Phosphatidylinositol 3,5-bisphosphate: A Molecular Switch of Vacuolar Fusion and Fission

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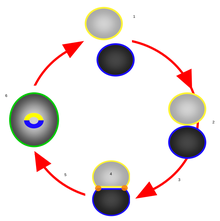
Abstract

Vacuoles purified from Saccharomyces cerevisiae are a well-studied model for membrane fusion and fission as the machinery is highly conserved throughout eukaryotes. Vacuole membranes undergo cycles of fusion and fission which have distinct mechanisms but are in part controlled by overlapping regulators. Both processes are dependent on proteins, ion concentrations, and lipid composition, highlighting the complex regulation of vacuole homeostasis.

Previous studies have shown the lipid $PI(3,5)P_2$ (phosphatidylinositol 3,5-bisphosphate) is a crucial activator of vacuolar fission. $PI(3,5)P_2$ is generated by the PI3P 5-kinase Fab1 . Fab1 is activated in response to osmotic stress leading to a sharp rise in $PI(3,5)P_2$ levels. Increases in $PI(3,5)P_2$ activates the calcium channel Yvc1, causing calcium to efflux from the vacuole. This, along with $PI(3,5)P_2$ activation of Vph1 (a subunit of a vacuolar V-ATPase) results in fragmentation of the vacuole.

Here we show that $PI(3,5)P_2$ is a novel inhibitor of vacuolar fusion. Additionally we found $PI(3,5)P_2$ does not prevent fusion by inhibiting priming and trans-SNARE pairing (the early steps of fusion). In order to look at the later steps of fusion, we conducted lipid mixing experiments to measure it's effects on hemi-fusion, the precursor step to vacuolar fusion. Our results show that $PI(3,5)P_2$ inhibits hemi-fusion, but the exact mechanism is still unclear. We also hypothesized that $PI(3,5)P_2$ acts to inhibit fusion through either the Yvc1 calcium efflux or the Vph1 V-ATPase pathway. Vph1 interactions promote either vacuolar fusion or fission depending on binding partners. We predicted $PI(3,5)P_2$ to disrupt Vph1 fusion complexes while promoting fission complexes. But, experiments using TAP-tagged Vph1 were inconclusive. Also, experiments with Yvc1 knockout yeast retained sensitivity to $PI(3,5)P_2$ - interestingly, the calcium *influx* pump Pmc1 *was* found to be enhanced by $PI(3,5)P_2$, which may explain decreased calcium influx.

Stages of Fusion



1.Priming: cis-SNAREs disrupted by Sec17/18
2.Tethering: Ypt7-dependent contact
3.Docking: Vacuoles drawn together
4.Vertex formation: Fusion factors become enriched at vertices and release of calcium
5.Lipid mixing
6.Content mixing

Why $PI(3,5)P_2$?

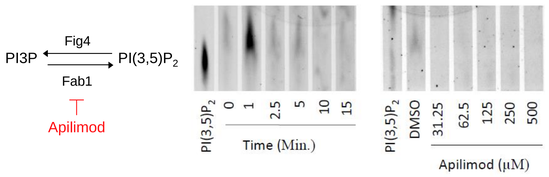


Figure 1. Vacuoles generate $PI(3,5)P_2$ under fusion conditions and Apilimod acts to block Fab1 activity (A) Vacuoles produce $PI(3,5)P_2$ under fusion conditions. Vacuoles were incubated with BODIPY-TMR C6- and 1mM sodium orthovanadate at 27°C for the indicated times after which lipids were extracted and resolved by TLC. (B) Apilimod inhibits Fab1 production of PI(3,5)P2. Vacuoles were incubated with BODIPY-TMR C6-PI3P in the presence or absence of Apilimod at the indicated concentrations at 27°C for 1 minute after which lipids were extracted and resolved by TLC

$PI(3,5)P_2$ Inhibits Fusion Independent of Yvc1

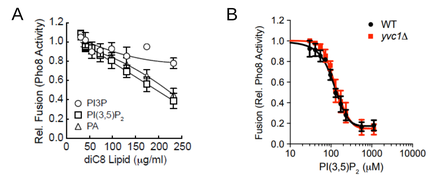


Figure 2. Wildtype and *yvc1Δ* vacuolar fusion are equally sensitive to $PI(3,5)P_2$. (A) $PI(3,5)P_2$ inhibits wildtype vacuolar fusion with the same effectiveness as PA, up to 70%. (B) Vacuolar fusion of *Yvc1Δ* yeast knockouts were treated with wildtype as control. Both *yvc1Δ* and wildtype were inhibited by $PI(3,5)P_2$ with no significance difference in inhibition. Fusion reactions were incubated for 90 min at 27°C. Error bars represent S.E.M. (n=3).

$PI(3,5)P_2$ Does Not Inhibit Priming

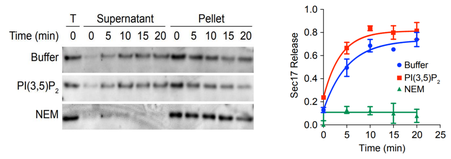


Figure 3. $PI(3,5)P_2$ does not effect the release of Sec17 during priming. (A) Fusion was conducted at 27°C and vacuoles were pelleted by centrifugation at the indicated times and proteins in the supernatant fraction were resolved by SDS-PAGE and imaged by Western blot. . Reactions were conducted with PS buffer, 232μM $PI(3,5)P_2$, or 1mM NEM. Western blots with antibody for Sec17 were used to visualize. (B) Normalized average (n=3). Error bars represent S.E.M. (n=3).

$PI(3,5)P_2$ Inhibition Occurs After Trans-SNARE Pairing

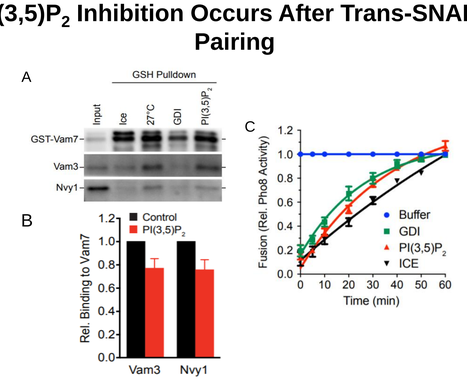


Figure 4. SNARES Vam3 and Nyv1 used in Trans-SNARE pairing are not inhibited by $PI(3,5)P_2$. (A) Fusion was conducted at 4°C or 27°C for 90 minutes with PS buffer, GDI, or 232μM $PI(3,5)P_2$. Reactions were centrifuged and incubated with glutathione beads, eluted, and visualized via western blotting. (B) is a normalized average of (A). (C) Fusion was conducted at 27°C for 60 minutes, inhibitors were added at the indicated times. Error bars represent S.E.M. (n=3).

Lipid mixing is Inhibited by $PI(3,5)P_2$

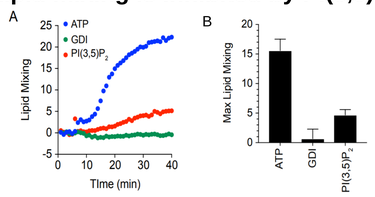


Figure 5. Lipid mixing during hemi-fusion is inhibited by $PI(3,5)P_2$. (A) Lipid mixing reactions were made according to fusion reactions, ATP was added after an initial reading was taken. Reactions were performed in the presence of Buffer (ATP), 0.5μMGDI, or 232μM $PI(3,5)P_2$. (B) is an average of (A) (n=3). Error bars represent S.E.M. (n=3).

$PI(3,5)P_2$ Does Not Inhibit Vph1-Nyv1 Interactions

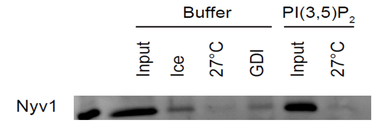


Figure 9. Nyv1-Vph1 interactions occur before trans-SNARE pairing. TAP-Vph1 were used to observe the effects of $PI(3,5)P_2$ on Vph1-Nyv1 interactions. Incubation occurred at at 27° C or 4° C for 90 minutes with PS buffer, 0.5μM GDI or 232μM $PI(3,5)P_2$. After incubation, reactions were centrifuged to isolate the membrane fraction. Membranes were solubilized, and TAP-Vph1 complexes were isolated with IgG-sepharose. Complexes were probed by immunoblotting for Nyv1.

$PI(3,5)P_2$ Inhibits Calcium Efflux by Activating Pmc1

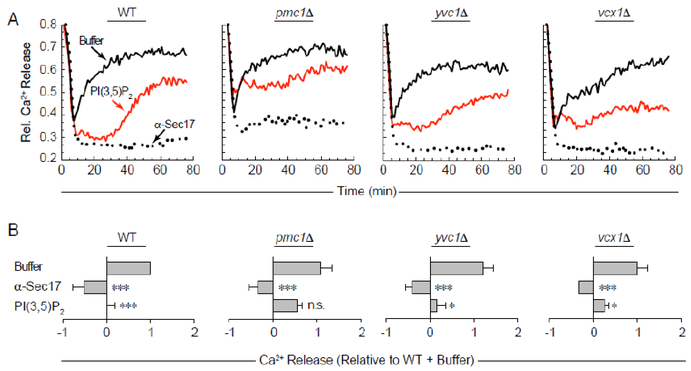


Figure 8. $PI(3,5)P_2$ regulates Ca^{2+} transport through Pmc1 during fusion. (A) Ca^{2+} transport assays were performed with WT, *pmc1Δ*, *yvc1Δ* or *vcx1Δ* vacuoles as described. After 6 min of incubation, select reactions were treated with DMSO, 116 μM $PI(3,5)P_2$. (B) Average Ca^{2+} efflux of multiple experiments as performed in panel A. Error bars are S.E.M. (n=3). Statistical significance is shown for ATP (column 2) versus $PI(3,5)P_2$ (column 6) *p<0.05, **p<0.01, ***p<0.001, n.s. (not significant).

Inhibition of Fab1 Activity Enhances Calcium Efflux

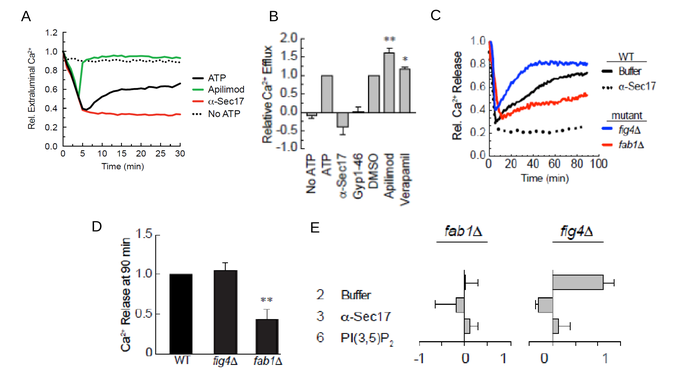


Figure 8. $PI(3,5)P_2$ activates calcium influx. (A) Calcium efflux measured in the presence of apilimod. (B)Quantitation of the effects of Apilimod, Verapamil and DSMO as well as Gyp1-46 on Ca2+ efflux. (C) Ca^{2+} transport assays were performed with WT, *fab1Δ* or *fig4Δ* vacuoles. (D) Average of multiple experiments shown in panel A where the relative amounts of Ca^{2+} released are compared between strains. (E) Average of multiple experiments where the relative amounts of Ca^{2+} released are compared for control reactions performed with *fab1Δ* or *fig4Δ* vacuoles. Error bars are S.E.M. (n=3). *p<0.05, **p<0.01, ***p<0.001.

$PI(3,5)P_2$ Enhances Nyv1 Association with Pmc1

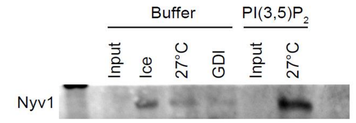
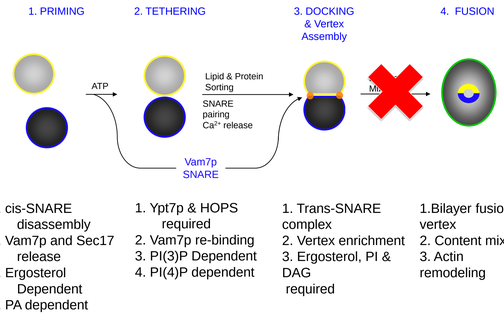


Figure 6. $PI(3,5)P_2$ regulates Pmc1-Nyv1 Association. We monitored the association of Pmc1 with its known binding partner Nyv1 utilizing TAP-Pmc1 vacuoles. Vacuoles were incubated at 27° C or 4° C for 90 minutes in the presence of PS buffer, 0.5μM GDI, or 232μM $PI(3,5)P_2$. After incubation, reactions were centrifuged to isolate the membrane fraction. Membranes were solubilized, and TAP-Pmc1 complexes were isolated with IgG-sepharose. Complexes were probed by immunoblotting for Nyv1.

$PI(3,5)P_2$ Inhibits Between Docking and Hemifusion



1. cis-SNARE disassembly
2. Vam7p and Sec17 release
3. Ergosterol Dependent
4. PA dependent

1. Ypt7p & HOPS required
2. Vam7p re-binding
3. PI(3)P Dependent
4. PI(4)P dependent

1. Trans-SNARE complex
2. Vertex enrichment
3. Ergosterol, PI & DAG required

1.Bilayer fusion at vertex
2. Content mixing
3. Actin remodeling

Conclusions

- **$PI(3,5)P_2$ is a novel inhibitor of vacuolar fusion**
- **$PI(3,5)P_2$ inhibition occurs after trans-SNARE pairing but before or at the hemifusion stage**
- **$PI(3,5)P_2$ inhibits calcium efflux indicating trans-SNARE pairing and calcium efflux are distinct events**
- **$PI(3,5)P_2$ appears to modulate calcium transport through activation of Pmc1 by promoting Pmc1-Nyv1 interactions**

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