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# TIP47 Associates with Lipid Droplets*

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**Most mammalian cells package neutral lipids into droplets that are surrounded by a monolayer of phospholipids and a specific set of proteins including the adipose differentiation-related protein (ADRP; also called adipophilin), which is found in a wide array of cell types, and the perilipins, which are restricted to adipocytes and steroidogenic cells. TIP47 was initially identified in a yeast two-hybrid screen for proteins that interact with the cytoplasmic tail of the mannose 6-phosphate receptor, yet its sequence is highly similar to the lipid droplet protein, ADRP, and more distantly related to perilipins. Hence, we hypothesized that TIP47 might be associated with lipid droplets. In HeLa cells grown in standard low lipid-containing culture media, immunofluorescence microscopy revealed that the cells had few lipid droplets; however, TIP47 and ADRP were found on the surfaces of the small lipid droplets present. When the cells were grown in media supplemented with physiological levels of fatty acids, the amount of neutral lipid stored in lipid droplets increased dramatically, as did the staining of TIP47 and ADRP surrounding these droplets. TIP47 was found primarily in the cytosolic fractions of HeLa cells and murine MA10 Leydig cells grown in low lipid-containing culture medium, while ADRP was undetectable in these fractionated cell homogenates. When HeLa and MA10 Leydig cells were lipid-loaded, significant levels of ADRP were found in the floating lipid droplet fractions and TIP47 levels remained constant, but the distribution of a significant portion of TIP47 shifted from the cytosolic fractions to the lipid droplet fractions. Thus, we conclude that TIP47 associates with nascent lipid droplets and can be classified as a lipid droplet-associated protein.**

TIP47 was first identified by a yeast two-hybrid screen using the cytosolic domain of the cation-dependent mannose 6-phosphate receptor (M6PR)[1] as a bait to screen an expression library from human Jurkat cells (1). A glutathione *S*-transferase TIP47 fusion protein was subsequently shown to bind to glutathione *S*-transferase fusion proteins of the cytosolic tails of both the cation-dependent and cation-independent M6PRs *in vitro* and thus, this protein was named TIP47 (tail-interacting protein of 47 kDa). Diaz and Pfeffer (1) have proposed that TIP47 directs the retrieval of M6PRs from a prelysosomal compartment with delivery back to the *trans*-Golgi network through the interaction of TIP47 with the cytoplasmic tails of M6PRs. An essentially identical cDNA, $PP17a_1$ (2), and a truncated version of this cDNA, $PP17a_2$, have been obtained by screening a human placental expression library with an antibody raised against a 38-kDa protein purified from human placenta (3). Interestingly, the amino acid sequence of TIP47 (PP17a) is highly similar to sequences from the members of a growing family of lipid droplet-associated proteins that includes perilipins and the adipose differentiation-related protein (ADRP), also called adipophilin. The amino acid sequence of TIP47 (1) is 43% identical to ADRP throughout the length of the sequence and bears higher homology (60% identity, 80% similarity to ADRP) in an amino-terminal region that is shared by the perilipins; hence, these proteins may all be members of a common family. The perilipins are encoded by a single gene; alternative splicing yields multiple mRNAs that are translated into three different isoforms that have similar amino termini but differ in the amino acid sequences of the carboxyl termini. The perilipins are specifically associated with lipid droplets in adipocytes (4, 5) and steroidogenic cells (6) and, to date, have been found neither in any other cell type nor in any other intracellular compartment. ADRP (7) is found on lipid droplets in many cultured cell lines and tissues (8, 9) and is also found on secreted milk lipid globules (10). The perilipins and ADRP have similar amino-terminal sequences; 105 consecutive amino acids are 32% identical, and 65% are similar (11). Detailed subcellular localization studies are lacking for several additional recently described family members. S3–12 (12) is a protein of 1403 amino acids that has a 33-amino acid segment that shares sequence similarity with ADRP; this segment is repeated 29 times. The region of ADRP that is similar to the S3–12 repeats lies outside of the sequence that is conserved between perilipins and ADRP, and hence, it appears that the perilipins lack strong similarity to the 33-amino acid repeated sequence of S3–12. Finally, a Blast search (13) of GenBank™ reveals two predicted sequences from *Drosophila* that share similar amino termini with ADRP and perilipins. The function and tissue distribution of these newly identified *Drosophila* proteins have not yet been addressed.

Lipid droplets are largely uncharacterized subcellular organelles found in the cytosol of most mammalian tissues and cultured cell lines as well as in the adipose tissue of other chordates, the fat body of insects, and the seeds of plants. Lipid droplets are spherical structures composed of a core of triacylglycerols and cholesterol esters covered by a monolayer of phospholipid. While large lipid droplets in adipose tissue store the body's major energy supply as triacylglycerols, histological sections of other nonadipose tissues including liver, intestine, muscle, kidney, heart, adrenal gland, testes, ovary, and mammary gland (14–16) also demonstrate the presence of small

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[1] The abbreviations used are: M6PR, mannose 6-phosphate receptor; ADRP, adipose differentiation-related protein; ER, endoplasmic reticulum; LDH, lactate dehydrogenase.

lipid droplets. In many of these tissues, the lipid droplets are primarily composed of cholesterol esters that play an important role in maintaining cellular cholesterol homeostasis by providing a source of cholesterol for membrane synthesis when circulating cholesterol levels are low. Additionally, steroidogenic cells such as those found in the adrenal cortex, testes, and ovaries use stored cholesterol esters as a source of substrate for steroid hormone synthesis. Most nonadipose cultured cell lines package neutral lipids into small lipid droplets, and the numbers of these lipid droplets are largely determined by the composition of the culture media (17). Typical culture medium is very low in lipid content, and the cells grown in these lipid-starved conditions have few, if any, lipid droplets. By contrast, the number of cytoplasmic lipid droplets increases dramatically upon supplementation of culture media with physiologic levels of free fatty acids or lipoproteins (17, 18). Thus, the formation of lipid droplets is controlled, at least in part, by the availability of exogenous or circulating substrates for neutral lipid synthesis. The release of neutral lipids from lipid droplets is regulated by extracellular hormones or intracellular mechanisms that signal the need for energy or building materials for membrane synthesis. Thus, it is reasonable to expect that the lipid droplet surface contains proteins involved in controlling this flux, given that the flux of lipids both to and from lipid droplets are regulated processes. Nonetheless, few lipid droplet-associated proteins have been identified, and the study of the proteins associated with lipid droplets is an emerging area of inquiry.

Due to the striking similarity between the amino acid sequences of TIP47 ($PP17a_1$), ADRP, and the perilipins, we hypothesized that TIP47 may associate with lipid droplets. Using polyclonal antibodies raised against TIP47 (1), we investigated the subcellular localization of TIP47 and found that it associates with lipid droplets. These observations raise the question as to whether or not TIP47 functions exclusively as a component of a selective sorting mechanism that directs proteins to vesicular endosomal compartments and imply a potential function for TIP47 in the regulation of the metabolism of neutral lipids.

## EXPERIMENTAL PROCEDURES

*Antibodies*—The anti-human TIP47 polyclonal antiserum was donated by Dr. Suzanne Pfeffer (1). The anti-mouse ADRP (mADRP) polyclonal antiserum was donated by Dr. Charles Schultz (National Institutes of Health, Bethesda, MD). The anti-human ADRP (hADRP) monoclonal antibody and anti-calnexin antibody were purchased from Research Diagnostics Inc. (Flanders, NJ) and Transduction Laboratories, Affiniti Research Products Ltd. (Mamhead, UK), respectively. The polyclonal anti-M6PR antibody raised against the cation-independent M6PR was donated by Dr. Thomas Braulke (Georg-August-University, Gottingen, Germany). Tetramethyl rhodamine-conjugated anti-goat and fluorescein-conjugated anti-rabbit IgGs were obtained from Jackson Immunoresearch (West Grove, PA). Alexa 594-conjugated anti-rabbit and Alexa 488-conjugated anti-mouse IgGs were obtained from Molecular Probes, Inc. (Eugene, OR).

*Cell Culture*—Human HeLa cells, human U937 monocytes and human K562 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin. Murine MA10 Leydig cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% horse serum, 2 mM glutamine, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin. When cells were grown in media supplemented with fatty acids, oleic acid was complexed to bovine serum albumin at a ratio of 6 mol of oleic acid/mol of albumin; the final concentration is indicated in the figure legends. All cells were cultured in a 5% $CO_2$ atmosphere at 37 °C.

*Northern Blot Analysis*—RNA was extracted using an RNeasy mini-kit (Qiagen) following the protocol recommended by the manufacturer. The extracted RNA was resolved by agarose gel electrophoresis, transferred to charged nylon membranes (Hybond N+; Amersham Pharmacia Biotech), and probed following the manufacturer's protocol for the NorthernMax-Gly kit (Ambion, Austin, TX).

*Fluorescence Microscopy*—HeLa cells were cultured on glass coverslips and were fixed with 2% formaldehyde in phosphate-buffered saline for 20 min. Primary and secondary antibodies were added sequentially to the fixed cells in phosphate-buffered saline containing 0.1% saponin and 0.1% bovine serum albumin. The fluorophore Bodipy 493/503 (Molecular Probes) specifically stains neutral lipids (19) and has narrow absorption and emission spectra, thus allowing for the use and detection of a second fluorophore. Bodipy 493/503 was dissolved in ethanol at 1 mg/ml and then added to secondary antibody solutions to a final concentration of 10 $\mu$g/ml. Coverslips were mounted on glass slides using Fluoromount G (Southern Biotechnology Associates Inc., Birmingham, AL). Images from the samples were acquired using a Zeiss LSM 410 confocal microscope (Carl Zeiss Inc., Thornwood, NY).

*Metabolic Labeling of Cells and Immunoprecipitation of TIP47*—For each condition, HeLa cells from confluent monolayers grown in 150-mm culture dishes were incubated with [$^{35}$S]methionine and [$^{35}$S]cysteine (Easytag$^{TM}$ EXPRE$^{35}$S$^{35}$S; PerkinElmer Life Sciences) in suspension in Dulbecco's modified Eagle's medium lacking methionine and cysteine, supplemented with 25 mM HEPES, pH 7, for 30 min at 37 °C. When incubation was required after labeling, cells were resuspended in complete Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES, pH 7. Cells were collected by low speed centrifugation, and pellets were solubilized in 50 mM Tris, pH 7.4, with 1% Triton X-100, 0.5% sodium deoxycholate, 300 mM NaCl, 1 mg/ml iodoacetamide, 10 mg/ml leupeptin, 100 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1 mM benzamidine, and 1 mM EDTA. Immunoprecipitations of TIP47 were performed, as described previously (20). The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis and revealed by fluorography.

*Disruption of Cells by Hyperosmotic Shock*—HeLa or MA10 Leydig cells were scraped into phosphate-buffered saline and pelleted by low speed centrifugation. The cell pellets were dispersed by vortexing, concurrent with the dropwise addition of 70% (w/w) sucrose dissolved in lysis buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 10 $\mu$g/ml leupeptin, 100 $\mu$M 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1 mM benzamidine, 1 mM EDTA) at room temperature. The cells were then incubated on ice for 10 min, during which time they were vortexed for 30 s every 2 min. To maximize the osmotic shock, 3 ml of lysis buffer were added rapidly while vortexing the sample. The homogenate was incubated for an additional 10 min on ice and vortexed for 30 s every 2 min; the cells were then further disrupted by passing them through a 27-gauge needle four times.

*Fractionation of Cells by Centrifugation of Sucrose Gradients*—Confluent monolayers of HeLa or MA10 Leydig cells were disrupted by hyperosmotic shock, as described above, and then centrifuged for 10 min at 1000 $\times$ *g* at 4 °C. The supernatant was mixed 1:1 with 70% sucrose (w/w) and layered onto a cushion of 0.6 ml of 50% sucrose (w/w). A sucrose gradient, as described in the figure legends, was layered over the sucrose-cell supernatant. The gradients were centrifuged for 4 h at 154,000 $\times$ *g* in a Beckman SW41Ti rotor at 4 °C. The buoyant fraction was collected by slicing off the tops of the tubes with a Beckman tube slicer, and this fraction was brought to 1 ml with lysis buffer. Eleven additional 1-ml fractions were collected.

*Fractionation of HeLa Cells by Differential Centrifugation*—HeLa cells from a confluent 150-mm dish were disrupted by hyperosmotic shock, and the homogenate was centrifuged for 10 min at 1000 $\times$ *g* at 4 °C. The supernatant was collected, adjusted to a volume of 4 ml with lysis buffer, and centrifuged in a SW60Ti rotor for 1 h at 165,000 $\times$ *g* at 4 °C. The buoyant fraction was collected with a tube slicer, as described previously.

*Analysis of Cellular Fractions*—Immunoblotting was performed as described previously (8). Neutral lipids were extracted in solvents and separated by thin layer chromatography and revealed, as described previously (21). Gradient fractions were assayed for activity of the cytosolic enzyme lactate dehydrogenase (22) and the integral endoplasmic reticulum enzyme NADPH- dependent cytochrome *c* reductase (23).

## RESULTS

*Characterization of TIP47 mRNA and Protein in HeLa Cells*—Since the amino acid sequence of TIP47 indicates a close relationship of TIP47 to a family of lipid droplet-associated proteins (24), we investigated the hypothesis that TIP47 localizes to lipid droplets. Previously, when a TIP47 ($PP17a_1$) cDNA was used to probe Northern blots of total HeLa RNA, multiple mRNAs were detected, and when immunoblots were probed with an antibody raised against a 38-kDa protein from placenta

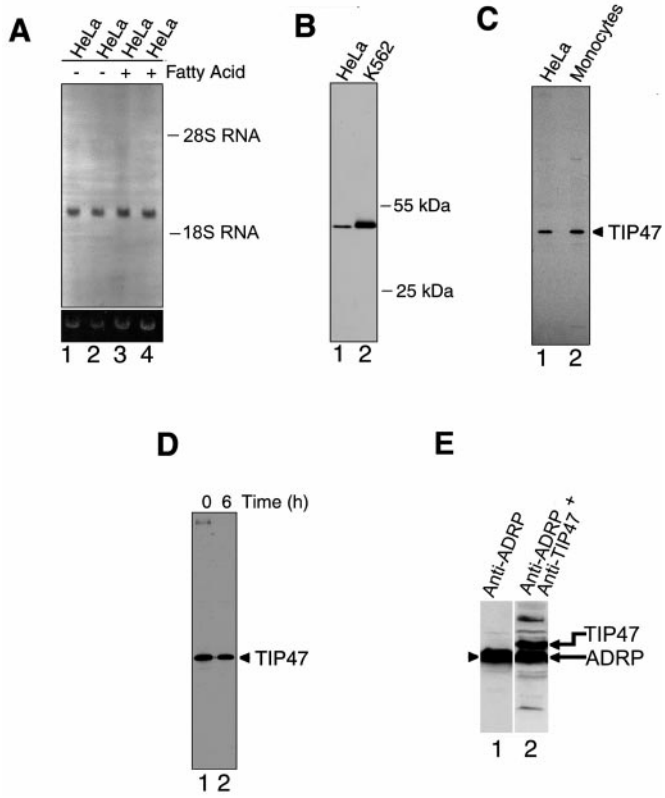


FIG. 1. **HeLa cells have a single mRNA encoding TIP47; HeLa cells, k562 cells, and U937 monocytes express a single protein isoform of TIP47.** *A*, Northern blot of total RNA from HeLa cells hybridized with a radiolabeled cDNA probe for TIP47 (*upper panel*) and the 18 S ribosomal subunit from the lanes stained with ethidium bromide (*lower panel*). Each *lane* contains 10 $\mu$g of total RNA extracted from HeLa cells. *Fatty Acid* + indicates that the cells were grown with 400 $\mu$M oleic acid complexed to albumin for 19 h before harvest. *B*, immunoblots of proteins from HeLa cells (*lane 1*) and K562 cells (*lane 2*) separated by SDS-polyacrylamide gel electrophoresis and probed with an anti-TIP47 polyclonal antiserum. *C*, autoradiograph of proteins from HeLa cells and U937 monocytes that were labeled for 4 h with [$^{35}$S]methionine and [$^{35}$S]cysteine, immunoprecipitated with anti-TIP47 antiserum, and resolved by SDS-polyacrylamide gel electrophoresis. *D*, autoradiograph of SDS-polyacrylamide gels containing proteins from HeLa cells labeled with [$^{35}$S]methionine and [$^{35}$S]cysteine for 30 min (*lanes 1* and *2*) and chased without label for 6 h (*lane 2*) before immunoprecipitation with anti-TIP47 antiserum. *E*, immunoblot of proteins from lipid-loaded MA10 Leydig cell homogenates probed with anti-mADRP antiserum (*lane 1*) or anti-TIP47 antiserum and anti-mADRP antiserum (*lane 2*).

that shares sequence identity with TIP47, multiple proteins were detected in most of the tissues that were tested (2). To characterize the expression of TIP47 in HeLa cells, we used Northern blotting, immunoblotting, and immunoprecipitation techniques to show that a single mRNA encodes a single protein isoform of TIP47 (Fig. 1). A Northern blot of total RNA from HeLa cells probed with a human TIP47 cDNA probe showed an mRNA consistent with a size of 1998 base pairs (Fig. 1*A*), the predicted size of the TIP47 mRNA (1). Immunoblots of proteins from HeLa cells probed with anti-TIP47 antiserum revealed a single protein of ~46 kDa (Fig. 1*B*). Human K562 cells, which were used in the initial characterizations of TIP47 (1), also displayed a single protein of ~46 kDa in TIP47 immunoblots (Fig. 1*B*). Additional immunoblots of proteins from cultured murine 3T3-L1 adipocytes (not shown) and rat MA10 Leydig cells (Fig. 5) showed a single TIP47 protein isoform. Furthermore, anti-TIP47 antiserum immunoprecipitated a single protein of ~46 kDa from homogenates of HeLa cells and human U937 monocytes (Fig. 1*C*), and rat MA10 Leydig cells (not shown) radiolabeled with [$^{35}$S]methionine and [$^{35}$S]cysteine. Thus, several cultured cell lines express a single isoform of TIP47.

Since ADRP and TIP47 share sequence similarity, it is possible that the anti-TIP47 antiserum recognizes ADRP. To test this, immunoblots of proteins from MA10 Leydig cell homogenates were probed with anti-mADRP antiserum (Fig. 1*E*) and then reprobed with the anti-TIP47 antiserum, without stripping the anti-mADRP antibody from the blots. The anti-mADRP antiserum recognized a single protein band and the anti-TIP47 antiserum recognized a single band of a slightly higher molecular weight, thus demonstrating that the TIP47 antibody and the ADRP antibody recognize discrete proteins.

The expression of perilipins (21) and ADRP (8, 25), lipid droplet-associated proteins related to TIP47, are regulated by the addition of fatty acids to the medium of cultured cells. By contrast, we observed no change in the level of TIP47 mRNA (Fig. 1*A*) or protein (Fig. 6) following the addition of oleic acid to the culture medium. We did, however, observe a dramatic change in the subcellular compartmentalization of TIP47 following the addition of fatty acids to the culture medium (Figs. 4–6). Additionally, the immunoprecipitation of TIP47 under nondenaturing conditions yielded a single radiolabeled band (Fig. 1, *C* and *D*), thus suggesting that TIP47 may not form stable complexes with other cellular proteins Finally, when using a pulse-chase protocol, greater than 50% of radiolabeled TIP47 was stable throughout a 6-h chase period (Fig. 1*D*), thus demonstrating that cellular TIP47 has a relatively long half-life.

*Antibody to TIP47 Decorates Neutral Lipid Droplets*—The subcellular localization of TIP47 was examined by immunofluorescence microscopy of HeLa cells grown in both the absence and presence of supplemental lipids. Subcellular lipid droplets were visualized with Bodipy 493/503, a stain for neutral lipids. Furthermore, since TIP47 has been described as a protein that interacts with the M6PR (1), HeLa cells were doubly stained with anti-M6PR antiserum and anti-TIP47 antiserum, and the localization of the two proteins was examined by immunofluorescence microscopy. The staining pattern for M6PR in HeLa cells was greatest in the juxtanuclear region with some staining of peripheral vesicles and the plasma membrane (Fig. 2*A*) and resembled that previously shown for M6PR in embryonic bovine trachea cells and COS cells (1); by contrast, the staining for TIP47 was both diffuse through the cytoplasm and specifically localized to punctate structures but was rarely coincident with the staining for M6PR when cells were doubly stained for both proteins (Fig. 2*A*). When the cells were supplemented with fatty acids to increase neutral lipid storage, the anti-TIP47 antiserum stained primarily larger, uniformly spherical structures that were not localized to the regions of most intense M6PR staining (Fig. 2*B*).

Cells grown under normal culture conditions and doubly stained for neutral lipids and TIP47 revealed that the punctate structures detected by the anti-TIP47 antiserum are lipid droplets, since the two stains colocalized (Fig. 2*C*). Lipid loading of the cells increased the sizes of the lipid droplets slightly and allowed the detection of a distinct ring of TIP47 staining surrounding the neutral lipid core (Fig. 2*D*). Interestingly, we observed that ~80% of HeLa cells grown in the standard low lipid-containing culture medium apparently lacked lipid droplets, while 20% of these cells contained detectable lipid droplets; in the cells lacking lipid droplets, the TIP47 staining appeared to be diffuse throughout the cytoplasm (data not shown). When the cells were grown in culture medium containing supplemental fatty acids, all of the cells displayed lipid droplets, most of which were stained with the anti-TIP47 antiserum.

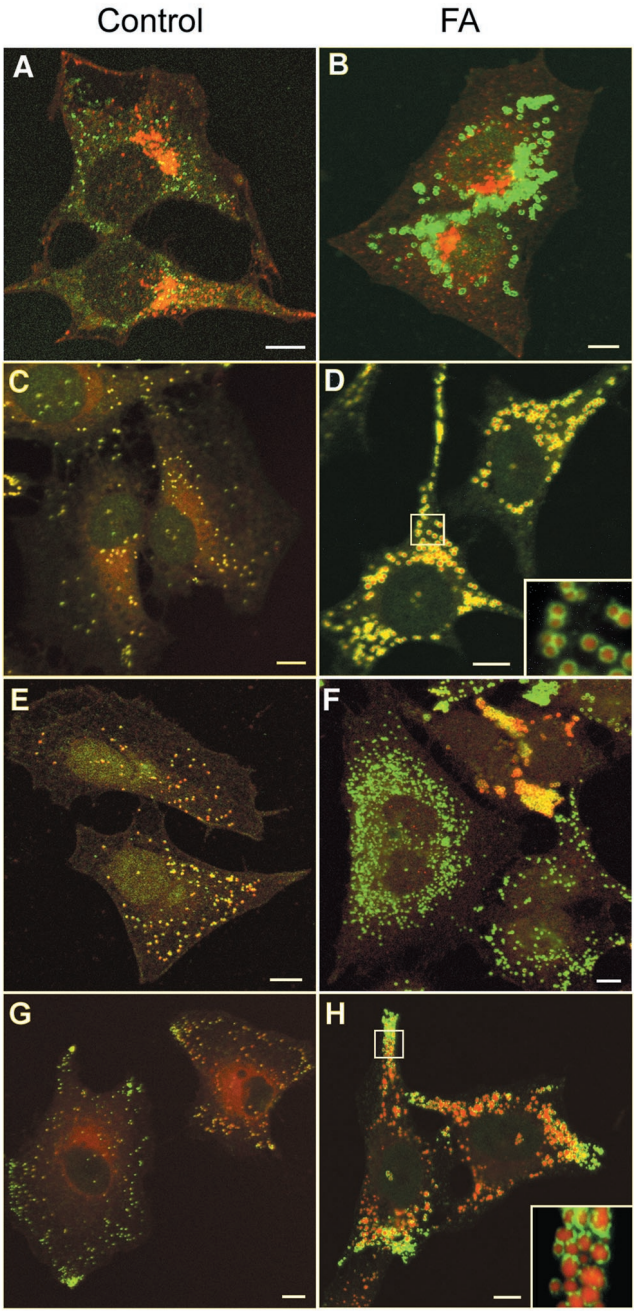


FIG. 2. **Anti-TIP47 polyclonal antiserum stains lipid droplets in HeLa cells.** HeLa cells were fixed with formaldehyde, permeabilized with saponin, and stained. *A*, *C*, *E*, and *G* show HeLa cells grown under normal low lipid-containing culture conditions; *B*, *D*, *F*, and *H* show HeLa cells grown with 600 $\mu$M supplemental oleate for 19 h. *A* and *B* show staining with anti-TIP47 antiserum in green and staining with anti-M6PR antiserum in *red*. *C* and *D* show staining with anti-TIP47 antiserum in *green* and neutral lipid staining in *red*; coincident staining is depicted in *yellow*. The *inset* to *D* shows a 3.4-fold magnification of the indicated region of the lower cell. *E* and *F* show staining with anti-TIP47 antiserum in *green* and anti-hADRP antiserum in *red*; coincident staining is depicted in *yellow*. Many cells showed staining for TIP47 but not ADRP; when both stains were observed in a cell, they were usually coincident. *G* and *H* show HeLa cells stained with anti-hADRP antiserum in *green* and neutral lipid staining in *red*. *Inset* to *H* shows a 4.1-fold magnification of the indicated region of the cell to the *left*. *Bars*, 10 $\mu$m.

The lipid droplet protein ADRP, which shares 43% identity with TIP47, covers the surfaces of lipid droplets in a number of types of cells (8, 9); this localization was confirmed in HeLa cells doubly stained for neutral lipid and ADRP in the current

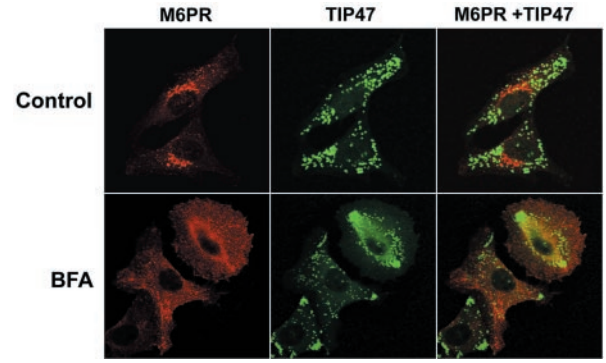


FIG. 3. **Brefeldin A alters the distribution of M6PR but fails to perturb the localization of TIP47.** HeLa cells were cultured in medium supplemented with 600 $\mu$M oleate for 18 h. Cells shown in the *lower panels* were then treated for 20 min with 5 $\mu$g/ml brefeldin A; cells in the *upper panels* were incubated in the absence of brefeldin A. The cells were immediately fixed and doubly stained with anti-TIP47 antiserum (*green*; *center panels*) and anti-M6PR antiserum (*red*; *left panels*). The *right panels* show superimposed staining for TIP47 and M6PR.

study (Fig. 2, *G*, *H*, and *inset* of *H*). To compare the subcellular distribution of ADRP and TIP47, HeLa cells were doubly stained with anti-hADRP monoclonal antibody and anti-TIP47 antiserum. In cells grown under normal culture conditions without lipid supplementation, most of the cells that contained detectable lipid droplets displayed staining for both TIP47 and ADRP (Fig. 2*E*). In lipid-loaded cells, the lipid droplets of almost all of the cells stained strongly with anti-TIP47 antiserum, while the lipid droplets of approximately half of the cells were simultaneously stained with anti-hADRP antibody (Fig. 2*F*); occasional cells showed lipid droplets that were decorated solely with anti-ADRP antibodies. The coincident staining of lipid droplets with both antibodies suggests colocalization of these highly related proteins on the surfaces of lipid droplets. Additionally, these observations imply that HeLa cells are a heterogeneous population of cells with regard to the expression of lipid droplet-associated proteins. Furthermore, this experiment provides additional proof that the anti-hADRP antibody and the anti-TIP47 antiserum recognize distinct proteins despite the similarities of the amino acid sequences between the two proteins, since the lipid droplets of some cells stain only with anti-TIP47 antiserum, while others stain only with anti-hADRP antibody.

Finally, the association of TIP47 with lipid droplets is not unique to HeLa cells; lipid droplets in primary mouse fibroblasts, human melanocytes, and 3T3-L1 adipocytes all show surface immunostaining for TIP47 (data not shown).

*The Subcellular Distribution of TIP47 Is Unchanged When Cells Are Treated with Brefeldin A*—The treatment of cells with the fungal metabolite brefeldin A causes extensive rearrangements of membranous compartments in the secretory pathway, including the recruitment of endosomal structures into a tubular reticulum (26). As expected, the treatment of HeLa cells with brefeldin A caused a redistribution of M6PR staining from a primarily juxtanuclear region to more peripheral structures, while having no obvious effect on TIP47 staining (Fig. 3), thus demonstrating that the bulk of the TIP47 is located in a brefeldin A-insensitive compartment, while the bulk of M6PR is in a brefeldin A-sensitive compartment.

*TIP47 Cofractionates with Neutral Lipids*—To assess the subcellular localization of TIP47 biochemically, HeLa cells and MA10 Leydig cells were fractionated by flotation on sucrose density gradients. Lipid droplets have a neutral lipid core comprising most of their volume and rendering them buoyant in aqueous solution, while membranous subcellular compartments have a higher protein/lipid ratio, giving them greater

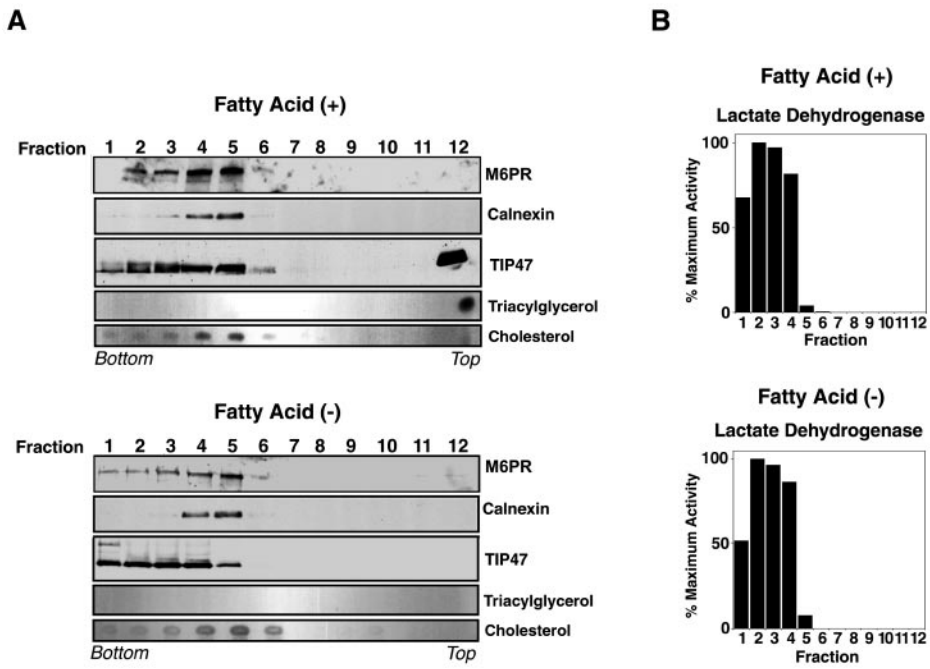


FIG. 4. **Lipid loading of cells increases the accumulation of triacylglycerols in lipid droplets; TIP47 becomes buoyant and fractionates with lipid droplets.** HeLa cells were grown in culture medium supplemented with 600 $\mu$M oleic acid, (*Fatty Acid* (+)), or normal low lipid-containing culture medium (*Fatty Acid* (−)), for 19 h before being harvested, homogenized, and fractionated by centrifugation of sucrose gradients. Each gradient had a 0.6-ml 50% (w/w) sucrose cushion, 3.4 ml of postnuclear supernatant corresponding to 7 mg of cellular protein in 40% (w/w) sucrose and 1 ml of 35% sucrose, overlaid with a 7-ml linear 0–30% sucrose gradient. *A* shows immunoblots depicting the distributions of the endosomal marker, M6PR, ER protein, calnexin, and TIP47, and thin layer chromatography data depicting the distributions of triacylglycerol (lipid droplets) and cholesterol (membranes) across the fractions from the gradients. *B* shows the distribution of the activity of the cytosolic enzyme lactate dehydrogenase across the gradients.

density. In HeLa or MA10 Leydig cells grown in standard low lipid-containing culture media, TIP47 (Fig. 4*A*, *Fatty Acid* (−), and Fig. 5*A*, *Fatty Acid* (−)) fractionated with lactate dehydrogenase (Figs. 4*B* and 5*B*), an enzyme used as a marker for cytosol. Low levels of TIP47 also cofractionated with calnexin, a marker for endoplasmic reticulum, in HeLa cells (Fig. 4*A*), and with cytochrome *c* reductase activity, an alternative marker for endoplasmic reticulum, in MA10 Leydig cells (Fig. 5, *A* and *B*). In fractions from HeLa cell homogenates, the distributions of TIP47 and M6PR overlapped in the lower portion of the sucrose gradients (Fig. 4*A*). These findings are consistent with the subcellular distribution described previously for TIP47 in COS cells (1). In the current studies, when HeLa cells and MA10 Leydig cells were grown under standard low lipid-containing culture conditions, no neutral lipid was detected in the gradients (Fig. 4*A* or 5*A*, *Triacylglycerol panels*, *Fatty Acid* (−)), thus indicating a lack of detectable lipid droplets in cellular fractionation experiments. Clearly, some of these cells contain lipid droplets, as shown in microscopy experiments, as depicted in Fig. 2, *C* and *G*.

Most cultured cells store small amounts of triacylglycerols and cholesterol esters in a few lipid droplets, and when the cells are incubated with fatty acids, the mass of triacylglycerol stored in the droplets increases. The lipid droplets of Leydig cells contain primarily cholesterol esters; this cholesterol serves as a substrate for the synthesis of steroid hormones (27). In the current studies, when MA10 Leydig cells were grown in media supplemented with fatty acids, they accumulated both cholesterol esters (data not shown) and triacylglycerols (Fig. 5*A*, *Fatty Acid* (+), *Triacylglycerol*). By contrast, HeLa cells accumulated primarily triacylglycerols. For both cell types, triacylglycerols were detected predominantly in the most buoyant fractions (Figs. 4*A* and 5*A*, *Fatty Acid* (+), *Triacylglycerol panels*, *lane 12*). Under these culture conditions, the most buoyant fractions also contained the greatest mass of TIP47, thus demonstrating that TIP47 localizes to newly synthesized lipid droplets.

Following the incubation of either HeLa cells or MA10 Leydig cells with fatty acids, ~70 or 25% of the total cellular TIP47 localized to lipid droplets in subcellular fractions from HeLa cells or MA10 Leydig cells, respectively; an additional portion of the TIP47 shifted from the cytosol to fractions containing markers for the endoplasmic reticulum in each type of cell. In fractions from HeLa cells grown in the absence of supplemental lipids, TIP47 was found primarily in fractions 1–5 (Fig. 4*A*, *Fatty Acid* (−)) that contained the highest levels of lactate dehydrogenase (Fig. 4*B*), a cytosolic marker. When these cells were incubated with fatty acids, 20% of the mass of TIP47 was found in fractions 4 and 5 that contained the majority of calnexin, a marker for endoplasmic reticulum, and cholesterol, a marker for cellular membranes (Fig. 4*A*, *Fatty Acid* (+)), with a corresponding decrease in the mass of TIP47 found in fractions 1–3. Likewise, in fractions from MA10 Leydig cells grown in the absence of supplemental lipids, TIP47 colocalized with lactate dehydrogenase in fractions 1–6 (Fig. 5, *A* and *B*, *Fatty Acid* (−)), and lipid loading shifted ~10% of TIP47 to fractions 6–8, which contained the majority of cholesterol and activity of NADPH-dependent cytochrome *c* reductase, a marker for endoplasmic reticulum (Fig. 5, *A* and *B*, *Fatty Acid* (+)). Thus, when cells are incubated under conditions that promote the synthesis of neutral lipids and the packaging of these lipids into lipid droplets, TIP47 moves into the most buoyant fraction that contains lipid droplets, as well as into the fractions that contain the bulk of the endoplasmic reticulum, the site of neutral lipid synthesis (28–30), and, presumably, the initial site of lipid droplet formation.

To confirm the observation of the colocalization of TIP47 and ADRP at the surfaces of lipid droplets obtained in the microscopy studies, the subcellular fractions from the sucrose gradients were immunoblotted for ADRP. MA10 Leydig cells, which show more homogeneous ADRP expression than HeLa cells (Fig. 2), were used to compare the subcellular distribution of TIP47 and ADRP. ADRP was not detected in fractions from cells grown in standard low lipid-containing culture media (Fig. 5*A*, *Fatty Acid* (−), *ADRP panel*); however, when the cells were supplemented with fatty acids, ADRP became easily detectable and was found almost exclusively in the most buoyant fractions (Fig. 5*A*, *Fatty Acid* (+), *ADRP panel*). By contrast, lipid loading of the cells did not change the total cellular mass of TIP47 but, rather, dramatically altered the subcellular distribution of TIP47 from a predominantly cytosolic localization onto both lipid droplets and membranes.

*Lipid Loading Induces the Movement of TIP47 from the Cytosol onto Newly Synthesized Lipid Droplets*—Culturing cells

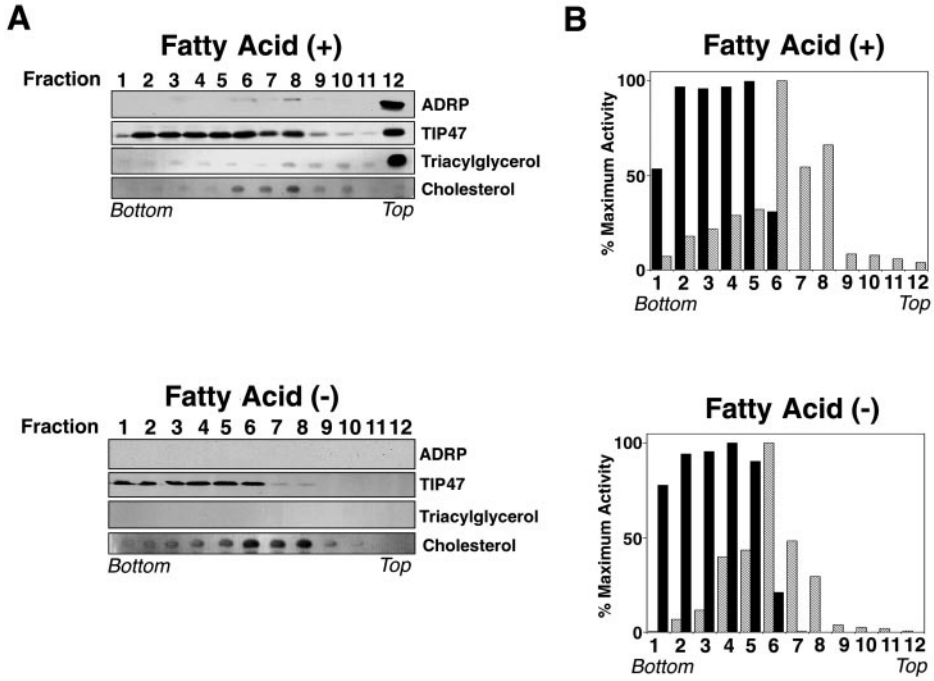


FIG. 5. **When triacylglycerols accumulate, TIP47 is recruited from the cytosolic fraction onto lipid droplets, while ADRP is found exclusively on lipid droplets.** MA10 Leydig cells were cultured in 600 $\mu$M supplemental oleate (*Fatty Acid* (+)) or normal low lipid-containing culture medium (*Fatty Acid* (−)) for 19 h before being harvested, homogenized, and fractionated by the centrifugation of sucrose gradients. Each gradient had a 0.6-ml 50% (w/w) sucrose cushion, 5 ml of postnuclear supernatant corresponding to 15 mg of cellular protein in 40% (w/w) sucrose, 1.5 ml of 35% sucrose, 1.5 ml of 20% sucrose, and 1.5 ml of 10% sucrose, overlaid with 1.9 ml of lysis buffer. *A* shows immunoblots depicting the distributions of ADRP and TIP47 and thin layer chromatography data depicting the distributions of triacylglycerol (lipid droplets) and cholesterol (membranes). *B* shows the distributions of the activities of the cytosolic enzyme lactate dehydrogenase (■) and the ER membrane marker NADPH-dependent cytochrome *C* reductase (▨).

with supplemental fatty acids causes TIP47 to associate with lipid droplets, as characterized by both immunofluorescence microscopy and subcellular fractionation experiments (Figs. 2, 4, and 5). To characterize the recruitment of TIP47 from the cytosol onto lipid droplets, HeLa cells were cultured in media supplemented with fatty acids for 0–8 h before harvest and subcellular fractionation. During this time, the total cellular mass of TIP47 was minimally changed (Fig. 6, *Total TIP47*). By 1 h of culture in the presence of supplemental fatty acids, the levels of triacylglycerols in the most buoyant fraction increased to clearly detectable levels concomitant with the appearance of TIP47 in this fraction (Fig. 6, *Triacylglycerol* and *Buoyant TIP47*). During the 8-h incubation of the cells with fatty acids, both the mass of triacylglycerol and the mass of TIP47 in the most buoyant fraction increased proportionally and in parallel, while the cytosolic pool of TIP47 was depleted (data not shown); these observations suggest that TIP47 moves onto nascent lipid droplets.

## DISCUSSION

The primary finding of this study is that TIP47 is a lipid droplet-associated protein. In microscopy experiments, TIP47 staining was observed on the surfaces of small, rare lipid droplets in HeLa cells grown in low lipid-containing media as well as on the somewhat larger and more numerous lipid droplets of cells grown in media supplemented with fatty acids to increase neutral lipid storage. Furthermore, TIP47 colocalized with ADRP, a lipid droplet-specific protein (8, 9, 10), on the surfaces of lipid droplets in HeLa cells grown under both conditions. Subcellular fractionation of both human HeLa cells and murine MA10 Leydig cells grown under lipid-enriched conditions showed that much of the cellular TIP47 localizes to the buoyant lipid droplet fraction along with the majority of the cellular triacylglycerol, cholesterol ester, and ADRP.

TIP47 shares sequence homology with at least two other lipid droplet-associated proteins, ADRP and perilipins. Perilipins localize exclusively to lipid droplets in adipocytes and steroidogenic cells (4–6) and have been found in no other subcellular compartment. ADRP has also been localized to lipid droplets in a wide variety of cells and tissues (8–10). By contrast, TIP47 is abundant in the cytosol of HeLa cells and MA10 Leydig cells cultured in typical low lipid-containing medium lacking supplemental lipids, as well as associated with the few small lipid droplets that occur under these culture conditions. Interestingly, the addition of physiological levels of fatty acids to the culture medium leads to the rapid synthesis of triacylglycerols, and the accumulation of TIP47 on lipid droplets with a concurrent loss of TIP47 from the cytosol, thus suggesting that TIP47 is recruited from the cytosol onto nascent lipid droplets.

TIP47 has been proposed to be a cargo selection device for the sorting of M6PRs into transport vesicles (1). Several observations make it difficult to reconcile our data with an exclusive role for TIP47 in determining the specificity of M6PR sorting in the cell. First, in addition to having a cytosolic localization, TIP47 associates with lipid droplets in a manner that is responsive to the status of neutral lipid synthesis and storage in the cell. Second, while the subcellular localization of M6PR is dramatically altered by the treatment of cells with brefeldin A, the distribution of TIP47 is unaffected; thus, the two proteins appear to occupy different subcellular compartments. Third, TIP47 has 43% sequence identity with ADRP over the entire length of the proteins; thus, it is likely that these proteins maintain a similar secondary structure. It is unlikely that ADRP plays a general role in protein trafficking, since ADRP has been found only in cells storing neutral lipid, with most or all of the protein on lipid droplets. Fourth, we have found that strong alkaline solutions of 100 mM sodium carbonate fail to extract both TIP47 and ADRP from isolated lipid droplets (data not shown); this treatment has been shown to disrupt electrostatic interactions while leaving hydrophobic interactions intact (23). Thus, both ADRP and TIP47 display similar characteristics of proteins integrally associated with lipid droplets. In conclusion, the current data describing the localization and tight association of TIP47 with lipid droplets, together with the sequence similarity between TIP47 and other lipid droplet-associated proteins, support the hypothesis that TIP47 plays a role in the metabolism of intracellular neutral lipids and not in the sorting of secretory proteins.

Does a portion of cellular TIP47 associate with endosomes? Immunofluorescence microscopy experiments conducted in

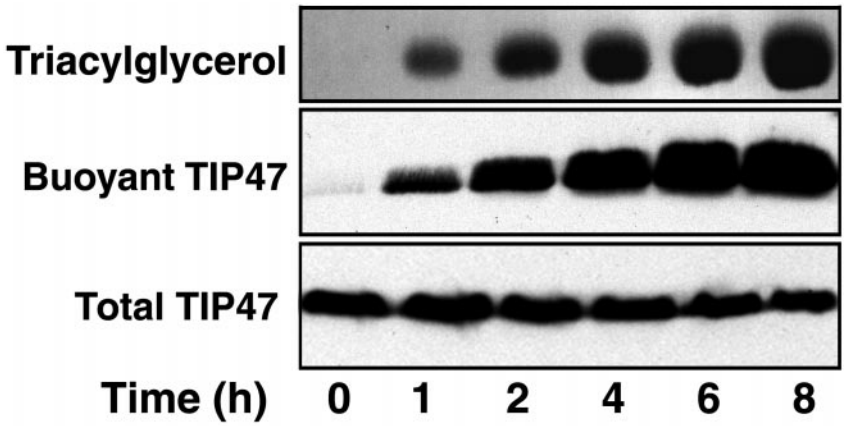


FIG. 6. **TIP47 accumulates in parallel with triacylglycerol in the most buoyant fraction.** HeLa cells were incubated with supplemental 600 $\mu$M oleate for the times indicated before being disrupted by hyperosmotic shock and fractionated by differential centrifugation. The most buoyant fraction was collected from each gradient and assayed for triacylglycerol (*Buoyant TG*; *upper panel*) by thin layer chromatography and TIP47 (*Buoyant TIP47*; *middle panel*) by immunoblotting. The lower panel (*Total TIP47*) shows an immunoblot of TIP47 in 0.9% of the starting postnuclear supernatants prior to fractionation; this *panel* indicates that the total mass of cellular TIP47 is equivalent at all times. The exposure time for the immunoblot in the *middle panel* was different from that of the *lower panel*; exposure times were selected to best compare the TIP47 levels within a given *panel*.

both the presence and absence of brefeldin A failed to support the localization of TIP47 to M6PR-containing endosomes. While subcellular fractionation experiments indicated that a portion of TIP47 colocalized with membrane fractions that contained endosomal markers, it is important to note that these fractions also contained markers for other membranous compartments such as ER; thus, the fractions containing membranes were not adequately resolved to unambiguously identify which membrane contains a small portion of the total cellular TIP47. Furthermore, the observation that the association of TIP47 with membranes is dependent upon lipid availability is suggestive of a role for TIP47 in lipid trafficking or lipid droplet assembly. Although these data do not preclude the possibility that a small amount of TIP47 is on an endosomal compartment, they clearly show that the bulk of TIP47 is either soluble or associated with neutral lipid droplets.

Most mammalian cells efficiently sequester and store neutral lipids in droplets, yet very little is known about this process. The final steps of neutral lipid synthesis occur in the endoplasmic reticulum (28–30). It is highly likely that the initial events that lead to the formation of lipid droplets occur in the endoplasmic reticulum and that the droplets then dissociate from these membranes, since these droplets are coated by a unique subset of cellular proteins that are not found in other compartments (5, 8, 9). In the current study, TIP47 moved from the cytosol to both membranes and lipid droplets during conditions that promoted the rapid synthesis of neutral lipids and the formation of lipid droplets. These observations raise the possibility that TIP47 has a role in the packaging of neutral lipids into droplets.

We have found TIP47 to be widely expressed and localized to lipid droplets, solidifying the notion that the perilipin/ADRP/TIP47 family of proteins may function in regulating the storage of neutral lipids. Chordates store much of the chemical energy required for their existence in triacylglycerol-cored lipid droplets. Failure to have or to mobilize this energy store is perilous when food supplies are scarce. Conversely, in the human population, food consumption often exceeds energy requirements, and the resulting obesity is a major factor in the development of cardiovascular disease and diabetes. Thus, the appropriate storage and mobilization of neutral lipid is critical for viability. The lipid droplet proteins, ADRP and perilipins, are located on the surfaces of the lipid droplets, between the stored neutral lipids and the soluble lipases that mobilize these lipids; both ADRP and perilipins have been proposed to play roles in maintaining lipid homeostasis (31, 32). Given that TIP47 is found on the surfaces of lipid droplets and that its cellular distribution is dependent upon the storage of neutral lipids, it is likely that TIP47 may also play a role in lipid metabolism.

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