LIPID COMPOSITION AND MEMBRANE PHYSICAL PARAMETERS REGULATE PRIMING AND SNARE FUNCTION IN YEAST VACUOLE MEMBRANE FUSION

BY

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DISSERTATION

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ABSTRACT

Fusion at highly specialized membrane microdomains is a prerequisite for processes like hormone and neurotransmitter release, antigen presentation and viral infection. Apart from a family of highly conserved proteins called SNAREs, membrane fusion also requires a set of regulatory lipids that includes ergosterol, diacylglycerol, phosphatidic acid and phosphoinositides. Although a lot is known about the SNARE protein machinery, the role of lipids and the lipid environment of the membrane are not clear. For long, lipids had been thought to play a passive role by binding protein factors through their lipid binding domains. However, recent studies have shown that a subset of lipids play critical regulatory role by modulating the SNARE fusion machinery.

The regulatory role of lipids on the fusion machinery can be divided into at least two mechanisms. The first mechanism depends on the direct chemical interaction of lipids with the protein fusion catalysts. Here, we report that phosphatidic acid serves to bind a key AAA+ family ATPase, Sec18p that effects priming in yeast vacuolar membrane fusion. The yeast PA phosphatase Pah1p alters the ratio of PA to DAG on the membrane during priming and serves to recruit Sec18p to the cis-SNARE complexes at the site of priming.

The second mechanism is indirect and physical and is dictated by the physical parameters of the membrane such as curvature, fluidity and lateral tension. We report that inducing negative curvature on the vacuole membrane lowers the force threshold required for fusion, thus allowing non-canonical SNARE complexes to support fusion. On the other hand, increasing the positive curvature of the membrane increases the force threshold such that even canonical SNARE complexes do not support fusion. We also report that the formation of the SNARE bundle and the presence of a transmembrane domain serve to transmit the pulling force generated from the SNARE bundle to the membrane, which serves to distort the lipid bilayer leading to fusion. Either a defect in the SNARE complex or the absence of a transmembrane leads to a break in force transmission and thus results in fusion stalled at the hemifusion stage.
The knowledge gained from this study sheds more light on the mechanism by which membrane lipids regulate fusion. We have demonstrated that regulatory lipids are important during distinct stages of fusion and serve to recruit key protein catalysts at the site of fusion. The lipid composition of the membrane also dictates membrane parameters such as curvature and fluidity. We demonstrate that membrane curvature, fluidity and lateral tension are essential factors that can affect the core fusion machinery. The contribution of the membrane in membrane fusion is only beginning to be understood and we hope that this study will contribute to a growing sea of knowledge in the field of membrane fusion.
Luxury it is, to choose the road less travelled
But there are no roads leading to your own little hollow
    Get ready to pave your own path
    And leave signs for those who follow

Dedicated to my family,
Who have always encouraged me to forge a new path ahead.
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CHAPTER I

INTRODUCTION
OVERVIEW

The transport of cargo through the endocytic and secretory pathways is controlled by machinery that is conserved throughout eukaryotes [1]. In addition, membrane fusion is essential for the release of neurotransmitters, antibodies, as well as the turnover of receptors, the destruction of pathogens and the generation of antigens [2-6]. The final stage in these pathways is the fusion of membranes and transfer of cargo through a mechanism catalyzed by NEM-sensitive factor attachment protein receptor (SNARE) proteins. All SNAREs contain a heptad-repeat termed a “SNARE motif” that is often flanked by various N-terminal domains and C-terminal membrane anchors [7]. SNAREs form parallel four helical bundles through their SNARE motifs, which are primarily composed of hydrophobic residues with the exception of a conserved central glutamine (Q), or arginine (R) that interact in the ionic zero-layer [8] (Fig 1.1). Each functional SNARE bundle is composed of 3 Q-SNARE coils and 1 R-SNARE coil. Yeast vacuole fusion depends on the Q-SNAREs Vam3p, Vam7p and Vti1p, and the R-SNARE Nyv1p. Vacuole fusion also requires the Rab GTPase Ypt7p and its effector complex HOPS [9].

These shared elements of the trafficking systems provide the basic outline for a conserved fusion mechanism. Fusion at highly specialized membrane microdomains is a prerequisite for processes like hormone and neurotransmitter release, antigen presentation and viral infection. Thus, studying and understanding the regulation and deregulation of these pathways in disease states and in infection will shed more light on the various biological processes that share the conserved fusion machinery.
STAGES OF MEMBRANE FUSION

Vacuole fusion occurs through a series of phases that have been experimentally defined. The fusion cascade is initiated when the AAA+ protein Sec18pp (NSF) and Sec17p (α-SNAP) prime inactive cis-SNARE complexes [9,10]. Disruption of cis-SNARE complexes leads to the dissociation of Sec17p as well as the soluble SNARE Vam7p [11,12]. Between priming and fusion, membranes undergo the tethering and docking stages that are controlled by a Rab GTPase and its effector molecules, which include tethering molecules, nucleotide exchange factors, GTPase activating proteins, and Sec/Munc proteins [13]. In yeast, primed vacuoles become reversibly docked through the function of Ypt7p and its effector complex HOPS [14,15]. Vacuole fusion occurs at highly organized membrane rafts termed vertex microdomains that form at the point of contact between partner vacuoles, and become enriched in the regulatory lipids and proteins required for fusion [16,17]. During the docking stage Vam7p rebinds through its interactions with the hetero-hexameric tethering complex HOPS and the lipid phosphatidylinositol 3-phosphate (PtdIns(3)P), and enters a trans-SNARE complex [18]. The formation of the trans-SNARE complexes triggers the release of Ca^{2+} from the vacuole lumen [19]. The penultimate step in fusion is a hemifusion intermediate where the closely apposed outer membrane leaflets fuse without fusing the inner leaflets [20]. The fusion pathway culminates with the fusion of inner leaflets and content mixing.
TOOLS TO STUDY MEMBRANE FUSION

A wide range of assay methods have been developed to study and monitor yeast vacuole membrane fusion. The most widely used assay makes use of the two yeast strains BJ3505 and DK6281. To assay fusion, vacuoles are isolated from these two strains, one with normal vacuolar proteases but deleted of the major phosphatase encoding gene Phop8 and the other deleted of the vacuolar protease Pep4p and hence unable to activate the catalytically-inactive pro-Pho8p. Neither population of vacuoles has phosphatase activity (Fig 1.2). Upon fusion, the vacuolar proteases gain access to the inactive pro-Pho8p and converts it to the active form. The activity of the active Pho8p can be assayed colorimetrically by using p-nitrophenylphosphate as the substrate, which is converted to p-nitrophenylate.

Another widely used assay to monitor membrane fusion, involves the dequenching of rhodamine labeled Phosphatidylethanolamine following membrane fusion and lipid mixing. Here, the outer leaflets of vacuoles are labeled with Rh-PE at levels that cause self-quenching of the fluorophore. Labeled vacuoles are mixed with an excess of unlabeled vacuoles and the fusion of the outer leaflets is measured by an increase in fluorescence due to the dilution and dequenching of Rh-PE in the hemifused vacuoles. Other widely used assay methods involve monitoring the increase in vacuole diameter by microscopy, reconstitution of the two subunits of the β-lactamase enzyme upon fusion.
REGULATION OF THE MEMBRANE FUSION MACHINERY

Vacuole fusion also requires a group of regulatory lipids that include ergosterol, diacylglycerol (DAG), phosphatidic acid (PA), as well as multiple phosphoinositides [12,16,21-24]. Moreover, the modification of these lipids plays an important role throughout the fusion pathway. For instance, DAG is produced by the phospholipase C activity of Plc1p on Phosphatidylinositol(4,5)P$_2$, or through the phosphatase activity of Pah1p on PA [21,25]. In both pathways the inhibition of DAG production severely inhibits vacuole fusion. The direct binding of lipids by soluble ligands can also perturb fusion at different stages of the pathway. Binding ergosterol or PtdIns(4,5)P$_2$ is known to inhibit at the priming stage, whereas binding PtdIns(3)P inhibits at the docking stage by preventing the rebinding of Vam7p to the membrane via its Phox domain [12,22]. The role of lipids on the fusion machinery can be divided into at least two mechanisms. The first mechanism depends on the direct chemical interaction of lipids with the protein fusion catalysts, which is exemplified by the interaction of the Vam7p PX domain with PtdIns(3)P [26]. The second mechanism is indirect and physical [27]. This is demonstrated by the dependence of SNARE function on the assembly of metabolically active membrane raft microdomains [16]. The assembly of ergosterol-rich microdomains affects the local microviscosity of the membrane and can potentially exert allosteric effects on the transmembrane domains (TMDs) of Vam3p, Vti1p, and Nyv1p. The concentration of specific lipids into small domains, which may increase avidity, also regulates direct protein-lipid interactions and the dynamics of vacuole associated actin [16,24].
Fig 1.1 A canonical 3Q:1R SNARE complex showing the 0-ionic layer. Figure reproduced with permission from © Nature Reviews, Molecular Cell Biology, Volume 7, September 2006, pg: 633.
Fig 1.2: Alkaline phosphatase maturation assay to study membrane fusion.
REFERENCES


CHAPTER II

ROLE OF LIPID COMPOSITION, CURVATURE, AND FLUIDITY OF THE \textit{S. cerevisiae} VACUOLE IN REGULATING SNARE FUNCTION AND HEMIFUSION-FUSION TRANSITION
SUMMARY

Yeast vacuole fusion requires the formation of SNARE bundles between membranes. Although the function of vacuolar SNAREs is controlled by regulatory lipids, the exact role of the membrane in regulating fusion remains unclear. Because SNAREs are membrane-anchored and transmit the force required for fusion to the bilayer, we hypothesized that the lipid composition and curvature of the membrane aid in controlling fusion. Here, we examined the effect of altering the activation energy of fusion on the functionality of fusion-incompetent SNARE mutants that are thought to generate insufficient force to trigger the hemifusion-fusion transition. The hemifusion-fusion transition was inhibited by disrupting the 3Q:1R stoichiometry of SNARE bundles with the mutant SNARE Vam7p^{Q283R}. Similarly, replacing the transmembrane domain of the syntaxin homolog Vam3p with a lipid anchor allowed hemifusion, but not content mixing. Hemifusion-stalled reactions containing either of the SNARE mutants were stimulated to fuse with chlorpromazine, an amphipathic molecule that alters membrane fluidity and curvature, and affects the activation energy of fusion. The activity of mutant SNAREs was also rescued by the overexpression of SNAREs, thus multiplying the force transferred to the membrane. Thus, we conclude that either lowering the activation energy, or multiplying SNARE-generated energy restored the fusogenicity of mutant SNAREs that are stalled at hemifusion. We also found that regulatory lipids differentially modulated the complex formation of wild-type SNAREs. Together, these data indicate that the physical properties and the lipid composition of the membrane regulate the function of SNAREs in promoting the hemifusion-fusion transition.
INTRODUCTION

The direct role of SNARE proteins on fusion is generally thought to occur through the transfer of energy generated from the formation of trans-SNARE complexes to the membrane and reaching the activation energy of the fusion reaction [28]. Fusion is attenuated when the TMD of Vam3p or Nyv1p are exchanged for lipid anchors, or when the 3Q:1R paradigm is altered by mutating Vam7p or Nyv1p at the ionic zero layers of their SNARE motifs [29-31]. The defects in triggering fusion lie downstream of trans-SNARE complex formation, leading to the hypothesis that these SNARE complexes exert insufficient force to overcome the activation energy of fusion and may be stalled at the hemifusion stage. In this study we examined whether modifications in membrane fluidity (lateral mobility) and curvature (membrane bending), or the overexpression of SNAREs could compensate for the inability of non-fusogenic mutant SNAREs in triggering fusion. We report that non-canonical 2Q:2R SNARE complexes as well as those containing lipid-anchored Vam3p that normally do not support vacuole fusion can be stimulated to trigger fusion by lowering the activation energy required for the hemifusion-fusion transition. We also found that a subset of regulatory lipids controls the assembly and function of wild type SNARE complexes and their interactions with the HOPS complex.
RESULTS

The central ionic residue of SNARE motifs is conserved throughout eukaryotes and is essential for fusion, and each fusogenic SNARE bundle is composed of 3 Q-SNAREs and 1 R-SNARE (3Q:1R). Previously, we found that point mutations yielding 2Q:2R complexes were fusion incompetent and that fusion was restored when membrane fluidity was increased with chlorpromazine (CPZ) [30]. CPZ is an amphipathic small molecule that traverses the lipid bilayer and inserts itself into the inner leaflet of membranes thereby increasing the negative curvature of the outer leaflet of the bilayer [32]. A wealth of knowledge on the effects of CPZ on membrane physical properties has been gained from force measurements on the outer hair cell of the mammalian cochlea. CPZ decreases the steady state tethering force and increases lateral lipid mobility in outer hair cell membrane tethers [33]. It also decreases the lateral cell wall tension required for vesicle generation in the plasma membrane of the outer hair cell [34]. We theorize that these changes effectively lower the activation energy of the fusion reaction to a level attainable for the 2Q:2R SNARE complex. Here, we continue to examine the regulatory role of lipid composition and curvature on SNARE function. We first compared the effects of CPZ on the ability of recombinant Vam7p to bypass anti-Sec17 antibody inhibited fusion. In these experiments, SNARE priming was inhibited with antibody against Sec17p, after which increasing concentrations of recombinant wild type Vam7p or Vam7p^{Q283R} was added to stimulate fusion. Similar to previous findings [30], we found that Vam7p^{Q283R} was unable to bypass the anti-Sec17p block (Fig. 2.1A, solid squares), whereas wild type Vam7p demonstrated the characteristic biphasic curve of bypass fusion with maximum activity between 50 and 100 nM (Fig. 2.1A, solid circles). Treating fusion reactions with CPZ prior to adding Vam7p^{Q283R}
restored fusion to near wild type levels (Fig. 2.1A, open squares); however, this required a 10-fold increase in Vam7p\textsuperscript{Q283R}. Interestingly, CPZ inhibited the fusion bypass expected with low concentrations (10-50 nM) of wild type Vam7p (Fig. 2.1A, open circles). The inhibitory effect of CPZ on wild type Vam7p was overcome at higher concentrations without losing the biphasic characteristic of the curve.

Because CPZ reduced the efficacy of Vam7p at low concentrations during bypass reactions, we next tested whether CPZ altered the binding of Vam7p to the membrane. To determine this we added a dose curve of GST-Vam7p to anti-Sec17p antibody-blocked reactions in the presence or absence of CPZ. After incubation, the membrane fractions were re-isolated and probed for bound GST-Vam7p by immunoblotting. In Figure 2.1B we show that treatment with CPZ did not alter the binding of Vam7p to vacuoles, indicating that the differences observed in fusion were likely due to the effect of CPZ on membrane curvature and lateral tension. However, these data do not indicate whether Vam7p entered into the required level of functional SNARE complexes. Vacuole extracts were also probed for the presence of membrane-anchored Ypt7p as a control for gel loading. It is possible that CPZ decreases lateral bilayer tension such that low concentrations of Vam7p, and hence low numbers of trans-SNARE pairs, were insufficient to stimulate fusion, and that this block was overcome at higher concentrations of Vam7p. To address this, we tested whether the order in which the Vam7p and CPZ were added altered the efficacy of Vam7p in stimulating fusion. We hypothesized that the addition of Vam7p prior to CPZ would allow the soluble SNARE to form sufficient numbers of fusion competent protein complexes. Consistent with our hypothesis, we found that adding Vam7p before CPZ improved bypass fusion when compared with reactions where CPZ was added prior
to Vam7p (Fig. 2.1C). When CPZ was added to fusion reactions that lacked anti-Sec17 antibody, we found that CPZ moderately reduced fusion whether it was added before or after exogenous GST-Vam7p (Fig. 2.1D). Together these data suggest that the physical properties of a membrane (e.g. lateral bilayer tension and curvature changes due to membrane bending) can regulate the efficiency of SNARE mediated fusion.

Our previous studies showed that WT and Vam7p\(^{Q283R}\) form trans-SNARE complexes successfully [30], yet fusion required a 10-fold excess of Vam7p\(^{Q283R}\) to reach WT levels of fusion (Fig. 2.1A). Thus, we hypothesize that Vam7p\(^{Q283R}\) forms weaker SNARE bundles that do not exert sufficient force to reach the activation energy of fusion. Fusion was activated by CPZ, which in addition to selectively incorporating into the inner leaflets of membranes, has been reported to alter membrane curvature, lateral lipid mobility and bilayer tension [35]. CPZ activity has also been linked with promoting the transition of membranes from a hemifused to a fully fused state and lowering the energy required for pore formation [36]. Taken together, our data suggests that Vam7p\(^{Q283R}\) SNARE complexes exert insufficient force to trigger fusion following the trans-SNARE step and may be stalled in the hemifusion stage. To determine whether fusion mediated by Vam7\(^{Q283R}\) SNARE complexes is stalled before or after the hemifusion state, and to rule out vacuole lysis as the reason for observed differences between the WT and mutant Vam7p, we used a lipid-mixing assay that measures outer leaflet fusion alone [20,37]. Here, the outer leaflets of vacuoles were labeled with Rh-PE at levels that cause self-quenching of the fluorophore. Labeled vacuoles were mixed with an excess of unlabeled vacuoles and the fusion of the outer leaflets was measured by an increase in fluorescence due to the dilution and dequenching of Rh-PE in the hemifused vacuoles. We found that following a priming block
using anti-Sec17 antibody, both WT and Vam7p\textsuperscript{Q283R} equally supported lipid mixing in the absence of CPZ (Fig. 2.1E-F), suggesting that Vam7p\textsuperscript{Q283R} SNARE complexes are stalled at or after hemifusion. As a control, we used reactions that were inhibited with antibody against Vam3p or a mixture of Gyp1-56p and GDI to inhibit Ypt7p function. These control wells exhibited low levels of lipid mixing, indicating that since an earlier step in fusion (priming) was inhibited, these vacuoles could not move to the lipid mixing stage (Fig. 2.1E). This further suggests that the effect of CPZ on content mixing occurs after the hemifusion state. In addition, these data suggest that the ionic zero-layer of SNARE complexes serves as a checkpoint that needs to be passed to effect a transition from hemifusion to fusion. It should be noted that although the final extent of lipid mixing is the same, the kinetics of our experiment seem slower that the one reported in [38]. We attribute this difference to the fact that in our case, lipid mixing was monitored after the addition of recombinant Vam7p following a priming block with anti-Sec17, whereas the previous report was an uninhibited reaction. It is possible that when priming is inhibited in these reactions, there is a lag between subsequent rounds of fusion and lipid mixing and hence displays slower kinetics.

To further determine the effects of curvature and elasticity on vacuole fusion we compared the effects of CPZ with lysophosphatidylcholine (LPC), which is an inverted cone shaped lipid and intercalates into the outer leaflet of membranes leading to an increase in both positive curvature and tension [39]. Treating standard fusion reactions with LPC has been shown by others to inhibit vacuole fusion downstream of trans-SNARE complex formation [20]. This was likely due to the increase of activation energy to levels above those reached by SNARE complexes. Here, we compared the contrasting physical effects of CPZ and LPC in the bypass of
anti-Sec17 antibody blocked reactions by recombinant Vam7p. Fusion reactions were treated with buffer or anti-Sec17p antibody to block SNARE priming. Next, fusion reactions were further treated with buffer, CPZ, or LPC. As seen previously in Fig. 2.1, the addition of CPZ activated the fusogenic potential of Vam7p^{Q283R} (Fig. 2.2). In contrast to the effects of CPZ, treating with LPC blocked bypass fusion by both wild type and Vam7p^{Q283R}.

Because the order of addition altered the effects of CPZ on Vam7p-mediated bypass fusion, we tested whether pre-treating vacuoles with CPZ would nullify the inhibitory effect of LPC. When CPZ pre-treated vacuoles were subsequently treated with LPC we observed that fusion was completely inhibited to levels seen with LPC alone. Similarly, when LPC was added before CPZ treatment, bypass fusion was blocked with both wild type and mutant Vam7p (Fig. 2.2). Membrane fusion can itself be viewed as a two-step process: an initial fusion of the outer leaflets and stalk formation, followed by the fusion of the inner leaflets and fusion pore formation [36]. Hemifusion could then be placed as an intermediate between these two steps, where stalk formation has occurred but inner leaflet fusion and pore formation have not. LPC has been reported to inhibit fusion by inhibiting stalk formation [40], whereas CPZ acts during the second stage of lipid mixing by promoting hemifusion-fusion transition. According to our data, the effects of LPC are dominant over the effects of CPZ because LPC inhibits the first step in lipid mixing, regardless of the order of incorporation of LPC or CPZ. The above experiment emphasizes how the shape of a lipid and hence its effect on the membrane curvature can be vital in supporting hemifusion at the sites of membrane fusion.
The previous experiments suggested that non-canonical SNARE complexes did not exert sufficient force on the membrane and were stalled in the hemifusion stage. Thus, we hypothesized that increasing the numbers of these weaker SNARE complexes (2Q:2R) on a membrane should result in enough pulling force to activate the hemifusion-fusion transition. To test this hypothesis, we used yeast strains that overexpressed the four vacuolar SNARE proteins to assay the efficacy of wild type and Vam7p\textsuperscript{Q283R} in the bypass of an anti-Sec17 antibody block. When using wild type vacuoles we found that 400 nM Vam7p rescued fusion as much as 70% of the uninhibited fusion, whereas, the Vam7p\textsuperscript{Q283R} mutant only partially rescued fusion (Fig. 2.3A; black bars). Consistent with previous figures, CPZ increased the bypass fusion by the Vam7p\textsuperscript{Q283R} mutant. When using vacuoles from SNARE overexpressing strains, we found that both the wild type and Vam7p\textsuperscript{Q283R} rescued fusion well beyond the positive control by approximately 225% and 150%, respectively (Fig. 2.3A; gray bars). The addition of CPZ further augmented fusion triggered by both wild type and Vam7p\textsuperscript{Q283R}. These data demonstrate that non-canonical SNARE complexes can overcome the activation energy barrier required for hemifusion-fusion transition when the surface density of the mutant SNARE complexes is multiplied.

To determine if more SNARE complexes were formed by exogenous GST-Vam7p, SNARE complexes were affinity isolated using glutathione agarose beads. We observed a clear increase in GST-Vam7p containing complexes in the presence of excess SNAREs. However, there were no observable differences between the wild type and Vam7p\textsuperscript{Q283R} in the presence or absence of CPZ (Fig. 2.3B), indicating that the enhanced fusion was due to an increase in SNARE complex concentration. An earlier report suggests that the HOPS complex plays a more
direct role in fusion by proofreading and ensuring the integrity of the trans-SNARE complexes. The authors indicate that HOPS proofreads in two stages: it inhibits the formation of mismatched 0-layer SNARE complexes and can suppress fusion with vacuoles bearing 0-layer mutations after the formation of these mismatches complexes [41]. In our experiments, curiously, the increase in SNARE complexes observed with SNARE overexpression vacuoles did not correlate with an increase in the concentration of associated HOPS complex, suggesting that the excess SNARE complexes bypass the requirement for the HOPS complex and the proofreading mechanism.

Our data thus far has indicated that the physical state (e.g. curvature and lateral tension) of the membrane regulates the efficiency of the SNARE-mediated hemifusion-fusion transition. This suggests that the membrane determines the activation energy level that SNARE-induced force is required to reach to stimulate fusion. It is widely accepted that the formation of the helical SNARE bundle pulls and distorts the apposed membranes together to activate fusion. Here the energy generated from the formation of the SNARE complexes is transmitted to the membrane through the TMDs at the point of contact between the bilayers. Previous work by others has shown that the TMD of the vacuolar SNARE Vam3p is essential for productive priming and supporting fusion [31]. Also, cells carrying a GPI anchored hemagglutinin (HA) were stalled in the hemifusion stage and could not achieve complete fusion with red blood cells. The defect in fusion has been attributed to the absence of a TMD and the fact that the GPI anchor only traverses the outer leaflet of the bilayer [42-44]. Although the sequence is not as critical, there is a stringent requirement on the length of the TMD to achieve full functionality. Truncation mutants in the TMD of HA revealed a strict requirement of at least 17 amino acids to
successfully effect fusion. TMDs smaller than the critical length could not span the entire length of the bilayer and were stalled at the hemifusion stage [45]. On the other hand, increase in the separation between the coiled-coil and the transmembrane domain (by insertion of a flexible linker) also drastically reduced fusion, possibly by relieving the strain generated on the membrane [46].

The alpha-helical TMD of Vam3p was replaced with the isoprenylation sequence (CCIIM) of Ykt6p to make Vam3p-CCIIM. The lipid-anchored form of Vam3p-CCIIM is inserted into the vacuole and is present at levels similar to wild type Vam3p. Vam3p-CCIIM vacuoles accumulate cis- and trans-SNARE complexes indicating that Sec18pp-dependent priming is attenuated and that trans-SNARE pairs are non-productive resulting in nearly an 80% reduction in fusion relative to wild type [31]. We hypothesize that Vam3p-CCIIM fails to function due to a break in energy transfer between the SNARE complex and the membrane. Thus, it is likely that this mutant SNARE complex cannot transfer sufficient energy to reach the activation energy needed for hemifusion-fusion transition. This may be due to the shortened span of the lipid anchor, which only crosses one leaflet of the bilayer. Another possibility is that the specific interactions between the TMD of Vam3p and certain lipids in the bilayer are abolished leading to reduced fusion. Furthermore, the defective priming seen with this mutation is likely due to the inability of Sec17p and Sec18pp to correctly disrupt the cis-SNARE complex, suggesting that Vam3p-CCIIM complexes may adopt an altered conformation compared to wild type complexes.
Because membrane fusion requires the generation of a specific activation energy to drive lipid rearrangement during the hemifusion-fusion transition, we hypothesized that lowering the activation energy would stimulate fusion of Vam3p-CCIIM containing vacuoles as well. To this aim, we used CPZ to lower the energy barrier and help catalyze fusion. We used vacuoles from DKY6281 and BJ3505 strains that produced Vam3p-CCIIM in standard fusion assays. In Fig. 2.4A we show that treatment with CPZ led to a greater than two-fold increase in fusion relative to the untreated control. This is also in accord with the idea that CPZ lowered the activation energy of fusion to a level that matched the force generated by Vam3p-CCIIM. This supports the idea that the membrane regulates SNARE mediated fusion. Others have shown that the interactions of Vam3p TMDs promote the hemifusion-fusion transition [47]. Thus, we posited that replacing the TMD of Vam3p with a lipid anchor would also lead to an arrest at the hemifusion-fusion state. To examine this we examined the ability of Vam3p-CCIIM to stimulate hemifusion using the aforementioned lipid-mixing assay and found that Vam3p-CCIIM vacuoles reached hemifusion similar to what was observed with WT vacuoles (Fig. 2.4B-C). As a control, we used reactions that were inhibited with antibody against Vam3p or a mixture of Gyp1-56p and GDI to inhibit Ypt7p function. Thus, the lack of a TMD inhibits the hemifusion-fusion transition.

Next, we performed Vam7p bypass experiments using two combinations of vacuoles. One combination contained wild type and Vam3p-CCIIM vacuoles at a 1:1 ratio, where the DKY6281 reporter strain contained wild type Vam3p and the BJ3505 reporter strain harbored Vam3p-CCIIM (Fig. 2.5A). The second combination used both reporter strains that harbored Vam3p-CCIIM (Fig. 2.5B). When we tested the effect of adding exogenous Vam7p, we found
that this SNARE was unable to activate fusion using either vacuole combination. However, fusion was activated when vacuoles were treated with CPZ. Here, Vam7p\textsuperscript{Q283R} was also able to stimulate fusion in the presence of CPZ, albeit less efficiently that wild type Vam7p. Importantly, no combination of Vam7p and CPZ was able to stimulate fusion when both vacuole partners contained Vam3p-CCIIM (Fig. 2.5B).

It should be noted that the vacuoles carrying the Vam3p-CCIIM mutation accumulate cis-SNARE complexes due to a defect in priming and require the addition of exogenous Sec18pp for efficient disassembly [31]. An alternative explanation for Fig 2.5B could be construed based on the fact that in these mutants, a higher proportion of SNAREs are stuck in the cis-SNARE configuration thus resulting in fewer free SNAREs available for trans-SNARE complex formation during the Vam7p bypass reaction. If this were true, the reduced numbers of free SNAREs available for trans pairing, on either or both of the fusing partners would serve as the limiting factor for fusion and we would expect to see identical fusion profiles for Vam7p bypass reactions in the presence of CPZ with both the mixed (WT fused with Vam3p-CCIIM) and the pure Vam3p-CCIIM populations of vacuoles. We reason that the trans-SNARE complexes formed in 2.5A and 2.5B are equivalent in numbers and only differ in the presence or absence of at least one functional TMD. This difference is reflected in their ability to respond to CPZ treatment. Thus, we rule out priming defect as an explanation for the above observation and conclude that CPZ enhances fusion with a mutant Vam3p defective in transmitting force to the bilayer.
Collectively, these data suggest that the correct SNARE complex conformation together with the TMD act to transmit the force generated by the SNARE complex to the lipid bilayer. This force could be used to generate the activation energy required for lipid rearrangement to complete fusion. The incorrect SNARE conformation or the absence of a TMD result in insufficient force transmitted to the membrane and hence an inability to progress to complete fusion from hemifusion. Again, CPZ helps promote the hemifusion-fusion transition, but is unable to do so in the absence of a TMD to transmit the force to the membrane.

Thus far, our data corroborates the hypothesis that the physical properties of membranes regulate fusion via the TMDs of SNAREs. In native biologically active membranes, the physical properties of the bilayer are controlled by the lipid composition and the lateral segregation of microdomains where regulatory lipids and SNAREs interdependently assemble to promote fusion [16]. These regulatory lipids are likely to control the local curvature and fluidity at the site of fusion. In vacuoles, the fusion machinery is gathered into membrane rafts regulated by a small group of chemically important lipids that include DAG, PA, ergosterol, and a panel of phosphoinositides. Here we tested whether regulatory lipids controlled trans-SNARE complex formation and interactions with the HOPS tethering complex. We used well-characterized lipid-binding ligands to specifically bind and sequester lipids. PtdIns(3)P was ligated with either FYVE or PX domains. The Ent3p-ENTH domain was used to bind PtdIns(3,5)P₂, PtdIns(4,5)P₂ was either bound with the Epsin 1 ENTH domain or converted to PtdIns(4)P with the bacterial PtdIns phosphatase SigD. FappPH was used to bind PtdIns(4)P. DAG was bound with C1b, and ergosterol was bound by the polyene macrolide filipin. To isolate trans-SNARE complexes, we performed fusion assays using vacuoles isolated from yeast that lacked Nyv1p and expressed
Vam3p containing an internal calmodulin-binding peptide (CBP) (CBP-Vam3p nyv1Δ), which were incubated with DKY6281 vacuoles (VAM3 NYV1) [48]. This ensured that the observed CBP-Vam3p-Nyv1p complexes were formed in trans. Following incubation CBP-Vam3p protein complexes were affinity isolated with calmodulin agarose and analyzed by immunoblotting. All of the ligands and phosphatases used resulted in potent fusion inhibition (Fig. 2.6A), consistent with previous reports [12,16,49]. The inhibition of fusion by Ent3p-ENTH is novel and suggests that PtdIns(3,5)P₂ plays an important role in vacuole fusion [50].

Through these experiments we found that regulatory lipids had distinct roles in the formation of trans-SNARE complexes as well as their interactions with the HOPS complex. We found that interaction of CBP-Vam3p with Nyv1p was most sensitive to FYVE, PX, and FappPH, illustrating that PtdIns(3)P and PtdIns(4)P were crucial for trans-SNARE pairing (Fig. 2.6B), in accord with findings reported by Collins and Wickner [48]. We also found that C1b and filipin reduced the pairing of CBP-Vam3p and Nyv1p, demonstrating that DAG and ergosterol were also required for optimal complex formation. The PX domain blocked fusion and reduced CBP-Vam3p-Nyv1p interaction, yet had no effect on Vam7p incorporation into the remaining complex. The effect on fusion was likely due to the binding of PtdIns(3)P and HOPS complex by the Vam7p PX domain [12,49]. Surprisingly, blocking PtdIns(4)P with FappPH had a striking effect on Vam7p incorporation into SNARE complexes while the total Vam7p associated with the membrane was unaffected (not shown). This suggests that PtdIns(4)P was important for the stabilization of the 3Q SNARE complex. Although Ent3p-ENTH potently inhibited fusion (Fig. 2.6A), it had no effect on trans-SNARE complex formation indicating that PtdIns(3,5)P₂ regulated fusion either downstream of docking or in a parallel pathway. The exact mechanism of
inhibition by Ent3p-ENTH remains uncertain. Others have found that the Ent3p-ENTH domain interacts with the N-terminal domains of Vti1p, Pep12p and Syn8p where it promotes the fusion of late endosomes [51,52]. Thus, the inhibitory nature of this domain on vacuole fusion is likely not due to SNARE binding but rather to its ability to bind PtdIns(3,5)P2 [50]. The role of PtdIns(4,5)P2 was examined using the Epsin1-ENTH domain and the bacterial phosphatase SigD. We found that each reduced trans-SNARE pairing and HOPS binding to the complex. C1b and filipin both reduced Nyv1p-Vam3p interaction and blocked the incorporation of Vam7p into SNARE complexes demonstrating that DAG and ergosterol control the formation of Vam7p-containing trans-SNARE complexes. This also suggests that Nyv1p does not require a complete 3Q-SNARE bundle to interact with Vam3p. This is keeping with a recent report showing the existence of an alternative pathway in trans-SNARE complex formation [53]. Other reports have shown that blocking DAG with C1b has no effect on bulk association of Vam7p with the membrane [16], suggesting that DAG is directly required in the stabilization of the 3Q-SNARE complex and subsequent trans complex. Curiously, treating vacuoles with C1b also caused an upward mobility shift in Vps33p; however, it is unclear whether protein modification occurred.

Regarding the role of regulatory lipids and the association of HOPS with SNARE complexes, we must integrate our results with previous studies investigating the overall binding of HOPS to vacuoles. Previous studies have shown that both Vam7p and HOPS can dissociate from vacuoles when treated with specific lipid binding ligands. One study showed that HOPS was released from membranes when vacuoles were treated with FYVE, FappPH or Epsin1-ENTH, indicating that PtdIns(3)P, PtdIns(4)P, and PtdIns(4,5)P2, respectively aid in retaining the tethering complex on the vacuole [49]. This is in keeping with our data showing that FYVE,
FappPH and Epsin1-ENTH reduced the amount of Vps33p associated with CBP-Vam3p protein complexes. Thus, the reduction of HOPS associated with SNARE complexes may be due to the overall loss of HOPS from the membrane. However, it is unclear whether HOPS or Vam7p continue to bind phosphoinositides after trans-SNARE complex assembly. It is not known whether the membrane association of HOPS is affected by ligands that target other regulatory lipids. The reduction of Vam7p in trans-SNARE complexes is also likely to be affected by the overall level of Vam7p present on membranes treated with lipid binding ligands. Previously, we showed that Vam7p was released from vacuole membranes when regulatory lipids were blocked with ligands that targeted PtdIns(3)P, PtdIns(4,5)P2, and ergosterol [16].

Because some lipids function in fusion before SNARE complex formation while others function downstream, we next examined whether a block on the early acting lipids could be bypassed by adding soluble Vam7p. Fusion reactions were blocked with different lipid ligands and subsequently treated with exogenously added soluble Vam7p. In Figure 2.7A we show that Vam7p rescued fusion reactions where PtdIns(3)P was blocked with PX, but not FYVE domain. This is likely due to the direct competition between PX and full length Vam7p. We also found that Vam7p could partially bypass blocks mediated by SigD, Epsin1-ENTH and FappPH. These data suggest that PtdIns(3)P, PtdIns(4)P, and PtdIns(4,5)P2 function upstream of Vam7p. In contrast, Vam7p was unable to bypass blocks by C1b and filipin, suggesting that DAG and ergosterol, respectively function downstream or in parallel to Vam7p. We next examined if modulating membrane curvature and fluidity could enhance the Vam7p bypass of lipid ligand inhibition. To this end we tested whether CPZ could aid Vam7p bypass of four different lipid ligands (Fig. 2.7B). We found that fusion blocked by FYVE or filipin were not affected by the
addition of CPZ. In contrast, CPZ enhanced the bypass of a PX block. Importantly, CPZ allowed Vam7p to bypass a C1b block. Because C1b prevents the function of DAG, which induces negative curvature and destabilizes lipid bilayers [54,55], it is possible that the effect of CPZ could overcome the effect of DAG by promoting negative curvature and lowering the energy barrier at the site of fusion. Together these data indicate that different regulatory lipids operate upstream and downstream of Vam7p. It also illustrates that some of the lipids that act downstream of Vam7p (e.g. DAG) could have an important role in maintaining the curvature of the membrane during fusion.
DISCUSSION

In this study we examined the role of the lipid bilayer in the regulation of SNARE-mediated fusion. The effects of lipid composition and the physical properties of membranes on SNARE function have been largely overlooked. The lipid bilayer exerts two major influences on membrane-spanning proteins at a non-bonding chemical level, and non-specific interactions that occur at a physical level. In terms of physical effects, the bilayer can serve as a regulator of membrane-spanning proteins, where protein function can be affected by numerous parameters including bilayer thickness, fluidity, curvature, acyl chain packing, lateral compression, hydrophobic mismatching, and line tension [27]. Membrane thickness itself can be modulated by sterol content as well as acyl chain length, and can have profound effects on protein function. Membrane raft microdomains are enriched in cholesterol/ergosterol and sphingolipids that phase separate from the bulk of the membrane [56]. We hypothesize that the core fusion machinery is controlled by the local physical state of the vacuolar membrane. This concept is consistent with studies showing that SNARE proteins are often found in clusters in cholesterol-rich membrane rafts as shown by microscopy, and isolation of detergent resistant rafts [57]. Moreover, a growing number of studies have shown that specific lipids that influence the physical properties of membranes regulate SNARE-dependent fusion, including omega-3 docosahexaenoic acid [58], sphingosine [59], cholesterol [60-62], phosphoinositides [12,16,23,63,64], DAG [21], and PA [25,65].

SNARE-mediated fusion occurs when the formation of four alpha-helical coiled-coils release free energy. The energy is transferred through the membrane-proximal linkers and into
the TMD domains, which then deform and destabilize the bilayer to surpass the activation energy threshold required for fusion. When SNARE complexes are mutated such that the canonical 3Q:1R ratio is changed (e.g. 2Q:2R), membrane fusion is attenuated. We surmise that the mutant SNARE complex exerts insufficient force on the membrane leading to fusion stalled at the hemifusion stage [30]. We further tested this hypothesis by examining SNARE complexes where one TMD is exchanged for a lipid anchor (e.g. Vam3p-CCIIM) [29,31]. Here we showed that matching the energy barrier needed to stimulate lipid mixing with the energy released by the SNARE bundle could activate otherwise non-functional SNARE complexes. One method used in this study was the chemical treatment of membranes with the cationic amphipath CPZ, which stimulated the hemifusion-fusion transition mediated by 2Q:2R SNARE complexes as well as those containing Vam3p-CCIIM. Because CPZ lowers membrane tension our results are in accord with the notion that lowering the activation energy threshold of lipid mixing enables weaker SNARE bundles to stimulate the hemifusion-fusion transition. We also augmented Vam7p$^{Q283R}$-mediated fusion by increasing the copy number of SNARE complexes on the vacuole, suggesting that the energy generated by each mutant SNARE complex is additive in the bypass of the membrane energy barrier to stimulate fusion.

Although we have shown that mutant SNAREs can be activated by chemically modulating membrane tension and curvature, it is still unclear how the assembly of the vertex microdomain controls SNARE mediated fusion. Lipid composition can change the local physical properties of the bilayer, yet the mechanisms by which these lipids regulate fusion remain uncertain. Regulatory lipids such as sterols and sphingolipids serve to thicken and stiffen raft microdomains, whereas others are essential for the recruitment and activation of soluble proteins
A third function of regulatory lipids in these microdomains is the focused destabilization of the bilayer. Lipids such as DAG are known to induce negative curvature and destabilize bilayers [54, 55]. The negative curvature induced by DAG and its enrichment at the vertex ring is consistent with the occurrence of a hemifusion intermediate [20]. This is also in keeping with the stalk hypothesis of hemifusion where the fusion stalk contains negative curvature structures [68]. Formation of the stalk is energetically disfavored by the insertion of LPC and creation of positive curvature in the outer layer of the membrane. Our finding that CPZ can overcome the inhibited fusion caused by C1b emphasizes this importance of DAG in creating negative curvature and destabilizing the membrane. We posit that binding DAG with C1b inhibits the lipid from inducing negative curvature and that CPZ restores the negative curvature needed for fusion.

The mechanism by which the membrane regulates SNAREs through a single TMD connecting to the SNARE motif via a short linker remains uncertain. However, new insights have been provided by the crystal structure of the neuronal cis-SNARE complex [69]. Several new aspects of SNARE interactions were revealed, including the continuity of the alpha helices. These helices begin in the SNARE motifs and continue undisrupted through the linkers and into the TMDs. With this knowledge, it is much easier to envision how changes in the membrane could be transferred to the SNARE motif. Furthermore, specific interactions were found between the TMDs of syntaxin-1 and synaptobrevin-2, suggesting that interactions between the TMDs could affect the conformational arrangement of the cytoplasmic domains and regulate cis-SNARE priming. The continuity of the alpha helices coupled with the interactions of TMDs sheds some light on other studies showing that TMD of synatxin-1 contains residues that regulate its cytoplasmic interactions with SNAP-25, a lipid anchored SNARE that lacks an alpha helical
TMD [70], indicating that the syntaxin-1 TMD allosterically regulates the interaction of SNAP-25 with the cytoplasmic domain of syntaxin-1. Ungermann and colleagues showed that the TMD of the vacuolar SNARE Vam3p is essential for productive priming and supporting fusion [31]. Lipid-anchored Vam3p-CCIIM vacuoles accumulate cis- and trans-SNARE complexes indicating that Sec18pp-dependent priming is attenuated and that trans-SNARE pairs are non-productive resulting in a striking inhibition in fusion relative to wild type Vam3p. The lack of a TMD would inhibit the interactions of Vam3p-CCIIM with the TMDs of other vacuolar SNAREs and potentially alter the conformational structure of the cytoplasmic SNARE bundle and its productive interactions with the priming machinery. There is mounting evidence, including the results of the current study, indicating that the membrane serves as an a regulator of SNARE function.

One way that membranes can modulate the allosteric effects on proteins is through changing its physical properties, which are regulated through modifications in lipid composition. This is consistent with vacuole fusion as the stoichiometry of lipid species composing the vertex microdomains are not static. Numerous lipid modifications occur through the actions of lipid kinases, phosphatases, and lipases. For example, during vacuole fusion DAG is produced by Plc1p (phospholipase C) as well as the phosphatidic acid phosphatase Pah1p [21,25]. In addition, various lipid kinases produce phosphoinositides during vacuole fusion that include PtdIns(3)P, PtdIns(4)P, PtdIns(3,5)P_{2}, and PtdIns(4,5)P_{2} [23,71]. The dephosphorylation of phosphoinositides has also been shown to disrupt vertex microdomains and disperse SNAREs from the site of fusion [16]. In addition, the genetic deletion of various phosphoinositide phosphatases yields abnormal vacuolar phenotypes, a hallmark of defective vacuole homeostasis. 
Among these phosphatases is the PtdIns(3)P phosphatase Ymr1p, the synaptojanin-like phosphatases Sjl2p and Sjl3p, and the PtdIns(4,5)P$_2$ 5-phosphatase Inp54p [72,73]. Together, these reports underscore the dependence of vacuole function and lipid modification. Although we have yet to determine the exact roles of lipid modification on vacuole SNARE function, there is growing evidence that the two are integrated in the regulation of vacuole function.
MATERIALS AND METHODS

Reagents

All reagents were diluted in PS buffer (20 mM PIPES-KOH pH 6.8, 200 mM sorbitol) to a working concentration before use in any experiment. Antibodies against Nyv1p [74], Sec17p [75], Sec18pp [11], Vam3p [76] have been described previously. Chlorpromazine (Sigma) was stored as a 4.5 mM stock solution in DMSO. Lysophosphatidyl choline (Sigma) was stored at a stock concentration of 1% (wt/vol) in 100% ethanol. Filipin was dissolved in DMSO at a stock concentration of 10 mM and used at a final concentration of 20 μM.

Recombinant proteins

Recombinant GST-Vam7p (wild type and Q283R) was purified and dialyzed into PS buffer with 125 mM KCl as described [30,77]. The lipid binding domains GST-C1b [78], GST-FYVE [79], GST-PX [12], GST-Ent3p-ENTH (a gift from S. Friant, Institut de biologie et chimie des proteins, Lyon, FR) [50], GST-Epsin1-ENTH [80], MBP-FappPH [49], His6-MTM-1 [81], His6-SigD [82], His6-Gyp1-56 [83], GDI [84] were purified as previously described.

Strains

BJ3505 (MATα pep4::HIS3 prb1-Δ1.6R his3-200 lys2-801 trp1Δ101(gal3) ura3-52 gal2 can1) and DKY6281 (MATα leu2-3 leu2-112 ura3-52 his3-Δ200 trpΔ1-901 lys 2-801) were used for standard vacuole fusion assays [85]. The Vam3p-CCIIM strains carry a chimeric Vam3p where the transmembrane domain was replaced with the farnesylation site from Ykt6p [31]. Yeast strains overexpressing the four vacuolar SNAREs under the control of an ADH promoter (A gift
from V. Starai, University of Georgia) were used in Vam7p bypass fusion reactions [84]. BJ3505 CBP-Vam3p nyv1Δ was used for isolating trans-SNARE complexes [48].

**Vacuole Isolation and in vitro vacuole fusion**

Vacuoles were isolated from yeast strains by floatation [86]. Fusion reactions (30 µl) contained 3 µg each of vacuoles from BJ3505 (pep4Δ PHO8) and DK6281 (PEP4 pho8Δ), fusion assay buffer (125 mM KCl, 5 mM MgCl₂, 20 mM PIPES-KOH pH 6.8, 200 mM sorbitol), ATP regenerating system (1 mM ATP, 29 mM creatine phosphate, 0.1 mg/ml creatine kinase), 10 µM CoA and 283 nM IB2. Reactions were incubated at 27°C for 90 minutes and the Pho8p activity was measured in 250 mM Tris-Cl pH 8.5, 0.4% Triton X-100, 10 mM MgCl₂, 1 mM p-nitrophenylphosphate. During fusion, the Pep4p protease gains access to the pro-alkaline phosphatase (proPho8p) and cleaves it to form mature alkaline phosphatase. The quantity of p-nitrophenylate produced was measured at 400 nm. One unit of fusion was defined as 1 µmol of p-nitrophenylate produced minute⁻¹ µg⁻¹ of pep4Δ vacuoles.

**Vam7p bypass fusion reaction**

Fusion reactions were incubated at 27°C with 85 µg/ml α-Sec17 antibody to block priming. After 15 minutes, 150 µM chlorpromazine (CPZ) or 0.033% lysophosphatidylcholine (LPC) was added and the reactions were incubated for 5 minutes before the addition of Vam7p or Vam7pQ283R. The reactions were incubated for an additional 70 minutes and assayed for alkaline phosphatase activity.
Trans-SNARE complex Isolation

Trans-SNARE complexes were isolated and analyzed as described with some minor modifications [48,87]. Large-scale 16X (480 μl) fusion reactions containing 48 μg of vacuoles from BJ3505 CBP-Vam3p nyvΔ and 48 μg of vacuoles from DK6281 backgrounds were incubated in the presence of buffer or different lipid binding domains (10 μM Ent3p-ENTH, 10 μM GST-Epsin1-ENTH, 25 μM GST-PX, 2 μM His6-SigD, 1 μM MBP-FappPH, 20 μM Filipin, 10 μM GST-C1b or 2 μM GST-FYVE). After incubation at 27°C for 90 minutes, reactions were placed on ice for 5 minutes and 30 μl was withdrawn from each sample to assay Pho8p activity. The remaining samples were centrifuged (16,000 g, 15 minutes, 4°C) to sediment the vacuoles and the supernatants were discarded. Vacuole pellets were overlaid with 200 μl ice-cold solubilization buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 0.5% Triton X-100 and 20% glycerol) with protease inhibitors (0.46 μg/ml leupeptin, 3.5 μg/ml pepstatin, 2.4 μg/ml Pefabloc-SC and 1 mM PMSF) and gently resuspended. Solubilization buffer was added to a final volume of 400 μl and the extracts were mixed by nutating at 4°C. After 20 minutes, insoluble debris was removed by centrifugation (16,000 g, 20 minutes, 4°C). Supernatants were transferred to fresh tubes and 10% of the extract was saved for input samples. The remaining extracts were brought up to 2 mM CaCl₂ and incubated with 40 μl calmodulin Sepharose beads equilibrated with solubilization buffer (GE Healthcare; 4°C, 12 h, nutating). The beads were collected by centrifugation (3,000 g, 2 minutes, 4°C) and washed five times with 1 ml of solubilization buffer containing 1% Triton and 2 mM CaCl₂ followed by bead centrifugation. Bound proteins were eluted with solubilization buffer containing 0.1% Triton X-100 and 5 mM EGTA. The eluents were mixed with SDS sample buffer and used for SDS-PAGE analysis and immunoblotting.
Lipid mixing

Lipid mixing assays were conducted using Rhodamine conjugated phosphatidylethanolamine (Rh-PE; Invitrogen) as described with minor modification [25]. BJ3505 background vacuoles (300 µg) were incubated in 400 µl of PS buffer containing 150 µM Rh-PE (10 minutes, 4°C, nutating). Samples were mixed with 15% Ficoll in PS buffer (wt/vol) and transferred to an ultracentrifuge tube (11 x 60 mm). Samples were overlaid with 1.0 ml each of 8%, 4%, and 0% Ficoll. Labeled vacuoles were re-isolated by centrifugation (105,200 g, 25 minutes, 4°C) and recovered from the 0 - 4%-Ficoll interface. Lipid mixing assays (90 µl) contained 2 µg of Rh-PE-labeled vacuoles and 16 µg of unlabeled vacuoles in fusion buffer. Reaction mixtures were transferred to a black, half-volume 96-well flat-bottom microtiter plate and Rhodamine fluorescence was measured using a fluorometer at 27°C. Measurements were taken every minute for 60 minutes, yielding fluorescence values at the onset ($F_0$) and during the reaction ($F_t$). The final 10 measurements of a sample after adding 0.33% (vol/vol) Triton X-100 were averaged and used as a value for the fluorescence after infinite dilution ($F_{TX100}$). The relative total fluorescence change $\Delta F_t/F_{TX100} = (F_t - F_0)/F_{TX100}$ was calculated.
Figure 2.1. Non-canonical SNARE complexes exert insufficient force on the membrane.

(A) Individual fusion reactions were blocked at the priming stage by treating vacuoles with 85 μg/ml anti-Sec17p antibody for 15 minutes. Recombinant Vam7p (closed circles) or Vam7pQ283R
(closed squares) was added to bypass the priming block. In parallel, Vam7p and Vam7pQ283R were added to reactions blocked with anti-Sec17p antibody and before treatment with CPZ. (B) Fusion reactions were treated with anti-Sec17p antibody in the presence or absence of CPZ. Next, recombinant Vam7p was added to reactions at the indicated concentrations. After incubation (10 minutes, 27°C), membranes were pelleted (16,000 g, 10 minutes, 4°C) and the unbound Vam7p was discarded with the supernatant. Bound Vam7p was examined by immunoblotting. Membranes were also probed for Ypt7p as a control for loading. (C) Fusion reactions were treated with anti-Sec17 antibody after which Vam7p alone (closed circles) was added to bypass the fusion block. In parallel, reactions were treated with CPZ either before (open triangles) or after addition of Vam7p (open squares). (D) Uninhibited fusion reactions were treated with Vam7p alone (closed circles). In parallel, reactions were treated with CPZ either before (open triangles) or after addition of Vam7p (open squares). (E) Lipid mixing (hemifusion) assays were performed using vacuoles blocked with anti-Sec17p at 4°C. After 15 minutes, 400 nM Vam7p (WT or Q283R) was added to the indicated reactions and incubated for 60 minutes at 27°C. The increase in fluorescence occurred when the outer leaflets of vacuoles were mixed during hemifusion. Shown is a representative of 3 trials. (F) Shown is the average lipid dequenching at 60 minutes. Error bars indicate SEM (n=3).
Figure 2.2. Lysophosphatidylcholine inhibits the Vam7p bypass of anti-Sec17p IgG blocked fusion reactions. Fusion reactions were treated with buffer or blocked with anti-Sec17p antibody. Reactions were further treated with 150 μM CPZ or 0.033% LPC for 5 minutes preceding the addition of recombinant Vam7p or Vam7p\textsuperscript{Q283R}. In parallel, anti-Sec17p antibody blocked vacuoles were treated with CPZ for 5 minutes followed by LPC for 5 minutes or vice versa before the addition of 400 nM Vam7p or Vam7p\textsuperscript{Q283R}. Error bars represent SEM (n=3)
Figure 2.3. Non-canonical SNARE complexes support fusion when their surface density is increased. (A) Standard fusion reactions containing vacuoles from wild type strains (black bars) or SNARE overexpressing strains (gray bars) were blocked at the priming stage with 85 μg/ml anti-Sec17p antibody for 15 minutes. Recombinant GST-Vam7p (400 nM, black bars) or Vam7pQ283R (gray bars) was added to bypass the priming block. Error bars represent SEM (n=3). (B) GST-Vam7p protein complexes were isolated as described in the Materials and Methods section and examined by immunoblotting for the indicated proteins.
Figure 2.4. CPZ augments fusion of vacuoles containing lipid-anchored Vam3p.
(A) Standard fusion reactions were performed using vacuoles from DKY6281 and BJ3505 strains harboring Vam3p-CCIIM. Fusion reactions were treated with buffer (Control) or CPZ and incubated for 90 minutes and fusion was measured by content mixing and Pho8p activity. (B) Lipid mixing (hemifusion) assays were performed using WT or Vam3p-CCIIM vacuoles as described in the Materials and Methods section. WT vacuoles were also treated with anti-Sec17 to block SNARE priming. Reactions were incubated for 60 minutes at 27°C. Shown is a representative of 3 trials. The inset represents the average lipid dequenching at 60 minutes. Error bars indicate SEM (n=3). (C) Shown is the average lipid dequenching at 60 minutes. Error bars indicate SEM (n=3).
Figure 2.5. CPZ and Vam7p bypass anti-Sec17p IgG-blocked fusion of vacuoles containing lipid-anchored Vam3p.

(A) Vacuoles from wild type DKY6281 were incubated with BJ3505 vacuoles harboring Vam3p-CCIIM. Priming was blocked with anti-Sec17p antibody for 15 minutes and reactions were treated with 150 μM CPZ for 5 minutes before the addition of 400 nM Vam7p or Vam7p^{Q283R}. (B) Fusion reactions were performed with DKY6281 and BJ3505 vacuoles harboring Vam3p-CCIIM. Fusion reactions were treated with CPZ and Vam7p as described in (A). Error bars represent SEM (n=3).
Figure 2.6. Regulatory lipids control trans-SNARE complex formation and interactions with the HOPS complex.

16X standard fusion reactions containing 48 μg of BJ3505 CBP-Vam3p nyv1Δ vacuoles and 48 μg DK6281 vacuoles were treated with inhibitory concentrations of the following lipid binding domains: 2 μM GST-FYVE, 25 μM GST-PX, 1 μM MBP-FappPH, 10 μM Ent3p-ENTH, 10 μM Epsin1-ENTH, 10 μM GST-C1b, 2 μM His6-SigD or 20 μM filipin for 90 minutes at 27°C. (A) Samples (30 μl) were withdrawn from each reaction to measure Pho8p activity following fusion. Error bars represent SEM (n=3). (B) The reaction remainders were centrifuged to isolate the vacuoles. Membranes were then solubilized and CBP-Vam3p complexes were affinity purified.
Figure 2.7. Vam7p and CPZ can bypass fusion blocked by specific lipid ligands.

(A) Fusion reactions were treated for 5 minutes with lipid binding domains including GST-FYVE, GST-PX, GST-C1b, Filipin, His$_6$-SigD, MBP-FappPH and Ent3p-ENTH as described in Fig. 5. Next, Vam7p (400 nM) was added to the reactions and incubated for a total of 90 minutes. Fusion was tested by content mixing and Pho8p activity. (B) Fusion reactions were treated for 5 minutes with lipid binding domains including. Next, 150 μM CPZ was added and the reactions were incubated for an additional 5 minutes before the addition of 400 nM Vam7p. Error bars represent SEM (n=3).
REFERENCES


CHAPTER III

ROLE OF THE YEAST PA PHOSPHATASE Pah1p IN
THE RECRUITMENT OF THE AAA+ ATPase Sec18p TO
CIS-SNARE COMPLEXES DURING PRIMING
SUMMARY

Yeast vacuole membrane fusion relies on a set of regulatory lipids and lipid modifying enzymes. One such enzyme (Pah1p) controls the dephosphorylation of phosphatidic acid (PA) to yield diacylglycerol (DAG). Here, we examined the role of Pah1p in the priming stage of fusion. Specifically, we found Pah1p as the factor responsible for recruitment of Sec18p, a AAA+ ATPase that disassembles cis-SNARE complexes during priming. We demonstrate a direct interaction between Sec18p and the lipid phosphatidic acid. This interaction was dictated by the nucleotide bound state of Sec18p. ADP bound population of Sec18p was primarily found associated with phosphatidic acid, whereas the ATP-bound active configuration of Sec18p bound the cis-SNARE complexes. We also show Pah1p as the factor that triggers a switch from inactive phosphatidic acid-bound Sec18p to the active cis-SNARE bound Sec18p during priming in yeast vacuole membrane fusion. These findings demonstrate that modifying the lipid composition of the membrane can be an important trigger for recruitment of fusion factors.
INTRODUCTION

Membrane fusion involves the fusion of two distinct lipid bilayers into a continuous membrane followed by mixing of the luminal contents. Biological membrane fusion is an underlying recurrent theme in a variety of cellular processes including endocytosis, neurotransmission, antigen presentation and cargo sorting (1-3). The basic scheme of lipid bilayer fusion is conserved throughout eukaryotes (4) and requires regulatory lipids and conserved proteins like the GTPases of the Rab/Ypt family, NSF/Sec18p ATPase and its co-chaperone α-SNAP/Sec17p and the essential protein fusion machinery called the SNAREs (soluble NSF attachment protein receptors (5).

Yeast homotypic vacuole fusion is an exemplary model to study membrane fusion and is characterized by a series of experimentally distinct, well-defined phases. The fusion cascade is initiated when the AAA+ protein Sec18pp (NSF) and Sec17p (α-SNAP) disassemble unproductive cis-SNARE complexes during priming (5,6). Disruption of cis-SNARE complexes leads to the dissociation of Sec17p as well as the soluble SNARE Vam7p (7,8). In the next stages of tethering and docking, primed vacuoles become reversibly docked through the function of the Rab family GTPase Ypt7p and its effector complex HOPS (9). The activity of Ypt7p is regulated by its cognate nucleotide exchange factor Mon1p and the GTPase activating factor Gyp7p. During the docking stage Vam7p rebinds through its interactions with the heterohexameric tethering complex HOPS and the lipid phosphatidylinositol 3-phosphate (PI3P), and enters a trans-SNARE complex (10). The Sec/Munc proteins play a vital role ensuring the correctness of the trans-SNARE complexes (11). Vacuole fusion occurs at highly organized membrane rafts termed vertex microdomains that form at the point of contact between partner
vacuoles, and become enriched in the lipids and proteins required for fusion (12,13). The formation of the trans-SNARE complexes triggers the release of $\text{Ca}^{2+}$ from the vacuole lumen (14). The penultimate step in fusion is a hemifusion intermediate where the closely apposed outer membrane leaflets fuse without fusing the inner leaflets (15). The fusion pathway culminates with the fusion of inner leaflets and content mixing.

The interplay of interaction between fusion proteins and lipids is a cornerstone in the progression and regulation of membrane fusion. The lipids could serve two functions; they could serve as a scaffold for the binding and recruitment of various protein factors. This function depends on the direct chemical interaction of lipids with the protein fusion catalysts, which is exemplified by the interaction of the Vam7p PX domain with PI(3)P (16). The second mechanism is indirect and physical (17). This is demonstrated by the dependence of SNARE function on the assembly of metabolically active membrane raft microdomains (12). The assembly of ergosterol-rich microdomains affects the local microviscosity of the membrane and can potentially exert allosteric effects on the transmembrane domains (TMDs) of Vam3p, Vti1p, and Nyv1p. The concentration of specific lipids into small domains, which may increase avidity, also regulates direct protein-lipid interactions and the dynamics of vacuole associated actin (12,18).

The interconversion of regulatory lipids is catalyzed by various lipid modifying enzymes. A recent study focused on a certain lipid metabolic pathway that leads to the generation of DAG from PA by the action of a PA phosphatase enzyme Pah1p (19). Both PA and DAG are lipids essential for membrane fusion and play a vital role in various stages of fusion. DAG has been reported to be involved in Golgi to ER transport and the generation of COPI vesicles (20).
Elevated levels of PA have been reported to be essential for sporulation and stimulates fusion with a Q-SNARE Spo20 (21,22). Among the three major PA phosphatases present in yeast, neither Lpp1p or Dpp1p are implicated in vacuole fusion. However, deletion of Pah1p resulted in severe vacuole fragmentation which upon further investigation revealed two major defects in the vacuole fusion machinery: a defect in priming and protein sorting to the vacuole. *PAHI* deleted vacuoles were characterized by a failure in Sec17p release and drastically reduced levels of the Sec18p, a AAA+ ATPase responsible for *cis*-SNARE disassembly, at the site of priming (19). In this study, we delve deeper into the mechanism of Pah1p action during priming. We report that like its mammalian homologue NSF, Sec18p also binds PA. This binding was specific and could be blocked by PA specific binding domain TGD2. Sec18p interaction with PA was also dependent on its nucleotide bound state. We found two distinct populations of Sec18p based on its nucleotide bound state: ADP bound Sec18p predominantly bound PA on the membrane and the ATP bound state predominantly bound *cis*-SNARE complexes. Based on these observations we hypothesize that Sec18p is held on the membrane at PA rich domains prior to the start of fusion. During priming, following the conversion of PA to DAG by Pah1p, Sec18p falls off the PA rich domain and is recruited to the *cis*-SNARE complexes. Thus, we conclude that Pah1p plays an important role in recruiting Sec18p to the *cis*-SNARE complexes during priming.
RESULTS

In an earlier study from our group (19), we uncovered a role for the yeast homologue of Lipin1 (Pah1p) in the regulation of membrane fusion. We used propranolol, a known small molecule inhibitor of PAP activity to inhibit Pah1p and demonstrated an essential role for the metabolic reaction synthesizing DAG from PA in membrane fusion (31,32). We found that propranolol inhibited vacuole fusion, specifically in the priming stage. Vacuoles treated with propranolol were defective in priming and Sec17p release during priming (Fig 3.1). PAHI deleted vacuoles also showed low levels of Sec18p bound to cis-SNARE complexes during priming (data not shown). Together, these observations underline a role for Pah1p during priming but the exact mechanism involved is yet to be unraveled.

The mammalian homologue of Sec18p (NSF) is a well studied AAA+ ATPase protein and its interaction with PA has been the crux of a few studies so far. NSF binds to PA and this binding is dependent on its ATPase activity and the nucleotide bound state of NSF. NEM inhibits the ATPase activity of NSF and locks it in a predominantly PA bound state. Whereas, the ATP-bound form of NSF displays reduced binding to phosphatidic acid (33). Drawing parallels from earlier NSF studies and weighing in our own findings from the Pah1p inhibition studies, we posit that Sec18p could also bind phosphatidic acid. This binding event on the membrane might serve to sequester Sec18p on the membrane before priming occurs. Phosphatidic acid phosphatase activity of Pah1p converts PA to DAG, hence releasing the bound Sec18p, which can now be recruited to the cis-SNARE complexes to effect priming.
Our model requires a demonstration of interaction between PA and Sec18p. To test if Sec18p can bind to PA, we first performed a protein lipid overlay assay. Increasing amounts of the two lipids, PA or DAG were spotted on Hybond-C membrane and the lipid strips were incubated with purified recombinant Sec18p. The extent of bound Sec18p protein was visualized by western blotting using an antibody against Sec18p. Sec18p exhibited binding to PA in the protein-lipid overlay assay (Fig 3.2A). Surprisingly, Sec18p also bound equally well to DAG. Neither Sec18p nor the antibodies used for western blotting bound non-specifically to the membrane (Fig 3.2A lane 1). As a positive control, we also incubated the lipid strips with recombinant TGD2, a PA specific binding domain (24,25) and observed specific binding between TGD2 and PA. Thus, we conclude that Sec18p interacts with both PA and DAG and this interaction is specific.

To further confirm our results from the protein-lipid overlay assays, we performed liposome association assays. Recombinant Sec18p was incubated with liposomes made from lipid mixtures of the four different lipids phosphatidylcholine, phosphatidylethanolamine, PA and DAG. Liposome-bound Sec18p was separated from the unbound protein by density gradient centrifugation followed by western blotting to probe for the bound Sec18p. Sec18p did not bind to PC/PE alone liposomes.However, the presence of either PA or DAG was enough to solicit Sec18p interaction (Fig 3.2B). It is also important to note that the presence of phosphatidylethanolamine in the lipid mixture did not have any significant effect of Sec18p binding (Fig 3.2B; comparing lane 2 to 3 and 4 to 5). As seen before, Sec18p bound equally well to liposomes containing either PA or DAG. This observation is in agreement with the results from the protein-lipid overlay assays and once again demonstrates a direct interaction.
between Sec18p and PA. Next, we tested whether the binding of Sec18p to either lipids PA or DAG was affected by the membrane lipid composition. To this effect, we made liposomes containing increasing mole percentages of either PA or DAG. The concentration of the bulk lipid phosphatidylcholine was maintained at 60 mole percent and the concentration of phosphatidylethanolamine and PA or DAG was varied to account for the rest of the 40 percent. There was steady increase in Sec18p associated with the liposomes when we increased the mole percent of phophatidic acid in the lipid mixture from 5% to 20% (Fig 3.2C). Surprisingly, increasing the mole percent of DAG in the lipid mixture resulted in reduced Sec18p binding. We had not observed this inverse relationship between Sec18p binding and the concentration of DAG in previous protein-lipid overlay assays. We reason that the liposomes were prepared with a mixture of lipids as opposed to pure lipids such as ones used in the protein-lipid overlay assays and this difference could account for the observed disparity in results. Also, with liposomes factors such as curvature and fluidity come into play, which are non-existent in lipid arrays such as the ones used in protein-lipid overlay assays. DAG has been reported to induce negative curvature in membranes (34,35). It is also possible that increasing the DAG concentration in these liposomes increases negative curvature such that it affects Sec18p binding.

Next, to ascertain the specificity of Sec18p binding to either of the lipids, we performed a competition binding assay. The liposome association assay was modified, wherein the liposomes containing PA or DAG were incubated first with a PA binding protein domain TGD2 or DAG binding domain C1b. Following which, Sec18p binding was analyzed as described above. As reported earlier, Sec18p binds to liposomes containing either PA or DAG but not to PC/PE alone.
liposomes. Blocking either PA or DAG in these liposomes resulted in abolishment of the Sec18p-lipid interaction (Fig 3.2D). Based on the data above, we conclude that Sec18p does indeed bind both PA and DAG on membranes. This interaction is specific to the lipids and dependent on the lipid composition of the membrane.

NSF, the mammalian homologue of Sec18p has been reported to bind PA. This binding was found to be dependent on the nucleotide bound state of NSF. By manipulating the nucleotide state of NSF, the authors found that the inactive ADP bound form of NSF predominantly interacted with phosphatidic acid, but the active ATP bound form did not (33). Since, in our experiments Sec18p exhibited specific binding to PA; we asked if this interaction could also be dependent on the nucleotide bound state of the AAA+ ATPase Sec18p. We used the following reagents to manipulate the nucleotide state of Sec18p: N-ethylmaleimide (NEM) is known to alkylate NSF and inhibit its ATPase activity, MgCl$_2$ to stabilise the ATP bound form of Sec18p and slowly-hydrolysable ATP$_7$S to lock Sec18p in the ATP bound state. We also used propranolol to inhibit Pah1p activity and hence alter the ratio of PA to DAG on the membrane and study the effect membrane lipid composition on Sec18p binding. As NEM has not been characterized in the context of yeast vacuole membrane fusion, we started out by testing it in a standard fusion reaction. NEM potently inhibited vacuole membrane fusion at concentrations as low as 1 mM (Fig 3.3A). Based on the inhibition curve we chose 3 mM as an effective inhibitory concentration of NEM to use in all our future experiments.

Since Sec18p is a homologue of NSF and NEM inhibits the ATPase activity of NSF, we asked if NEM could also inhibit the ATPase activity of Sec18p. A previous study comparing the
effects of NEM on NSF and Sec18p reported that 3 mM NEM inhibits the ATPase activity of NSF but Sec18p remained insensitive to inhibition by NEM (Morgan et al; Biochemistry 1999). We also performed ATPase activity assays on Sec18p in the presence of NEM and did not observe any significant inhibition (data not shown). It is really interesting to note that NEM does not inhibit Sec18p ATPase activity but is effective in inhibiting membrane fusion (Fig 3.3).

To further characterize the mechanism of inhibition by NEM, we performed fusion assays to measure the extent of resistance gained towards different inhibitors in a standard fusion reaction. Inhibitory concentrations of propranolol, NEM and Vam3p IgG were added to standard fusion reactions at specified times after the start of fusion. Reactions were also placed on ice at these indicated times to stop fusion. Fusion reactions gain resistance early to inhibitors that act early in the fusion pathway but remain sensitive longer to reagents that inhibit the later stages in fusion. This assay gives a good indication of the time frame within which a reagent or protein acts during fusion. Fusion reactions gained resistance to propranolol and NEM as early as 10 minutes but remained sensitive to Vam3p IgG inhibition indicating that both propranolol and NEM inhibit an early stage but Vam3p IgG inhibits a late stage of fusion (Fig 3.3B). An earlier report also demonstrated a similar inhibition kinetics for propranolol and confirmed that it does in fact inhibit the priming stage (19). Since, the inhibition kinetics for NEM overlaps well with that for propranolol, we conclude that NEM should also act at the priming stage of fusion although, the mechanism of inhibition still remains to be elucidated.

We next used the effective concentrations of all the above mentioned reagents in a standard fusion assay. As expected, 2 mM Propranolol and 3 mM NEM abolished fusion presumably by inhibiting priming (Fig 3.3C). MgCl₂ at a concentration of 2 mM was expected to
stabilize the ATP bound form of Sec18p and hence activate priming which is reflected in fusion values as high as the positive control. ATPγS is a slowly-hydrolysable form of ATP and hence would lock Sec18p in the ATP bound form and inhibit nucleotide exchange. Interestingly, the inhibition observed with ATPγS was only partial. We attribute this observation to the fact that purified vacuoles already carry all the components required for an active fusion. It is possible that a fraction of Sec18p on these purified vacuoles is already bound to ATP which accounts for the residual Sec18p activity and hence fusion even in the presence of ATPγS.

We next asked, if like NSF, altering the nucleotide bound state of Sec18p could also affect its interaction with PA. We performed the aforementioned liposome association assay using PC/PE liposomes containing 20 mole percent PA. The Sec18p-liposome binding assay was slightly modified, wherein, Sec18p was first incubated with one of following reagents, 2 mM propranolol, 3 mM NEM, 2 mM MgCl2 or ATPγS for 30 minutes before binding PA containing liposomes. Propranolol treatment or NEM treatment did not affect Sec18p binding to PA liposomes (Fig 3.3D). We reason that since the target of propranolol inhibition, Pah1p, was not present in the liposomes and NEM has no effect on the ATPase activity of Sec18p, it is expected that neither treatments would not affect Sec18p-PA binding. MgCl2 and ATPγS treatment on the other hand, reduced Sec18p binding to PA marginally. It should be noted that in an earlier report, similar treatment of NSF MgCl2 and ATPγS also reduced NSF-phophatidic acid binding. Based on these data, we conclude that the ATP bound form of Sec18p, which is also the active pool of Sec18p, displays reduced binding to PA on membranes and hence that the nucleotide bound state of Sec18p dictates its interaction with PA.
Sec18p is an AAA ATPase and serves to disassemble *cis*-SNARE complexes during priming. We next asked if modifying the nucleotide bound state and hence the ATPase activity of Sec18p would affect its function during priming. To answer this question, we isolated *cis*-SNARE complexes during the first 10 minutes of fusion and analyzed Sec18p associated with these complexes. Fusion reactions were stopped after the first 10 minutes of fusion, allowing fusion to proceed only to the priming stage and hence ensuring that the SNARE complexes isolated would predominantly be in the *cis* configuration. We analyzed the Sec18p associated with *cis*-SNARE complexes at the start (0 minutes) and at the end of the priming (10 minutes) following treatment with one of the following reagents: 2 mM propranolol, 3 mM NEM, 2 mM MgCl$_2$ or ATP$\gammaS$. Inhibition of Pah1p activity with propranolol, resulted in a loss of Sec18p associated with the SNARE complex (Fig 3.4A) indicating that Pah1p activity was required for the recruitment of Sec18p to *cis*-SNARE complexes during priming. Based on the data so far, this can be explained as follows; the inhibition of Pah1p activity and hence the inhibition of PA to DAG conversion results in more Sec18p locked in the PA bound pool and hence less of it is available to be recruited to the *cis*-SNARE complexes. NEM treatment slightly reduced Sec18p association at the start of priming and this defect was further exaggerated by the end of 10 minutes. Interestingly, as noted earlier, NEM treatment does not inhibit the ATPase activity of Sec18p but inhibits priming (Fig 3.3B) and also resulted in reduced Sec18p recruitment to the *cis*-SNARE complex. It is possible that NEM could alkylate Sec18p thus altering the conformation of the protein, which affects its ability to interact with the SNARE proteins without affecting its ATPase activity. As expected, both MgCl$_2$ and ATP$\gamma$S treatments resulted in decreased PA binding (Fig 3.3D) and increased Sec18p recruitment to the *cis*-SNARE complexes during priming (Fig 3.4A).
It is important to consider the possibility that the above treatments might alter membrane association ability or even the stability of Sec18p. To rule out this possibility, we next analyzed the levels of total membrane-associated Sec18p following treatment with propranolol, NEM, MgCl$_2$ or ATP$_7$S. Fusion reactions were treated with the above-mentioned reagents for 10 minutes. The total membrane fraction was sedimented by centrifugation and probed for Sec18p by western blotting. None of the above treatments had any effect on the levels of membrane associated Sec18p (Fig 3.4B). This observation taken together with the data so far leads us to the conclusion that nucleotide bound state of Sec18p dictates its distribution between the ATP bound-cis-SNARE associated and the PA bound pools. This observation demonstrates an inverse relationship between PA associated and cis-SNARE associated pools of Sec18p, which further lends credence to our hypothesis that Sec18p is released from a PA bound pool and then recruited to cis-SNARE complexes.

Lastly, since Pah1p dephosphorylates PA to DAG and the deletion of Pah1p results in elevated levels of PA on the membrane, we asked if altering the ratio of these two lipids on the membrane by deleting Pah1p in vacuoles would affect Sec18p association. Dgk1 encodes a DAG kinase, which is the enzyme responsible for phosphorylation of DAG to PA. We reasoned that if the deletion of Pah1p results in PA accumulation, the Dgk1 deletion would result in the accumulation of DAG on the vacuole membrane and would provide us a good tool to study the effect of vacuole lipid composition on Sec18p association. Vacuoles from the above mentioned strains were used in a standard fusion reaction and incubated in the presence of 100 nM GST-Vam7p. cis-SNARE complexes were isolated by immunoprecipitating the GST tagged Vam7p
and associated Sec18p was analyzed. Pah1p deletion results in reduced Sec18p recruitment to the cis-SNARE complexes, presumably due to an increased amount of Sec18p in the PA bound pool and thus leaving less of it available to be recruited to the cis-SNARE complexes (Fig 3.5). The levels of associated Sec18p returned to WT levels in strains where the Pah1 deletion was complemented with a PAH carrying plasmid indicating that the Pah1p is critical for Sec18p recruitment during priming. Interestingly, we observed increased Sec18p recruitment in Dgk1 deleted vacuoles. We reason that when the DAG kinase is deleted, PA bound Sec18p would be released by the PAP activity of Pah1p but the reverse reaction producing PA from DAG is abolished thus resulting in diminished reserves of PA on the vacuole membrane. These diminished reserves might not be sufficient to sequester enough Sec18p and hence more of the Sec18p is available to be recruited to the cis-SNARE complexes during priming. Based on the data above, we conclude that Pah1p is the factor responsible for the recruitment of Sec18p to cis-SNARE complexes during priming and the change in the membrane lipid composition is the trigger responsible for the distribution of Sec18p between the PA bound and cis-SNARE bound pools.
DISCUSSION

In this study, we delved deeper into the role of the yeast PA phosphatase Pah1p during membrane fusion. In an earlier study, we reported \( PAHI \) as a factor essential for priming and Sec17p release during priming (19). It was also reported that the deletion of \( PAHI \) resulted in a drastic reduction in the amount of Sec18p associated with the SNARE complex during priming. Here, we shed light and provide a deeper understanding of the role of Pah1p during priming in membrane fusion. We demonstrate that the PAP activity of Pah1p is responsible for the recruitment of Sec18p to \( \text{cis-SNARE} \) complexes during priming. The absence of Pah1p or inhibition of its PAP activity results in a failure of Sec18p recruitment to the site of fusion which translates to a defect in priming and hence membrane fusion.

We have demonstrated a specific interaction between the Sec18p and PA using both protein-lipid overlay and liposome association assays. With the use of lipid specific protein domains we have successfully demonstrated that Sec18p interaction with phosphatidic is a direct physical interaction and possibly serves to sequester Sec18p on the membrane. Another overlooked aspect of the Sec18p-PA interaction could be in part due to the local curvature changes induced by the presence of PA on the membrane (36). It is possible that islands of PA rich regions on the membrane display significantly different local curvature than the bulk of the membrane and Sec18p serves to sense this difference in curvature in order to bind the membrane.

In protein lipid overlay and liposome association assays, Sec18p bound equally well to both PA and DAG. The only difference in Sec18p interaction with either lipid was observed when the mole percent of either of these lipids was increased on the membranes. The increase in
mole percent of PA resulted in increased Sec18p association with the membrane. On the other hand, increasing the percentage of DAG resulted in reduced Sec18p association. In the context of membrane fusion, these experiments mirror the events immediately preceding priming and SNARE disassembly. The activity of Pah1, preceding priming, would result in more and more PA being converted to DAG and over the course of time result in a decrease in the local concentration of PA and an increase in the local concentration of DAG. Based on our data from the liposome association assays, it is possible that as the conversion of PA to DAG proceeds, progressively less Sec18p is bound to these PA rich regions and hence more Sec18p is available to be recruited to \textit{cis}-SNARE complexes.

It has been reported that NEM inhibits the ATPase activity of NSF, which is the mammalian homologue of Sec18p, but was ineffective against Sec18p itself (23). We demonstrate that NEM does in fact inhibit membrane fusion and more specifically the priming stage of fusion. By isolating \textit{cis}-SNARE complexes, we found that NEM inhibits the recruitment of Sec18p to \textit{cis}-SNARE complexes during priming. The effect of NEM on Sec18p recruitment seems to be unrelated to its ATPase activity. It is possible that NEM changes the conformation of Sec18p such that it affects \textit{cis}-SNARE binding without affecting its ATPase activity.

Propranolol, a small molecule inhibitor of the PAP activity of Pah1p has been shown to inhibit membrane fusion by inhibiting priming (19). Here, we demonstrate a defect in Sec18p recruitment to -SNARE complex during priming in the presence of propranolol. Taken together, we conclude that the PAP activity of Pah1p is essential for the recruitment of Sec18p during priming. The phosphatase activity of Pah1p results in the production of DAG from PA. Deletion
of Pah1p resulted in a defect in Sec18p recruitment. On the contrary, deletion of Dgk1, the DAG kinase, results in an increased Sec18p recruitment. Both Pah1p and Dgk1, function by altering the lipid composition of the membrane during membrane fusion. Deletion of Pah1 results in the accumulation of PA whereas a deletion of Dgk1 results in the accumulation of DAG on the membrane. Taken together, it is possible that Pah1p serves to kick start priming by altering the lipid composition of the membrane, which then results in Sec18p being recruited to the cis-SNARE complexes at the site of priming. This study underlines the role of membrane lipid composition in the recruitment of fusion factors to the site of fusion.
MATERIALS AND METHODS

Reagents

All reagents were diluted in PS buffer (20 mM PIPES-KOH pH 6.8, 200 mM sorbitol) to a working concentration before use in experiments. Antibodies against, Sec17p and Sec18pp have been described previously. NEM, propranolol hydrochloride and ATPyS were bought from Sigma Aldrich and dissolved in PS buffer. All the lipids used in this study: POPA (palmitoyl oleoyl PA), POPC (palmitoyl oleoyl phosphatidylcholine), POPE (palmitoyl oleoyl phosphatidylethanolamine), and POG (palmitoyl oleoyl glycerol) were bought from Echelon biosciences as stock solutions dissolved in chloroform and stored at -20°C.

Recombinant proteins

Recombinant His tagged Sec18p was purified from the cytosolic fraction of BL-21 derived Rosetta competent cells (EMD Biosciences) as described before (23). Briefly, CBB214 strain of XL-1 blue competent cell carrying the PQE9-His Sec18p plasmid were streaked LB agar plates containing 100 µg/ml ampicillin and 12 µg/ml tetracycline. A single colony was used to start an overnight 100 ml preculture in LB. The next day, culture flasks containing 1L terrific broth were inoculated with 50 ml of the preculture and the cells were grown at 37°C. Once the optical density reached 0.9, cells were induced with 200 µM IPTG at 37°C overnight for 4 hours. The next day, cells were harvested in 50ml lysis buffer (10 mM HEPES-KOH pH 7.0), 500 mM KCl, 5 mM ATP, 5 mM MgCl₂, 2 mM β-mercaptoethanol, 1 mM PMSF and protease inhibitor cocktail). The cell suspension was subjected to lysis in a french pressure cell and the lysate cleared by centrifugation at 16,000 rpm, 4°C for 30 minutes in a JA-20 rotor. For further clarification, the lysate was subjected to centrifugation at 250,000g for 1 hour at 4°C in a
60Ti rotor. The cleared lysate was loaded onto a Ni-NTA agarose column and the protein was allowed to bind for 2 hours at 4°C. The column was washed using buffer A (20 mM HEPES-KOH pH 7.0, 200 mM KCl, 1 mM MgCl₂, 0.5 mM ATP, 2 mM β-mercaptoethanol, 10% glycerol and 50 mM Imidazole). His-Sec18p was eluted from the column using buffer A containing 500 mM Imidazole. The eluted protein was further purified on a Gel filtration column pre-equilibrated with GF buffer (20 mM PIPES-KOH pH 6.8, 200 mM Sorbitol, 125 mM KCl, 5 mM MgCl₂, 2 mM ATP, 2 mM DTT and 10% glycerol) and 1ml fractions were collected. Sec18p elutes from the column as two distinct populations: the hexameric pool with a molecular mass of 640 kDa elutes early (fraction peak around 50 ml post injection) and the later dimeric pool with estimated molecular mass of 140 kDa. The hexameric pool of Sec18p was used in all out experiments. Recombinant His-TGD2 was purified according to the protocol described earlier (24,25) dialysed into PS buffer containing 125 mM KCl, aliquoted and stored at -80°C.

**Strains**

BJ3505 (**MAT**α **pep**4::**HIS**3 **prb**1-Δ1.6R **his**3-200 **lys**2-801 **trp**1Δ101(**gal**3) **ura**3-52 **gal**2 **can**1) and DKY6281 (**MAT**α **leu**2-3 **leu**2-112 **ura**3-52 **his**3-Δ200 **trp**Δ1-901 **lys** 2-801) were used for standard vacuole fusion assays (26). BJ3505 and DK6281 CBP-Vam3p strains harboring a Nyv1deletion were used for the isolation of *cis*-SNARE complexes (27). Pah1p deleted strains RFY 17 and RFY 18 and WT PAH1 plasmid complemented strain RFY 19, 20 have previously been described (19). Yeast strains where DAG kinase, Dgk1 has been deleted (SS1144) and where both Dgk1 and Pah1p have been deleted (SS1147) have been described earlier (28).
Vacuole Isolation and in vitro vacuole fusion

Vacuoles were isolated from yeast strains by floatation (29). Fusion reactions (30 μl) contained 3 μg each of vacuoles from BJ3505 (pep4Δ PHO8) and DK6281 (PEP4 pho8Δ), fusion assay buffer (125 mM KCl, 5 mM MgCl₂, 20 mM PIPES-KOH pH 6.8, 200 mM sorbitol), ATP regenerating system (1 mM ATP, 29 mM creatine phosphate, 0.1 mg/ml creatine kinase), 10 μM CoA and 283 nM IB2. Reactions were incubated at 27°C for 90 min and the Pho8p activity was measured in 250 mM Tris-Cl PH 8.5, 0.4% Triton X-100, 10 mM MgCl₂, 1 mM p-nitrophenylphosphate. During fusion, Pep4p protease gains access to the pro-alkaline phosphatase (proPho8p) and cleaves it to form mature alkaline phosphatase. The extent of fusion and hence alkaline phosphatase maturation was measured by measuring the quantity of p-nitrophenylate produced was measured at 400 nm. One unit of fusion was defined as 1 μmol of p-nitrophenylate produced min⁻¹ μg⁻¹ of pep4Δ vacuoles.

Protein Lipid Overlay Assay

Stock lipids of POPA (palmitoyl oleoyl phophatidic acid) and POG (palmitoyl oleoyl glycerol) were diluted to the indicated concentration in a methanol, chloroform and water mixture (2:1:0.8). 5 μl of each lipid from each dilution was spotted on a Hybond—C extra membrane and allowed to dry at room temperature for 1 hour. The membrane was blocked with 5% BSA solution in TBST for 1 hour at room temperature. Recombinant His-Sec18p was diluted to 2.5 nM concentration in blocking buffer and the membrane incubated with the protein at 4°C overnight. The membrane was washed 3 times over 30 minutes followed by incubation with the primary antibody directed against Sec18p for 1 hour. Again, the membrane was washed 3 times
over 30 minutes and incubated with secondary antibody (goat anti-rabbit IgG) for 1 hour. After a final wash, Sec18p bound to the membrane was detected by development of a fluorescence signal following incubation of the membrane with the ECF substrate (GE healthcare).

**Liposome Preparation and Co-Floatation Assay**

SUV liposomes containing different lipid compositions were prepared using the sonication method. Briefly, stock lipids in chloroform were mixed to produce a lipid mixture with the desired lipid mole percentages containing 2.6 µmoles of total phospholipids. The lipid mixture was dried under a gentle stream of nitrogen and dried in a speed-vacuum for an additional 60 minutes until a milky white layer of lipids appears on the glass walls. The tubes were left under a steady vacuum stream in a dessicator overnight. To the dried lipids, 2.6 ml of 1X PBS solution was added, the tubes were covered with parafilm and allowed to sit at room temperature for an hour. The tubes were vortexed well to resuspend the lipids completely and sonicated in a water bath sonicator for 30 minutes, till a slightly hazy suspension appears. The result was a uniform suspension of liposomes containing 1 mM phospholipids in PBS. To measure protein binding to the liposomes, 2 µg of recombinant His -Sec18p was incubated with 1 ml of the 1 mM liposome suspension for 2 hours at room temperature. Following which, the bound and unbound Sec18p were separated on a 5-20% sucrose gradient by ultracentrifugation at 55000 rpm for 3 hours in a Ti60 rotor. The liposomes floated up to the interface between 0% and 10% sucrose. Liposome-bound Sec18p was then analysed by western blotting. To study the effect of different inhibitors on Sec18p binding, the protein was incubated with each of the different inhibitors 2 mM propranolol, 3 mM NEM, 2 mM MgCl2 or 1X ATP regenerating system containing ATPγS for 1 hour at 4°C before allowing it to bind liposomes. For competition assays, 10 times molar excess
of His-TGD2 or GST-C1b was allowed to bind the liposomes at room temperature for 2 hours before Sec18p binding and gradient centrifugation.

**CBP Pull-down assay**

Sec18p-SNARE complexes were isolated and analyzed as described with some minor modifications (30). Large-scale 16X (480 μl) fusion reactions containing 48 μg of vacuoles from BJ3505 CBP-Vam3p and 48 μg of vacuoles from DK6281 CBP-Vam3p backgrounds were incubated in the presence of buffer or different fusion modulators (2 mM propranolol, 3 mM NEM, 2 mM MgCl2 and 1X ATP regenerating system containing ATPγS). After incubation at 27°C for 10 min, reactions were placed on ice for 5 min and 30 μl was withdrawn from each sample to assay Pho8p activity. The remaining samples were centrifuged (16000 g, 15 min, 4°C) to sediment the vacuoles and the supernatants were discarded. Vacuole pellets were overlaid with 200 μl ice-cold solubilization buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM MgCl2, 0.5% Triton X-100 and 20% glycerol) with protease inhibitors (0.46 μg/ml leupeptin, 3.5 μg/ml pepstatin, 2.4 μg/ml Pefabloc-SC and 1 mM PMSF) and gently resuspended. Solubilization buffer was added to a final volume of 400 μl and the extracts were mixed by nutating at 4°C. After 20 min, insoluble debris was removed by centrifugation (16,000 g, 20 min, 4°C). Supernatants were transferred to fresh tubes and 10% of the extract was saved for input samples. The remaining extracts were brought up to 2 mM CaCl2 and incubated with 40 μl calmodulin Sepharose beads equilibrated with solubilization buffer (GE Healthcare; 4°C, 12 h, nutating). The beads were collected by centrifugation (3,000 g, 2 min, 4°C) and washed five times with 1 ml of solubilization buffer containing 1% Triton and 2 mM CaCl2 followed by bead centrifugation. Bound proteins were eluted with solubilization buffer containing 0.1% Triton X-
100 and 5 mM EGTA. The eluents were mixed with SDS sample buffer and used for SDS-PAGE analysis and immunoblotting.

**GST Pull-down assay**

Sec18p associated with *cis*-SNARE complexes was analysed by modifying the GST-Vam7p pulldown assays described earlier. Briefly, 10X fusion reactions containing WT, *pah1Δ*, *dgk1Δ* or the double mutant *pah1Δ dgk1Δ* vacuoles were incubated with 100 nM GST-Vam7p at 27°C for 10 min. At the end of 10 min, vacuoles were sedimented by centrifugation at 13000 rpm for 10 minutes. The pellets were solubilized with 200 µl solubilization buffer (20 mM HEPES-KOH pH 7.4, 100 mM NaCl, 2 mM EDTA, 20% glycerol, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, 1X PIC) and the insoluble debris pelleted by centrifugation at 13,000 g for 10 min. To the supernatant, 80 µl of 25% GSH bead slurry (equilibrated in solubilisation buffer) was added and incubated overnight at 4°C. The beads were pelleted and washed 5 times with 1 ml of solubilisation buffer. After the final wash, bound proteins were eluted with 40 µl of SDS sample loading buffer. GST-Vam7p associated Sec18p was analyzed by western blotting.
Fig 3.1. Pah1p activity is required for effective priming. (A) Standard fusion reaction were incubated with increasing amounts of propranolol at 27°C for 90 minutes and membrane fusion measured in an alkaline phosphatase fusion assay. (B) 10X standard fusion reaction containing WT or Pah1 deleted vacuoles were incubated at 27°C for 90 minutes. At the end of 90 minutes, vacuoles were reisolated by centrifugation, resuspended in SDS sample buffer, resolved by SDS-PAGE and immunoblotted for Sec17 and Actin.
Fig 3.2. Sec18p interacts with both PA and DAG on liposomes.
(A) Protein lipid overlay assay: Increasing dilutions of the two lipids POPA and POG were spotted on a nitrocellulose membrane and protein binding (TGD2 or Sec18p) to the lipids was examined by immunoblotting as detailed in the Materials and Methods section. (B-D) Liposomes containing the indicated lipid composition were prepared as described, incubated with 2µg of purified recombinant His-Sec18p and the liposome bound Sec18p was reisolated using a 5-20% Sucrose gradient. Immunoblots showing Sec18p bound to PA and DAG containing liposomes (B), bound to liposomes containing increasing mole percentages of PA or DAG (C) and bound to liposomes pre-treated with 10 times molar excess of TGD2 (to block PA) or GST-C1b (to block DAG) (D) are shown. Immunoblots are representative of 3 independent trials.
Fig 3.3. NEM and TGD2 inhibit membrane fusion.

(A) Standard fusion reactions were treated with increasing concentrations of NEM for 90 minutes at 27°C. Fusion was tested by content mixing and measuring Pho8p activity. (B) 2 mM propranolol, 3 mM NEM or anti-Vam3p IgG were added to standard fusion reactions at indicated times, after the start of fusion. The extent of resistance gained to these inhibitors was measured by measuring fusion at each of these time points. (C). Standard fusion reactions were treated
with 2 mM Propranolol, 3 mM NEM, 2 mM MgCl₂ or 1X ATP regenerating system containing ATPγS at 27°C for 90 minute and membrane fusion measured with the alkaline phosphatase maturation assay. (D) 2 µg of Sec18p was treated with buffer, 2 mM propranolol, 3 mM NEM, 2 mM MgCl₂ or ATPγS for 30 minutes at room temperature before subjecting it to the liposome association assay. Error bars represent SEM (n=3).
Fig 3.4. The nucleotide bound state of Sec18p dictates its recruitment to the cis-SNARE complexes during priming.

(A) Standard 10X fusion reactions were treated with 2 mM Propranolol, 3 mM NEM, 2 mM MgCl₂ or 1X ATP regenerating system containing ATPγS. The reactions were either left on ice or incubated at 27°C for 10 min. Following which, vacuole membranes were sedimented by centrifugation and subjected to immunoblot analysis to isolate the membrane bound fraction of Sec18p following various treatment conditions. (B) 16X standard fusion reactions containing 48 µg of BJ3505 CBP-Vam3p nvyΔ vacuoles and 48 µg DK6281 vacuoles were treated with 2mM Propranolol, 3 mM NEM, 2 mM MgCl₂ or 1X ATP regenerating system containing ATPγS. The
reactions were immediately placed on ice or allowed to incubate at 27°C for 10 minutes and centrifuged to isolate the total membrane fraction. Membranes were then solubilized and CBP-Vam3p complexes were affinity purified using calmodulin agarose as described in the Materials and Methods. Trans-SNARE complexes were detected by immunoblotting for Vam3p, Nyv1p and Vam7p. The Image shown is representative of 3 independent trials.
Fig 3.5. *Pah1p* is essential for the recruitment of *Sec18p* to cis-SNARE complexes during priming.

Standard 10X fusion reactions containing vacuoles isolated from wildtype, *pah1p* deleted, *pah1p* complemented or *Dgk1* deleted yeast strains were incubated for 10 min at 27°C in the presence of 100 nM GST-Vam7p. Vacuoles were sedimented by centrifugation, the membrane solubilized and incubated with Glutathione sepharose beads overnight at 4°C. GST-Vam7p containing complexes were eluted from the Glutathione beads and the bound *Sec18p* was analysed by immunoblotting.
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CHAPTER IV

GENERAL DISCUSSIONS
Overview

In this study, we have attempted to redefine the role of the membrane in vacuole membrane fusion. We have focused on two distinct properties of the membrane arising from its constituent lipids: the physical properties such as curvature, lateral membrane tension and fluidity which are an indirect result of the different lipid shapes and their lateral distribution on the membrane and the chemical property such as lipid composition, which is a direct result of the equilibrium and conversion between different lipids on the membrane. In chapter II, we manipulate the curvature and lateral tension of the membrane using curvature altering agents such as Chlorpromazine and Lysophosphatidylcholine and study the effect of these parameters on the function of the SNARE complex in effecting fusion. In chapter III, we zoom in and focus on a single metabolic reaction responsible for maintaining the levels of phosphatidic acid and DAG on the membrane and study the role this lipid metabolic pathway in recruitment of a key ATPase protein during priming.

Role of Membrane Curvature in Vacuole Membrane Fusion

CPZ is an amphipathic small molecule that traverses the lipid bilayer and inserts itself into the inner leaflet of membranes thereby increasing the negative curvature of the outer leaflet of the bilayer (1). A wealth of knowledge on the effects of CPZ on membrane physical properties has been gained from force measurements on the outer hair cell of the mammalian cochlea. CPZ decreases the steady state tethering force and increases lateral lipid mobility in outer hair cell membrane tethers (2). It also decreases the lateral cell wall tension required for vesicle generation in the plasma membrane of the outer hair cell (3). Lysophosphatidylcholine (LPC), on
the other hand, is an inverted cone shaped lipid and intercalates into the outer leaflet of membranes leading to an increase in both positive curvature and tension (4). LPC has been reported to inhibit fusion by inhibiting stalk formation (5). Using these two small molecules to manipulate membrane curvature and lateral tension, we report that decreasing the membrane lateral tension with negative-curvature inducing agents such as CPZ, decreases the effectiveness of the SNARE complex during fusion (Fig 2.1.C). In the presence of non-canonical SNARE complexes, which exert insufficient pulling force to effect force, decreasing the membrane tension with CPZ, allows more of the non-productive SNARE complexes to reach the force threshold for fusion by lowering the threshold (Fig 2.1.A). On the other hand, increasing the membrane curvature and lateral tension with LPC, resulted in increasing the force threshold such that even the canonical, productive SNARE complexes could not effect membrane fusion (Fig 2.2). Interestingly, the opposing effects of positive and negative-curvature inducing molecules did not simply cancel each other in the context of membrane fusion. Instead, we report that the membrane experiences local regions of either positive or negative curvature during different stages of fusion. The positive curvature inducing agent, LPC, inhibits the formation of a fusion stalk whereas, negative-curvature inducing CPZ promotes the transition from hemifusion to complete fusion.

Role of Pulling-Force Generation by the trans-SNARE Complex

The central ionic residue of SNARE motifs is conserved throughout eukaryotes and is essential for fusion, and each fusogenic SNARE bundle is composed of 3 Q-SNAREs and 1 R-SNARE (3Q:1R). Previously, we found that point mutations yielding 2Q:2R complexes were fusion incompetent and that fusion was restored when lateral membrane tension was reduced
with chlorpromazine (CPZ) (6). Our previous studies showed that WT and Vam7p^{Q283R} form trans-SNARE complexes successfully (6), yet fusion required a 10-fold excess of Vam7p^{Q283R} to reach WT levels of fusion. Thus, we hypothesized that Vam7p^{Q283R} forms weaker SNARE bundles that do not exert sufficient force to reach the activation energy of fusion. We further report that the 2Q:2R complexes can still be made fusion competent by increasing their surface density (Fig 2.3.A). These data demonstrate that non-canonical SNARE complexes can overcome the activation energy barrier required for hemifusion-fusion transition when the surface density of the mutant SNARE complexes is multiplied. Interestingly, the overexpression of SNARE proteins on the vacuole surface, bypasses the requirement for the HOPS complex and the proofreading mechanism, thus allowing the non-canonical complexes to effect fusion.

Role of the SNARE Transmembrane Domain in Hemifusion-Fusion Transition During Membrane Fusion

It is widely accepted that the formation of the helical SNARE bundle pulls and distorts the apposed membranes together to activate fusion. Here the energy generated from the formation of the SNARE complexes is transmitted to the membrane through the TMDs at the point of contact between the bilayers. Previous work by others has shown that the TMD of the vacuolar SNARE Vam3p is essential for productive priming and supporting fusion (7). Also, cells carrying a GPI anchored HA were stalled in the hemifusion stage and could not achieve complete fusion with RBCs. The defect in fusion has been attributed to the absence of a TMD and the fact that the GPI anchor only traverses the outer leaflet of the bilayer (8,9). Although the sequence is not as critical, there is a stringent requirement on the length of the TMD to achieve full functionality. Truncation mutants in the TMD of HA revealed a strict requirement of atleast
17 amino acids to successfully effect fusion. TMDs smaller than the critical length, could not span the entire length of the bilayer and were stalled at the hemifusion stage (10). On the other hand, increase in the separation between the coiled-coil and the transmembrane domain (by insertion of a flexible linker) also drastically reduced fusion, possibly by relieving the strain generated on the membrane (11).

We report that the TMD in the Q-SNARE Vam3p serves to transmit the pulling force generated by the trans-SNARE complex to the membrane. The TMD was replaced with a flexible lipid anchor, which resulted in successful inner leaflet mixing but defective outer leaflet mixing and hence the fusion reactions were stalled in the hemifusion stage (Fig 2.4.B). These hemifusion stalled fusion reactions could then be pushed to make the hemifusion to fusion transition by lowering the lateral membrane tension with Chlorpromazine. We also report that the absence of a TMD combined with a defective 2Q:2R SNARE complex, produces insufficient pulling-force which is not transmitted to the membrane and hence lowering the lateral membrane tension with CPZ had not effect on this particular combination (Fig 2.5.A). As expected, the absence of the Q-SNARE TMD in both the partner vacuoles resulted in a severe break in force transmission that any combination of the SNARE complex (3Q:1R or 2Q:2R) with CPZ could not effect fusion (fig 2.5.B).

**Role of Pah1p in Sec18p Recruitment During Priming**

Among the three major PA phosphatases present in yeast, neither LPP1 nor DPP1 are implicated in vacuole fusion. However, deletion of Pah1p resulted in severe vacuole fragmentation which upon further investigation revealed two major defects in the vacuole fusion machinery: a defect in priming and protein sorting to the vacuole. Pah1 deleted vacuoles were
characterized by a failure in Sec17 release and drastically reduced levels of the Sec18p, a AAA ATPase responsible for cis-SNARE disassembly, at the site of priming (12). In chapter III, we delve deeper into the mechanism of Pah1p action during priming.

Propranolol, a small molecule inhibitor of the PAP activity of Pah1p has been shown to inhibit membrane fusion by inhibiting priming (12). Here, we demonstrate a defect in Sec18p recruitment to cis-SNARE complex during priming in the presence of propranolol (Fig 3.4.B). We report that the PAP activity of Pah1p is essential for the recruitment of Sec18p during priming. Inhibition of Pah1p activity also resulted in a defective Sec18p recruitment to the cis-SNARE complexes (Fig 3.4 and Fig 3.5.). We envision a scenario wherein, Sec18p is held on the membrane at PA rich domains prior to the start of fusion. During priming, following the conversion of PA to DAG by Pah1p, Sec18p falls off the PA rich domain and is recruited to the cis-SNARE complexes

**Role of the Membrane Lipid-Composition in Membrane Fusion**

Deletion of Pah1p, the PA phosphatase resulted in defective Sec18p recruitment to the cis-SNARE complexes during Priming. On the contrary, deletion of Dgk1, the DAG kinase, results in an increased Sec18p recruitment (Fig 3.5). Both Pah1p and Dgk1 function by altering the membrane lipid-composition during fusion. Deletion of Pah1 results in the accumulation of PA whereas a deletion of Dgk1 results in the accumulation of DAG on the membrane. We hypothesise that Pah1p serves to kick start priming by altering the lipid composition of the membrane, which then results in Sec18p being recruited to the cis-SNARE complexes at the site
priming. This study underlines the role of membrane lipid composition in the recruitment of fusion factors to the site of fusion.

Lipid composition can change the local physical properties of the bilayer, yet the mechanisms by which these lipids regulate fusion remain uncertain. Regulatory lipids such as sterols and sphingolipids serve to thicken and stiffen raft microdomains, whereas others are essential for the recruitment and activation of soluble proteins (13,14). A third function of regulatory lipids in these microdomains is the focused destabilization of the bilayer. Lipids such as DAG are known to induce negative curvature and destabilize bilayers (15,16). The negative curvature induced by DAG and its enrichment at the vertex ring is consistent with the occurrence of a hemifusion intermediate (17). This is also in keeping with the stalk hypothesis of hemifusion where the fusion stalk contains negative curvature structures (18). Formation of the stalk is energetically disfavored by the insertion of LPC and creation of positive curvature in the outer layer of the membrane. Our finding that CPZ can overcome the inhibited fusion caused by C1b emphasizes this importance of DAG in creating negative curvature and destabilizing the membrane. We posit that binding DAG with C1b inhibits the lipid from inducing negative curvature and that CPZ restores the negative curvature needed for fusion.
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