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Proteomic Analysis of Proteins Associated with Lipid Droplets of Basal and Lipolytically Stimulated 3T3-L1 Adipocytes*

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Adipocytes hold the body's major energy reserve as triacylglycerols packaged in large lipid droplets. Perilipins, the most abundant proteins on these lipid droplets, play a critical role in facilitating both triacylglycerol storage and hydrolysis. The stimulation of lipolysis by β-adrenergic agonists triggers rapid phosphorylation of perilipin and translocation of hormone-sensitive lipase to the surfaces of lipid droplets and more gradual fragmentation and dispersion of micro-lipid droplets. Because few lipid droplet-associated proteins have been identified in adipocytes, we isolated lipid droplets from basal and lipolytically stimulated 3T3-L1 adipocytes and identified the component proteins by mass spectrometry. Structural proteins identified in both preparations include perilipin, S3-12, vimentin, and TIP47; in contrast, adipophilin, caveolin-1, and tubulin selectively localized to droplets in lipolytically stimulated cells. Lipid metabolic enzymes identified in both preparations include hormone-sensitive lipase, lanosterol synthase, NAD(P)-dependent steroid dehydrogenase-like protein, acyl-CoA synthetase, long chain family member (ACSL) 1, and CGI-58. 17-β-Hydroxysteroid dehydrogenase, type 7, was identified only in basal preparations, whereas ACSL3 and 4 and two short-chain reductase/dehydrogenases were identified on droplets from lipolytically stimulated cells. Additionally, both preparations contained FSP27, ribophorin I, EHD2, diaphorase I, and ancient ubiquitous protein. Basal preparations contained CGI-49, whereas lipid droplets from lipolytically stimulated cells contained several Rab GTPases and tumor protein D54. A close association of mitochondria with lipid droplets was suggested by the identification of pyruvate carboxylase, prohibitin, and a subunit of ATP synthase in the preparations. Thus, adipocyte lipid droplets contain specific structural proteins as well as lipid metabolic enzymes; the structural reorganization of lipid droplets in response to the hormonal stimulation of lipolysis is accompanied by increases in the relative mass of several proteins and the recruitment of additional proteins.

In vertebrate animals the most abundant energy reserve is stored as triacylglycerol in the lipid droplets of adipocytes. These lipid droplets can be as large as 100 μm and are composed of a core of triacylglycerol surrounded by a phospholipid and cholesterol monolayer into which numerous proteins are embedded. Most other types of cells contain tiny lipid droplets that store primarily cholesterol esters and serve as a reservoir of cholesterol for the synthesis and maintenance of membranes; steroidogenic cells of the adrenal cortex, testes, and ovaries use stored cholesterol additionally as a source of substrate for steroid hormone synthesis. Little is known about the mechanisms that control the flux of neutral lipids into and out of lipid droplets in any type of cell, but it is clear that the processes that control lipid traffic in adipocytes are central to the regulation of whole body energy metabolism.

The first lipid droplet-associated proteins to be identified were members of the PAT family of proteins that includes perilipins, adipophilin (also called ADRP for adipose differentiation-related protein), TIP47, and S3-12; PAT family proteins are the major structural proteins of lipid droplets. Although adipophilin and TIP47 are ubiquitously expressed (1–5), perilipins and S3-12 are selectively expressed in adipocytes from white and brown adipose tissue (6, 7) and steroidogenic cells (8). Furthermore, perilipins, adipophilin, and S3-12 localize selectively to lipid droplets (1, 6, 7, 9), whereas TIP47 localizes to both cytosol and lipid droplets (3–5) and, thus, differs from the other family members in being exchangeable between lipid-associated and soluble forms.

Three protein isoforms of perilipin, perilipins A, B, and C, are translated from alternatively spliced forms of mRNA from a single gene; perilipin A is the most abundant protein on the lipid droplets of adipocytes. Studies in perilipin null mice (10, 11) and in various cultured cell models (12–18) show that perilipins play an important role in regulating both the storage and hydrolysis of triacylglycerol in adipocytes. Under basal conditions, when the body is in the fed state, glucose and fatty acids are taken up by adipocytes and used to synthesize triacylglycerols; perilipin A facilitates the storage of triacylglycerols in lipid droplets by providing a barrier against lipolysis (10–12). When energy is required, hormonal stimulation of β-adrenergic receptors on the plasma membranes of adipocytes triggers the phosphorylation of perilipin A by cAMP-dependent protein kinase, promoting lipolysis (15–18) at least in part by facilitating the docking of cAMP-dependent protein kinase-phosphorylated hormone-sensitive lipase on lipid droplets (16).

Lipolytic stimulation of adipocytes initiates a complex program manifested by substantial morphological changes of the lipid droplets. The phosphorylation of perilipins and hormone-sensitive lipase occurs rapidly upon hormonal stimulation, and nearly quantitative translocation of hormone-sensitive lipase from the cytoplasm to the lipid droplet surface occurs in the

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first 5 min (19). Visible by 30 min and escalating over several hours, a dramatic fragmentation of lipid droplets occurs[1] (20, 21) that vastly increases the surface area and dispersion of the myriad micro-lipid droplets that are formed. Although initial studies have demonstrated these morphological changes in cultured 3T3-L1 adipocytes, recent evidence suggests that they also occur in lipolytically stimulated white adipose tissue *in situ* (22). Thus, lipolytic stimulation of adipocytes triggers both rapid and more gradual but sustained changes in the protein content and morphology of lipid droplets.

The protein composition of lipid droplets from yeast (23, 24), Chinese hamster ovary fibroblasts (25), cultured human HuH7 hepatoma cells (26), cultured human A431 epithelial cells (27), and mouse mammary glands (28) have been studied using techniques of proteomic analysis. These studies have revealed that lipid droplet-associated proteins include enzymes involved in many aspects of lipid metabolism; additionally, the mammalian lipid droplet preparations contained the ubiquitously expressed PAT family members adipophilin and TIP47. The protein composition of adipocyte lipid droplets has not yet been reported. Furthermore, unlike the other types of cells for which this information has been collected, the signaling mechanisms that stimulate lipolysis and the consequent remodeling of lipid droplets are unique to adipocytes. For these reasons we investigated the protein composition of lipid droplets isolated from cultured 3T3-L1 adipocytes incubated under both basal conditions that foster triacylglycerol storage and stimulated conditions when lipolysis is activated and fatty acids are released from the cells. To capture both rapid and more gradual changes to protein composition that might occur, we stimulated lipolysis for 2 h before the isolation of the lipid droplets.

EXPERIMENTAL PROCEDURES

Materials

POROS 20 R2 beads were purchased from Applied Biosystems (Foster City, CA). C_{18} Zip-tips were from Millipore (Bedford, MA). Tris(2-carboxyethyl)phosphine hydrochloride and trifluoroacetic acid were from Pierce. Ammonium bicarbonate, iodoacetamide, formic acid, insulin, isobutylmethylxanthine (IBMX),[2] dexamethasone, biotin, and heat-inactivated fetal bovine serum were from Sigma-Aldrich. Dulbecco's minimal essential medium, 100× penicillin and streptomycin solution, 200 mM glutamine stock for cell culture media, and HPLC grade acetonitrile and methanol were purchased from Fisher.

Methods

Culture and Differentiation of 3T3-L1 Adipocytes—3T3-L1 preadipocytes (American Type Culture Collection, Herndon, VA) were cultured in 100-mm culture dishes from Corning-Costar as described previously (1); confluent monolayers of 3T3-L1 cells, used within 6–10 passages, were induced to differentiate into adipocytes by the daily addition of Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 8 μg/ml biotin, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.5 mM IBMX, 10 μg/ml insulin, and 10 μM dexamethasone for 72 h followed by the daily addition of culture medium containing biotin but without insulin, IBMX, and dexamethasone for up to 3 more days.

Incubation of 3T3-L1 Adipocytes in Lipolytically Stimulating Conditions and Isolation of Lipid Droplets—Six days after the initiation of differentiation, 40 dishes of 3T3-L1 adipocytes were incubated either with 10 μM isoproterenol and 0.5 mM IBMX for 2 h at 37 °C for lipolytically stimulated conditions or without the additions for basal conditions before harvest. Culture medium was removed, and cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) before scraping cells into PBS using Sarstedt cell scrapers. Cells from sets of 10 dishes of cells were pooled into 15 ml conical screw-capped tubes (Falcon) and centrifuged at 500 *x g* for 5 min to pellet the cells. Cell pellets were resuspended in a hypotonic 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 by pipetting and incubated on ice for 10 min before homogenization by 10 strokes in a Teflon-glass homogenizer. Cell lysates were centrifuged at 26,000 *x g* for 30 min at 4 °C in a SW41Ti rotor (Beckman), and the rotor was allowed to coast to a stop. The floating lipid droplet layers were harvested using a Beckman tube slicer, and the harvested fractions were adjusted to 25% sucrose and 100 mM sodium carbonate, pH 11.5, using a 60% sucrose stock solution and a 1 M sodium carbonate stock solution with protease inhibitors followed by gentle mixing by pipetting. The density-adjusted fractions (~4 ml) were layered into centrifuge tubes containing 1-ml cushions of 60% sucrose and then overlaid with ~5 ml of 100 mM sodium carbonate, pH 11.5, with protease inhibitors followed by ~3.5 ml of the hypotonic lysis medium with protease inhibitors. Tubes were centrifuged at 26,000 × *g* for 30 min at 4 °C in a SW41Ti rotor, and the rotor was allowed to coast to a stop. Floating lipid droplets were harvested using a Beckman tube slicer into 1.5-ml microcentrifuge tubes. Residual carbonate solution was removed by centrifuging tubes at 14,000 × *g* for 20 min at 4 °C in an Eppendorf microcentrifuge; infranatant was removed with an 18-gauge needle from below the floating lipid droplet fraction, and the lipid droplet fraction was rinsed once with hypotonic lysis solution containing protease inhibitors.

Delipidation of Lipid Droplets, Preparation of Component Proteins, and SDS-PAGE—Lipid droplet fractions in microcentrifuge tubes were delipidated with 1.5 ml of cold acetone overnight at −20 °C followed by centrifugation at 14,000 × *g* for 30 min at 4 °C and removal of solvent from the protein pellet. The pellet was further extracted with room temperature acetone followed by 1:1 acetone:ether (v:v) and ether. Residual solvents were evaporated under nitrogen, and proteins were solubilized in 2× Laemmli sample buffer (29) by incubation in a bath sonicator at 65 °C for 4–5 h with frequent mixing using a vortex mixer. Additional β-mercaptoethanol was added to samples before loading onto SDS-PAGE gels. Lipid droplet proteins from 28 dishes of adipocytes were loaded onto 30-cm-long SDS-PAGE gels for staining and further identification; proteins from 2 dishes of cells were loaded onto gels for transfer to nitrocellulose membranes and immunoblotting. Gels containing greater protein loads were stained for 2 h in 0.25% Coomassie Blue G250 in 10% acetic acid, 50% methanol and then destained in 7% acetic acid, 5% methanol for 4–6 h. Proteins on gels containing samples from two dishes of adipocytes were electrophoretically transferred to nitrocellulose membranes.

Lipid droplets were isolated from 8 separate sets of 40 dishes of adipocytes grown and differentiated at different times; 4 sets of dishes were used for basal conditions, and 4 sets of dishes were used for lipolytically stimulated conditions. Proteins from lipid droplet preparations from 3 sets of 28 dishes of basal adipocytes were separated in 3 lanes of a single SDS-PAGE gel. Coomassie-stained bands were compared and found to be equivalent for all lanes; bands from one lane were excised for analysis by mass spectrometry. Proteins from lipid droplet preparations from 3 sets of 28 dishes of lipolytically stimulated adipocytes were separated in 3 lanes of a single SDS-PAGE gel and compared with a single lane containing proteins from 28 dishes of basal adipocytes on the same gel. Coomassie-stained bands were compared and found to vary slightly between the three preparations from the lipolytically stimulated adipocytes and significantly between basal and stimulated preparations; bands were excised for all stained proteins from two lanes of stimulated preparations and analyzed by mass spectrometry. Because some differences were obtained throughout the analysis, bands from an additional lane of proteins from 28 dishes of lipolytically stimulated adipocytes separated on a new gel were analyzed separately; all proteins reported were identified in at least 2 of the 3 analyzed preparations of proteins.

In-gel Tryptic Digestion of Lipid Droplet-associated Proteins—Coomassie-stained protein bands were excised from the gels and destained with 45% acetonitrile in 100 mM ammonium bicarbonate. The resulting gel slices were incubated with 10 mM tris(2-carboxyethyl)phosphine hydrochloride, alkylated by the addition of 50 mM iodoacetamide, and then digested *in situ* with trypsin (100 ng per band in 50 mM ammonium bicarbonate). The tryptic peptides were extracted using POROS 20 R2 beads (Applied Biosystems) in 0.2% trifluoroacetic acid in 5% formic acid. The extracted peptides were concentrated using C_{18} zip-tips and eluted with 0.1% trifluoroacetic acid in 30% acetonitrile followed by 0.1% trifluoroacetic acid in 75% acetonitrile. The eluates were dried under vacuum using a Speed Vac concentrator.

Mass Spectrometry—The resulting peptides were dissolved in 2–25 μl of HPLC sample solvents containing water:methanol:acetic acid:

[1] A. Marcinkiewicz, D. Gauthier, and D. L. Brasaemle, unpublished observations.

[2] The abbreviations used are: IBMX, isobutylmethylxanthine; ACSL, acyl-CoA synthetase long chain family member; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry.

trifluoroacetic acid (70:30:0.5:0.01, v/v/v/v); the volume used was proportional to the staining intensity of the given band. Micro-HPLC-MS/MS analysis was conducted on an LCQ electrospray ionization ion trap mass spectrometer (Thermo Finnigan) coupled with an online MicroPro-HPLC system (Eldex Laboratories). Two microliters of tryptic peptide solution was injected into a Magic C18 column (0.2 × 50 mm, 5 μm, 200 Å, Michrom BioResources) which had been equilibrated with 70% solvent A (0.5% acetic acid and 0.01% trifluoroacetic acid in water: methanol (95:5, v/v)) and 30% solvent B (0.5% acetic acid and 0.01% trifluoroacetic acid in methanol:water (95:5, v/v)). Peptides were separated and eluted from the HPLC column with a linear gradient from 30 to 95% solvent B in 15 min at a flow rate of 2.0 μl/min. The eluted peptides were sprayed directly into the LCQ mass spectrometer (2.8 kV). The LCQ mass spectrometer was operated in a data-dependent mode for measuring the molecular masses of peptides (parent peptides) and collecting MS/MS peptide fragmentation spectra.

Database Search and Protein Identification—The measured molecular masses of parent peptides and their MS/MS data were used to search National Center for Biotechnology Information nonredundant DNA/protein sequence database (nr) using the program KNEXUS (Genomic Solutions). The mass error tolerance used in the database search was ±3 Da for the parent ions and ±0.5 Da for the fragment ions, respectively. Protein identifications were made based on expectation values $<1 \times 10^{-2}$ or the quality of MS/MS spectra of peptides identified. BLAST searches were performed for hypothetical and unknown proteins.

Immunoblotting of Lipid Droplet Protein Preparations—To confirm the identification of known lipid droplet-associated proteins, nitrocellulose membranes containing lipid droplet proteins were probed with antisera raised against the amino terminus of rat perilipin A (1), mouse adipophilin (Research Diagnostics Inc., Flanders, NJ), mouse TIP47 (Research Diagnostics), mouse S3-12 (kindly donated by Dr. Perry Bickel (7)), and caveolin-1 (kindly donated by Dr. Michael Lisanti).

Microscopy—3T3-L1 adipocytes were grown and differentiated on glass coverslips; before fixation, some cells were incubated with 10 μM isoproterenol and 0.5 mM IBMX for 2 h. Cells were fixed in 3% paraformaldehyde in phosphate-buffered saline, and cells were prepared for immunofluorescence microscopy, as described previously (9). Primary antibody incubations included antisera raised against perilipin (1), adipophilin, S3-12 (7), CGI-58 (50), and calnexin (StressGen Biotechnologies) for 1–3 h at room temperature. Secondary antibodies included rhodamine-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, Denver, PA) for 1–2 h at room temperature; BODIPY 493/503 (Molecular Probes, Inc., Eugene, OR) was added to secondary antibody solutions to detect neutral lipids (30). Cells were visualized with a Nikon Eclipse E800 fluorescence microscope equipped with a Hamamatsu Orca digital camera interfaced to a Macintosh G4 computer. Images were captured in monochrome and then processed using Improvision Openlab software; doubly stained cells are depicted in the opposite colors to those observed for esthetic reasons.

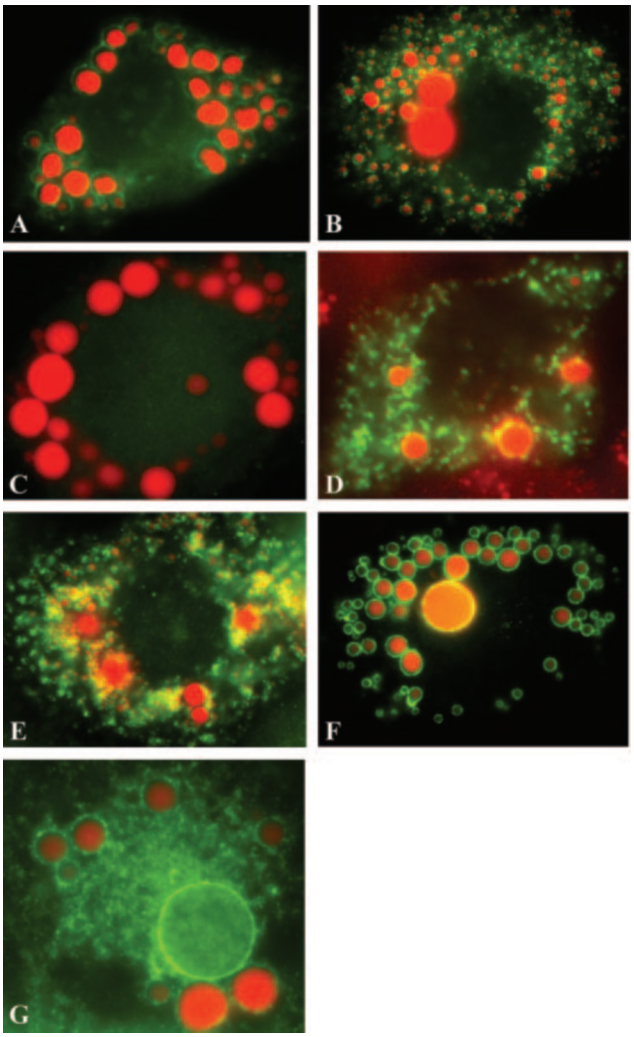

FIG. 1. **Microscopy of lipid droplets of 3T3-L1 adipocytes under basal and lipolytically stimulated conditions.** Differentiated 3T3-L1 adipocytes (day 6) were incubated under basal conditions (*A*, *C*, *F*, and *G*) or with isoproterenol and IBMX for 2 h to stimulate lipolysis (*B*, *D*, and *E*) before fixation in 3% paraformaldehyde and staining with polyclonal antisera raised against perilipin (*A* and *B*), adipophilin (*C* and *D*), S3-12 (*E*), CGI-58 (*F*), and calnexin (*G*); all cells were co-stained with rhodamine-conjugated secondary antibodies (*green*), and BODIPY 493/503 (*red*) for the detection of neutral lipid. Images were captured in monochrome, and cells are depicted in the opposite colors to those observed for esthetic reasons.

RESULTS

Isolation of Lipid Droplets—Cultured 3T3-L1 adipocytes package stored triacylglycerol into multiple lipid droplets of varying sizes (Fig. 1*A*); as differentiation progresses, the lipid droplets increase in size and fuse until a few very large lipid droplets fill the majority of the cytoplasm (not shown). In preliminary experiments we found that the large lipid droplets that characterize differentiated adipocytes at 10–14 days are fragile and difficult to keep intact during isolation. Thus, to avoid these problems we isolated lipid droplets from fresh, unfrozen cells collected at 6 days after the initiation of differentiation. At 6 days, the expression of many of the proteins that characterize fully differentiated adipocytes such as perilipin, hormone-sensitive lipase, and adipose fatty acid-binding protein has been highly induced (31). To study alterations in the protein content of lipid droplets that occur during lipolytic stimulation, we incubated day 6 differentiated adipocytes with isoproterenol, an agonist of β-adrenergic receptors, and IBMX, a phosphodiesterase inhibitor, for 2 h; these conditions were selected to capture both rapid and more gradual alterations in the protein content of lipid droplets as they begin to fragment and disperse (Fig. 1*B*). Finally, to minimize the nonspecific adherence of soluble proteins to the lipid droplets, isolated lipid droplets were centrifuged through 100 mM carbonate to disrupt electrostatic interactions while preserving hydrophobic interactions (32) that anchor integral proteins into the hydrophobic environment of the lipid droplet.

Protein Profiles from Basal and Lipolytically Stimulated Lipid Droplets Are Dramatically Different—Coomassie-stained SDS-PAGE gels revealed that the protein composition of lipid droplets isolated from adipocytes incubated under basal conditions is dramatically different from the composition of lipid droplets after the stimulation of lipolysis (Fig. 2). Although perilipin A is by far the most abundant protein on lipid droplets isolated from basal adipocytes (Fig. 2*A*, excised bands 17–19), the pair of bands containing phosphorylated perilipin A from lipid droplets of lipolytically stimulated adipocytes (Fig. 2*B*, excised bands 16 and 17) represent a smaller fraction of the total protein content. Numerous additional strongly stained protein bands were detected on lipid droplets isolated from lipolytically stimulated adipocytes.

We excised stained bands as depicted in Fig. 2 from the gels,

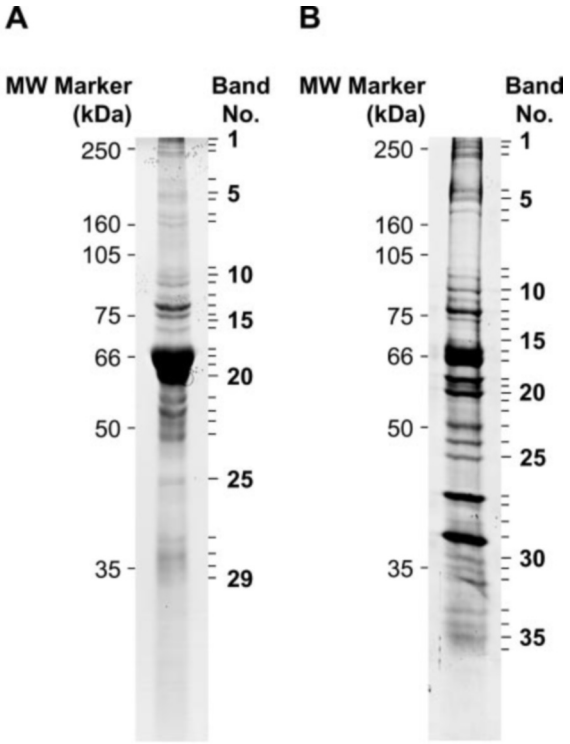

FIG. 2. **SDS-PAGE of protein pattern of lipid droplet-associated proteins.** Coomassie Blue-stained SDS-PAGE gels of proteins from lipid droplet preparations used for proteomic analysis from basal (*A*) and lipolytically stimulated (*B*) 3T3-L1 adipocytes. Position of molecular mass markers is denoted on the *left sides* of the *panels*; location of excised bands is denoted on the *right sides* of the *panels*.

separated tryptic peptides of the bands by HPLC, and identified the protein components by mass spectrometry; Tables I and II list the proteins identified in preparations of lipid droplets from basal and lipolytically stimulated adipocytes, respectively. Although PAT family members perilipin, S3-12, and TIP47 were found on lipid droplets isolated from adipocytes incubated under both conditions, adipophilin was found selectively on lipid droplets from lipolytically stimulated adipocytes. Based on previous observations of the relative mobility of perilipins A and B on SDS-PAGE gels, it is likely that the perilipin detected in bands 17–19 of the preparations from basal adipocytes (Table I; Fig. 2*A*) and bands 16–17 of the preparations from lipolytically stimulated adipocytes (Table II; Fig. 2*B*) is perilipin A, whereas the perilipin detected in bands 22 (Table I; Fig. 2*A*) and 23 (Table II; Fig. 2*B*) is perilipin B. Vimentin, an intermediate filament protein, was identified in both preparations, consistent with the previous localization of vimentin to lipid droplets in adipocytes (9, 33). Proteins with known or postulated roles in lipid metabolism, including lanosterol synthase, hormone-sensitive lipase, NAD(P)-dependent steroid dehydrogenase-like protein, CGI-58, and acyl-CoA synthetase, long chain family member 1 (ACSL1), were also found in both preparations (Tables I and II), although the staining intensity of the bands that contained these proteins was greater in preparations of lipid droplets isolated from lipolytically stimulated adipocytes (Fig. 2). Additional proteins that were identified in both preparations included ancient ubiquitous protein, calnexin, EHD2, fat specific protein FSP27, ribophorin I, diaphorase I, and several chaperone proteins.

Several proteins were identified in lipid droplet preparations from either basal adipocytes or lipolytically stimulated adipocytes but not both. Proteins that were selectively isolated with lipid droplets from basal adipocytes included 17-β-hydroxysteroid dehydrogenase type 7 and CGI-49, a protein of unknown function (Table I). Proteins that were selectively isolated with lipid droplets from lipolytically stimulated adipocytes included adipophilin, caveolin-1, tubulin, ACSL3 and -4, short chain dehydrogenase/reductase family member 1, aldehyde dehydrogenase ALDH3B1, tumor protein D54, an unstudied protein (expressed sequence AI462440), and several Rab GTPases (Table II).

Some probable contaminant proteins were isolated with the lipid droplet preparations. Several species of collagen were detected in high molecular weight bands of both preparations (Tables I and II); likewise, the identification of fatty acid translocase (CD36) was most likely due to contamination of the preparations with plasma membrane fragments. The most significant contaminating proteins were from mitochondria, including prohibitin, ATP synthase, and pyruvate carboxylase (Tables I and II); this is perhaps unsurprising since mitochondrial biogenesis is increased 20–30-fold during the differentiation of 3T3-L1 adipocytes (34), and mitochondria are closely apposed to lipid droplets in adipocytes (9, 35).

Confirmation of Lipid Droplet Association of Proteins by Immunofluorescence Microscopy—The localization of some of the identified proteins to lipid droplets was confirmed by immunofluorescence microscopy of paraformaldehyde-fixed 3T3-L1 adipocytes. Lipid droplets were identified by staining the fixed cells with BODIPY 493/503, a fluorophore that detects neutral lipid (30). Perilipins localized to all visible lipid droplets in basal adipocytes (Fig. 1*A*) and to both large and micro-lipid droplets containing neutral lipid cores in adipocytes incubated with isoproterenol and IBMX (Fig. 1*B*). In contrast, adipophilin was not detected on the surfaces of lipid droplets in basal adipocytes (Fig. 1*C*) but localized to the surfaces of both large and micro-lipid droplets in lipolytically stimulated adipocytes (Fig. 1D), thus supporting the proteomic identification of adipophilin only in lipid droplet preparations from lipolytically stimulated adipocytes (Table II). S3-12 was most easily visualized on micro-lipid droplets in lipolytically stimulated adipocytes (Fig. 1*E*). Polyclonal antisera raised against recombinant CGI-58 (50) detected a strong signal for CGI-58 at the surfaces of lipid droplets of basal adipocytes (Fig. 1*F*). Calnexin, an integral membrane protein that localizes to the endoplasmic reticulum, was detected in both types of preparation (Tables I and II), suggesting that fragments of endoplasmic reticulum co-fractionated with the lipid droplets. Interestingly, basal adipocytes stained for calnexin showed both a reticular staining pattern and rings of fluorescence loosely surrounding nuclei and lipid droplets (Fig. 1*G*), suggesting that endoplasmic reticulum membranes closely associate with lipid droplets.

Confirmation of Protein Identifications by Immunoblotting of Lipid Droplet Fractions—Immunoblotting of the lipid droplet preparations was used to confirm the identifications of some of the proteins. Under basal conditions, perilipin A was detected as a heavy band running at ~62 kDa (Fig. 3*A*), consistent with the appearance of the major band in the Coomassie-stained gel (Fig. 2*A*), whereas perilipin B appeared as a doublet of ~53 and 54 kDa (Fig. 3*A*). The stimulation of lipolysis leads to the phosphorylation of perilipin A on as many as 6 cAMP-dependent protein kinase-site serines and perilipin B on as many as 3 serines; cAMP-dependent protein kinase-phosphorylated perilipin A was detected as a doublet of doublets at 65–67 kDa, whereas perilipin B was detected as a triplet of bands at 51–53 kDa (Fig. 3*B*). Adipophilin was detected as a band of ~46 kDa on blots of lipid droplet proteins from lipolytically stimulated adipocytes (Fig. 3*C*); TIP47 was detected in a band of ~48 kDa when the same blots were stripped and re-probed (Fig. 3*D*), showing that the migration of these two related proteins was

TABLE I
Lipid droplet-associated proteins from 3T3-L1 adipocytes incubated under basal conditions

Identification	Gl number[a]	Molecular mass	Expect[b]	Band	Previously found in lipid droplet preparations	Reference
		kDa				
Known lipid droplet-associated proteins						
Perilipin	28316726	55.9	5.7E-72	17–19, 22	Yes	6, 9
S3−12	10181204	140.2	3.2E-43	5	Yes	7, 25
TIP47	12849312	47.3	7.0E-06	21	Yes	3–5, 26, 27
Vimentin	31982755	53.7	1.4E-41	20	Yes	9, 33
Lipid metabolism or transport						
17-β-Hydroxysteroid dehydrogenase type 7	20385196	33.7	3.1E-04	26	Yes	25–27
Acyl-CoA synthetase long-chain family member 1	31560705	78.9	9.9E-59	13, 14	No	
CGI-58	13385690	39.5	3.9E-31	24	Yes	25, 27, 47
Hormone-sensitive lipase	1708847	84.0	1.7E-07	11	Yes	16, 19
Lanosterol synthase	22122469	84.4	5.9E-07	12	Yes	25–27
NAD(P)-dependent steroid dehydrogenase-like	18043286	40.9	2.5E-29	25	Yes	26, 27, 44, 45
Miscellaneous						
Ancient ubiquitous protein	6671604	46.5	2.7E-14	23	No	
BiP	121570	72.5	2.9E-09	12	Yes	25, 27
Calnexin	3123183	68.0	2.7E-06	9	No	
CGI-49	20832116	47.7	5.1E-06	23–24	Yes	26
Diaphorase 1 (NADH)	19745150	34.3	6.8E-19	27–29	Yes	25–27
EHD2	23346469	57.4	1.1E-38	16	No	
Fat-specific gene 27	30410022	27.5	3.1E-02	28	No	
HSP 70	31981690	71.0	2.3E-33	14	Yes	27, 28
Ribophorin I	31543605	68.6	1.9E-02	15	No	
Probable contaminants						
ATP synthase β-subunit	2623222	56.3	3.1E-02	21	No	
B-cell receptor-associated protein 37	28526501	33.3	2.0E-12	26	Yes	25
Collagen, type IV, α3	4104232	185.8	2.5E-12	2	No	
Collagen, type VI, α2	420193	42.2	8.1E-05	5	No	
Collagen, type VI, α3	31791061	288.2	5.5E-13	3	No	
Fatty acid translocase/CD36	31982474	53.1	1.4E-05	10	No	
Lipoprotein lipase	387406	52.8	2.5E-05	20	No	
Polymerase I and transcript release factor	6679567	43.9	1.8E-09	20	No	
Pyruvate carboxylase	6679237	130.3	1.0E-14	7	Yes	28

[a] Sequence identification number in GenBank™.
[b] The expectation value is a statistical term that allows the comparison of the reliability of results. Low expectation values ($\ll 1$) correspond to confident identifications.

clearly resolved by SDS-PAGE. S3-12 was detected as a diffuse band at ~120 kDa (Fig. 3*E*). Caveolin-1 was detected as a band of ~27 kDa on blots containing lipid droplet-associated proteins from lipolytically stimulated adipocytes.

DISCUSSION

The lipid droplets of adipocytes are unique among lipid droplets of many types of cells with respect to size, the perilipin-enriched protein composition, and the dynamic rearrangements in structure that occur in response to stimulation of the β-adrenergic signaling pathway. Although perilipin A is the most abundant protein associated with the large lipid droplets of basal adipocytes, when lipolysis is stimulated, these droplets fragment into myriad micro-lipid droplets. Interestingly, the total mass of perilipin does not increase in proportion to the greatly increased surface area of the micro-lipid droplets. Some of the excess surface becomes coated with other PAT family members, adipophilin, TIP47, and S3-12; additionally, the association of numerous unrelated proteins with the lipid droplets increases.

Adipophilin was found by proteomic analysis of proteins on lipid droplets isolated from lipolytically stimulated 3T3-L1 adipocytes but not basal adipocytes; this finding was confirmed by immunofluorescence microscopy experiments. The expression pattern of adipophilin in 3T3-L1 adipocytes is unusual; levels of adipophilin mRNA increase during adipocyte differentiation, whereas protein levels of adipophilin disappear as lipid droplets expand and acquire a surface coating of perilipin A (1). Consistent with these observations, adipophilin is absent from the surfaces of lipid droplets of mouse adipocytes *in situ* even though mRNA levels of adipophilin are high in adipose tissue. In contrast, the mature lipid droplets of adipocytes in perilipin null mice are coated with adipophilin (11). Furthermore, the ectopic expression of perilipin A in fibroblasts reduces levels of adipophilin on lipid droplets (17), suggestive of competitive displacement of adipophilin by perilipin. Interestingly, adipophilin mRNA is efficiently translated in adipocytes, but the nascent protein is rapidly degraded[3]; hence, the adipocyte is poised to rapidly produce and utilize adipophilin. The appearance of adipophilin on the lipid droplets of lipolytically stimulated adipocytes may be a consequence of increased availability of binding sites on micro-lipid droplets to stabilize nascent adipophilin. The results strongly suggest that adipophilin plays a role in lipolysis.

The identification of caveolin-1 on lipid droplets isolated from lipolytically stimulated but not basal adipocytes is consistent with previous observations (35). Caveolin-1, perilipin, and the catalytic subunit of cAMP-dependent protein kinase were co-immunoprecipitated from lysates of adipocytes preincubated with β-adrenergic receptor agonists but not from basal adipocytes (35); thus, the three proteins form a complex in stimulated cells. Furthermore, perilipin A is poorly phosphorylated in lipolytically stimulated adipocytes from caveolin-1 null mice due to the inability of this complex to form in the absence of caveolin-1 (35). Thus, caveolin-1 bridges between perilipin and the catalytic subunit of cAMP-dependent protein kinase to facilitate the phosphorylation of perilipin. Interestingly, caveolin-1 associates with lipid droplets in several different types of cells when the cells are incubated with oleate to

[3] D. L. Brasaemle, unpublished observations.

TABLE II
Lipid droplet-associated proteins from 3T3-L1 adipocytes incubated under conditions that stimulate lipolysis

Identification	Gl number[a]	Molecular mass	Expect[b]	Band	Previously found in lipid droplet preparations	Reference
		kDa				
Known lipid droplet-associated proteins						
Adipophilin (ADRP)[c]	1168362	46.9	6.7E-24	23	Yes	1, 2, 25–28
Perilipin	28316726	55.9	2.7E-55	16–17, 23	Yes	6, 9,
S3-12	10181204	140.2	1.1E-49	4	Yes	7, 25
TIP47	13385312	47.3	1.1E-62	20	Yes	3–5, 26, 27
Vimentin	55408	53.7	7.2E-57	18	Yes	9, 33
Lipid metabolism or transport						
Acyl-CoA synthetase long-chain family member 1	729927	78.9	1.2E-37	12	No	
Acyl-CoA synthetase long-chain family member 3	20455039	81.5	7.9E-06	12	Yes	25–27
Acyl-CoA synthetase long-chain family member 4	6172341	75.6	4.3E-02	10	Yes	25, 26
Aldehyde dehydrogenase ALDH3B1	18028981	50.6	8.3E-07	19	No	
CGI-58	13385690	39.5	9.1E-08	25	Yes	25, 27, 47
Hormone-sensitive lipase	6754552	84	6.1E-23	9,10	Yes	16, 19
Lanosterol synthase	22122469	84.4	4.4E-32	11	Yes	25–27
NAD(P)-dependent steroid dehydrogenase-like	8473695	40.9	8.6E-09	26, 27	Yes	26, 27, 44, 45
Short-chain dehydrogenase/reductase member 1	13278172	34.5	1.5E-06	28	Yes	25
Miscellaneous						
Ancient ubiquitous protein	6671604	46.5	6.0E-02	24	No	
BiP	2598562	72.5	1.6E-13	12	Yes	25, 27
Calnexin	6671664	67.6	2.5E-05	8	No	
Caveolin-1	6705981	7.4	4.5E-03	36	Yes	25, 36–40
Diaphorase 1 (NADH)	19745150	34.3	7.6E-04	29	Yes	25–27
dnaK-type molecular chaperone hsc73	2119718	71.0	7.9E-77	13	No	
EHD2	18203333	61.2	1.3E-06	15	No	
Expressed sequence AI462440	23600211	52.8	7.7E-16	20	No	
Fat-specific protein FSP27	2829467	27.5	8.0E-02	32	No	
Rab14	18390323	24.1	1.1E-03	35	Yes	25
Rab18	6755258	23.3	6.0E-05	35	Yes	25, 27
Rab5c	20072723	22.4	1.2E-06	33	Yes	25, 26
Rab7	6679599	23.8	4.1E-05	34	Yes	25, 27
Ribophorin I	16359229	68.6	5.1E-10	14	No	
Tubulin, β5	7106439	50.1	5.6E-08	22	Yes	25
Tumor protein D54 (hD54)	12850393	24.1	2.8E-05	31	Yes	27
Probable contaminants						
ATP synthase, β subunit	25052136	56.2	4.6E-37	20	No	
Collagen, type VI, α1	6753484	109.5	7.2E-27	5	No	
Collagen, type VI, α2	3913189	110.9	1.3E-45	4	No	
Collagen, type VI, α3	3236370	288.2	2.7E-109	1–3	No	
Fatty acid translocase/CD36	3273897	53.2	7.2E-11	9	No	
Lipoprotein lipase	15030193	53.7	3.7E-06	18	No	
Polymerase I and transcript release factor	6679567	43.9	4.7E-32	19	No	
Prohibitin	6679299	29.8	1.6E-09	30, 31	No	
Pyruvate carboxylase (EC 6.4.1.1)	200246	103.8	2.7E-20	6	Yes	28

[a] Sequence identification number in GenBank™.
[b] The expectation value is a statistical term that allows the comparison of the reliability of results. Low expectation values ($\ll 1$) correspond to confident identifications.
[c] ADRP, adipose differentiation-related protein.

promote triacylglycerol synthesis and storage (25, 36, 37) or treated with brefeldin A (36–40).

The mechanisms contributing to the fragmentation and dispersion of the micro-lipid droplets into the periphery have not been studied; however, the association of tubulin with lipid droplets in lipolytically stimulated adipocytes may be important for this dispersion. Recent studies show that motility of lipid droplets in various types of cultured cells depends upon the presence of both intact microtubules (37, 41) and caveolin (37); thus, the observed association of caveolin-1 and tubulin with lipid droplets in stimulated cells may facilitate dispersion of the micro-lipid droplets. Similarly, the localization of various Rab GTPases to lipid droplets in lipolytically stimulated cells may be required for these dramatic structural rearrangements, although recent studies identify several of the same Rab GTPases on lipid droplets isolated from Chinese hamster ovary fibroblasts (25), human epithelial cells (27), and human hepatoma cells (26), which do not show these morphological changes. It may be significant, however, that lipid droplets in these other types of cells are tiny, dispersed, and most likely motile.

Several enzymes with roles in aspects of lipid metabolism were identified on lipid droplets isolated from both basal and lipolytically stimulated adipocytes. Hormone-sensitive lipase was found in both preparations, although previous studies show that hormone-sensitive lipase is primarily cytosolic in basal adipocytes and nearly completely localized to lipid droplets in stimulated adipocytes (19, 42). Two enzymes from the sterol biosynthetic pathway, lanosterol synthase and NAD(P)-dependent steroid dehydrogenase-like protein, were found in both preparations, consistent with previous reports (25–27; 43–45). ACSL1 was found in both preparations, whereas ACSL3 and -4 were found selectively on lipid droplets from lipolytically stimulated adipocytes. Mutations in CGI-58, a member of the lipase subfamily of α/β-hydrolase fold enzymes, were recently shown to be the cause of an inherited neutral lipid storage disorder characterized by excessive triacylglycerol deposition in many types of cells (46). Thus, the localization of CGI-58 to lipid droplets in adipocytes, as confirmed by immunofluorescence microscopy, implies a role for CGI-58 in adipocyte triacylglycerol metabolism. We (50) and others (47) have recently reported that the association of CGI-58 with adipocyte

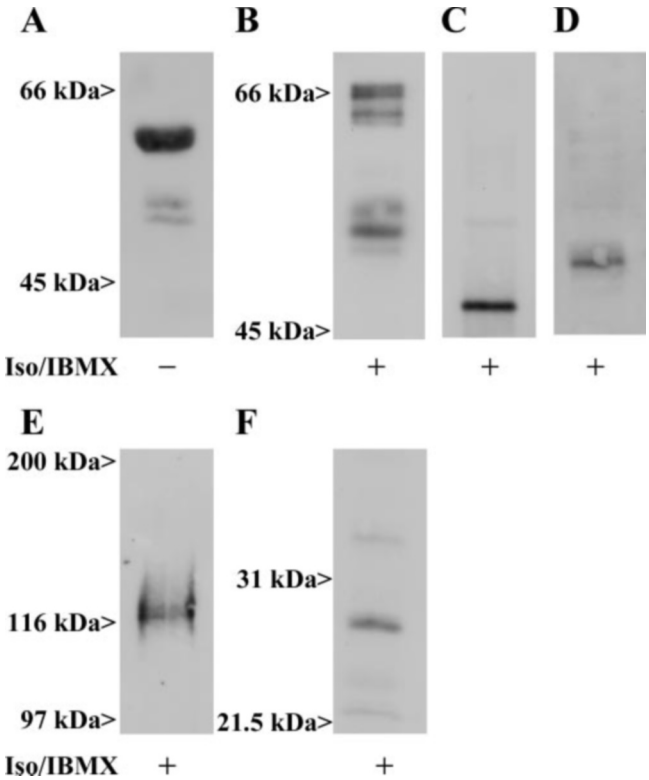

FIG. 3. **Confirmation of lipid droplet-associated proteins by immunoblotting.** Immunoblots of lipid droplet-associated proteins from 3T3-L1 adipocytes incubated under basal conditions (*A*) or with isoproterenol (*Iso*) and IBMX for 2 h to stimulate lipolysis (*B*, *C*, *D*, *E*, and *F*) were probed with polyclonal antisera raised against perilipin (*A* and *B*), adipophilin (*C*), TIP47 (*D*), S3-12 (*E*), and caveolin-1(*F*). Each lane is representative of lanes from at least three preparations, and the same preparation is depicted for all lanes of lipolytically stimulated proteins; proteins from basal and lipolytically stimulated conditions were run on separate SDS-PAGE gels.

lipid droplets occurs through a binding interaction with perilipin. Additionally, multiple members of the reductase/dehydrogenase family of lipid metabolic enzymes were found in one or both preparations. The identifications of lipid metabolic enzymes on the lipid droplets of adipocytes and several other types of cells shows that these structures are not passive repositories for neutral lipids but, instead, comprise a dynamic pool of lipids.

The identifications of several proteins in the preparations carry implications regarding the immediate subcellular neighborhood of lipid droplets. The intermediate filament protein vimentin was identified in both preparations. Previous studies show that fibrous vimentin intermediate filaments collapse into a cage structure around developing lipid droplets in differentiating adipocytes (33); the disruption of vimentin cage formation halts lipid droplet formation (48). Identification of the Eps-15 homology domain protein EHD2, a protein that binds to actin filaments in differentiated 3T3-L1 adipocytes (49), provides additional evidence of a close link between lipid droplets and the cytoskeleton. The identification of calnexin in both lipid droplet preparations, as confirmed by immunofluorescence microscopy, suggests that segments of endoplasmic reticulum come into close contact with lipid droplets. Finally, several mitochondrial proteins were identified in the preparations, including a subunit of ATP synthase, prohibitin, and pyruvate carboxylase, indicating that mitochondria are closely associated with and difficult to separate from lipid droplets; several studies have illustrated the tight packing of mitochondria around lipid droplets in adipocytes (9, 35).

Several relatively unstudied proteins were identified on lipid droplets. The significance of the isolation of ancient ubiquitous protein, tumor protein D54, ribophorin I, fat-specific protein FSP27 (a member of the CIDE family of apoptosis effectors), and others on lipid droplet preparations is unknown. Although some of these proteins have been identified in subcellular compartments other than lipid droplets, there are no conclusive data regarding subcellular localization for others. Because we have found evidence of the co-purification of other membranous structures, particularly mitochondria, with lipid droplets, it is possible that some of these proteins are major components of contaminating membranes.

In conclusion, surface proteins on adipocyte lipid droplets include enzymes involved in aspects of lipid metabolism and members of the Rab family of GTPases, as found on lipid droplets in other types of cells; however, unlike other types of lipid droplets, perilipins are a major protein component that controls lipid traffic and the association of adipophilin (1, 11, 17), hormone-sensitive lipase (16), CGI-58 (47, 50), and likely other proteins with the droplets. The stimulation of lipolysis is accompanied by the fragmentation of large lipid droplets into myriad dispersed micro-lipid droplets accompanied by major changes in the overall protein composition. Although most of the changes appear to affect the relative mass of various proteins that also associate with basal lipid droplets, adipophilin and caveolin-1 selectively associate with droplets in stimulated cells. The current study has characterized proteins that associate with lipid droplets through hydrophobic interactions and, thus, withstand alkaline carbonate treatments; undoubtedly, additional proteins of interest bind to protein and lipid components of lipid droplets through electrostatic interactions and remain to be elucidated.

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