The TIP30 Protein Complex, Arachidonic Acid and Coenzyme A Are Required for Vesicle Membrane Fusion

Chengliang Zhang\(^1,2\), Aimin Li\(^1,3\), Shenglan Gao\(^1\), Xinchun Zhang\(^2\), Hua Xiao\(^1\)*

\(^1\) Department of Biomedical and Integrative Physiology, Michigan State University, East Lansing, Michigan, United States of America, \(^2\) Genetics Program, Michigan State University, East Lansing, Michigan, United States of America, \(^3\) Department of Oncology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, People’s Republic of China

Abstract

Efficient membrane fusion has been successfully mimicked in vitro using artificial membranes and a number of cellular proteins that are currently known to participate in membrane fusion. However, these proteins are not sufficient to promote efficient fusion between biological membranes, indicating that critical fusogenic factors remain unidentified. We have recently identified a TIP30 protein complex containing TIP30, acyl-CoA synthetase long-chain family member 4 (ACSL4) and Endophilin B1 (Endo B1) that promotes the fusion of endocytic vesicles with Rab5a vesicles, which transport endosomal acidification enzymes vacuolar (H\(^+\))-ATPases (V-ATPases) to the early endosomes in vivo. Here, we demonstrate that the TIP30 protein complex facilitates the fusion of endocytic vesicles with Rab5a vesicles in vitro. Fusion of the two vesicles also depends on arachidonic acid, coenzyme A and the synthesis of arachidonyl-CoA by ACSL4. Moreover, the TIP30 complex is able to transfer arachidonyl groups onto phosphatidic acid (PA), producing a new lipid species that is capable of inducing close contact between membranes. Together, our data suggest that the TIP30 complex facilitates biological membrane fusion through modification of PA on membranes.

Introduction

Membrane fusion is well known as one of the most fundamental cellular processes in living organisms. It generally requires cellular factors to bring donor and recipient membranes into close proximity, increase membrane curvature, and disturb lipid bilayers [1,2]. Extensive studies during the past decades have led to the identification of a number of fusogenic proteins and lipids. In addition to lipids such as acyl-CoA [3,4], arachidonic acid [5,6], phosphoinositides, phosphotidic acid and diacylglycerol [7,8,9], many proteins including Rab5, Rab5 effectors, SNARE proteins and SNARE accessory factors are necessary for various membrane fusion events [10,11].

Recently, efficient endosome fusion was successfully mimicked using protoliposomes reconstituted with 17 recombinant core fusion proteins [2,12]. Notably, however, these proteins are not sufficient to promote efficient fusion between biological membranes, indicating that biological membrane fusion requires more cellular factors than artificial membrane fusion. Given that purified endosomes contain the core SNARE proteins Syntaxin6, Syntaxin13, Vti1a and VAMP4, it is likely that factors needed for initiating membrane fusion have yet been identified.

Among the lipids that play key roles in membrane fusion, arachidonic acid has been shown to be required for endosome fusion in vitro [6], and is the most effective fusogen in chromaffin granule fusion [13]. In addition, membrane-bound arachidonic acid can drive annexin II-mediated membrane fusion of the lamellar body with the plasma membrane during the exocytosis [5].

We have recently identified a protein complex containing TIP30, ACSL4 and Endo B1 that interacts with Rab5a and facilitates the loading of V-ATPases on endocytic vesicles. Inhibiting any of these proteins causes the mislocalization of V-ATPases, thus leading to the trapping of EGF-EGFR complexes in endocytic vesicles, delayed EGFR degradation and sustained EGFR endosomal signaling [14]. In addition, we have shown that Rab5a and V-ATPase reside in vesicles devoid of EGFR, the early endosomal marker EEA1 and the recycling endosomal marker transferrin receptor (TIR), suggesting that Rab5a functions as a identity tag for vesicles that deliver V-ATPases to endosomes [14]. Given that localization of integral membrane proteins to their target membranes requires vesicle membrane fusion [15], we therefore examined if the TIP30 protein complex can promote vesicle membrane fusion in vitro. Since ACSL4 was identified in the protein complex and it is an acyl-CoA synthetase that prefers arachidonic acid as the reaction substrate, we included both arachidonic acid and coenzyme A in the in vitro membrane fusion reactions. We found that the TIP30 complex and the synthesis of arachidonyl-CoA from arachidonic acid and coenzyme A are required for efficient fusion of Rab5a vesicles with endocytic vesicles. The TIP30 complex may initiate membrane fusion by modifying membrane PA.

Results

The TIP30 complex, arachidonic acid and coenzyme A promote fusion between endocytic and Rab5a vesicles

To investigate whether the TIP30 complex can promote membrane fusion in vitro, we used a confocal microscopy-based in vitro
fusion assay [16,17] to monitor fusion between endocytic and Rab5a vesicles in an initial pilot experiment. Endocytic and Rab5a vesicles were prepared from HepG2 cells that do not contain detectable endogenous TIP30 and EGFR. Rab5a vesicles were labeled by expressing EYFP-Rab5a fusion proteins and prepared from serum-starved cells. Endocytic vesicles were labeled by expressing EGFR-DsRed fusion proteins and prepared from EGF treated cells. The two types of vesicles that contain equal amount of proteins were incubated in the fusion buffer at 37°C followed by examination with confocal microscopy. Vesicle fusion and aggregation were represented by the fluorescence overlap between EGFR-DsRed and EYFP-Rab5a.

Since ACSL4 is an acyl-CoA ligase with high substrate preference for arachidonic acid (C20:4) [18], we first tested if arachidonic acid and coenzyme A are needed. Vesicles resulting from reactions that were kept on ice were evenly distributed on the slides, appearing as small vesicles with low fluorescence intensity and no fluorescence overlap (Figure 1A, lane 1; Figure 1B). Similarly, no fluorescence overlap was observed in the absence of arachidonic acid or in the presence of triacsin C (10 μM), a potent inhibitor of ACSL4 (Figure 1A, lanes 4 and 6; Figure 1B). In contrast, in the presence of arachidonic acid and coenzyme A, immunopurified TIP30 complex caused vesicle enlargement and significantly increased fluorescence overlap (TIP30 complex: 50±6%; control eluates: 20±3%; omitting coenzyme A: 17±2%; omitting GTP: 16±4%; n = 6 representative confocal images of 143×143 μm, p<0.01 versus TIP30 complex; Figure 1A and 1B), thereby resulting in much intensified fluorescence. Rab5a vesicles attached to aggregated endocytic vesicles, but remained as small particles when the TIP30 complex, coenzyme A, or GTP was omitted. The effect of GTP exclusion is consistent with the fact that GTP is a known membrane fusion factor and required for Rab5a function. We next screened for other fatty acids that might promote membrane fusion, including palmitic, palmitoleic, oleic, linoleic, linolenic, eicosapentaenoic, and docosahexaenoic acids. None of these fatty acids could promote vesicle enlargement and fluorescence overlap (data not shown). Furthermore, arachidonic acid significantly increased fluorescence overlap in the presence of HeLa cell cytoplasmic S100 extracts that containing the TIP30 complex (HeLa S100: 2±2%; HeLa S100+arachidonic acid: 50±5%; n = 6 representative confocal images of 143×143 μm, p<0.01 versus HeLa S100; Figure 1C). These results suggest that arachidonic acid and the TIP30 complex are involved in membrane aggregation and/or fusion, which are consistent with our in vivo data [14] and previous reports that arachidonic acid is essential for vesicle membrane fusion [6,13].

To determine whether that vesicle enlargement and fluorescence overlap were due to membrane fusion or aggregation, we examined the vesicles that resulted from fusion reactions by transmission electron microscopy (TEM). Without the addition of arachidonic acid, all vesicles appeared spherical and ranged in diameter from 50 to 300 nm. In contrast, the complete fusion reaction led to the production of vesicles enlarged to more than 1 μm in diameter (Figure 2A); the larger vesicles were not found in reactions without arachidonic acid. Ongoing fusion could also be observed. Quantitative analysis revealed a significant increase in the amount of enlarged vesicles (with arachidonic acid: 300–500 nm, 24±2%; >500 nm, 16.5±1.5%; without arachidonic acid: 300–500 nm, 1.2±0.2%; >500 nm, 0; n = 150, p<0.01; Figure 2B). Given the fluorescent overlap between two types of vesicles in the complete reactions shown in Figure 1, these results indicate that the TIP30 complex and synthesis of arachidonyl-CoA are essential for the fusion of Rab5a vesicles with endocytic vesicles.

Figure 1. The TIP30 protein complex, arachidonic acid and coenzyme A promote the fusion between endocytic and Rab5a vesicles. (A) Aliquots of isolated EGFR-DsRed and EYFP-Rab5a vesicles (both contain 20 μg of proteins) were mixed and incubated in reactions (20 μl) with the indicated components. The resulting fusion products were spotted on glass slides and images were taken using confocal microscope. Panels 1, 4 and 6 were scanned with 3× amplification gain setting due to lower fluorescence intensity of individual vesicles. Arachidonic acid (100 nmol) was used in the reactions. Images are single plane and are representative for at least three independent experiments. Scale bars, 5 μm. (B) Signal overlap was quantified using MBF_ImagelJ. Pearson’s colocalization coefficients were calculated from three independent experiments and were converted to percentages. Data represent means ± SEM. **P<0.01, ***P<0.001; t test. (C) Arachidonic acid promotes the vesicle fusion induced by HeLa cell S100. S100 fractions (4 mg/ml) of HeLa cells were incubated with isolated EGFR-DsRed and EYFP-Rab5a vesicles (both contain 20 μg proteins) in the absence or presence of 100 nmol arachidonic acid. Resulting vesicles were examined using confocal microscopy (left panel) and fluorescence overlaps were quantified (right panel). doi:10.1371/journal.pone.0021233.g001

Recombinant TIP30, ACSL4 and Endo B1 can replace the TIP30 complex in promoting fusion of endocytic vesicles with Rab5a vesicles

To exclude possible influences of other associated or contaminating proteins in immunopurified TIP30 complexes, we replaced
the immuno-complexes with bacterially-expressed recombinant TIP30, ACSL4 and Endo B1 in cell free assays. The three highly purified recombinant proteins (Figure 3A) can efficiently promote fluorescence overlap (75±6%), whereas lack of any of these proteins led to significantly less overlap (TIP30: 18±1%; TIP30 and ACSL4: 23±1%; Endo B1: 35±3%; ACSL4 and EndoB1: 8±2%; ACSL4: 20±5%; n = 6 representative images of 143±143 μm; p<0.01 versus TIP30, ACSL4 and Endo B1; Figure 3B and 3C). TIP30M, a TIP30 mutant with a mutated putative nucleotide binding motif (GXXGXXG) [19], only promoted 24±2% overlap (n = 6 representative images of 143±143 μm; p<0.01 versus TIP30, ACSL4 and Endo B1; Figure 3B and 3C; panel 4). These data suggest that TIP30, ACSL4 and Endo B1 together can substitute for the TIP30 complex in promoting the fusion between endocytic and Rab5a vesicles.

Vesicle tethering and stacking induced by acylation of phosphatidic acid

To gain further insight into how TIP30 and its interacting proteins mediate membrane fusion, we first used [3H]arachidonic acid to label membrane lipids and found that at least one lipid species in purified early endosomes was specifically labeled by the TIP30 complex (Figure 4A, lane 2). The production of the labeled lipid was significantly less in the reaction using control eluates (lane 1) or TIP30M immunoprecipitates (lane 3), and was blocked by triacsin C (lane 5). The new lipid species was not observed when Rab5a vesicles were used in the reactions (data not shown). These data indicate that lipids on endocytic vesicles are the specific recipients of the arachidonyl group.

To identify the lipids that are modified, we performed protein-lipid overlay assays using membrane strips prespotted with 15 cellular membrane lipids (Figure 4B). TIP30 and Endo B1 specifically bind phosphatidic acid (PA) and cardiolipin. Endo B1 also binds phosphatidylinositol 4-phosphate (PtdIns(4)P; Figure 4C). ACSL4 and Rab5a did not bind any lipids spotted on the strips (data not shown). Cardiolipin is found predominantly in the inner mitochondrial membrane, whereas PA has been implicated in the fusion of various intracellular membranes [7,20]. Therefore, we focused on PA and tested whether the TIP30 complex could convert PA to PA-derivatives. Lipids extracted from the acylation

**Figure 2. Transmission electron microscopy analysis of products from in vitro vesicle fusion assays.** (A) EGFR-DsRed and EYFP-Rab5a vesicles were incubated with immunopurified TIP30 complex in the fusion buffer with (right panel) or without (left panel) 100 nmol of arachidonic acid. Resulting vesicles were stained with uranyl acetate and examined using TEM. Scale bars, 500 nm. (B) The graphs show the percentages of vesicles with different diameters. At least 6 images from two independent experiments were counted. Data represent means ± SEM. n = 150; **p<0.01, t test.
doi:10.1371/journal.pone.0021233.g002
The TIP30 Complex Promotes Vesicle Fusion

Reactions with PA and arachidonic acid as substrates were subjected to MS/MS and LC-MS/MS spectrometry analyses (Figure 5A–5C). The flow injection precursor scan in both modes revealed one predominant peak with an m/z (mass-to-charge ratio) value of 940.2, which does not match the exact mass of any known lipids in the database (lipidmaps.org). Thus, the identity of lipid product of the TIP30 complex remains unknown. However, since its mass is very close to the mass (940.85) of triacylglycerol (C_{61}H_{112}O_{6}), we speculate that it might be a triacylglycerol.

To determine how PA derivatives per se would affect membrane fusion, we carried out acylation reactions by incubating PA or phosphoinositol (PI) with the TIP30 complex in the fusion buffer. The resulting lipids were purified, resuspended by sonication, and incubated with vesicles. Interestingly, the PA derivatives promoted dramatic fluorescence overlap among endocytic and Rab5a vesicles on ice (Figure 6). In contrast, PA incubated with control eluates did not promote significant fluorescence overlap among the vesicles, nor did phosphoinositol (PI) that was prepared with the same procedure as the PA derivative. A triacylglycerol (1,2-Dilinoleoyl-3-palmitoyl-sn-glycerol) with a palmitoyl tail at the sn-3 position also increases significant fluorescence overlap (Figure 6). These results indicate that the PA derivatives possibly promote membrane aggregation and/or fusion.

Finally, we used electron microscopy to examine the vesicles after incubation with PA derivatives that were generated by either immunopurified or recombinant protein-reconstituted TIP30 complexes. (Figure 7). PA derivatives caused vesicle tethering and stacking, which is marked by dramatic deformation (Figure 7B and 7C). In contrast, the vesicle aggregation caused by triacylglycerol seemed due only to tethering (Figure 7E), indicating that these specific commercially available triacylglycerols do not have the ability to induce vesicle stacking; therefore do not represent the new lipid species generated by the TIP30 complex. Consistent with the confocal microscopy data, PA treated with control eluates (Figure 7A) or PI treated with immunopurified TIP30 complex (Figure 7D) had no apparent effect.

Nonetheless, we did not observe complete fluorescence overlap and enlarged endosomes (>0.5 μm in diameter) as seen in Figure 2, suggesting that additional activities of the TIP30 complex or other proteins on the membranes, such as SNAREs, SNARE accessory factors, Rab5a and its effectors, are needed to accomplish the fusion steps following close membrane contact. Collectively, these data indicate that TIP30, ACSL4, and Endo B1 promote vesicle membrane fusion by fatty acylating PA.

**Discussion**

Elucidating the molecular basis of intracellular trafficking and protein sorting requires the identification of the critical components participating in these processes. Our earlier studies demonstrated that proteins in the TIP30 protein complex are required for the fusion of endocytic and Rab5a vesicles in vivo. In the present study, using both confocal microscopy and transmission electron microscopy, we demonstrated that the TIP30 complex promotes membrane fusion in vitro and that arachidonyl-CoA synthesis by ACSL4, a component in the TIP30 complex, is essential for membrane fusion.

Arachidonic acid has been shown to be involved in vesicle membrane fusion [6,13]. Previous reconstituted approaches in vitro have contributed greatly to our understanding of membrane fusion. However, to our knowledge, efficient fusion between biological membranes has hardly been achieved in vivo in cell free assays without using cytosol fractions or arachidonic acid, further emphasizing the important role of arachidonic acid during membrane fusion. How does arachidonic acid promote membrane fusion? Arachidonic acid has been proposed to stimulate SNARE complex assembly [21,22,23]. Our data seem support these observations by showing that endocytic vesicles recognize Rab5a vesicles only after arachidonic acid was added to the reactions. Interestingly, fusion did not occur in the absence of the TIP30 complex or in the presence of the ACSL4 inhibitor. These results suggest that in addition to directly promoting SNARE complex assembly, arachidonic acid must be activated by esterification to stimulate membrane fusion.

We further showed that the arachidonyl group is transferred to endosomal PA to generate a new lipid species that induce vesicle tethering and stacking. Thus, we suggest a hypothesis that arachidonic acid promotes membrane fusion by contributing to both PA
acylation and SNARE complex assembly. Consistent with this view, PA and its synthetase phospholipase D (PLD) are known to participate in membrane fusion during vesicle transport [20,24]. Moreover, as integral components of many biological membranes, triacylglycerols have been demonstrated to possess great potency to promote spontaneous curvature in an acyl chain length dependent manner [25], which may alter membrane structure to support membrane fusion [1,2]. We speculate that the addition of the hydrophobic acyl chain to endosomal PA may help to overcome the repulsive hydration force generated from water bound to the lipid headgroups. In addition, triacylglycerols can exhibit an extended conformation [26] with the 3-arachidonyl group in the opposite direction of the other two acyl chains, thereby allowing for attachment and fusion between two membranes [27,28]. Our results suggest that as an initiation event, acylation of endosomal PA enables the close contact between donor and recipient membranes, thus allowing for fusion to be accomplished by SNAREs, SNARE accessory factors, Rab5 and their effectors. Nevertheless, although we favor this hypothesis to explain the role of TIP30 in membrane fusion and endocytic trafficking, we do not rule out other possible hypotheses that may also explain the actions of the TIP30 complex. Undoubtedly, efficient fusion could be achieved using artificial membranes in the absence of arachidonic acid. This view is supported by the observation that the same set of proteins could promote efficient fusion of artificial membranes but incapable of inducing efficient biological membrane fusion [12]. Furthermore, our data suggest that the TIP30 complex modifies lipids to initiate membrane fusion. This step may have been bypassed during the processes of lipid extraction and membrane reconstitution. It would be interesting to find out whether addition of arachidonic acid, CoA and the TIP30 complex could enhance artificial membrane fusion.

Clearly, more work is needed to determine how fatty acylated PA and other lipid derivatives cooperate with the action of SNAREs, Rab5 and their effectors in membrane fusion. Future studies may also focus on finding out how the TIP30 complex acylates PA and what the exact identity of the fusion products is. It is expected that a more precise mechanism underlying membrane fusion will emerge by integrating information from studies of both lipids and proteins.

**Materials and Methods**

**Reagents**

DMEM, fetal bovine serum, trypsin, penicillin, and streptomycin were purchased from Invitrogen. Anti-HA agarose beads were from Roche Applied Science. Polyclonal rabbit anti-human Rab5a...
Figure 5. Arachidonyl group was transferred onto phosphatidic acid. (A) PA was incubated with the TIP30 protein complex in the fusion buffer. Resulting lipids were purified and subjected to flow injection negative scan in the range of 400–1400 u. A predominant peak of 699.5 was
detected. Atomic mass: arachidonic acid, 304.5 u; 18:1 PA, 699.5 u. The lipids in the smaller peaks have molecular weights that do not match any of the expected PA derivatives. (B) The flow injection negative precursor scan for 303.2 u over a mass range of 400–1400 u. (C) The LC-C18 negative precursor scan for 303.2 u over a mass range of 400–1400 u. The lipids after the acylation reaction was extracted and chromatographed on a C-18 column to reduce possible adduction mechanisms of compounds detected in the flow injection negative precursor scan of 303.2 u.

doi:10.1371/journal.pone.0021233.g005

antibody was from Cell Signaling. Polyclonal rabbit anti-human ACSL4 was a generous gift from Stephen Prescott (University of Utah). Polyclonal rabbit anti-human Endo B1, LPA, triacylglycerol (1,2-Dilinoleoyl-3-palmitoyl-glycerol), arachidonic acid and CoA were from Sigma-Aldrich. PA (1,2-dioleoyl-sn-glycero-3-phosphate) were purchased from Avanti Polar Lipids. [3H]-arachidonic acid was from PerkinElmer.

Cell culture

PLC/PRF/5 and HepG2 cell lines were purchased from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C.

Immunoprecipitation

Immunoprecipitations were performed as previously described [14].

Purification of endocytic and Rab5a vesicles

HepG2 cells were transduced by the lentivirus carrying vector pSin-EGFR-DsRed or pSin-EYFP-Rab5a and were selected for 4 days with 2 μg/ml puromycin. To prepare endosomes carrying EGFR-DsRed, we treated cells with 10 ng/ml EGF at 37°C for 10 minutes and purified early endosomes on the flotation gradient essentially as described [29]. Briefly, cells were incubated with 10 ng/ml EGF for 10 min at 37°C. After several washings, cells were scraped and pelleted at 4°C before being subjected to needle breakdown in homogenization buffer (250 mM sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA) at 4°C. Homogenates were centrifuged at 10000 rpm for 10 min at 4°C and post-nuclear supernatants (PNS) were collected. PNSs were then suspended in 40.6% sucrose by adding a stock solution (62% sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA) at the bottom. Centrifugation was done at 100000 g for 60 min at 4°C. Early endosomal fractions were collected at the 35%/25% sucrose interface.

Rab5 vesicles containing EYFP-Rab5a were prepared as described [30]. Briefly, HepG2 cells expressing pSin-EYFP-Rab5a were starved for 24 hours before being scraped and pelleted at 4°C. PNSs were then centrifuged at 10000 rpm at 4°C using a bench top centrifuge. The post mitochondria supernatants were loaded on top of homogenization buffer in SW40 centrifugation tubes with 0.5 ml cushion solution (62% sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA) at the bottom. Centrifugation was done at 100000 g for 60 min at 4°C and Rab5a vesicles were collected on top of the cushion solution.

In vitro vesicle fusion assay

The assay was performed as described previously with modifications [16,17]. Briefly, EGFR-DsRED endocytic vesicles and EYFP-Rab5a vesicles (aliquots of both contain 20 μg proteins) were gently mixed in a total volume of 20 μl fusion buffer (10 mM Hepes, pH 7.4, 1.5 mM MgOAc, 1 mM DTT, 50 mM KOAc, 100 nmol arachidonic acid, 1 mM coenzyme A, 5 mM GTP, complemented with 4 μl of an ATP-regenerating system containing 1:1 mixture of 100 mM ATP, 600 mM creatine phosphate, and 4 mg/ml creatine phosphokinase). After incubating with indicated purified proteins at 37°C for 40 min, a portion of the reactions were spotted on slides and examined using Zeiss LSM 510 Meta confocal microscope. All images are representative single optical sections. Colocalization analysis was done using MBF ImageJ. For reactions in Figure 5, PA and PI were incubated with control eluates or the TIP30 complex in a total volume of 200 μl fusion buffer. Resulting lipids were purified using Bligh-Dyer Method [31] and incubated with vesicles in homogenization buffer (250 mM sucrose, 5 mM Hepes, pH 7.4) on ice.

Figure 6. Fatty acylation of phosphatidic acid promotes vesicle aggregation. Lipids were extracted after incubating 100 nmol PA or phosphatidylinositol (PI) with 100 nmol arachidonic acid the TIP30 complex or control eluates. Lipids were resuspended in homogenization buffer by sonication and were mixed with EGFR-DsRed and EYFP-Rab5a vesicles in in vitro fusion buffer at 37°C. Resulting vesicles were spotted on glass slides and images were taken using confocal microscope. Scale bars, 5 μm.

doi:10.1371/journal.pone.0021233.g006
Electron microscopy studies was performed as described previ-
ously using TEM JEOL 100CX [12].

Protein-lipid overlay assays
Protein-lipid overlay assays were performed essentially as
previously described [32]. Briefly, membrane strips (Echelon
Biosciences Inc.) containing 15 pre-spotted lipids were incubated
overnight at 4°C with recombinant proteins (10 μg/ml) in TBST
with 5% milk. After being washed with TBST 10 min each for 3
times, the strips were incubated overnight at 4°C with specific
primary antibodies against the recombinant proteins in TBST with
5% milk. The strips then were washed again and incubated with
corresponding fluorescent secondary antibodies at room tempera-
ture for 1 hour. Images were acquired by scanning the strips using a
Li-Cor scanner.

Lipid acylation
Purified endocytic vesicles (aliquots containing 20 μg proteins)
were incubated with indicated proteins and [3H]-arachidonic acid
(PerkinElmer) in the presence or absence of 10 μM triacin C at
37°C for 1 hour in a total volume of 200 μl fusion buffer. Lipids
were extracted using Bligh-Dyer method [31] and resolved on silica
gel 60 TLC plate with chloroform/ethanol/water/triethylamine
(30/35/7/35) as the solvent. The TLC plate was exposed to Kodak
Tritium Sensitive Storage Phosphor Screen. Images were acquired
by scanning the screen using a Molecular Dynamics Storm 860. For
preparation of lipid derivatives from PA, PI or LPA, 100 nmol lipids
were incubated with immunopurified TIP30 complex or recombi-
nant TIP30, ACSL4 and Endo B1 in the fusion buffer. Resulting
lipids were purified using Bligh-Dyer Method [31].

MS/MS spectrometry analysis
MS/MS spectrometry analysis of PA derivatives were per-
duced according to Bligh-Dyer method and redissoved in
methanol/chloroform (35/18) with 10 mM NH₄OAC and 1 μg/ml
NH₄OH. First, standards of 17:0–20:4 PA and 17:0–20:4 PI
were prepared in the above solution and infused in the API 4000
QTrap triple quadrupole with linear ion trap instrument for opti-
mization of collisional energy to provide mass fragments of
arachidonic acid (20:4) at 303.2 u. Then samples were flow
injected into the MS/MS in negative scan mode to discover
products from the described reaction. The m/z value was used to
search the most likely molecular species at http://www.lipidmaps.

Statistical analysis
All statistical tests were two-tailed t-test. Data represent means
± SEM. *p<0.05, **p<0.01.

Acknowledgments
We are grateful to Stephen Prescott for generously sharing ACSL4
antibody. We thank Drs. Hans Cheng, Jerry Dodgson, Karen Friderici and
Richard Schwartz for critical reading of the manuscript and Alicia Pastor
for electron microscopy analysis.

Author Contributions
Conceived and designed the experiments: CZ HX. Performed the
experiments: CZ AL SG XZ. Analyzed the data: CZ HX XZ. Wrote
the paper: CZ HX.

Figure 7. Fatty acylation of phosphatidic acid induce vesicle tethering and stacking. Effects of acylated PA on vesicle fusion were
determined using electron microscope. Lipids were extracted after incubating PA with control immunoprecipitates (A), PA with immunopurified
TIP30 complex (B), PA with recombinant TIP30, ACSL4, and Endo B1 (C) or PI with immunopurified TIP30 complex (D). Each of these lipids or
triacylglycerol (E) was suspended in homogenization buffer and incubated on ice with EGRF-DsRed and EYFP-Rab5a vesicles. The resulting vesicles
were examined using TEM. Scale bars, 500 nm.
doi:10.1371/journal.pone.0021233.g007
References

1. Martens S, McMahon HT (2008) Mechanisms of membrane fusion: disparate players and common principles. Nat Rev Mol Cell Biol 9: 545–556.
2. McMahon HT, Koolov MM, Martens S (2010) Membrane Curvature in Synaptic Vesicle Fusion and Beyond. Cell 140: 661–665.
3. Pfanner N, Glick BS, Arden SR, Rothman JE (1990) Fatty acylation promotes fusion of transport vesicles with Golgi cisternae. J Cell Biol 110: 95–961.
4. Pfanner N, Orti I, Glick BS, Amherdt M, Arden SR, et al. (1995) Fatty acetyl-coenzyme a is required for budding of transport vesicles from Golgi cisternae. Cell 59: 95–102.
5. Chattopadhyay N, Sun P, Wang P, Abonyo B, Cross NL, et al. (2003) Fusion of Lamellar Body with Plasma Membrane Is Driven by the Dual Action of Annexin II Tetramer and Arachidonic Acid. Journal of Biological Chemistry 278: 39675–39683.
6. Mayorga LS, Colombo MI, Lennartz M, Brown EJ, Rahman KH, et al. (1993) Inhibition of endosome fusion by phospholipase A2 (PLA2) inhibitors points to a role for PLA2 in endocytosis. Proc Natl Acad Sci U S A 90: 10253–10259.
7. Hauke V, Di Paolo G (2007) Lipids and lipid modifications in the regulation of membrane traffic. Curr Opin Cell Biol 19: 426–435.
8. Jun Y, Fratti RA, Wickner W (2004) Diacylglycerol and Its Formation by Phospholipase C Regulate Rab- and SNARE-dependent Yeast Vacuole Fusion. Journal of Biological Chemistry 279: 53186–53195.
9. Mima J, Hickey CM, Xu H, Jun Y, Wickner W (2008) Reconstituted membrane fusion requires regulatory lipids, SNAREs and synergistic SNARE chaperones. EMBO J 27: 2031–2042.
10. Steenmark H (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10: 513–525.
11. Wickner W, Schekman R (2008) Membrane fusion. Nat Struct Mol Biol 15: 636–644.
12. Ohya T, Miacyrantsa M, Coskan U, Lommen B, Runge A, et al. (2009) Reconstitution of Rab- and SNARE-dependent membrane fusion by synthetic endosomes. Nature 459: 1091–1097.
13. Creutz CE (1981) cis-Unsaturated Fatty Acids Induce the Fusion of Chromaffin Granules Aggregated by Synexin. The Journal of Cell Biology 91: 247–256.
14. Zhang C, Li A, Zhang X, Xiao H (2011) A novel TIP30 protein complex regulates EGF receptor signaling and endocytic degradation. J Biol Chem 286: 9373–9381.
15. Pryer NK, Wuestehube LJ, Schekman R (1992) Vesicle-mediated protein sorting. Annu Rev Biochem 61: 471–516.
16. Bethani I, Werner A, Kadian C, Geumann U, Jahn R, et al. (2009) Endosomal fusion upon SNARE knockdown is maintained by residual SNARE activity and enhanced docking. Traffic 10: 1543–1559.
17. Brandhorst D, Zwilling D, Rizzoli SO, Lippert U, Lang T, et al. (2006) Homotypic fusion of early endosomes: SNAREs do not determine fusion specificity. Proc Natl Acad Sci U S A 103: 2871–2876.
18. Cao Y, Trzeciak S, Zimmerman GA, McIntyre TM, Prescott SM (1998) Cloning, Expression, and Chromosomal Localization of Human Long-Chain Fatty Acid-CoA Ligase 4 (FACL4). Genomics 49: 327–330.
19. Xiao H, Palhan V, Yang Y, Roeder RG (2000) TIP30 has an intrinsic kinase activity required for up-regulation of a subset of apoptotic genes. Embo J 19: 956–963.
20. Jenkins G, Frohman M (2005) Phospholipase D: a lipid centric review. Cellular and Molecular Life Sciences 62: 2303–2316.
21. Connell E, Darios F, Breslin K, Gansby N, Peak-Chew SY, et al. (2007) Mechanism of arachidonic acid action on syntaxin-Munc18. EMBO Rep 8: 414–418.
22. Latham CF, Osborne SL, Cyle MJ, Memurru FA (2007) Arachidonic acid potentiates exocytosis and allows neuronal SNARE complex to interact with Munc18a. J Neurochem 100: 1543–1554.
23. Rickman G, Davletov B (2005) Arachidonic acid allows SNARE complex formation in the presence of Munc18. Chem Biol 12: 545–553.
24. Jones D, Morgan C, Cookson S (1999) Phospholipase D and membrane traffic: Potential roles in regulated exocytosis, membrane delivery and vesicle budding. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1439: 229–244.
25. Lee YC, Zheng YO, Taraschi TF, James N (1996) Hydrophobic alkyl headgroups strongly promote membrane curvature and violate the headgroup volume correlation due to “headgroup” insertion. Biochemistry 35: 3677–3684.
26. Fahey DA, Small DM (1986) Surface properties of 1,2-dipalmitoyl-sn-glycerols. Biochemistry 25: 4468–4472.
27. Kinmune PK, Helouani JM (2000) Mechanisms of initiation of membrane fusion: role of lipids. Biosci Rep 20: 465–482.
28. Kinmune PK (1992) Fusion of lipid bilayers: a model involving mechanistic connection to HII phase forming lipids. Chem Phys Lipids 63: 251–258.
29. Grevot JP, Escola JM, Stang E, Bakke O (1995) Invariant chain induces a delayed transport from early to late endosomes. J Biol Chem 270: 2741–2746.
30. Fuchs R, Ellinger I (2002) Free-flow electrophoretic analysis of endosome subpopulations of rat hepatocytes. Curr Protoc Cell Biol Chapter 3: Unit 3 11.
31. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917.
32. Dowler S, Kular G, Alessi DR (2002) Protein lipid overlay assay. Sci STKE 2002: p6.