Trends in Biochemical Sciences

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Review

The Participation of Regulatory Lipids in Vacuole Homotypic Fusion

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In eukaryotes, organelles and vesicles modulate their contents and identities through highly regulated membrane fusion events. Membrane trafficking and fusion are carried out through a series of stages that lead to the formation of SNARE complexes between cellular compartment membranes to trigger fusion. Although the protein catalysts of membrane fusion are well characterized, their response to their surrounding microenvironment, provided by the lipid composition of the membrane, remains to be fully understood. Membranes are composed of bulk lipids (e.g., phosphatidylcholine), as well as regulatory lipids that undergo constant modifications by kinases, phosphatases, and lipases. These lipids include phosphoinositides, diacylglycerol, phosphatidic acid, and cholesterol/ergosterol. Here we describe the roles of these lipids throughout the stages of yeast vacuole homotypic fusion.

Highlights

Vacuole fusion is spatiotemporally controlled by regulatory lipids and their modifications.

The phosphatidic acid (PA) phosphatase Pah1/Lipin1 regulates vacuole maturation by affecting the recruitment of the phosphatidylinositol (PI) 3-kinase Vps34 and its production of PI 3-phosphate (PI3P). PI3P recruits the nucleotide exchange factor Mon1–Ccz1, which then recruits and activates the Rab Ypt7.

PA sequesters Sec18 protomers from *cis*-SNARE complexes to prevent priming. The conversion of PA to diacylglycerol (DAG) by Pah1 releases Sec18 from the membrane to allow SNARE priming.

The soluble Qc-SNARE Vam7 contains a polybasic region (PBR) in its middle domain that regulates the PI3P binding of the adjacent PX domain as part of an autoregulatory mechanism.

The DAG kinase Dgk1 converts DAG to PA and its deletion augments vacuole fusion. The increase in fusion is linked to enhanced Ypt7 activity in the presence of elevated DAG concentrations.

Membrane Fusion in Eukaryotic Cells

In eukaryotes, cargo is trafficked between organelles and the plasma membrane through the creation, movement, and **fusion** (see Glossary) of transport vesicles. The fusion of these vesicles is essential for cellular homeostasis and to execute specialized functions, such as neurotransmitter release and hormone secretion. Defects in cellular pathways that rely on membrane fusion are linked to many human pathologies, including diabetes, lipodystrophy, polycystic ovary syndrome, neurodegenerative diseases, and some cancer types [1–5]. The fusion process for organelles and vesicles is carried out through a series of highly conserved events, utilizing mechanisms that are found throughout eukarya [6]. Because of this conservation, vacuoles from *Saccharomyces cerevisiae*, organelles similar to mammalian lysosomes, serve as an ideal model for studying the mechanisms and regulation of membrane fusion. The stages of homotypic vacuole fusion have been experimentally defined and the core protein machinery necessary for the process is well known [7]. In fact, the use of purified intact organelles has allowed for extensive characterization of necessary fusion components *in vitro*. Additionally, the system has been reconstituted using all-purified components, allowing for simple manipulation of individual components to identify specific roles for both the proteins and lipids included [8–10]. However, the roles of key regulatory players in the process, including membrane lipids, remain largely under-characterized. In recent years, we and others have probed deeper into understanding how membrane trafficking and fusion is affected by specific lipids and their modifications in spatial and temporal manners. Thus, a focused review of how lipids affect the fusion machinery of the vacuole homotypic fusion model system is needed, as the field continues to elucidate the mechanisms underlying the regulatory effects of lipids. In particular, it is important to summarize how each stage of the fusion pathway is affected by the composition and modification of vacuolar lipids.

The Stages of Homotypic Vacuole Fusion

Yeast vacuole fusion occurs in a series of stages that operate in a cycle (Figure 1, Key Figure). At the end of each fusion cycle, membranes are left with inactive *cis*-**SNARE** complexes that need

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to be dissociated to start a new round of fusion. This process, called **priming**, is carried out by the AAA+ protein **Sec18/NSF** and its co-chaperone **Sec17/α-SNAP**. Sec18 hydrolyzes ATP to use Sec17 as molecular wedge to disrupt SNARE bundles into individual proteins that can subsequently interact in *trans* with those on a new membrane partner [11]. This leads to the release of Sec17 and the soluble SNARE **Vam7** from the membrane. Once priming is completed, membranes initially interact in the **tethering** stage, which is driven through the interactions of the Rab **Ypt7** and its effector complex **HOPS** [12–14]. At this stage, Vam7 reassociates with the vacuole through its interactions with HOPS and the lipid PI3P [15,16]. Unlike the fusion pathway of synaptic vesicles, where priming follows tethering, the vacuole fusion pathway requires priming to occur first. This has been established through gain of resistance experiments showing that the pathway gains resistance to reagents that target priming (e.g., anti-Sec18 IgG) early on, while remaining sensitive to those that target tethering (e.g., anti-Ypt7 IgG) [11,13,17–19]. Moreover, blocking tethering does not affect SNARE priming efficiency, whereas blocking priming completely blocks tethering. After tethering is complete, vacuoles enter the **docking** stage in which *trans*-SNARE complexes form. These include one R-SNARE and three Q-SNAREs from apposing membranes that bring the two vacuoles into close contact [12]. The tight apposition of the membranes forms distinct morphological features. The contact interface of the vacuoles forming a disc is termed the boundary membrane, and the point of contact between the membranes at the edge of the boundary is the vertex ring, where proteins and lipids that regulate fusion become enriched (Figure 2) [20–22]. The fusion pathway then enters the stage where the path diverges into direct bilayer fusion through a fusion pore, or through a **hemifusion** intermediate in which the outer leaflets of the membrane bilayers mix prior to full fusion and content mixing [23–26]. Hemifusion occurs around the vertex ring, leading to the internalization and subsequent degradation of the boundary membrane, which facilitates selective cargo transport [24,27].

Lipids in Membrane Trafficking

Although the stages of membrane trafficking and fusion have been well described with respect to the different protein components of the machinery, the interplay between proteins and their lipid environment is less clear. Regulatory lipids serve an important role in various membrane trafficking pathways. Functions have been described in cargo sorting, membrane budding, membrane fission, actin organization, membrane transport via cytoskeletal elements, and membrane fusion [15,20,28–33]. For example, phosphoinositides and diacylglycerol (DAG) promote association of necessary protein factors with the membrane. Elsewhere in the cell, phosphatidic acid (PA) is required for Glut4 trafficking, regulated exocytosis, and mitochondrial fusion and in yeast membrane fusion during spore formation [34–38]. An asymmetric distribution of PA and phosphoinositides promote vesicle fusion at the plasma membrane in regulated exocytosis in neuronal systems [39]. In higher eukaryotes, cholesterol has been shown to mediate SNARE protein conformation and affect the organization of SNAREs in biological membranes [40]. Because these lipids are essential for processes throughout the stages of fusion across multiple pathways, it is vital to understand how they influence the proteins that drive the process.

Overview of Lipid Requirements for Vacuole Fusion

Through the work of many groups it is now clear that the complex lipid compositions of organelle membranes exquisitely regulate their fusion with vesicles and each other through their biophysical properties and interactions with key proteins. As such, in this review we will focus on the lipids that regulate vacuole fusion. Vacuole fusion requires a growing list of lipids and lipid modifications that reflect the fine tuning necessary for optimal fusion efficiency. The regulatory lipids that were first discovered to be required for fusion are PI3P, PI(4,5)P_2, ergosterol, and DAG [20,31,32] (Table 1). These studies were performed using purified vacuoles and

Glossary

Dgk1: the yeast kinase that converts diacylglycerol to phosphatidic acid.
Docking: the irreversible stage of fusion in which SNARE complexes form between partner compartments.
Fusion: the complete merging of two membrane lipid bilayers into one continuous membrane, resulting in luminal content mixing.
Guanine nucleotide exchange factor (GEF): mediates the exchange of GDP for GTP to activate Rab GTPases.
Hemifusion: merging of the outer leaflets of docked membranes while the inner leaflets remain intact, thus preventing luminal content mixing.
Hexamerization: the complex formation of six copies of a protein into an active enzyme.
HOPS: a heterohexameric tethering complex that binds the Ypt7, SNAREs, and regulatory lipids.
Membrane curvature: the lipid composition of a lipid bilayer can influence how flat or curved a membrane can be.
Pah1: the yeast ortholog of the PA phosphatase Lipin1 that produces diacylglycerol.
Priming: the mechanical separation of *cis*-SNARE bundles by Sec18 and Sec17.
Protomer: a single copy of a protein that can organize into a multicopy functional enzyme.
Reconstituted proteoliposome (RPL): a defined group of lipids and proteins that are assembled into a vesicle.
Sec17/α-SNAP: the co-chaperone of Sec18/NSF used as a molecular wedge to disrupt *cis*-SNARE bundles during priming.
Sec18/NSF: the AAA+ protein that hydrolyzes ATP to prime SNAREs with its co-chaperone Sec17.
SNAREs: membrane anchored/bound proteins that form four-alpha helical bundles between membranes. Each membrane donates one R-SNARE (containing a central arginine) that interacts with three Q-SNAREs (containing a central glutamine) donated by the partner membrane.
Tethering: the reversible attachment of compartments mediated by an organelle specific Rab GTPase and its effector tethering factor(s).
Vam7: a soluble Qc-SNARE that associates with membranes through

determined which lipids showed a required availability for fusion to reach untreated, wild type conditions. Their required presence in fusion was determined through the use of specific ligands that sequestered lipids from their site of action, or through their modification with phosphatases or lipases. For instance, the requirement of $PI(4,5)P_2$ during priming and docking was determined through sequestration with the Epsin ENTH domain or antibody, dephosphorylation with the bacterial phosphatase SigD, or the activity of phospholipase C, all which blocked measured fusion [20,32,41]. Similarly, the activity of PI3P during tethering and docking was observed through use of the FYVE ligand and the MTM-1 phosphatase [20]. DAG is a known fusogenic lipid, whose role in fusion is blocked with the DAG-binding protein domain C1b or by phosphorylating it with the DAG kinase DgkB to make it PA [20,42,43]. Ergosterol extraction from vacuoles and supplementation of the lipid both affected fusion, and later the ergosterol ligand filipin was shown to inhibit fusion during priming [20,31].

the interaction of its PX domain with the lipid PI3P.
Ypt7: the late endosome and vacuolar Rab7 homolog that uses the HOPS complex to execute the tethering stage of fusion.

The minimal vacuole lipid requirements for vacuole fusion were later defined through the use of **reconstituted proteoliposomes (RPLs)** containing purified vacuolar protein components and specific lipid compositions at physiologically relevant levels. This cell-free approach allowed for the determination of the isolated effects of specific lipids on vacuole membrane fusion without introducing pleiotropic effects that may be seen in whole cell studies. RPL experiments verified the need for PI3P, or $PI(4,5)P_2$, DAG, and ergosterol, and showed the additional need for phosphatidylethanolamine (PE) and PA for vacuole membrane fusion [9,44]. PA is necessary for full association of HOPS and Sec18 to RPL membranes, and both PA and PE are required for

Key Figure

The Vacuole Fusion Cycle

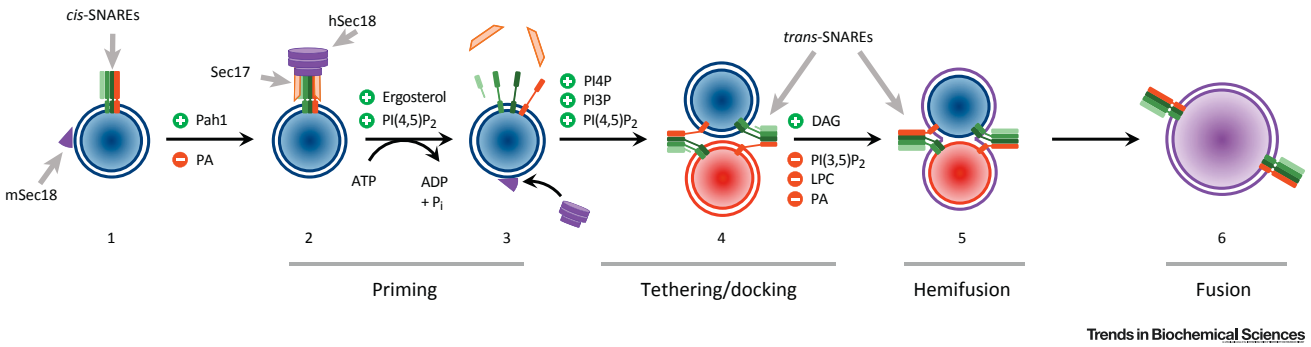

Figure 1. Depicted are the stages of vacuole fusion. *(1)* Isolated vacuoles contain inactive *cis*-SNARE complexes composed of the 3Q-SNAREs (Vam7, Vam3, and Vti1, shades of green) and 1R-SNARE (Nyv1, red). Also pictured is a PA-bound monomeric Sec18 (mSec18, purple wedge) associated with the membrane. Pah1 activity promotes moving to the next stage while excess PA blocks the progression. *(2)* Hexameric Sec18 (hSec18) associates with *cis*-SNARE through the use of Sec17 (orange trapezoids). This is promoted by ergosterol and $PI(4,5)P_2$. *(3)* Sec18 hydrolyzes ATP to use Sec17 as a molecular wedge to separate the *cis*-SNARE bundle into individual proteins. Sec17 and Vam7 are released from the membrane. Individual SNAREs are shown to partially acquire secondary structure in their SNARE motifs, depicted as elongated lines extending from the ovals. Sec18 is thought to reassociate with the membrane as a PA-bound protomer/monomer. *(4)* During vacuole docking SNAREs interact in *trans* and partially zipper. This is promoted by PI3P, PI4P, and $PI(4,5)P_2$. *(5)* *trans*-SNARE complexes continue to fully zipper, leading to hemifusion. The outer leaflets of both compartments merge (purple), while the inner leaflets remain separated to prevent content mixing. This is promoted by DAG, while it is inhibited by $PI(3,5)P_2$, LPC, and PA. *(6)* Inner leaflets fuse to fully merge the compartments into a continuous membrane and mix luminal content (purple). The postfusion SNAREs are shown now in *cis* and the vacuole returns to step 1 of the cycle. DAG, Diacylglycerol; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PI3P, phosphatidylinositol 3-phosphate; PI4P, phosphatidylinositol 4-phosphate; $PI(3,5)P_2$, phosphatidylinositol 3,5-bisphosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

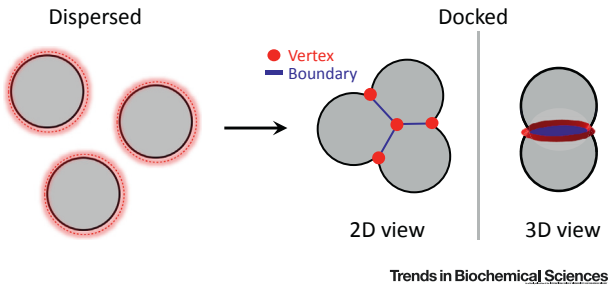

Figure 2. Formation of the Vertex Ring on Docked Vacuoles and Spatial Lipid-Binding Probes. Individual dispersed vacuoles labeled with a spatial probe (red). Upon docking, vacuoles form distinct morphological features. Vacuoles become tightly apposed, forming a flattened disc termed the boundary membrane. The edge of the boundary where membranes come into contact is termed the vertex ring, where lipids and proteins that drive fusion become enriched (red dots). The docked vacuoles are shown in both 2D and 3D projections. Our previous work has shown that phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2], diacylglycerol, and ergosterol become enriched at the vertex [17].

SNARE complex assembly [44]. Removing DAG, ergosterol, PI3P, or PI(4,5)P_2 from RPLs significantly reduced their capacity for fusion, confirming each as a requirement for optimal fusion [9]. Later work using RPLs found that neutral lipids that promote non-bilayer structure formation, including DAG, PE, and ergosterol, are also important for vacuole fusion [45].

While not considered necessary for vacuole fusion, other lipids found at the organelle have been shown to have potential effects on fusion. Changes to the availability of these lipids in vacuole fusion reactions have been shown to have effects on aspects of fusion, but are not necessary requirements for efficient RPL fusion to occur. For example, sequestering PI4P with FAPP-PH inhibits *trans*-SNARE complex formation but is not required for RPL fusion [9,46]. Additionally, the equilibrium between vacuole fusion and fission processes are closely linked to the synthesis of the vacuole lipid PI(3,5)P_2 [18,47,48]. Another example is cardiolipin, which was at first considered a vacuole membrane component and key lipid regulator of fusion [9,44]. However, cardiolipin is only made in mitochondria and later work using RPLs showed it is not necessary for fusion if the tethering protein Ypt7 is present [49]. Its inclusion in the original vacuole lipid composition may have been due to mitochondrial contamination in vacuole isolations or from mitophagy [7].

Table 1. Regulatory Lipids in Vacuole Fusion

Lipid	Stage of action	Protein interactions	Protein interaction domains	Refs
PI3P	Tethering	1) Mon1 2) Vam7 3) HOPS	1) Longin 2) PX 3) Unknown	[16,63,70]
PI4P	Tethering	HOPS	Unknown	[16]
PI(3,5)P_2	Postdocking	1) Vph1 2) HOPS	1) Unknown 2) Unknown	[16,71]
PI(4,5)P_2	1) Priming 2) Postdocking	1) Unknown 2) HOPS	1) – 2) Unknown	[16,32,72]
PA	Priming Tethering/ docking	1) Sec18 2) Vam7	1) Unknown 2) PX	[19,51]
DAG	Docking	Unknown	–	[20,42]
Ergosterol	1) Priming 2) Docking	1) Unknown 2) Unknown	1) – 2) –	[20,31]

Modulation of SNARE Function by Regulatory Lipids

The lipid regulation of SNAREs first occurs with their organization during docking. It has long been known that the soluble SNARE Vam7 associates with vacuole membranes through interactions between its PX domain and PI3P [15]. This assists in both the formation of active 3Q-SNARE complexes and in tethering apposed membranes [50]. More recently it was determined that a polybasic region of amino acids in the middle domain of Vam7 reduces its affinity for PI3P [51]. Mutating these basic amino acids to alanine increases binding of the protein to PI3P while reducing its association with SNAREs and HOPS, leading to decreased fusion. This likely reflects an autoregulatory mechanism to optimize Vam7 function.

Lipids, including sterols, PE, and DAG, can have significant effects on membrane fluidity [52,53]. SNAREs must transmit force to the membrane to catalyze fusion, and it is reasonable to speculate that the biophysical characteristics, such as fluidity and curvature, of a membrane would affect their activity [54]. Regulation of SNARE function by lipids occurs during the fusion stage and is thought to be tied to local membrane fluidity and curvature because of some key observations. Noncanonical SNARE complexes containing the mutant Vam7^{Q283R}, which forms stable but weakened SNARE complexes, or lipid-anchored Vam3–CCIIM are unable to facilitate fusion, leading to a buildup of hemifused intermediate compartments [55,56]. The inability of these mutant SNAREs to trigger fusion is restored by chemically changing the fluidity and increasing the negative curvature of the vacuole with the cationic amphiphile chlorpromazine. In contrast, lysophosphatidylcholine, which induces positive curvature, blocks wild type SNAREs from triggering hemifusion [26]. Together, these results showed that the fluidity and curvature of the vacuole membrane, which is dependent on its lipid composition, likely mediate the ability of SNAREs to catalyze fusion.

PA Phosphatase Activity and Vacuole Maturation

Aside from phosphoinositides and their modification, we found that the PA phosphatase **Pah1**, the ortholog of Lipin1, plays a significant role in endosome to vacuole maturation and vacuole fusion [57]. Deletion or inhibition of Pah1 activity leads to increased PA levels at the vacuole membrane. Vacuoles lacking Pah1 show dramatically reduced fusion that is linked to two independent pathways. One pathway involves the regulation of SNARE priming though the direct interaction of PA with Sec18 (see below). The second pathway affected by Pah1 function is the maturation, or acidification and changed Rab protein activity and presence, of the late endosome to the vacuole [57]. Vacuoles isolated from *pah1*Δ cells lack a number of factors necessary for fusion, including the PI 3-kinase Vps34, the HOPS subunit Vps39, Mon1-Ccz1, and Ypt7, which can be linked together under the Rab cascade model for the endolysosomal pathway. Here, Vps34 produces PI3P to recruit the Ypt7 **guanine nucleotide exchange factor (GEF)** Mon1–Ccz1, which in turn recruits and activates Ypt7 [58,59]. Thus, the role of PA conversion to DAG and PI3P converges in the development of active vacuoles. What remains to be seen is how Pah1 activity directly affects Vps34 recruitment to the vacuole. One possible mechanism could be related to vacuole acidification. This notion is bolstered by studies showing that *pah1*Δ vacuoles are hyperacidified in yeast [60], while in mammalian cells the hyperacidification of phagosomes leads to the loss of Vps34 and reduced PI3P production [61].

Regulation of the Ypt7 Nucleotide Exchange Factor Mon1–Ccz1 by PI3P

In the Rab cascade model, early pathway Rabs recruit the GEF for the downstream Rab in order to convert it to its GTP-bound form [62]. This allows for an overall Rab identity conversion of a compartment, which helps to define organelle identity and coordinate the directionality of membrane trafficking and fusion [62]. For example, work with *Caenorhabditis elegans* showed that the early endosomal Rab5 recruited the Mon1 ortholog SAND-1, which in turn recruits and

activates the late endosomal Rab7 [59]. This work also showed that Mon1/SAND-1 recruitment required PI3P. Later work in yeast also showed that Mon1–Ccz1 activity required PI3P to help facilitate nucleotide exchange and ultimately tethering by the Rab Ypt7 [58,63]. In addition, we showed that Mon1 could be released from vacuoles when PI3P was blocked with the FYVE domain or dephosphorylated with the MTM-1 phosphatase. Separately, we showed that after its GEF activity is complete, Mon1 is phosphorylated by the casein kinase Yck3, leading to the release of Mon1–Ccz1 from the vacuole. These results were consistent with initial studies that showed Mon1 is released from vacuole membranes in an ATP-dependent manner [22]. Together these studies showed that PI(3)P association of Mon1–Ccz1 is a necessary means of regulating Ypt7 tethering activity at the vacuole.

Regulation of SNARE Priming by PA

Above we discussed the results of a chronic loss of Pah1 in vacuole development; however, the acute inhibition of its activity has an independent effect on vacuole fusion. Early on we found that inhibiting PA phosphatase Pah1 activity on isolated vacuoles blocks the association of Sec18 with SNARE complexes, while remaining bound to the vacuole [57]. The blocked recruitment of Sec18 to *cis*-SNARE complexes, without decreasing its abundance on the vacuole membrane, led us to hypothesize that Pah1 could regulate the transition of Sec18 from a lipid-bound state to an active SNARE-bound state. This hypothesis was supported by previous unrelated work that unexpectedly identified NSF, the Sec18 mammalian homolog, as a PA-binding protein [64]. Further investigation showed that Sec18, like NSF, is a PA-binding protein and that its regulation could be tied to PA levels at the vacuole [19]. This indicated that Pah1 activity reduces PA concentrations, leading to the release of Sec18 from the membrane and allowing it to bind to *cis*-SNARE complexes. The notion that increased PA blocked SNARE priming is bolstered by the ability of exogenous short chain dioctanoyl PA (C8-PA) to block Sec18 binding to *cis*-SNAREs and inhibit vacuole fusion [19]. It is important to point out that this regulation of Sec18 has so far only been shown at the vacuole and this observed effect may not be present throughout the cell. Ongoing work suggests organelle-specific **membrane curvature** could play a role in priming regulation by PA [65].

Thus far we have discussed how PA binds Sec18 to inhibit SNARE priming. However, the binding mechanism or the effects of binding on Sec18 dynamics remained unclear. In subsequent studies we showed that PA binding significantly alters the Sec18 conformation and preferentially binds to it in its monomeric form [65]. This is thought to be incompatible with **hexamerization**, as putative binding sites are masked in the holoenzyme. This is in accordance with previous data showing that Sec18 recruitment was decreased when PA levels at the vacuole membrane are elevated. When Sec18, or its mammalian homolog NSF, binds to inactive SNAREs, it does so as a homohexamer [66]. In our current model, elevated PA concentrations bind to Sec18 **protomers** to prevent hexamerization and binding *cis*-SNARE bundles.

PA: Beyond Priming

We have shown that the acute inhibition of Pah1 blocks priming, an effect that is reproduced by the addition of C8-PA. That said, we found that C8-PA also blocks fusion at a stage after priming. The role for PA downstream of priming is supported by RPL experiments that bypass priming yet require PA [44]. These experiments show a second role of Sec18 after priming where it prevents SNAREs from pairing incorrectly. Occasionally SNARE complexes do not form properly during docking and are reactivated by Sec18 and Sec17 [67]. However, if this step becomes unregulated or occurs in the presence of excess Sec18, all SNARE complexes are disassembled, whether active or inactive, and fusion cannot proceed [9,12,49]. In the cell, this proofreading process is tightly controlled by the HOPS subunit Vps33 [68]. In addition to

tethering, HOPS prevents Sec18 association with active, properly formed *trans*-SNARE complexes during docking. Thus, it stands to reason that an excess of PA could block the proofreading mechanism of Sec18. In addition to inhibiting Sec18, it is possible that PA interacts with other factors to block fusion downstream of priming and independent of Sec18-mediated proofreading. For example, Vam7 binds PA in addition to PI3P, thus it is possible that the downstream effect of PA affects how Vam7 interacts with other components of the fusion machinery [51].

DAG Increases the Fusogenicity of Vacuoles

DAG is a fusogenic lipid that localizes to the vacuole vertex domain and boundary membrane immediately before the final fusion stage occurs [20,41]. It is known to be necessary for vacuole fusion to occur and is considered one of the minimum lipid requirements for RPL fusion [9,20,41]. One way DAG is generated at the membrane is through the PA phosphatase activity of Pah1. DAG levels are in part modulated by the reverse pathway, whereby DAG is phosphorylated to PA by the DAG kinase **Dgk1** [69]. Other groups have found that deleting the DAG kinase Dgk1 compensates for the effect(s) of lacking Pah1 on the architecture of the endoplasmic reticulum and nuclear envelope [69]. Surprisingly, deletion of Dgk1 does not reverse the fusion defect seen for *pah1*Δ vacuoles [42]. This was attributed to the temporal differences in the need for DAG and PA during the stages of fusion. Deletion of Dgk1 alone, however, had the opposite effect on fusion compared with deletion of Pah1. Vacuoles lacking Dgk1 show a marked increase in fusion levels. This is linked to an increase in the fusogenic lipid DAG in vacuole membranes that was accompanied by an increase in resistance of *dgk1*Δ vacuoles to dioctanoyl-PA (C8-PA). There was also an observed increase in Ypt7 activity, which manifested in a decreased sensitivity to the inhibitors anti-Ypt7 IgG, GDP dissociation inhibitor (GDI), and the GTPase-activating protein Gyp1-46. Together, these results showed that increased DAG levels augment the fusogenicity of vacuoles. This was supported by the increase in wild type vacuole fusion when exogenous DAG was added. The increased fusion of *dgk1*Δ vacuoles can also be linked to the effect of DAG on supporting non-bilayer structure formation in membranes, which is a required component for membranes to completely fuse [45].

The effect of deleting *DGK1* on the sensitivity of Ypt7 to inhibitors remains unclear. This shift seen could be for two distinct reasons. First, increased membrane DAG could promote increased activation of lipid-anchored Ypt7 through bilayer destabilization, changing membrane curvature, or altering membrane fluidity. The second possibility is that increased membrane DAG could simply decrease the requirement for Ypt7 in the vacuole fusion process.

Concluding Remarks and Future Directions

The research discussed in this review provides evidence of previously undiscovered lipid regulation of the stages of vacuole fusion. Evidence is provided for the regulation of Ypt7-dependent tethering by DAG or PA levels in the membrane. Extensive work looking at the effects of PA at the vacuole suggests a regulatory mechanism in which PA specifically regulates Sec18 priming activity at the vacuole by decreasing its association with *cis*-SNAREs. This regulation of Sec18 by PA appears to be through a direct interaction that alters the overall architecture of the protein. Questions remain, including the effects other lipids or lipid modifications may have on the different stages of fusion (see Outstanding Questions).

Taken together, these studies show how specific lipids can exquisitely control distinct functions of the fusion machinery. These effects are not merely due to the absence or presence of a particular lipid species. In multiple examples, we now understand that a lipid can have biphasic effects linked to its local concentration. In such cases a lower threshold is stimulatory for fusion

Outstanding Questions

How does Pah1 and membrane DAG/PA levels affect the recruitment of Vps34 and the downstream maturation of the vacuole?

How does PA block vacuole fusion in a downstream stage from priming?

After SNARE priming is complete, how does Sec18 transition to a PA-bound state?

Does the hexamerization of Sec18 protomers require protein or lipid interactions?

Does PA in biological membranes prevent Sec18 from re-priming SNAREs to allow fusion to proceed?

How does DAG affect Ypt7 activity?

How does the PBR of Vam7 interact with the PX domain to control lipid binding and multimerization?

What is the role of PA binding by Vam7?

What specific lipids are needed in the final fusion step leading to content mixing?

while high concentrations result in fusion inhibition. Thus, it is not enough to consider when and where a lipid plays a role in fusion. We must also consider how much is present. The coming years will continue to be exciting, as new aspects of how lipids control the fusion pathway emerge.

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