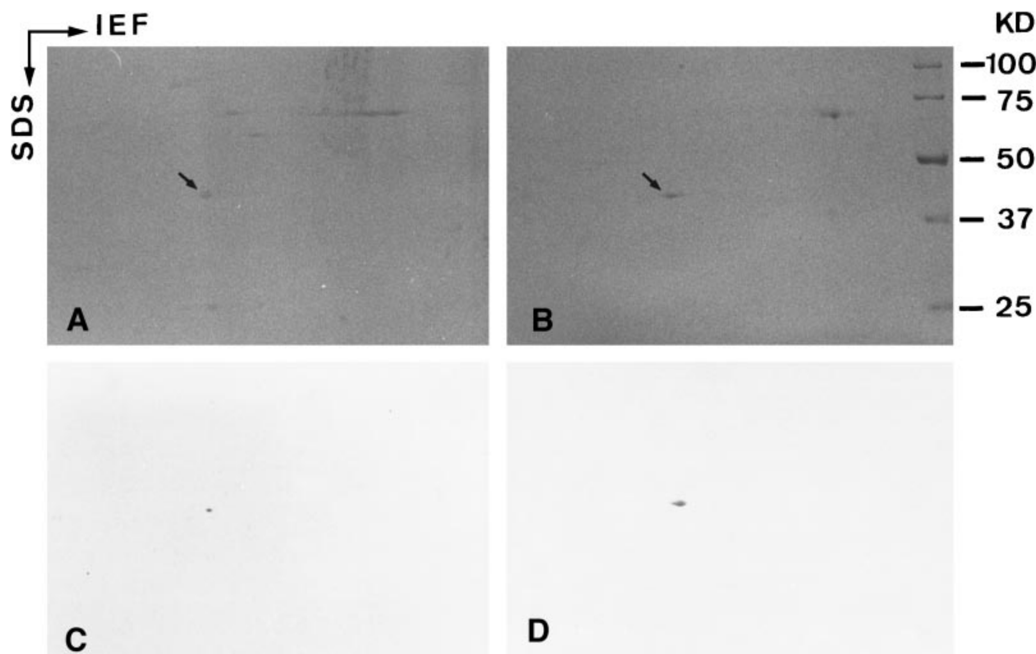

FIG. 3. Two-dimensional gels and immunoblot analysis of lipid droplet preparations of adrenocortical cells (A and C) and adipocytes (B and D). A and B: Coomassie blue-stained gels. C and D: Parallel gels immunoblotted with β-actin monoclonal antibody. Gels show that the actin (43 kD) in both lipid droplet preparations is only the beta isoform (arrows in A and B). Immunoblot shows the significant β-actin spots in both lipid droplet preparations (C and D).

concentration with Bio-Rad protein assay dye and methods. An equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8 containing 2% SDS, 10% glycerol, and 5% β-mercaptoethanol) was added, and the mixture was heated to 95°C for 5 min. After centrifugation (10,000 rpm for 3 min) the upper layer of lipid was discarded; proteins (30–40 μg/lane) in the lower layer were electrophoresed on 10% polyacrylamide gels and transferred to nitrocellulose paper (21). Strips cut from the nitrocellulose paper were blocked with 5% non-fat milk in PBS and then incubated in primary antibody at 4°C overnight. After washing with PBS-0.1% Tween 20, the strips were incubated with biotin-conjugated secondary antibody for 1 h at room temperature. After washing with PBS-0.1% Tween-20, peroxidase-conjugated streptavidin was added and incubated for another hour at room temperature. After washing with PBS, positive bands were visualized by using H_2O_2 as the substrate and diaminobenzidine (DAB) as the chromogen.

Two-dimentional electrophoresis. The Bio-Rad Mini Protean 2D-cell system and methods (Bio-Rad, Richmond, CA) were used to perform isoelectric focusing of capillary gels. First-dimension gels contained 1.6% ampholytes (pH 5–7) and 0.4% ampholytes (pH 3–10). Lipid droplet preparations from adrenocortical cells and adipocytes were supplemented with 9.5 M urea containing 1.6% ampholytes (pH 5–7) and 0.4% ampholytes (pH 3–10). Protein (5–10 μg/capillary) was loaded and run at 750 V for 3.5 h. Second dimension gels containing 10% polyacrylamide were electrophored and transferred to nitrocellulose paper, and then immunoblotting was performed as mentioned.

Primary culture of adrenocortical cells. Primary cultures of rat adrenocortical cells were previously described (15). Briefly, adrenal glands were isolated and cut into small fragments. Cells were obtained by incubating the fragments in 1 mg/ml of a collagenase solution of DMEM for 30 min at 37°C with gentle shaking, followed by mechanical dispersion by aspiration using a glass pipette. Cells were then washed, pelleted by low-speed centrifugation (1000 rpm for 5 min), resuspended, and cultured in DMEM/Ham's F-12 (1:1 v/v) medium supplemented with 12.5% horse serum, 2.5% fetal bovine serum, and 100 IU penicillin/streptomycin. Cells were grown and spread onto glass coverslips and maintained at 37°C in an atmosphere of 95% air and 5% CO_2.

Immunofluorescence. Isolated intracellular lipid droplets were placed on 10% poly-L-lysine-coated slides for 30 min at room temperature for adhesion, then fixed with 10% formalin in PBS for 5 min and blocked with 5% non-fat milk for 30 min. Cultured cells grown on coverslips were fixed and permeated with methanol (−20°C) for 10 min. Nonspecific binding sites were blocked by incubation of $NaBH_4$ (1 mg/ml in PBS) for 30 min. Purified lipid droplets and cultured cells were then incubated with rabbit polyclonal anti-actin antibodies and mouse monoclonal anti-beta-actin antibody for 1 h at room temperature, respectively. After washing with PBS, the samples were reacted with Texas-Red-conjugated goat anti-rabbit or FITC-conjugated goat anti-mouse secondary antibodies for another hour at room temperature. After PBS washing, samples were mounted with 2% n-propyl gallate and 50% glycerol in PBS (pH 8.0), sealed in place with nail polish, and examined with a Nikon epifluorescence microscope.

For FITC-phalloidin staining, cultured cells were fixed with 10% formalin for 10 min and then acetone (−20°C) treatment for another 3 min. After PBS rinsing, FITC-phalloidin was incubated for 20 min at room temperature to label the actin filaments. After PBS washing, samples were mounted and examined.

RESULTS

Actin Is Associated with Isolated Lipid Droplets

Using hypotonic lysis and discontinuous sucrose gradient centrifugation, we isolated intracellular lipid droplets from adipocytes and adrenocortical cells. The isolated steroidogenic intracellular lipid droplets of ad-

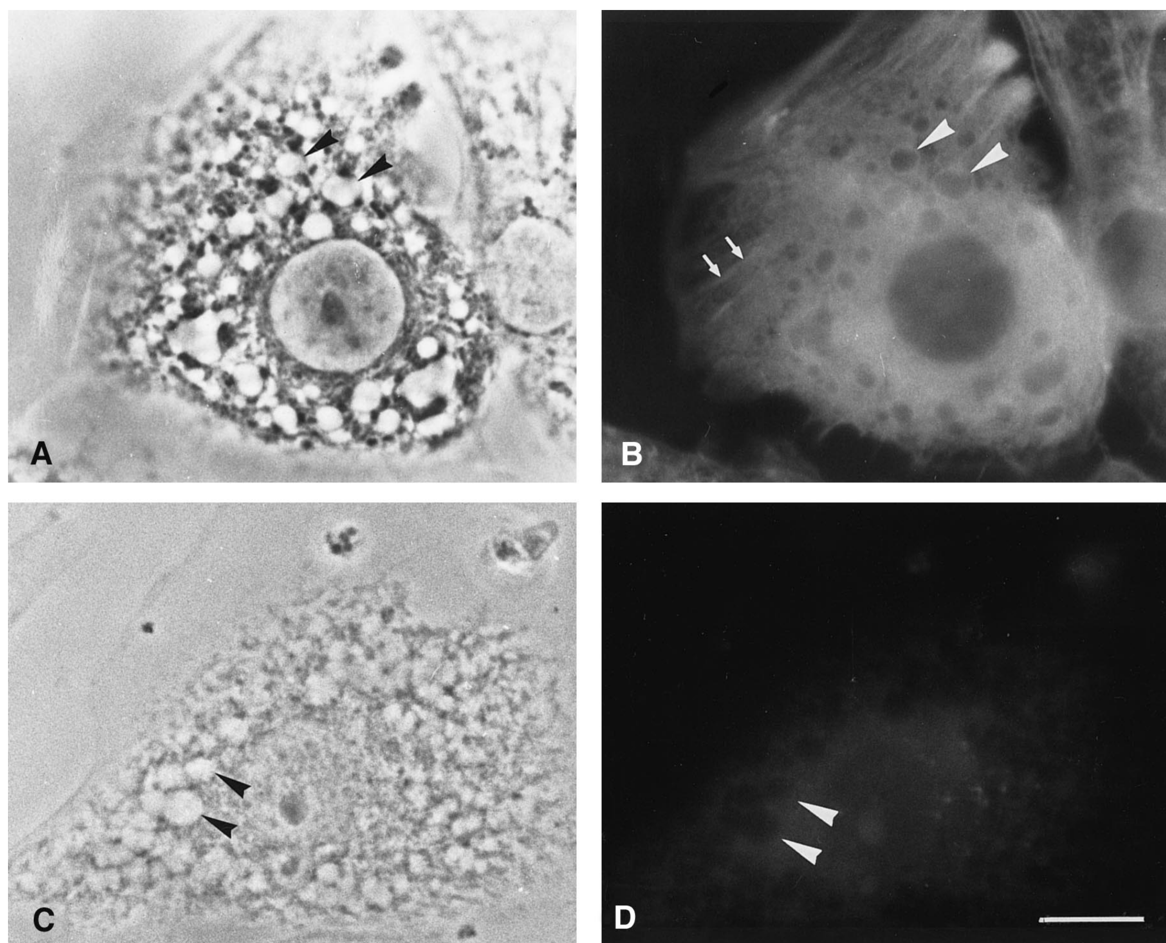

FIG. 4. Immunofluorescence staining of cultured adrenocortical cells. A and C are the phase images of B and D, respectively. Intracellular lipid droplets became clear vacuoles after methanol fixation and extraction (arrowheads in A). Note that bright rims labeled with β-actin monoclonal antibody enclose intracellular lipid droplets (arrowheads in B). Some stress fibers are also positive for β-actin (arrows in B). No fluorescent staining can be observed in blank control (arrowheads in D). Bar = 20 μm.

renocortical cells were small in bright field (Fig. 1A). Immunofluorescent staining with anti-actin polyclonal antibodies displayed peripheral staining of some of the lipid droplets (Fig. 1B). In addition, the isolated neutral lipid droplet from adipocytes was clear and large in bright field as shown in Fig. 1C. After the same immunostaining, a bright fluorescent rim structure was observed surrounding the large lipid droplet (Fig. 1D). There was no immunofluorescent reactivity without primary antibodies in the blank control (data not shown). These results indicate that actin was not only co-isolated with intracellular lipid droplets but also associated with the surface of isolated lipid droplets.

Lipid Droplet-Associated Actin Is in the β-Isoform

In the present study, we used SDS-PAGE and immunoblot analysis to identify the isoform of lipid droplet-associated actin (Fig. 2). The abundant myosin (200 kD) and alpha isoform of actin (43 kD) in myofibrils of rat soleus muscles were visualized by Coomassie blue R-250 staining (lane 1 of Fig. 2A). The proteins co-isolated with intracellular lipid droplets of rat adrenocortical cells (lane 2 of Fig. 2A) and adipocytes (lane 3 of Fig. 2A) were also separated by SDS-PAGE. Coomassie blue staining showed that the proteins in the lipid droplet preparation of adrenocortical cell differed from those of adipocyte. Interestingly, a 43 kD protein (asterisks in lanes 2 and 3 of Fig. 2A) having the same migration rate as the α-actin of myofibrils was visualized in both lipid droplet preparations. Immunoblot analysis showed that the 43 kD protein was indeed recognized by the anti-β-actin monoclonal antibody (visualized bands in lanes 2 and 3 of Fig. 2B), but the α-actin in myofibrils of rat soleus muscles was not labeled (lane 1 of Fig. 2B). As shown in Fig. 2C, there was a clear background when the primary anti-β-actin antibody were omitted. Furthermore, two-dimensional gel electrophoresis indicated that only β-type actin was visualized in lipid droplet preparations of adrenocortical cells (arrow in Fig. 3A) and adipocytes (arrow in

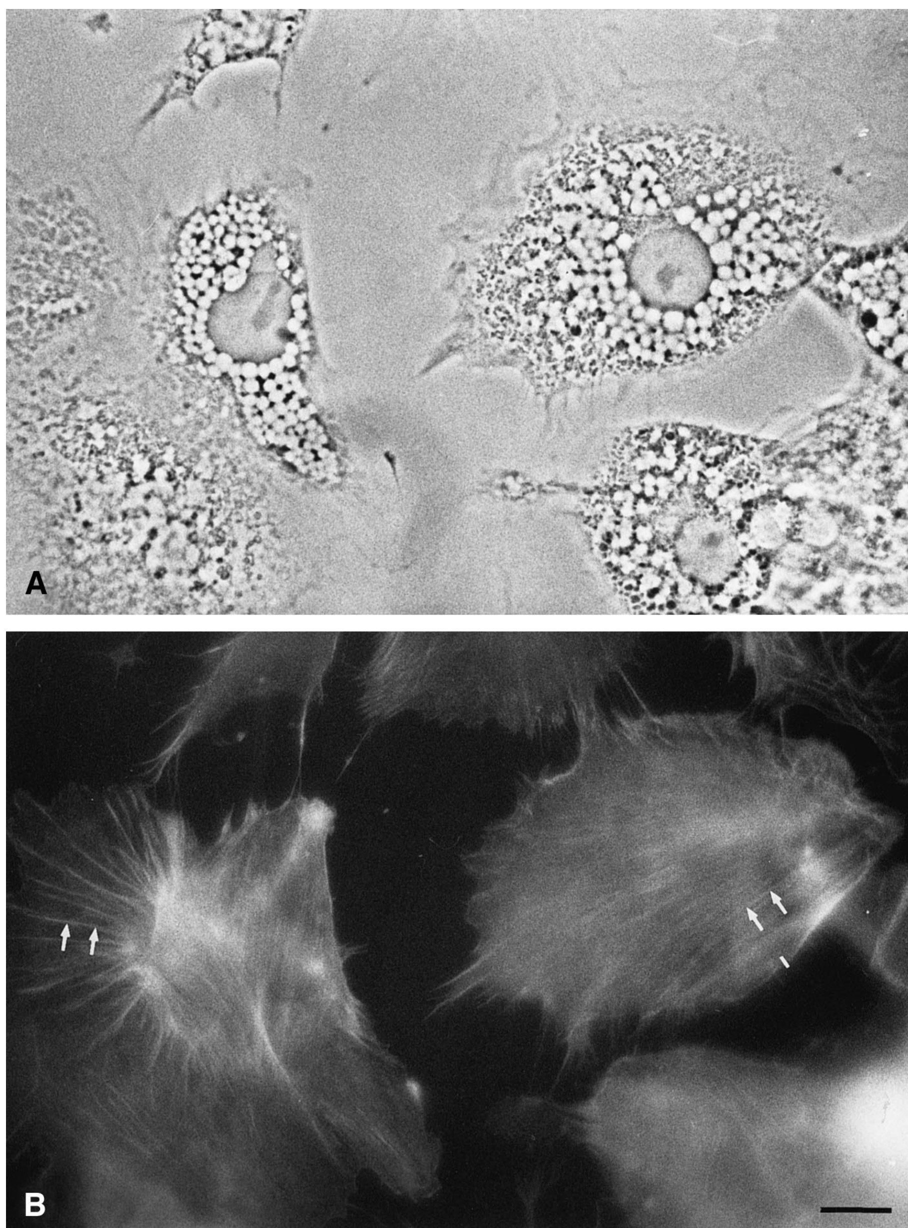

FIG. 5. FITC-phalloidin staining of cultured adrenocortical cells. A is the phase pair of B. Note that stress fibers (arrows in B) but not intracellular lipid droplets are labeled by FITC-phalloidin. Bar = 20 μm.

Fig. 3B) by Coomassie blue staining. Lipid droplet-associated actin was identified to be the beta-type by two-dimensional immunoblot of adrenocortical cells (Fig. 3C) and adipocytes (Fig. 3D).

Globular Type of β-Actin Is Associated with Intracellular Lipid Droplets

Immunofluorescence was used to investigate the localization of β-actin in cultured adrenocortical cells. After methanol fixation, cultured adrenocortical cells showed numerous intracellular lipid droplets that had a clear appearance (arrowheads in Fig. 4A). Anti-β-actin monoclonal antibody not only labeled the filamentous stress fibers (arrows in Fig. 4B) but also labeled the rim structures around the intracellular lipid droplets (arrowheads in Fig. 4B). With omission of the primary antibody, there was no staining of stress fibers or the periphery of lipid droplets (arrowheads in Figs. 4C and 4D). On the other hand, we utilized FITC-conjugated phalloidin to specify the characteristics of actin filaments in cultured adrenocortical cells. Stress fibers, the bundles of actin filaments, were strongly stained by FITC-phalloidin (arrows in Fig. 5B), which however did not mark the periphery of the lipid drop-

let. These data suggest that actin located on the surface of lipid droplet might be the globular type instead of the filamentous type.

DISCUSSION

Lipid droplet-associated proteins are potentially involved in mediating lipid metabolism. Among these proteins, perilipins were first described and were proposed to participate in lipid hydrolysis and lipid packaging in adipocytes, steroidogenic cells of the adrenal cortex, and testes (22). The adipose differentiation-related protein (ADRP) is another lipid droplet-associated protein that plays a role in the management of lipid stores in a wide variety of cells including adipocytes, steroidogenic Leydig cells, fibroblasts and hepatoma cells (23). The capsular proteins of lipid droplets are reported to mediate lipid hydrolysis in response to hormone stimulation in adrenocortical cells (14, 15) and Leydig cells (16). In addition, a novel vimentin-associated protein was proposed to protect the nascent lipid droplets because the protein might translocate from vimentin intermediate filaments to the surface of nascent lipid droplets during lipid accumulation in 3T3-L1 adipocytes (17). The above-mentioned proteins may act as barrier proteins enclosing the lipid droplets, but lipid transport into or out of droplets remains unclear (18).

Previous studies have shown that intracellular lipid droplets and mitochondria will attach to vimentin intermediate filament in steroidogenic adrenal cells (24, 25) and Leydig cells (26). Stimulation of acrylamide, which specifically disrupts vimentin intermediate filaments and shortens the distance between lipid droplets and mitochondria, improves steroid production in mouse adrenal tumor (Y-1) cells (27). Thus, the binding of lipid droplet and mitochondria with vimentin intermediate filaments provides a possible mechanism by which the transport of cholesterol takes place from lipid droplets to mitochondria (26, 27). On the other hand, the nascent lipid droplet of 3T3-L1 adipocytes was even enclosed and protected by a vimentin intermediate filamentous cage during adipose conversion (28). Vimentin, one of the intermediate cytoskeletal proteins, seems to be another type of lipid droplet-associated protein involved in the regulation of lipid metabolism.

Treatment with cytochalasins induced the disruption of actin filaments and inhibited the conversion of [^{3}H]cholesterol to 20alpha-[^{3}H]dihydroprogesteron (a major product of the mitochondrial cleavage enzyme) in adrenal tumor (Y-1) cells during ACTH stimulation. Intact actin filaments were proposed to be necessary for the transport of cholesterol from intracellular lipid droplets to mitochondria (29, 30). Moreover, the transport of cholesterol in adrenal tumor (Y-1) cells and Leydig cells responding to trophic hormone or dibutyryl cyclic AMP was also inhibited by liposomes containing anti-actin antibodies (31, 32) or by erythrocyte ghosts loaded with DNase I (33). It seems that steroidogenic cells maintain a pool of monomeric actin (globular type of actin) which is available for facilitating cholesterol transport (34, 35).

In the present study, we provide evidence that globular beta-actins are significantly associated with the surface of intracellular lipid droplets in adrenocortical cells and adipocytes by immunofluorescent staining and immunoblotting analysis. However, both cells greatly differ in their physiological functions. These data therefore suggest that beta-actin might be a universal protein located on the surface of intracellular lipid droplets. Based on the fact that actin filaments have a defined polarity, by showing that the addition of monomers occurs faster at the plus (+) end than the minus (−) end, we suspect that globular beta-actins associated with lipids may guide the direction or accelerate intracellular lipid transport during hormone stimulation.

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