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Review Article

Mechanism of membrane fusion: protein-protein interaction and beyond

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Abstract: Membrane fusion is a universal event in all living organism. It is at the heart of intracellular organelle biogenesis and membrane traffic processes such as endocytosis and exocytosis, and is also used by enveloped viruses to enter hosting cells. Regarding the cellular mechanisms underlying membrane fusion, pioneering studies by Randy Schekman, James Rothman, Thomas C. Südhof and their colleagues have demonstrated the function of specific proteins and protein-protein interactions as essential fusogenic factor to initiate membrane fusion. Since then, function of lipids and protein-lipid interaction has also been identified as important players in membrane fusion. Based on that NSF (NEM-sensitive factor where NEM stands for *N*-ethyl-maleimide) and acyl-CoA are required for the membrane fusion of transporting vesicles with Golgi cisternae, it is further suggested that the transfer of the acyl chain to a molecule(s) is essential for membrane fusion. Among the previously identified fusogens, phosphatidic acid (PA) is found as an acyl chain recipient. Functionally, acylation of PA is required for tethering the membranes of Rab5a vesicles and early endosomes together during membrane fusion. As certain threshold of proximity between the donor and acceptor membrane is required to initiate membrane fusion, fusogenic factors beyond protein-protein and protein-lipid interaction need to be identified.

Keywords: Acyl-CoA, ACSL4, Endo B1, endosome, lysosome, membrane fusion, NSF, phosphatidic acid, Rab5a, SNARE, Tip30

Introduction

Membrane fusion is initiated when separate membrane vesicles or compartments are brought into close proximity. To allow initiation, two membranes must overcome two dominant forces: a repulsive hydration force arising from water tightly bound to the hydrophilic lipid head groups and an attractive hydrophobic force between the hydrocarbon interiors of the bilayers [1-3]. Initiation of membrane fusion is then followed by hemifusion and formation of the fusion pore. The final stage of membrane fusion is triggered by expansion of the fusion pore [4]. To allow cells to function in a controlled and regulated manner, membrane fusion, which mediates exchange and trafficking among cellular compartments, requires activity-dependent and organized execution rather than being a random event. First of all, not all bilayer membranes are chemically and functionally equal. Lipid composition may define the efficiency of distinct fusion process. It has been shown that, while the inverted cone-shaped LPCs suppress hemifusion, they facilitate fusion pore formation. The cone-shaped PEs facilitate hemifusion but inhibit fusion pore formation [4, 5]. Conceptually, random and spontaneous membrane fusion is prevented by physical repulsion from hydration and charges when bilayer membranes are in close distance to each other. It is accepted that the energy required to overcome the repulsion may come from the formation of protein complex across the to-be-fused membranes. Molecular and biophysical studies have shown that a group of membrane bound SNARE (soluble NSF attachment protein receptor) proteins possesses a conserved coiled-coil region that can mediate strong inter-molecular interaction [6]. The formation of the inter-molecular bundle via physical association among the SNARE motifs triggers energy release to bring

membranes into close proximity. It is evident that the requirement for SNARE-mediated protein-protein interaction varies and may depend on lipid content and protein-lipid interaction. For example, bilayer with high PE/PS fuses more efficiently and requires less SNARE complexes [7]. It is further proposed that the fusion site may possess unique molecular characteristics including high density of SNAREs [8], lipid composition that facilitates membrane curvature [9], and enrichment of protruding lipids [10].

Fatty acids and vesicle membrane fusion

The last two decades witnessed an explosion of knowledge on a number of factors involved in vesicle membrane fusion, including proteins, fatty acids, acyl-CoA esters and membrane lipids. Arachidonic acid, a long-chain polyunsaturated fatty acid (PUFA), has been demonstrated to be an effective fusogen. It can significantly promote Ca^{2+}-triggered fusion of isolated chromaffin granules [11], endosome-endosome fusion [12] and GTP-dependent fusion of microsomes [13]. Moreover, membrane-bound arachidonic acid can drive annexin II-mediated membrane fusion of the lamellar body with the plasma membrane during exocytosis [14].

How arachidonic acid promotes membrane fusion remains unclear. In neuronal cells, arachidonic acids are capable of removing the inhibitory Munc18 protein from syntaxin *in vitro*, thus allowing the formation of the SNARE complex, which is composed of vesicle-associated membrane protein 2 (VAMP2), SNAP-25 (synaptosome associated protein-25), and syntaxin1 [15]. However, a later *in vitro* study shows that Munc18 still attaches to syntaxin1 after the arachidonic acid-stimulated formation of the SNARE complexes. Thus, the function of arachidonic acids in SNARE complex formation needs further investigation.

Fatty acyl-coenzyme A (CoA) esters are substrates for β-oxidation, which is critical for synthesis and remodeling of lipids and protein acylation reactions. One acyl-CoA, palmitoyl-CoA, is found indispensable for the budding of transport vesicles from Golgi cisternae and fusion of transport vesicles with Golgi cisternae [16-18]. Vesicle transport is blocked by inhibitor of long-chain acyl-CoA synthetase and a nonhydrolyzable analogue of palmitoyl-CoA, suggesting that fatty acid has to be activated by CoA to stimulate transport and that the acyl group has to be transferred to other molecules.

Many of the proteins that mediate synaptic vesicle fusion and trafficking are indicated as the recipients of the acyl groups [19, 20]. Palmitoyl groups are covalently linked to cysteine residues of synaptotagmin, α-SNAP (soluble Nethylmaleimide-sensitive-factor-attachment protein-α) and SNARE proteins Ykt6, VAMP and SNAP-25. Palmitoylation of these proteins may be required for anchoring them to membranes or sorting to particular membrane micro-domains such as lipid rafts. Palmitoylation of Ykt6 has been suggested to regulate the rate of intracellular membrane flow and vesicle fusion in the secretory pathway [21]. However, the significance of palmitoylation of these proteins in vesicle membrane fusion is unclear.

Phosphatidic acid (PA) is another fusogenic lipid that plays important roles in vesicle transport. It is proposed that PA, with a very small negatively charged head group, induces negative membrane curvature at the inward membrane curve [22]. Phospholipase D (PLD) hydrolyzes membrane phosphatidylcholine to produce PA. The two isoforms of PLDs, PLD1 and PLD2, are involved in vesicle trafficking during endocytosis and exocytosis [23, 24]. Depletion of PLD2 inhibits recycling of transferrin receptors in HeLa cells [25]. Endocytic trafficking and endosomal signaling of EGFR (epidermal growth factor receptor) are also regulated by PLD1 and its regulators, protein kinase Cα and RalA [26]. The role of PLD-derived PA has been shown to be required for key exocytotic processes in various cell types including adipocytes [27], neuroendocrine cells [28], mast cells [29] and pancreatic beta-cells [30]. Another fusogenic lipid is diacylglycerol (DAG), which can be generated at the membrane through the PA phosphatase activity of Pah1 [31, 32]. Cumulating evidence has suggested that DAG increases the fusogeneicty of vacuoles [33]. These observations strongly suggest that lipid modifications are essential for various vesicle membrane fusion events.

Role of SNARE and SM (Sec1/Munc18-like) proteins in membrane fusion

SNARE proteins are receptors for SNAP and NSF. They belong to a family of membrane teth-

ered coiled-coil proteins that are required for vesicle membrane fusion. SNARE proteins have been shown to mediate fusion of lipid bilayers in assays using reconstituted liposomes; therefore they are considered the best candidates for the cellular fusogens. It is proposed that vesicle-associated v-SNARE proteins syntaxin and SNAP-25 pair with cognate t-SNARE protein VAMP on the target membrane to form four-helix bundle (SNAREpin) that brings lipid bilayers into close proximity. The pairing starts at the N termini of the SNARE proteins and then proceeds in a zipper-like manner towards the C-terminal trans-membrane regions, thereby enabling bridging of donor and acceptor membranes. SNARE interactions may also increase their local concentration to help SNARE assembly or convert SNAREs into a fusion competent form [34]. The resulting mechanical force might overcome the energy barrier and bring the lipid bilayers close enough for fusion to occur [35, 36].

Fusion assays using *in vitro* reconstituted lipid bilayers have lead the hypothesis that SNARE proteins are the minimal fusion machinery. It has been shown that when synaptic vesicle membrane protein VAMP2, a v-SNARE protein, and two plasma membrane t-SNARE proteins syntaxin1A and SNAP25 are reconstituted into phospholipids to form donor and acceptor vesicles, respectively, they are sufficient to promote specific fusion between the two types of vesicles [36]. However, it is also evident that, in addition to the known SNARE proteins, many other proteins have permissive roles to allow vesicle fusion *in vivo* [3, 36]. In neuroendocrine cells, for instance, Munc-18 (mammalian uncoordinated-18) protein, a member of the SM protein family, has been shown to facilitate syntaxin trafficking to the cell surface by interacting with syntaxin and preventing premature SNARE complex formation between syntaxin and SNAP-25 [37].

Although lines of evidence have been obtained using artificial membranes, ample studies also indicate that artificial membrane fusion will not necessarily be mediated by the same mechanism used for fusion between biological membranes. Recently, endosome-endosome fusion was successfully mimicked using reconstituted proteoliposomes with up to 17 recombinant proteins purified from bacteria [38]. These proteins include Rab5, Rab5 effectors, SNARE proteins and SNARE accessory factors, and other proteins that are currently known to be important for vesicle fusion. They can promote fusion of proteoliposomes at physiologically meaningful rate. However, they are not capable of promoting efficient fusion between biological intact endosomes, suggesting that the SNARE proteins are not the fusogenic factors that are minimally required for membrane fusion [38]. In addition, high fusion rate can be reached with reconstituted proteoliposomes utilizing bacterially expressed proteins that essentially lack post-translational modifications, whereas many proteins that are involved in membrane fusion are post-translationally modified *in vivo*, such as the palmitoylation of synaptotagmin, α-SNAP and SNARE proteins Ykt6, VAMP and SNAP-25 [19-21], as well as the isoprenylation of Rab5 [39]. This further indicates that fusion of artificial lipid bilayers may be mechanistically different from the fusion of biological membranes.

The data from reconstitution assays also imply that some components critical to vesicle fusion are missing in the *in vitro* assay system and that these components are likely to function prior to SNARE-mediated cellular events [3, 38]. Supportively, while arachidonic acid is essential for fusions between many kinds of vesicles including endosome-endosome fusion, it is not needed for artificial membrane fusion [12-14].

Novel function of the TIP30-Rab5a-Endo B1-ACSL4 complex in regulating activity-dependent termination of EGFR signaling through endosome membrane fusion

Although the necessary machinery for membrane fusion is present in both transport and target vesicles, membrane fusion does not occur spontaneously but is rather activity dependent. With regard to the functional role of endosome/lysosome-mediated receptor degradation, it is known that, following EGF stimulation, the EGFR signaling is then terminated through receptor-mediated endocytosis. The internalized EGFR is initially delivered to the sorting station early endosomes, where they are either recycled back to the plasma membrane or transported to late endosomes and lysosomes for degradation [40-42]. Under cer-

tain conditions, the altered EGFR signaling may leads to pathological outcomes such as tumorigenesis.

The function of TIP30 in membrane fusion was discovered by an unbiased and non-hypothesis driven approach. TIP30, also called CC3 or HTATIP2, was initially identified as a metastasis suppressor [43]. It was independently isolated as an HIV-1 Tat-interacting protein that may enhance Tat-activated transcription [44]. Tip30-deficient mice with C57BL6/J and 129SvJ mixed genetic background spontaneously developed a spectrum of tumors, suggesting TIP30 as a tumor suppressor [45]. Further, aberrant expression of TIP30 has been associated with a variety of human cancers, including human liver [45, 46], lung [47], breast [48], prostate [49, 50], and gastric cancers [51], as well as colorectal carcinoma [52]. Mechanistically, TIP30 may function as a transcription repressor to inhibit ERα-mediated c-myc transcription by interacting with ERα-interacting coactivator NCOA5/CIA [53]. Unrelated to its transcription repressor function, TIP30 is also found in the cytosol and regulates EGFR-mediated Akt signaling [54]. Intriguingly, genetic depletion of Tip30 causes trapping of the EGF-EGFR complex in early endosome and, in turn, results in a much-delayed EGFR degradation [54]. The data suggests a surprising new function of TIP30 in regulating endocytic trafficking. Co-immunoprecipitation followed by mass spectrometric analysis reveals that TIP30 interacts with Rab5a, ACSL4 (acyl-CoA synthetase long-chain family member 4), and Endo B1 (Endophilin B1, also known as Bif-1), all of which co-exist in a complex [54]. Notably, within this complex, Rab5a and Endo B1 are known to regulate certain aspect of endocytic membrane fusion and trafficking [55, 56]. ACSL4 preferentially uses arachidonate as substrate and converts free long-chain fatty acids into fatty acyl-CoA esters, and thus may affect membrane lipid composition [57].

Rab5 is a member of the Rab GTPase family, which is anchored to the cytoplasmic face of all vesicles involved in intracellular transport via the prenyl groups covalently linked to two cysteines in the C-terminus. Based on the connection between lipid and Rab, it has been proposed that Rab proteins may act as identity tags for distinct transporting vesicles and bring transporting vesicles to specific recipient membranes and tether those membranes by recruiting multitude of effectors [58-61]. Although Rab5 and its effector EEA1 are required for the transition from early endosome to late endosome, endosomal tethering, and endocytic degradation of EGFR [54, 55, 62, 63], Ohya et al. found that Rab5 and EEA can only increase endosomal fusion by 3% and 10%, respectively [38]. This suggests that Rab5 and EEA may not be sufficient to trigger significant fusion between biological intact endosomes. With regards to endocytic trafficking and degradation of EGFR, a study by Zhang et al. found that the TIP30-ACSL4-Endo B1 complex recruits Rab5-positive vesicles [54], which harbor V-ATPase (vacuolar H^+-ATPase) but are devoid of EEA1 and EGFR, to early endosomes in response to EGF. Fusion of Rab5-positive vesicles with early endosomes introduces V-ATPase and in turn causes acidic luminal pH [64] to drive EGF and EGFR dissociation and termination of EGFR signaling.

Endophilins are a group of proteins that contain an N-terminal amphipathic helix, a BAR (Bin/Amphiphysin/Rvs) domain and a C-terminal SH3 domain. The BAR domain is highly conserved in many proteins that involve in membrane dynamics. The dimeric BAR domains of endophilins are banana shaped and can sense and bind membrane curvature via its concave face to remodel liposomes structure [65]. In *Drosophila* and *Caenorhabditis elegans,* endophilin is required for synaptic vesicle recycling [66-69]. In mammalian cells, endophilin is localized to synaptic vesicles and is required for neurotransmitter release from endocytic vesicles [56, 66, 70]. As for the function of a specific endophilin, Endo B1 along with TIP30 and ACSL4 recruits Rab5 vesicles in response to EGF stimulation. Further, knockdown of TIP30, ACSL4 or Endo B1 suppresses EGF-EGFR dissociation and EGFR degradation via delaying the endocytic trafficking and membrane fusion [54].

Novel function of the TIP30-Endo B1-ACSL4 complex in mediating lipid modification and conversion and vesicle stacking

It is recognized that, although reconstitution assays with SNAREs, Rabs and other effector proteins show successful fusion with artificial

membranes, efficient fusion of biological membranes requires additional factors [3, 38] such as arachidonic acid and co-enzyme A [11, 12]. Although how arachidonic acid plays an essential role in membrane fusion is unclear, the TIP30 complex, which mediates the fusion of Rab5a-positive vesicles with endosomes, contains ACSL4. Based on that ACSL4 is an acyl-CoA ligase, which preferentially uses arachidonic acid as substrate [71], how the TIP30 complex, coenzyme A, and arachidonic acid regulate fusion was directly tested by *in vitro* assay with endocytic and Rab5a vesicles [72]. The Rab5 vesicles were labeled with EYFP-Rab5a fusion proteins and purified from HepG2 cells that do not express detectable TIP30 and EGFR. The endocytic vesicles were labeled with EGFR-DsRed fusion proteins and purified from EGF-treated HepG2 cells. The Rab5a and endocytic vesicles were mixed along with the supplement of immune-purified TIP30 complex, arachidonic acid and co-enzyme A, and examined by confocal microscopy. Efficient vesicle fusion and aggregation were observed, as indicated by the co-localization of EGFR-DsRed and EYFP-Rab5a fluorescent signals, in a GTP-dependent manner. This is consistent with that GTP is required for Rab5a function. In stark contrast, when the TIP30 complex, or coenzyme A, or arachidonic acid, or GTP was excluded from the supplement, membrane fusion cannot be accomplished. Both endocytic and Rab5a vesicles remained as small particles; fluorescence co-localization was not detected. The function of these molecules in membrane fusion was further examined by transmission electron microscopy. With the supplement of arachidonic acid, small vesicles (50 to 300 nm in diameter) fused to form larger vesicles (more than 1 μm in diameter). Further, membrane fusion did not occur when arachidonic acid was replaced by other fatty acids including palmitic, palmitoleic, oleic, linoleic, linolenic, eicosapentaenoic, and docosahexaenoic acids. These *in vitro* results are consistent with function of TIP30 complex in regulating endocytic EGFR trafficking in living cells [54].

One remaining question is how arachidonyl-CoA, which is likely synthesized from arachidonic acid and co-enzyme A by ACSL4 in the TIP30 complex, facilitates membrane fusion. Conceptually, the arachidonyl group of the synthesized arachidonyl-CoA may be transferred to certain lipid and in turn trigger membrane fusion. In fact, the radioactivity of ^{3}H-arachidonic acid was transferred to a different lipid species when the TIP30 complex was supplemented. With a protein-lipid overlay assay, TIP30 and Endo B1 were found to specifically bind phosphatidic acid (PA), a lipid species involved in membrane fusion of various intracellular vesicles [73, 74]. A preliminary analysis of the lipid profile following MS/MS and LC-MS/MS spectrometry suggested that triacylglycerols may be the product of PA acylation. Strikingly, lipid fraction purified from reaction mixture of PA and the TIP30 complex promoted fusion of endocytic and Rab5a vesicles (as indicted by fluorescence co-localization). A specific triacylglycerol species, 1,2-Dilinoleoyl-3-palmitoyl-rac-glycerol with a palmitoyl tail at the sn-3 position also promoted vesicle fusion. However, transmission electron microscopy found that the PA derivatives only causes vesicle tethering and stacking but does not lead to the formation of large membrane fusion product (i.e. vesicles with diameter of >0.5 um).

Summary and future direction

Based on previous studies, we propose that, upon EGF stimulation, Rab5a vesicles move to peripheral regions where TIP30, ACSL4 and Endo B1 act in concert to facilitate the fusion of Rab5a vesicles with early endosomes. TIP30 presumably binds PA on early endosomes and tethers Rab5a, ACSL4 and Endo B1 together at the fusion sites. ACSL4 catalyzes the synthesis of arachidonyl-CoA from arachidonic acid and coenzyme A. Next, the arachidonyl chain of arachidonyl-CoA is substituted for the phosphate headgroup of PA to form triacylglycerol, which enables attachment of Rab5a vesicles with early endosomes. Subsequently, Rab5a, SNAREs and their associated proteins facilitate the following membrane fusion steps. We envision that the replacement of the phosphate headgroup of endosomal PA with an arachidonyl chain not only neutralizes the negative charge of PA, but also provides a hydrophobic group to insert into the membrane of Rab5a vesicles and perturb the lipid bilayers. Future studies to identify effective endogenous triacylglycerol that enables effective membrane fusion of Rab5 vesicles with early endosomes

would provide insight concerning the direct role of lipid to initiate membrane fusion. There are also challenges to elucidate mechanisms underlying fusion processes beyond the tethering and stacking step as well as fusion pore formation and expansion.

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Disclosure of conflict of interest

None.

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