

TIP47 Is Not a Component of Lipid Droplets*

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TIP47 functions in the delivery of mannose 6-phosphate receptors from endosomes to the trans-Golgi network both *in vitro* and *in vivo*. It binds directly and very specifically to the cytoplasmic domains of both the cation-independent and cation-dependent mannose 6-phosphate receptors. TIP47 is 43% identical to a lipid droplet-associated protein named adipophilin; much of the identity resides near the N termini of these proteins. It was recently reported in this journal, in a study using antiserum from this laboratory, that TIP47 is a constituent of lipid droplets (Wolins, N. E., Rubin, B., and Braasmele, D. L. (2001) *J. Biol. Chem.* 276, 5101–5108). We show here that the findings of Wolins *et al.* were likely due to either a cross-reactive, unidentified protein in HeLa cells that is recognized by our antiserum and/or the fact that our serum also cross-reacts with the adipophilin protein itself, shown directly by expression of adipophilin in *Escherichia coli*. Using antibodies specific for residues 152–434 of TIP47, we show that TIP47 is not a constituent of lipid droplets.

TIP47¹ is a protein of 47 kDa that binds to the cytoplasmic domains of the cation-dependent and cation-independent mannose 6-phosphate receptors (MPRs) (1). When TIP47 is depleted from cells using antisense oligonucleotides, MPRs are mis-sorted to the lysosome (1). Moreover, TIP47 is needed in cytosol to support endosome-to-trans-Golgi network transport of MPRs, both *in vitro* (1) and in living cells (1, 2).

TIP47 binds to a specific Phe-Trp motif in the cation-dependent MPR (1) that has been shown to be important for the normal trafficking of this receptor (3). Indeed, mutation of these residues in the cation-dependent MPR has the same consequence as depleting cells of TIP47; in both cases, the cation-dependent MPR is misdirected to the lysosome (1, 3). Binding of TIP47 to the cation-independent MPR is somewhat more complex and involves a membrane-proximal, hydrophobic motif located between residues 48 and 74 that is presented by more carboxyl-terminal residues that appear to contribute to the overall folding of the cytoplasmic domain (4). Finally, TIP47 is very specific in its interaction with MPRs in that it fails to bind to the cytoplasmic domains of the low density

lipoprotein receptor (1), furin, phosphorylated furin, carboxypeptidase D, and TGN 38 (5).

Given our extensive functional and structural analysis of TIP47, we were surprised by a recent report in this journal suggesting that TIP47 is present on lipid droplets (6). These workers used an antiserum from our laboratory, and we show here that those results were likely due to a cross-reactive, unidentified protein that is recognized by our antiserum and especially enriched in HeLa cells. In addition, our antiserum recognizes recombinant adipophilin. Using affinity-purified antibodies, we demonstrate that TIP47 is not present in lipid droplets.

EXPERIMENTAL PROCEDURES

Antibodies and Recombinant Proteins—Rabbit and mouse anti-MPR antibodies and rabbit anti-TIP47 antiserum were previously characterized (1). Rabbit anti- β -COP antibody was raised against the EAGE peptide of that protein (7). Anti-human adipose differentiation-related protein (ADRP) monoclonal antibody was purchased from Research Diagnostics Inc. (Flanders, NJ). The anti-TIP47 serum (1) was affinity-purified using either full-length protein or His-tagged TIP47 residues 152–434 (2) covalently attached to Affi-Gel 10 (Bio-Rad Laboratories). Human ADRP cDNAs (Incyte Genomics, Inc.) were used to produce two His-tagged, truncated ADRP forms (ADRP 1–75 and ADRP 1–202). Polymerase chain reaction products were ligated into pTrcHis2-TOPO (Invitrogen). His-tagged proteins were expressed in *Escherichia coli* as described (1, 4). Fluorescein-conjugated goat anti-Rabbit and Texas Red-conjugated goat anti-mouse IgGs were from Jackson ImmunoResearch Laboratories Inc., West Grove, PA.

Cell Culture—Human HeLa cells and African green monkey kidney epithelial (BS-C-1) cells were maintained in Dulbecco's modified Eagle's medium or minimal essential medium, respectively, supplemented with 7.5% fetal calf serum, penicillin, and streptomycin. For fatty acid treatment, cells were incubated for 18 h in media supplemented with 600 μ M oleic acid (Sigma) complexed to fatty acid-free bovine serum albumin (Sigma) at a molar ratio of 6:1 (6).

Immunoblot Analysis—HeLa or BS-C-1 cells were grown on 60-mm plates and treated for 18 h with oleic acid. After three washes with phosphate-buffered saline, cells were harvested in radioimmune precipitation buffer (1) supplemented with protease inhibitors (40 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 μ M pepstatin A, and 1 μ M phenylmethylsulfonyl fluoride). Protein extracts were centrifuged for 10 min, and supernatants were collected. Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose.

Immunofluorescence Microscopy—Immunofluorescence was performed as described by Warren *et al.* (8) using cells grown on collagen-coated glass coverslips. Brefeldin A treatment was for 20 min in media containing 5 μ g/ml brefeldin A. All primary antibodies were used at a 1:1000 dilution except affinity-purified anti-TIP47, which was used at 1:500 dilution. Texas Red- or fluorescein isothiocyanate-conjugated secondary antibodies were used at 1:1000. Images were obtained using a Nikon Diaphot 300 inverted microscope.

RESULTS

TIP47 (1) is 43% identical to a protein named adipophilin or ADRP. As shown in Fig. 1, much of the homology resides near the N termini of these proteins whereas similarity is seen over the length of both coding regions. Although adipophilin is found on lipid droplets, its function is currently unknown; moreover,

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¹The abbreviations used are: TIP47, tail-interacting protein of 47 kDa; MPR, mannose 6-phosphate receptor; ADRP (adipophilin), adipose differentiation-related protein; BS-C-1 cells, African green monkey kidney epithelial cells.

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ADRP : 1 MASVAVDP
TIP47: 1 MSADGAEADGSTQVTVEEPVQ

ADRP : 9 QPSVVTRVNLPLVSSTYDLMSAYLSTKDQYPYLKSVCEMAENGVKTTITSVAMTSALPI
TIP47: 22 QPSVVDRAVSMPLISSTCDMVSAAYASTKESYPHVKTVCDAAEKGVRTLTAADVSGAQPI
QPSVV RV PL SST D S AY STK YP K VC AE GV T T A A PI

ADRP : 69 IQKLEPQIAVANTYACKGLDRIEERLPILNQPSTQIVANAKGAVTGAKDAVTTTGTGAKD
TIP47: 82 LSKLEPQIASASEYHRGLDKLEENLPILQQPTEKVLADTKELVSSKVSQAQEMVSSAKD
KLEPQIA A YA GLD EE LPIL QP A K V V AKD

ADRP : 129 SVASTITGVMDKTKGAVTGSVEKTKSVVSGSINTVLGSRMMQLVSSGVENALTKSELLVE
TIP47: 142 TVATQLSEAVDATRGAVQSGVDKTKSVVTGGVQSVMGSRGQMVLSGVDTVLGKSEEWAD
VA D T GAV V KTKSVV G V GSR Q V SGV L KSE

ADRP : 189 QYLPLTEEELEKEAKKVEGFDLV-----QKPSYVRLGSLSTKLHSRAYQQALSRVKEA
TIP47: 202 NHLPLTDAELARIATSLDGFDAVSVQQRQEQSYFVRLGSLSERLRQHAYEHSGLKLRAT
LPLT EL A GFD Q SY VRLGSL L AY L

ADRP : 243 KQKSQQTISQLHSTVHLIEFARKNVYSANQKIQDAQDKLYLSWVEWKRISIGYDDTDESHC
TIP47: 262 KQRAQEALLQLSQALSLMETVKQGV---DQKLVGQEKHLQMWLSWNQKQLQGPEKEPPK
KQ Q QL L E V QK Q KL W W E

ADRP : 303 AEQFESRTLAIARNLTQQLQTTCHTLLSNIQGVQNIQDQAKHGMVMAGDIYSVFRNAAS
TIP47: 319 PEQVESRALTMFRDIAQQLQATCTSLGSSIQGLPTNVKDQVQQARRQVEDLQATFSSIHS
EQ ESR L R QQLQ TC L S IQG P N DQ D F S

ADRP : 363 FKEVSDSLLTSSKGQLQKMKESLDDVMDYLVNNTPLNWLVGPFYPQLTESQNAQDQGAEM
TIP47: 379 FQDLSSSILAQSRERVASAREALDHMVEYVAQNTPVTWLVGPFAPGITEKAPEEKK
F S S L S E LD Y NTP WLVGPF P TE

ADRP : 435 DKSSQETQRSEHKTH
    
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FIG. 1. Sequence alignment of human TIP47 and ADRP. The bold sequences indicate identities between the two sequences.

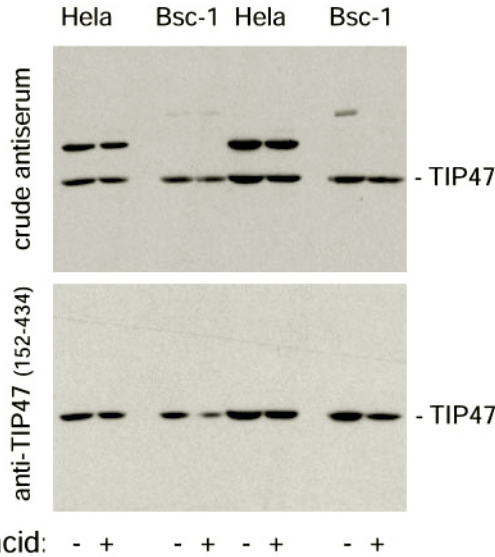


FIG. 2. Immunoblot analysis of HeLa and BS-C-1 cells using anti-TIP47 antibodies. The upper panel was probed with crude anti-TIP47 serum (1:1000); the lower panel was probed with affinity-purified anti-TIP47 residues 152–434. The cells were treated with oleic acid where indicated; cell extracts were loaded at 15 or 30 µg of total protein (left half and right half of the gels, respectively). The mobility of TIP47 is indicated at the right.

the functional basis for the similarity between TIP47 and adipophilin remains entirely unclear.

Wolins *et al.* (6) carried out immunofluorescence analyses using our crude polyclonal anti-TIP47 antiserum (1). We have shown previously that this antiserum recognizes a single 47-kDa polypeptide in immunoblots of K562 cells (1) and localizes by cell fractionation and light microscopy to MPR-containing

late endosomes in Chinese hamster ovary and bovine tracheal epithelial cells (1). Because of the unexpected report of Wolins *et al.* (6) we tested our serum on extracts from HeLa cells and also BS-C-1 cells. As shown in Fig. 2, the antiserum recognized two polypeptides in HeLa cells (47 kDa and ~60 kDa) and one polypeptide in BS-C-1 cells. In longer exposures, many additional polypeptides could be detected (not shown). Affinity purification of the antiserum using full-length TIP47 yielded the same results (not shown).

The above data suggested that lipid droplet staining using crude antiserum (6) could have been due to a cross-reacting protein, rather than TIP47. Therefore, we prepared an affinity-purified antibody specific for the C-terminal half of the protein. TIP47 residues 152–434 were expressed as a His-tagged protein (2) and used to affinity purify the antiserum. In contrast to the total serum, antibodies specific for residues 152–434 recognized a single polypeptide in both BS-C-1 and HeLa cell extracts (Fig. 2, lower panel).

With a more TIP47-specific antibody in hand, we compared the subcellular localizations of proteins recognized by either anti-full-length TIP47 antibodies or anti-TIP47 residue 152–434 antibodies. Lipid droplet formation was induced by incubating cells with oleic acid (6). As shown in Fig. 3, in the absence of oleic acid, TIP47 staining was detected on perinuclear structures that are likely to represent late endosomes (1). Upon treatment with oleic acid, BS-C-1 cells displayed significant lipid droplet accumulations. Importantly, whereas the anti-TIP47 (full-length) antibodies led to fluorescent decoration of lipid droplet structures in cells treated with oleic acid (white arrows, upper panels), the anti-TIP47-(152–434) antibody clearly did not (white arrows, lower panels). Indeed, TIP47 staining was excluded from regions of the cytoplasm enriched in lipid droplets. Thus in BS-C-1 cells, TIP47 is not present on lipid droplets.

Like TIP47, MPRs showed a perinuclear distribution that

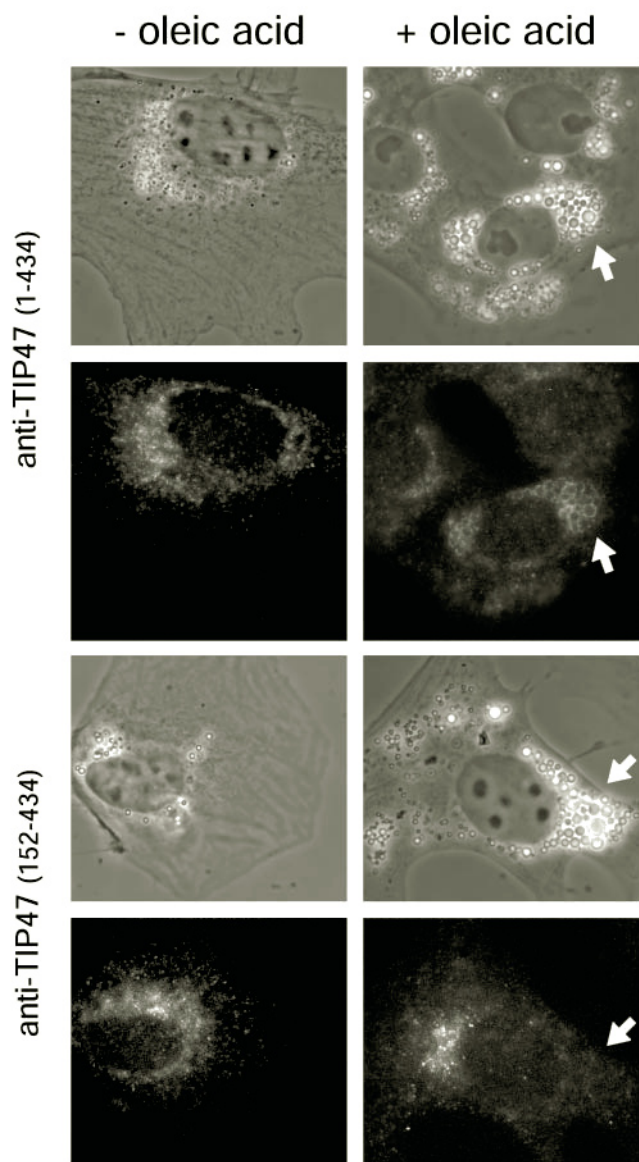


FIG. 3. **TIP47 is not on lipid droplets in BS-C-1 cells.** Cells were treated with or without oleic acid and analyzed by immunofluorescence microscopy using anti-TIP47 antibodies (affinity-purified against the full-length protein, 1:1000), upper 4 panels, or affinity-purified anti-TIP47 residues 152–434 (1:500), lower 4 panels. Lipid droplets were observed by differential interference contrast imaging (rows 1 and 3). White arrows point to cell regions containing lipid droplet accumulations.

was unchanged in oleic acid-treated cells (Fig. 4). In addition, in contrast to the findings of Wolins *et al.* (6) the localization of MPRs was unchanged in the presence of brefeldin A (Fig. 5). Whereas β -COP staining became rapidly diffuse, MPRs remained adjacent to the nucleus in a tight and discrete structure. Previous work has shown that endosomal structures tubulate in some types of brefeldin-A-treated cells (9, 10). Nevertheless, MPRs retain a concentrated, perinuclear staining (10) such as that shown here.

Wolins *et al.* (6) used HeLa cells for immunolocalization experiments. Our initial studies utilized BS-C-1 cells, which are flat and display beautiful cellular morphology. Attempts to localize TIP47 in oleic acid-treated HeLa cells using affinity-purified antibodies revealed a very low level labeling of lipid droplets (data not shown). Thus it was essential for us to determine whether the affinity-purified antibodies cross-reacted to some extent with adipophilin itself.

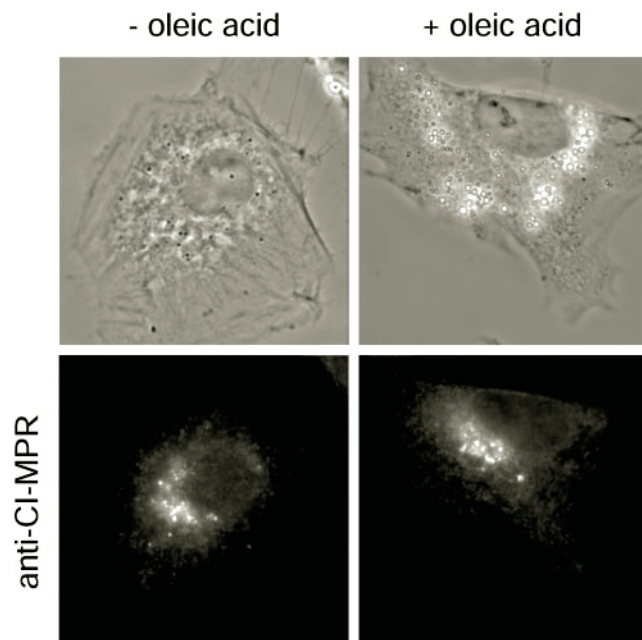


FIG. 4. **Mannose 6-phosphate receptors, like TIP47, fail to re-localize in cells treated with oleic acid.** Rabbit anti-cation-independent MPR antibodies were used at 1:1000 dilution.

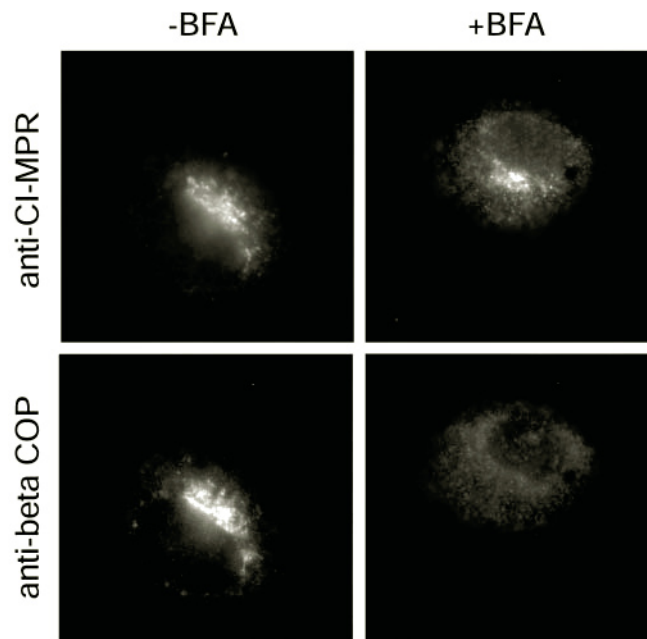


FIG. 5. **Brefeldin A does not alter MPR localization in BS-C-1 cells.** Cells were treated with or without brefeldin A (BFA) for 20 min. Mouse anti-cation-independent MPR culture supernatant was used undiluted; rabbit anti- β -COP serum was used at 1:1000.

Adipophilin cDNAs representing the N-terminal 75 or 202 residues were obtained commercially and expressed as His-tagged polypeptides in bacteria. Extracts from bacteria expressing adipophilin polypeptides were then analyzed by immunoblotting. As shown in Fig. 6, both anti-TIP47 serum and anti-TIP47-(152–434)-specific antibodies recognized adipophilin peptides. However, anti-TIP47 serum gave a much stronger reaction than the affinity-purified, anti-TIP47-(152–434) antibody; we have shown that the serum recognizes N-terminal epitopes as well as C-terminal epitopes (data not shown). Thus anti-TIP47 serum sees both TIP47 and adipophilin; antiserum affinity-purified against residues 152–434 of TIP47 lost signifi-

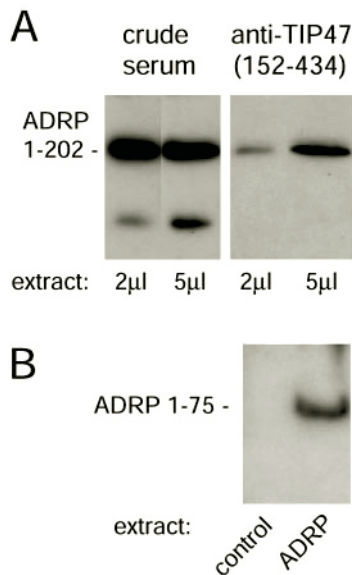


FIG. 6. TIP47 antibodies recognize adipophilin polypeptide. Bacterial extracts expressing either adipophilin residues 1–75 or 1–202 were analyzed by immunoblots using either anti-TIP47 serum (1:1000) or affinity-purified anti-TIP47 residues 152–434 antibodies (1:300). *A*, bacterial extract expressing ADRP residues 1–202; volumes analyzed are indicated. *B*, bacterial extract expressing either a control plasmid or ADRP residues 1–75. The blot in the lower panel was probed with anti-TIP47 antiserum at a dilution of 1:1000.

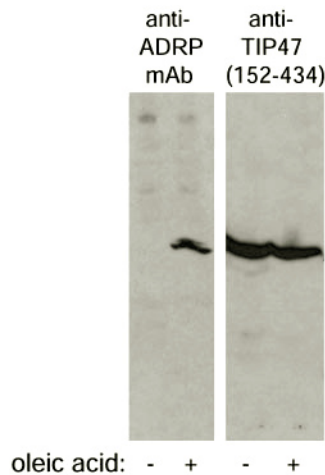


FIG. 7. Anti-adipophilin antibody detects a protein induced by oleic acid treatment of BS-C-1 cells. BS-C-1 cell extract (50 µg) was analyzed by immunoblot for the presence of polypeptides recognized by anti-ADRP monoclonal antibody (undiluted, lanes 1 and 2) or affinity-purified anti-TIP47 residues 152–434 (1:250, lanes 3 and 4). Oleic acid treatment was as indicated.

inant titer to adipophilin but still recognized the recombinant protein to some extent.

We would have liked to preadsorb our serum to remove all anti-adipophilin antibodies, but this was impossible because the expressed, recombinant, N-terminal fragments were diffi-

cult to express in significant quantities, and a full-length adipophilin cDNA clone was not available.

It is important to note that endogenous TIP47 and adipophilin polypeptides migrated identically in immunoblots of BS-C-1 cells (Fig. 7). When adipophilin was detected using a commercial monoclonal antibody, it was clearly induced in BS-C-1 cells treated with oleic acid. The mobility of the adipophilin protein was indistinguishable from endogenous TIP47 analyzed on the same gel and identified using affinity-purified anti-TIP47-(152–434) antibody. Unlike adipophilin, TIP47 levels were slightly decreased with oleic acid (Figs. 2 and 7). The comigration of adipophilin and TIP47, along with the cross-reactivity of our polyclonal antibody, make it difficult to distinguish between these functionally distinct protein molecules.

DISCUSSION

We have shown here that TIP47 and adipophilin proteins share epitopes that are recognized by a polyclonal anti-TIP47 antiserum. Moreover, whereas many of these epitopes seem to be concentrated near the closely related N termini of these proteins, at least one shared epitope resides between TIP47 residues 152 and 202 because antibodies directed against TIP47 residues 152–434 recognize bacterially expressed adipophilin residues 1–202. Examination of TIP47 residues 152–202 (Fig. 1) reveals many identities between TIP47 and adipophilin in this region that likely lead to cross-reactivity. Such cross-reactivity could easily have misled another research group to conclude, erroneously, that TIP47 is a constituent of lipid droplets (6). Unfortunately, cross-reaction with recombinant adipophilin protein was not tested in that study.

We have shown that TIP47 is not a component of lipid droplets in cells that accumulate large numbers of these structures and show oleic acid induction of adipophilin polypeptide. All of these results are consistent with our well documented functional analyses of TIP47 as a protein that mediates MPR trafficking in mammalian cells (1, 2). Further studies will be needed to help elucidate the function of adipophilin and the significance of its relationship to TIP47. The recent connection between lipid droplets and caveolin and cholesterol regulation surely adds to the potential interest in adipophilin and its role in lipid droplet structure and function (11).

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