Lipin proteins form homo- and hetero-oligomers

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INTRODUCTION

Lipin proteins are a novel family of Mg2+-dependent PAPs [PA (phosphatidic acid) phosphatases], which catalyse the dephosphorylation of PA into DAG (diacylglycerol) [1]. They are evolutionarily conserved and show similar primary organization [2]. Lipin proteins consist of a N-terminal domain (N-LIP) and a C-terminal domain (C-LIP), with the latter containing a conserved DXDXT motif that is required for the catalytic activity [1–3]. Distinct from fungi, nematodes and insects that only express one lipin isoform, mammals express three lipin isoforms named lipin 1, 2 and 3 [2]. In addition, three lipin 1 protein isoforms are produced by alternative mRNA splicing [4–6]. Mice lacking lipin 1 have a reduction in adipose tissue mass and are insulin-resistant [7]. Targeted overexpression of lipin 1 in mouse adipose tissue results in obesity, yet the mice maintain insulin sensitivity [8]. In humans, point mutations in the LPIN2 gene encoding lipin 2 cause Majeed syndrome [9], an autoinflammatory disorder, which may result from ablation of the PAP activity of the enzyme [10].

The lipin proteins primarily localize to the cytosol and are found in both the soluble and cytosolic membrane compartments, but can also be found in the nucleus [2,3,5,11]. Biochemical studies of PAP activities conducted prior to the identification of the gene encoding lipin 1 led to the hypothesis that this enzymatic activity transiently associates with membranes to dephosphorylate PA (reviewed in [12]). Subsequent work has suggested that phosphorylation controls lipin 1 movement between cellular compartments [3,11]. The yeast lipin Pah1p has been reported to associate with the promoters of genes involved in phospholipid biosynthesis [13]. In mammalian cells, lipin 1 can positively or negatively regulate gene expression via interaction with transcription factors, such as PPARα (peroxisome-proliferator-activated receptor α) and NFATc4 (nuclear factor of activated T-cells c4), as well as transcriptional co-regulators such as PGC-1α (PPARγ co-activator 1α) and HDACs (histone deacetylases) [14,15]. The PAP catalytic activity of lipin 1 appears to be separate from its activity in the regulation of transcription.

In the course of studies designed to identify lipin-1-interacting proteins by immunoprecipitation of epitope-tagged lipin isoforms we made the surprising discovery that lipin 1 can self-associate. The present study was conducted to investigate potential interactions between lipin isoforms. We show that lipin 1 forms stable homo-oligomers and hetero-oligomers with lipin 2 and lipin 3, suggesting that the function of these enzymes may be linked in a previously unappreciated manner.

EXPERIMENTAL

Chemicals

DAG (1,2-dioleoyl-sn-glycerol dioleoyl) and PA (1,2-dioleoyl-sn-glycero-3-phosphate) were purchased from Avanti Polar Lipids. NEM (N-ethylmaleimide) was purchased from Sigma. λPPase (λ-phosphatase) was purchased from New England Biolabs.

Key words: Förster resonance energy transfer (FRET), lipin, oligomer, phosphatidic acid phosphatase.

Abbreviations used: ACAT1, acyl-coA:cholesterol acyltransferase 1; ASBT, acceptor spectral bleed-through; BN-PAGE, blue-native PAGE; CFP, cyan fluorescent protein; C-LIP, lipin C-terminal domain; DAG, diacylglycerol; DAPI, 4′,6-diamidino-2-phenylindole; DGAT, DAG acyltransferase; DSBT, donor spectral bleed-through; DTT, dithiothreitol; FBS, fetal bovine serum; FLIM, fluorescence lifetime imaging microscopy; FRET, Förster resonance energy transfer; GST, glutathione transferase; HA, haemagglutinin; HEK, human embryonic kidney; HEK-293T, HEK-293 cells expressing the large T-antigen of SV40; HRP, horseradish peroxidase; LPP, lipid phosphate phosphatase; NEM, N-ethylmaleimide; Ni-NTA, Ni2+-nitritotriacetate; N-LIP, lipin N-terminal domain; PA, phosphatidic acid; PAP, PA phosphatase; PFRET, processed FRET; PGC-1α, peroxisome-proliferator-activated receptor γ co-activator 1α; λPPase, λ-phosphatase; PPARα, peroxisome-proliferator-activated receptor α; YFP, yellow fluorescent protein.

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Plasmids

V5-tagged lipin 1a, lipin 1b, lipin 2 and lipin 3 expression vectors were gifts from Dr Karen Reue (University of California, Los Angeles, CA, U.S.A.) [16]. HA (haemagglutinin)–lipin 1a, HA–lipin 1b (G64R), HA–lipin 1b (Lmut), HA–lipin 1b (D712E), GST (glutathione transferase)–lipin 1b (1–641) and GST–lipin 1b (642–924) have been described previously [14]. To construct V5–lipin 1b (N1, 1–171), V5–lipin 1b (N2, 1–641), V5–lipin 1b (C1, 121–924) and V5–lipin 1b (C2, 469–924), corresponding cDNA fragments were PCR-amplified from mouse lipin 1b and ligated into the pcDNA 3.1/D/V5-His-TOPO vector (Invitrogen). V5–LPP3 (lipid phosphate phosphatase 3) has been described previously [17]. EYFP [enhanced YFP (yellow fluorescent protein); Venus] and ECFP [enhanced CFP (cyan fluorescent protein); Cerulean]-tagged lipin 1b constructs were constructed by subcloning full-length lipin 1b upstream of the fluorescent tags. Both Cerulean and Venus contained the monomeric A206K mutation. CAAX–lipin 1b was generated by PCR using the reverse primer 5′-GAGCGGCCGCTA T-GAGCGGCCGCTATGAGGCTGAA TGC-3′. This removed the endogenous stop codon from lipin 1 and added the amino acid sequence KQPEDRQMCKCVELS, with the Ras-derived CAAX box at the C-terminus underlined. All of the clones were confirmed by DNA sequencing. Adenoviral constructs express anti-IgG antibodies (Sigma) and AP (alkaline phosphatase)-conjugated anti-IgG antibodies (Pierce) and AP (alkaline phosphatase)-conjugated anti-IgG antibodies (Sigma). Alexa Fluor® 488-, Alexa Fluor® 555- and Alexa Fluor® 568-conjugated anti-IgG antibodies (Invitrogen), HRP (horseradish peroxidase)-conjugated anti-IgG antibodies (Pierce) and AP (alkaline phosphatase)-conjugated anti-IgG antibodies (Sigma).

Antibodies

Anti-lipin 1 (Lab1 and Lab2) and anti-lipin 2 polyclonal antibodies have been described previously [4,18]. The following antibodies were obtained commercially: anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Abcam), anti-V5 (Invitrogen), anti-HA (Santa Cruz Biotechnology), anti-lipin 1 (Santa Cruz Biotechnology; C-15), anti-V5–agarose (Sigma), anti-HA–agarose (Sigma), Alexa Fluor® 488-, Alexa Fluor® 555- and Alexa Fluor® 568-conjugated anti-IgG antibodies (Invitrogen), anti-HA–agarose (Sigma), Alexa Fluor® 488-, Alexa Fluor® 555- and Alexa Fluor® 568-conjugated anti-IgG antibodies (Invitrogen).

Cell culture and transfection

COS-7 (A.T.C.C.), HEK-293T [HEK (human embryonic kidney)-293 cells expressing the large T-antigen of SV40 (simian virus 40); American Type Culture Collection] and HEK-293A (Invitrogen) cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% FBS (fetal bovine serum; Invitrogen) and antibiotics (penicillin/streptomycin). COS-7 cells were transfected with Lipofectamine™ 2000 (Invitrogen). 3T3-L1 cells were differentiated and maintained as described previously [3].

Generation of lipin baculovirus

Recombinant baculoviruses for expression of lipin 1b with an N-terminal tandem His6 and HA tag were generated using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer’s protocol. Viral stocks were obtained by transfection of Sf9 insect cells cultured in Grace’s insect medium with recombinant bacmids.

Purification of recombinant lipin protein

Recombinant lipin 1b protein tagged with both HA and His6 was extracted from a soluble fraction obtained from lysates of Sf9 cells that were infected with the recombinant lipin 1b baculovirus and cultured for 48 h post-infection. Lipin protein was purified with the Ni-NTA (Ni2+-nitrilotriacetate) purification system (Invitrogen). Briefly, pelleted cells were suspended in lysis buffer [50 mM sodium phosphate and 0.5 M NaCl (pH 8.0)] and disrupted by three freeze–thaw cycles using liquid nitrogen and a 37°C water bath. After centrifugation at 16000 g for 15 min, the supernatant was collected and mixed with the Ni-NTA resin for 1 h at 4°C with gentle agitation. The resin was allowed to settle under gravity, and washed with 8 ml of washing buffer [50 mM sodium phosphate, 0.5 M NaCl and 0.02 M imidazole (pH 8.0)] five times. Purified lipin protein was eluted with 6 ml of elution buffer [50 mM sodium phosphate, 0.5 M NaCl and 0.25 M imidazole (pH 8.0)]. The eluates were combined and concentrated to approx. 100 μl using Millipore Amicon ultra-4 centrifugal filter units.

Immunoprecipitation and immunoblotting

Cells were lysed by brief sonication in lysis buffer [50 mM Tris/HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 10% glycerol and Complete™ Protease Inhibitor Cocktail (Roche Diagnostics)]. Lysates were subjected to immunoprecipitation with anti-V5– or anti-HA–agarose. Immune complexes were washed five times with lysis buffer and subjected to immunoblotting with proper antibodies. Immunoblotting analysis was performed as described previously [3,19]. 3T3-L1 cells were homogenized with glass/Teflon in buffer A [50 mM 2-mercaptoethanol, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM sodium phosphate (pH 7.4) and 10 mM Triton X-100] supplemented with protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin and 0.25 μM microcystin) and cleared by centrifugation at 16000 g for 10 min. [3]. Lysates were subjected to immunoprecipitation using non-immune IgG, Lab2 [4] or anti-lipin 2 antibodies [18].

Immunofluorescence microscopy

Immunofluorescence staining was performed as described previously [20]. In brief, cells were fixed with 4% paraformaldehyde in PBS for 30 min. Following fixation, cells were treated with 0.2% Triton X-100 in PBS for 5 min. After blocking with 10% FBS in PBS for 20 min, cells were incubated for 1 h with primary antibody, followed by washing in PBS and incubation for 1 h with the relevant secondary antibody. Nuclei were stained with Hoechst 33342 (Invitrogen) or DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories). Pictures of cells were taken with a laser-scanning confocal microscope (Olympus FV500, Tokyo, Japan) or a Zeiss Axiovert 200 fluorescence microscope. Images were pseudo-colorized and merged with ImageJ or Adobe Photoshop.

FRET ( Förster resonance energy transfer) imaging

COS-7 cells on coverslips in six-well plates were transfected with 6 μg of plasmids. At 48 h after transfection, cells were fixed at room temperature (25°C) in 4% (w/v) paraformaldehyde for 15 min. The system used for spectral imaging was a Zeiss Axiovert 200 M epifluorescence motorized microscope coupled to a Zeiss 510 confocal-multiphoton imaging system at the W.M. Keck Center for Cellular Imaging (University of Virginia, Charlottesville, VA, U.S.A.). Details of imaging and the computational analysis used to separate DSBDT (donor spectral bleed-through) and ASBT (acceptor spectral bleed-through) from the signal acquired during excitation of the FRET channel have

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been published previously [21]. Briefly, reference spectra for untagged, monomeric Cerulean or Venus fluorescent proteins were subtracted from cells expressing both lipin–Venus and lipin–Cerulean fusion proteins using linear unmixing and the algorithm described previously [21–23]. All FLIM (fluorescence lifetime imaging microscopy) imaging was performed on live HEK-293T cells that were transfected 36–48 h previously with 3 μg of lipin 1b–Cerulean, or lipin 1b–Cerulean+lipin 1b–Venus. FLIM data were acquired using a multiphoton system consisting of a Nikon TE300 epifluorescence microscope coupled to a Bio-Rad Radiance 2100 confocal and multiphoton system. A 10 W Verdi tunable (700–1000 nm) pulsed Ti:sapphire laser tuned to 820 nm was used for two-photon excitation of the Cerulean fluorophore at 13 ns pulse intervals. Photons were collected using a PMT (photomultiplier tube) over a period of 120 s for each measurement. Data collection and analysis were performed using SPCImage software (Becker and Hickl), as well as ImageJ.

GST pull-down

The GST pull-down experiment was performed as described previously [24], with major modifications. In brief, HEK-293A cells transiently expressing various V5-tagged lipin 1b fragments were lysed in ice-cold lysis buffer [50 mM Tris/HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 10% glycerol and Complete™ Protease Inhibitor Cocktail]. Samples were briefly sonicated and centrifuged at 16,000 g at 4°C for 30 min. The supernatant was incubated with anti-V5–agarose at 4°C overnight. The beads were extensively washed with lysis buffer, and the recombinant lipin peptide was eluted by incubation with 70 μl of V5 peptide (100 μg/ml) in lysis buffer at 4°C for 1 h. After brief centrifugation, the supernatants were mixed with GST, GST–lipin 1b-(1–641) or GST–lipin 1b-(642–924) prebound to glutathione–Sepharose beads in 1 ml of binding buffer [50 mM Tris/HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 10% glycerol and Complete™ Protease Inhibitor Cocktail], and incubated with rotation for 4 h at 4°C. The beads were washed four times with 1 ml of binding buffer, separated by SDS/PAGE (4–12% gradient gels) and analysed by immunoblotting.

PAP activity assay

For the PAP activity assay, 10 cm plates of HEK-293T cells were transfected with Lipofectamine™ (2000) with 0.5 μg of Myc–lipin 1b, and 0.25, 0.5 or 1.0 μg of HA–lipin 1b (D712E) plasmids. At 48 h after transfection, cells were homogenized in glass/Teflon at 1000 rev./min in 1 ml of buffer A. Immunoprecipitations were performed as described previously [3] and divided into separate aliquots for electrophoresis and PAP activity measurement. PAP activity was determined by measurement of soluble [32P]Pi, release from [32P]PiPA using substrates presented in Triton X-100/PA mixed micelles as described previously [18].

Gel-filtration chromatography of 3T3-L1 adipocyte extracts

3T3-L1 adipocytes were homogenized by glass/Teflon in a minimal volume of TES buffer [255 mM sucrose, 1 mM EDTA, 0.1 mM DTT (dithiothreitol) and 1 mM Tris/HCl (pH 7.4)] supplemented with protease inhibitors. Extracts were centrifuged at 16,000 g for 10 min and the resulting supernatants subjected to centrifugation at 53,000 rev./min (in a Ti-90 rotor) for 60 min at 10°C. The soluble fraction was filtered through a 0.45 μm cellulose acetate syringe filter, and 400 μl representing 1.0–1.6 mg of total protein was loaded on a Superose 6 10/300 GL column equilibrated with buffer A. The sample was eluted at a flow rate of 0.1 ml/min and 1 ml fractions were collected. The columns were calibrated with molecular mass standards obtained from GE Healthcare/Pharmacia. PAP activity measurements were performed on 40 μl aliquots for 20 min at 37°C. In some cases samples were pre-incubated in 10 mM Triton X-100 or 1% fatty-acid-free BSA for 15 min at room temperature with continuous mixing before loading on to the column.

Gel-filtration chromatography of recombinant lipin 1b protein

Concentrated protein (50 μl) representing approx. 10 μg of purified lipin 1b was analysed by gel-filtration chromatography on a Superose 6 10/300 GL column. The flow rate used in the separation was 0.5 ml/min with PBS (pH 7.4). The columns were calibrated with molecular mass standards obtained from GE Healthcare/Pharmacia. After 14 min, 30 fractions of 0.5 ml of each were collected. PAP activity measurements of gel-filtration fractions of recombinant lipin 1 were performed essentially as described previously [16]. Briefly, 65 μl of column eluates were assayed for times up to 60 min (<20% substrate hydrolysis) at 30°C in 100 μl volumes of reaction buffer [50 mM Tris/HCl (pH 7.4), 1 mM MgCl2, 2 mM Triton X-100, 2 mg/ml fatty-acid-free BSA, and 0.1 mM PA (with ~20 000 d.p.m. of 32P-labelled PA made by phosphorylation of DAG with bacterial DAG kinase)] for the release of soluble [32P]Pi, from Triton X-100/[32P]PA mixed micelles.

BN-PAGE (blue-native PAGE)

HEK-293A cells cultured in 35 mm plates were transfected with pcDNA3 (vector), lipin 1b–V5 or lipin 1b–V5 (D712E). At 36 h later, cells were lysed in 100 μl of lysis buffer [50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 1% digitonin and Complete™ Protease Inhibitor Cocktail]. Following centrifugation, 15 μl of samples were loaded on to NativePAGE™ Novex Bis-Tris Gel system (Invitrogen) and transferred on to a PVDF membrane, according to the manufacturer’s protocol. The proteins on the membrane were fixed with 8% acetic acid and probed with an anti-V5 antibody and HRP-coupled secondary antibody.

RESULTS

Lipin proteins self-associate in mammalian cells

COS-7 cells were transiently transfected with plasmid constructs encoding N-terminally HA-tagged lipin 1b, with or without C-terminally V5-tagged lipin 1a, 1b, 2 or 3 respectively. At 36 h later, the cells were homogenized in stringent lysis buffer (0.5% Triton X-100 and 250 mM NaCl) and epitope-tagged proteins were precipitated with anti-V5–agarose. As shown in Figure 1(A), HA–lipin 1b was co-immunoprecipitated with all of the V5-tagged lipin isoforms. In a reciprocal co-immunoprecipitation experiment, HA-tagged lipin 1a or lipin 1b also associated with lipin 1b–V5 (Figure 1B). As reported previously [16], the V5-tagged lipin 2 was expressed at lower levels than V5-tagged lipin 1 or 3 (Figure 1A). However, despite decreased expression, V5-tagged lipin 2 still co-immunoprecipitated lipin 1 at levels proportionate to its expression. Next, since LPP family proteins are also PAPs and have been reported to form homo- or hetero-oligomers [25], we examined the possible interaction between lipin 1b and LPP3, a representative LPP. As shown in Figure 1(C), HA–lipin 1b did not co-immunoprecipitate with V5-tagged LPP3, arguing that lipin 1 associates with itself and other lipin isoforms, but not with LPPs. In the
subsequent experiment we found that endogenous lipin 2 was
immunoprecipitated with anti-lipin 1 antibodies from the lysates
of differentiated 3T3-L1 cells. This indicates that the lipin–lipin
associations are not a result of ectopic expression of the
proteins (Figure 1D). Whereas we failed to detect lipin 1 in
anti-lipin 2 immunoprecipitates from 3T3-L1 lysates, this could
be explained by the possibility that association with lipin 1
might prevent the accessibility of the lipin 2 epitope to the anti-
lipin 2 antibody. Finally, immunoprecipitation of adenovirally
delivered HA-tagged lipin 1 or lipin 2 in 3T3-L1 adipocytes
revealed that endogenous lipin 1 and 2 were able to interact
with recombinant lipin 1 and 2 (Figure 1E). Taken together, these
co-immunoprecipitation experiments reveal that lipin proteins can
form homo- or hetero-oligomers in vivo.

**Lipin 1 forms stable oligomers**

Next, we examined the biochemical properties of lipin oligomers
by identifying experimental conditions that would allow us to
reversibly assemble and disassemble these complexes in vitro.
Co-ordination of Mg\(^{2+}\) in the active site is necessary for the
PAP activity of lipin 1 [1]. However, lipin oligomerization was
observed in the presence of both EDTA and EGTA indicating
that these associations are divalent-cation-independent. PAP
Mapping the oligomerization of lipin 1

Oligomerization of lipin 1 is dependent on NEM-sensitive cysteine residues \[16,26\]. However, as shown in Figure 1(F), washing immunoprecipitates with a buffer containing either oxidants NEM or H\(_2\)O\(_2\), or the reducing agent DTT, did not affect lipin 1b self-association (Figure 1F, lanes 6, 7 and 8). This suggests that lipin 1 oligomerization is redox-insensitive, and does not involve formation of intermolecular disulfide bonds. Lipin 1 is a phosphoprotein, and phosphorylation of lipin 1 may play a regulatory role in mediated oligomerization. However, we found that lipin 1 oligomerization appears to be independent of its phosphorylation status, because incubation of the immunoprecipitates with \(\lambda\)PPase, which effectively dephosphorylated the protein as indicated by altered mobility on SDS/PAGE (Figure 1F), did not impair the lipin–lipin interaction (Figure 1F, lane 9). Finally, the interaction between V5–lipin 1b and HA–lipin 1b was resistant to a high concentration of salt (2 M NaCl) and moderate concentration (2 M) of urea (Figure 1F, lanes 5 and 10), suggesting that lipin 1 oligomers are stable even at increased ionic strength or in the presence of a denaturant.

Mapping the domains of lipin 1 in mediating oligomerization

We next investigated the regions of lipin 1 that are necessary for self-association. A series of N- or C-terminally truncated variants of lipin 1b with a C-terminal V5 tag was constructed and co-expressed with HA–lipin 1b in COS-7 cells. The interactions between the full-length and the truncated mutants of lipin 1b were determined by co-immunoprecipitation. As shown in Figures 2(A) and 2(B), full-length lipin 1b interacted with all of the N- and C-terminal fragments of lipin 1b employed, suggesting that the N-terminal (amino acids 1–171) and C-terminal (amino acids 469–924) regions of lipin 1b simultaneously mediate lipin 1 oligomerization. Using dimerization, the simplest organization form of lipin 1, as an example, there are two possible models for lipin–lipin association: (i) ‘head-to-head/tail-to-tail’, and (ii) ‘head-to-tail/tail-to-head’. To test these two possibilities, glutathione–Sepharose-bound GST–lipin 1b-(1–641) or lipin 1b-(642–924) from bacteria was incubated with V5–lipin 1b-(1–171) or V5–lipin 1b-(469–924) immunopurified from HEK-293A cells, and interacting proteins were evaluated by immunoblotting with anti-V5 antibody. As shown in Figure 2(C), the N-terminal part of lipin 1b only interacted with the N-terminus, whereas the C-terminal part preferred to associate with the C-terminus. Our results thus suggest a model that lipin 1 oligomerizes through ‘head-to-head/tail-to-tail’ interactions.

Co-localization of lipin family members in cells

Lipins exhibit variable subcellular localization patterns. As shown in Figure 3(A), except for lipin 2 which is mainly excluded from nucleus, other lipin isoforms including lipin 1a, 1b and 3 are targeted to both cytosol and nucleus in the COS-7 cells. Co-expression of HA-tagged lipin 1b with individual V5-tagged lipin isoforms revealed substantial co-localization (as shown by yellow immunofluorescence in the merged panels of Figure 3B). Lipin 1 also showed a co-localization with lipin 2 outside of the nucleus (Figure 3B). Co-localization of these lipin isoforms is consistent with their ability to self-associate as revealed by the experiments shown in Figure 1.

Demonstration of intermolecular interactions between lipin monomers in cells by FRET

The results shown in Figure 3 indicate that lipin isoforms are co-localized in cells, but provide no information about the proximity of these proteins. To address this issue, lipin 1 was tagged at the C-terminus with either Venus fluorescent protein...
or Cerulean fluorescent protein, and energy transfer from the donor (Cerulean–lipin 1) to the acceptor (Venus–lipin 1) was determined by spectral imaging. Venus–lipin 1 and Cerulean–lipin 1 showed extensive co-localization, as expected from the immunofluorescence imaging results (Figure 4A). In the event of close opposition of the fluorescent tags, such as occurs upon interaction of the tagged proteins, energy from the donor molecule can be transferred to the acceptor molecule by a process termed FRET. Because FRET is dependent on proximity, fluorophores must be within ∼1–10 nm of each other for energy transfer to occur, a distance that also typically occurs during protein–protein interaction. Excitation at 458 nm in cells where Venus–lipin 1 and Cerulean–lipin 1 were co-expressed led to an increase in emission at 540 nm when compared with either alone (Figure 4B). To quantify the amount of energy transferred by excitation of the donor to emission of the acceptor requires subtraction of the contaminating DSBT and ASBT. For example, excitation of either Cerulean or Cerulean–lipin 1 alone at 458 nm gives a peak emission at 500 nm; however, DSBT can be seen in the emission at 540 nm in double-labelled cells (Figure 4B). Whereas excitation of YFP or Venus–lipin 1 at 514 nm gives a peak emission at 540 nm, excitation at 458 nm also leads to ASBT emission at 540 nm, albeit at a reduced level compared with excitation at 514 nm. In order to subtract DSBT and ASBT from the actual FRET signal, linear unmixing was used to remove the DSBT from the FRET channel, and a computer algorithm was employed to identify and remove contaminating ASBT from the FRET signal [21,22]. The resulting values, termed PFRET (processed FRET) showed that the efficiency of energy transferred from the donor to the acceptor (E%) was 21.7% [± 4.66 (S.D., n = 5)] which is similar in magnitude to the energy transfer efficiency observed for a number of other well-characterized protein–protein interactions such as CFP- and YFP-tagged C/EBPα (CCAAT/enhancer-binding protein α) homodimers at 23% [± 5% (S.D.)] [21]. That the energy transfer E% values are not dependent on acceptor concentration is shown in Figure 4(C), where the calculated E% values from a single cell are plotted against the acceptor intensity (intensity/pixels). Non-specific transfer of energy (E%) that is dependent on acceptor intensity gives a correlation coefficient approaching 1, whereas the

Figure 3  Co-localization of lipin 1 and lipin 1, 2 and 3 in cells
(A) COS-7 cells were transfected with vectors for expression of V5-tagged lipin 1a, 1b, 2 or 3, or HA-tagged lipin 1b. At 36 h after transfection, the cells were fixed and subjected to immunostaining with an anti-HA or anti-V5 antibody. (B) COS-7 cells were co-transfected with HA-tagged lipin 1b and V5-tagged lipin 1a, 1b, 2 or 3. At 36 h after transfection, the cells were subjected to immunostaining with an anti-HA or anti-V5 antibody. Red, HA–lipin 1b; green, V5-tagged lipin isoforms; blue, Hoechst 33342.

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Figure 4  FRET analysis of fluorescent-tagged lipin 1

(A) Co-localization of Venus–lipin 1b and Cerulean–lipin 1b. Images were merged using ImageJ. (B) Single cell example of uncorrected emission spectra from co-transfected Venus–lipin 1b and Cerulean–lipin 1b (Co-transfection) at 514 or 458 nm, and single-transfected Venus–lipin 1b (Acceptor alone) or Cerulean–lipin 1b (Donor Alone) excited at 458 nm. (C) Upper panel: intensity map showing E% of a representative cell where spectral bleed-through of both donor and acceptor have been removed by linear unmixing and a computer algorithm (PFRET). Lower panel: graph of E% against the acceptor intensity of the individual cell shown above. (D) Decay curve for cells transfected with either Cerulean–lipin 1b (Cer-lip) alone or Cerulean–lipin 1b and Venus–lipin 1b (Cer-lip + Ven-lip). (E) Average decay time (ns) per cell for Cerulean–lipin 1b (Cer-lip) and Cerulean–lipin 1b + Venus–lipin 1b (Cer-lip + Ven-lip). *P < 0.00001.

correlation coefficient of the fluorescently tagged lipin 1 protein(s) in this representative example was −0.284, showing that the calculated E% was independent of acceptor concentration. Taken together, these results indicate that the association between lipin isoforms observed using biochemical approaches in cell lysates reflects close interactions between these proteins in live cells.

As a second measure of FRET we employed FLIM to measure changes in fluorescence decay of the donor in the presence of an acceptor. We measured the lifetime of the donor, Cerulean–lipin 1b, with and without the acceptor, Venus–lipin 1b, in live HEK-293T cells. In order to do so, the fluorophores were excited by a single laser pulse at 820 nm and the photons released during the return of the fluorophore to the ground state from the excited state were captured and displayed as data points. SPCImage software generated a fitted curve based on these data that verified an exponential decay of the fluorophore. Figure 4(D) compares the fluorescence decay of Cerulean–lipin 1 alone with Cerulean–lipin 1 plus Venus–lipin 1. In this example the presence of Venus–lipin 1 decreases the slope of the decay curve of Cerulean–lipin 1. Quantification of multiple cells reveals that this is significant with the average decay time of Cerulean–lipin 1 decreasing from 2.226 ns [± 0.05 (S.D., n = 14)] to 2.101 ns [± 0.09 (S.D., n = 16, P < 0.00001)] in the presence of Venus–lipin 1, providing additional support for the Venus and Cerulean fluorophores being in close enough apposition for energy to be transferred from the donor to the acceptor.

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Figure 5  Forced plasma membrane targeting of lipin 1 via a CAAX domain can recruit soluble lipin 1 to the plasma membrane

COS-7 cells were transfected with plasmids for expression of CAAX-tagged lipin 1b and/or Myc-tagged lipin 1b. At 48 h after transfection, cells were fixed and subjected to immunostaining with anti-HA and/or anti-Myc antibodies. Transfections are indicated to the left-hand side, and immunostaining is shown at the top. Red, HA–lipin 1b–CAAX; green, Myc–lipin 1b; blue, DAPI.

**Forced relocalization of lipin 1 in cells**

Subcellular fractionation studies indicate that lipin 1 translocates between the cytosol and the endoplasmic reticulum/microsomal membranes in a dynamic manner dependent on its phosphorylation state and fatty acid availability [3,27]. However, when visualized by immunofluorescence, intracytosolic changes in lipin 1 localization are subtle (results not shown). Therefore to test the hypothesis that lipin self-association could lead to redistribution of lipin isoforms in cells, we determined whether co-expression of a plasma-membrane-targeted lipin 1b variant (see below) could redirect cytosolic lipin 1b to the plasma membrane through oligomerization. We employed a Myc-tagged lipin 1, which is localized diffusely in transfected COS-7 cells, with undetectable plasma membrane staining (Figure 5, upper panels). In addition, we developed a plasma-membrane-targeted lipin 1b (lipin 1b–CAAX) construct which, upon overexpression, showed clear plasma membrane localization in addition to the whole-cell distribution (Figure 5, middle panels). The plasma-membrane-targeted lipin 1b was obtained by fusing a CAAX-box motif to the C-terminal tail of lipin 1b, to direct prenylation of lipin 1 [28]. Co-transfection of both constructs resulted in the easily observable relocalization of Myc–lipin 1 to the plasma membrane. A portion of the images are shown at a larger size at the bottom.

Similar results were seen with lipin 2 co-transfected with lipin 1–CAAX (results not shown). These results demonstrate that oligomerization between lipin isoforms can direct intracellular localization.

**Estimation of the native size of endogenous and recombinantly expressed lipin 1**

Taken together, the results presented indicate that lipin proteins can self-associate to form oligomeric complexes that can be isolated biochemically and visualized by fluorescence imaging. The NEM-sensitive PAP activity (a hallmark feature of the lipin enzymes) of rat liver cytosol was reported to elute with a molecular mass of 540 kDa during gel-filtration chromatography [29]. Because 3T3-L1 adipocytes express high levels of lipin 1 protein, we analysed soluble fractions of these cells by Superose 6 gel-filtration chromatography to determine the size of native lipin 1. As detected by Western blotting, lipin 1 and lipin 2 were eluted from the column with a molecular mass of 500–600 kDa; the same size observed for the majority of the PAP activity (Figures 6A and 6B). Although the predicted molecular mass of the lipin 1, 2 and 3 polypeptides is ~90–100 kDa, on SDS/PAGE these proteins migrate aberrantly with an apparent molecular mass of ~140–150 kDa. In the case of lipin 1, phosphorylation accounts for part,
Oligomerization of lipin 1

Figure 6 Lipin 1 exists as an oligomer in 3T3-L1 adipocytes

(A) Column fractions from Superose 6 gel-filtration chromatography of 3T3-L1 adipocyte cytosolic extract immunoblotted for lipin 1 and 2. The bottom panel is same extracts incubated with 10 mM Triton X-100 (TX-100) before loading on to the column. (B) Lipin 1 immunoreactivity (arbitrary units) in column fractions against Mg2+-dependent PAP activity (nmol/min per ml).

but not all, of the unexpected decrease in mobility on SDS/PAGE [3]. Gel filtration measures the hydrodynamic radius of a molecule, not the molecular mass, and the tertiary structure of lipin molecules could partially account for the discrepancy in its elution from the column. Alternatively, unidentified post-translational modifications other than phosphorylation could produce changes in lipin migration on SDS/PAGE and elution by size-exclusion chromatography. Indeed, a proportion of lipin 1 has been recently reported to be SUMOylated, a covalent modification of ~17 kDa [30]. Nevertheless, it is highly unlikely that post-translational modification or the shape of individual lipin molecules would change the elution profile from a molecular mass of 100 kDa to a molecular mass of 500–600 kDa. Based purely on the predicted molecular mass of lipin 1, tetramerization of lipin 1 would produce an molecular mass of approx. 400 kDa if the lipin tetramer adopted a mostly spherical shape. Therefore a combination of oligomerization, probably tetramerization, molecular shape and possibly post-translational modifications probably account for size of lipin 1, as measured by size-exclusion chromatography. Addition of 10 mM Triton X-100 to cytosolic extracts before loading on to the column caused a partial shift in the lipin 1 elution profile with the addition of the predicted molecular mass of a Triton X-100 micelle, whereas treatment of extracts with 1% BSA was without effect (Figure 6A and results not shown).

We also examined the molecular mass of recombinant lipin 1 that was expressed in insect cells and purified by metal-ion-affinity chromatography. Similar to our observations with endogenously expressed lipin 1 in 3T3-L1 cells, purified lipin 1 eluted as a discrete peak of molecular mass ~600 kDa, coincident with the elution pattern of PAP activity from the column (Figures 7A and 7B). Isolation of mono/di/tetrameric lipin 1 would allow functional studies to determine whether the different oligomeric forms of lipin 1 display differing enzymatic activities. In an attempt to obtain fractions containing lipin 1 species with molecular masses that are consistent with those of a monomer or dimer we employed a Superdex 200 column to increase the resolution of recombinant lipin 1b were collected and analysed for PAP activity. (B) Immunoblotting of column fractions. (C) HEK-293A cells transiently expressing V5–lipin 1b or V5–lipin 1b (D712E) were homogenized in digitonin-containing lysis buffer, the lysates were separated by BN-PAGE and subjected to immunoblotting with an anti-V5 antibody. The molecular mass in kDa is indicated on the left-hand side. WB, Western blot.

As an alternative method to gel-filtration chromatography, extracts from HEK-293A cells expressing V5-tagged lipin 1b were subjected to BN-PAGE in an attempt to preserve the native molecular structure. As shown in Figure 7(C), immunoblotting with an anti-V5 antibody clearly showed that native non-denatured lipin 1b existed in two forms with a molecular mass slightly more than 2- and 4-fold that of the predicted lipin 1b–V5 monomer (compared with the molecular mass with SDS/PAGE in Figure 1A). Like gel filtration, native PAGE measures hydrodynamic radius, and the more the divergence from a spherical shape the larger the molecule or molecular complex will appear by these chromatographic methods. These experiments suggest that both endogenous and recombinant lipin 1 exists primarily as tetrameric complexes both in cells and when purified in vitro. Although more detailed analyses will be required to rule out the presence of other non-lipin proteins in these complexes, our determination of the molecular mass of recombinant lipin 1 by gel-filtration chromatography and by non-denaturing electrophoresis argue that the major lipin 1 species is a tetramer.
The relationship between oligomerization and the catalytic activity of lipin 1

Clearly lipin 1 variants with altered self-association would be valuable reagents to probe the regulation and biological importance of this interaction. We therefore examined the self-association of a series of previously described lipin 1 mutants with defects in enzymatic activity of phosphorylation. As shown in Figure 8, lipin 1b (G84R), a natural mutation that results in a mouse fatty liver dystrophy phenotype and reduces lipin enzymatic activity [2,3], lipin 1b (D712E), a catalytic site mutation that eliminates PAP activity [3], and the coactivator-deficient mutant lipin 1b (Lmut) [14], retained the ability to associate with V5–lipin 1b. Similar results were obtained with other single (or double)-site lipin 1 mutants we generated (e.g. SUMOylation-deficient lipin 1 mutant [30], lipin phosphorylation site mutants and others), which are predicted to be deficient in various post-translational modifications (results not shown). Taken together with the results shown in Figures 1(F) and 2, these findings suggest that lipin 1 oligomers are stable and difficult to disrupt by single (or double) amino acid mutations. This could result from a ‘double security’ mechanism for oligomerization that utilizes both N- and C-terminal interactions. Our results also indicate that the loss-of-function phenotypes associated with the synthetic and naturally occurring lipin 1 mutations noted above do not result from defects in self-association.

We next determined how the lipin 1 monomers function as PAP enzymes in the context of oligomers. Since our findings suggest that lipin 1 self-associates in a ‘head-to-head/tail-to-tail’ orientation, we postulated that the PAP activity might require two functional catalytic sites in physical proximity to function. To test this we co-expressed catalytically deficient HA-tagged lipin 1b (D712E) with Myc-tagged lipin 1b in HEK-293T cells. The PAP activity in lysates of Myc-tagged lipin 1b trans-fected cells was unaffected by increasing amounts of the HA-tagged D712E lipin 1b mutant (results not shown), demonstrating that PAP-null lipin 1 does not act in a dominant manner to inhibit the PAP activity of its oligomeric partner(s).

We also verified that the measured PAP activity came from HA–lipin1b-D712E/mict–lipin 1b oligomers by immunoprecipitation (Figure 9A). Although tetrameric combinations preclude a simple 1:1 stoichiometry within each lipin 1 oligomer at equimolar amounts, the selective use of epitope tags suggests that there will be at least one mutant or wild-type lipin 1b within each immunoprecipitated oligomer. We speculated that if a catalytically inactive monomer of lipin 1b negatively interferes with the PAP activity of its wild-type lipin 1b oligomeric partner, then HA–lipin 1 (D712E) immune complexes would not contain detectable PAP activity. In fact, we detected a linear increase in the PAP activity contained in HA–lipin 1 (D712E) immune complexes with the increasing amount of transfected wild-type lipin 1b (Figure 9B). As a control, immunoprecipitated HA–lipin 1b (D712E) itself showed no detectable PAP activity and anti-HA antibodies did not immunoprecipitate catalytically active Myc-tagged lipin 1b (Figure 9B). These observations suggest that lipin 1 monomers function independently in catalysing dephosphorylation of PA (Figure 9C).

**DISCUSSION**

In the present study, we demonstrate that lipin proteins self-associate and appear to exist predominately as stable homo- and hetero-dimers/tetramers in vitro and in vivo. Our evidence...
for this includes observations from co-immunoprecipitation studies in which both overexpressed and endogenous lipin 1 robustly interacts with itself and other lipin isoforms. Gel-filtration and native protein electrophoresis suggests that both native and recombinant lipin 1 exists as a dimer and tetramer with little if any monomeric protein present. The three lipin isoforms are extensively co-localized in transfected cells, and the spatial distance between lipin monomers is close enough to allow for efficient FRET. Mutational studies combined with co-immunoprecipitation and in vitro GST-pull-down experiments support a model in which lipin monomers self-associate in a ‘head-to-head/tail-to-tail’ orientation. Catalytically inactive lipin 1 (D712E) mutant was able to efficiently pull down PAP activity when co-expressed with wild-type lipin 1 in cells, indicating that the active sites of individual lipin 1 monomers operate independently in the oligomer.

Our observations raise the interesting possibility that oligomerization plays an important role in lipin 1 function. It is conceivable that the lipin 1 oligomers may be more stable or trafficked differentially to the appropriate cellular locale. Alternatively, oligomerization could directly affect some of the functions of lipin 1. For example, oligomerization could facilitate the ability of lipin 1 to function as an adapter protein to regulate gene transcription. Lipin 1 is predicted to simultaneously bind PPARα, PGC-1α, and p300 [14]. In this model, it is feasible that the dimerization/tetramerization of lipin 1 allows it to interact with each component of the transcriptional machinery to exert its ‘scaffold’ functions. The observation that lipin 1, lipin 2 and lipin 3 can all interact with PPARα ([14] and H. B. Kim and T. E. Harris, unpublished work) suggests a possibility that they could be recruited to the gene promoter and activate transcription in a hetero-oligomeric form, perhaps in specific physiological contexts.

Our results also indicate that the catalytic activity of lipin 1 oligomerization is not necessary for its oligomerization. This conclusion is supported by several observations, including: (i) lipin 1 oligomerization does not require Mg2+: (ii) NEM, a thiol-targeting agent that abrogates the PAP activity of lipin 1, has no effect on oligomerization; (iii) enzymatically inactive lipin 1 mutants can still form oligomers; and (iv) wild-type lipin 1 and enzymatically inactive lipin 1 function independently in oligomers.

In lower organisms, such as yeast and worms, there is only one lipin isoform [2]. The present study suggests that in higher organisms that express more than one lipin isoform hetero-oligomerization could produce lipin complexes with unique functions. For example, mammalian lipin 2 and 3 show a lower Vmax for catalytic activity compared with lipin 1 [16]. Given that each lipin monomer functions independently as a phosphatase within the oligomeric complex, hetero-oligomers containing lipin 1 would be predicted to have a higher PAP activity than lipin 2 or 3 homo-oligomers. In liver, lipin 2 is expressed at much higher levels than lipin 1 [18]. However, lipin 1 is robustly induced by stimuli that increase hepatic PAP activity (e.g. fasting and glucocorticoids; [14]), which probably serves to increase lipin 1 content or proportion in hetero-oligomers. In addition, lipin 2 demonstrates a different intracellular localization when compared with lipin 1, and may be enriched in the nuclear envelope [31], where the lipin phosphatase Dullard/NET56 is localized [32,33]. Thus hetero-oligomerization between lipin 2 and lipin 1 in the tissues where they are both expressed (such as liver and adipocytes) could allow a lipin-2-mediated recruitment of lipin 1 on to the nuclear envelope in order to obtain a high level of PAP activity at the nuclear periphery. As expression of the lipin isoforms approach parity, it seems reasonable to conclude that there will be a great likelihood of forming heterodimers with resulting changes in specific activity as well as subcellular localization.

Homo-oligomerization may be related to substrate-induced lipin 1 activation. Allosterically activated enzymes are frequently multisubunit protein complexes. For example, ACAT1 (acyl-coA:cholesterol acyltransferase 1) is thought to have separate dimerization domains in the N- and C-termini, similar to what we have observed with lipin 1 [34]. In a mixed micelle assay system, purified ACAT1 exhibits allosteric activation by its substrate, cholesterol [35]. Purified Pah1p, the yeast lipin homologue, shows positive co-operative kinetics in its activation by its substrate PA, indicative of two PA-binding sites and suggestive of allosteric activation [36,37], although it remains to be demonstrated that Pah1p forms oligomers. Mammalian lipin 1 also shows positive co-operative kinetics with PA, although it is unclear whether this is due to co-operative interaction between lipin and its substrate or allosteric activation of lipin enzymatic activity by the substrate [6,16]. Interestingly, the inhibition of rat lipin 1 by acyl-CoAs and fatty acids has been described as occurring through a negative allosteric interaction [38]. Whereas the lipin 1 monomers function independently in dephosphorylating PA within the oligomeric complex, it is possible that full lipin catalytic activity, particularly with regard to substrate co-operativity, requires oligomerization. To definitively determine whether lipin 1 oligomerization regulates allosteric activation by substrate will require identification of lipin mutants that fail to oligomerize and/or the isolation and characterization of lipin monomers. Unfortunately our efforts to generate such mutants and to isolate monomeric lipin 1 have been unsuccessful, so further work will be required to test these interesting possibilities.

The last two enzymatic reactions in triacylglycerol synthesis are lipin-mediated dephosphorylation of PA to generate DAG, followed by the acylation of DAG to form triacylglycerol (reviewed in [39]). There are two DAG acyltransferases, DGAT1 and DGAT2. DGAT1 is structurally similar to ACAT1, whereas DGAT2 is unrelated [40]. Interestingly, DGAT1 also forms homo- and hetero-oligomers [41]. Although allosteric activation of DGAT1 by DAG or acyl-CoAs has not been shown, an N-terminal fragment of DGAT1 can bind to acyl-CoA in a cooperative fashion suggestive of allosteric activation [42,43]. Like lipin family members, dimerization of DGAT1 is not required for enzymatic activity, as heterodimerization with a truncated and inactive isoform of DGAT1 did not eliminate catalytic activity of the full-length form [41]. It is curious that the two proteins that catalyse the final two steps in triacylglycerol synthesis, lipin 1 and DGAT1, both form homo- and hetero-oligomers. Future questions should address whether oligomerization of these enzymes has any functional role in channelling lipid intermediates towards esterification.

In summary, the present study reveals for the first time that lipins can form homo- and hetero-oligomers and suggests that the three mammalian lipin isoforms are tightly linked with each other and serve as an integrated unit.

AUTHOR CONTRIBUTION
Guanghui Liu, Jing Qu, Anne Carmack, Hyun Bae Kim, Chang Chen, Hongmei Ren and Thurl Harris performed the experiments. Guanghui Liu, Andrew Morris and Thurl Harris conceived and designed the experiments. Guanghui Liu, Chang Chen, Andrew Morris, Brian Finck and Thurl Harris analysed the data and wrote the paper. All of the contributing authors critically reviewed the manuscript.

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