A small-molecule competitive inhibitor of phosphatidic acid binding by the AAA+ protein NSF/Sec18 blocks the SNARE-priming stage of vacuole fusion

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ABSTRACT

The homeostasis of most organelles requires membrane fusion mediated by soluble NSF attachment protein receptors (SNAREs). SNAREs undergo cycles of activation and deactivation as membranes move through the fusion cycle. At the top of the cycle, inactive cis-SNARE complexes on a single membrane are activated, or primed, by the hexameric ATPase associated with diverse cellular activities (AAA+) protein, N-ethylmaleimide-sensitive factor (NSF/Sec18), and its co-chaperone α-SNAP/Sec17. Sec18-mediated ATP hydrolysis drives the mechanical disassembly of SNAREs into individual coils, permitting a new cycle of fusion. Previously, we found that Sec18 monomers are sequestered away from SNAREs by binding phosphatidic acid (PA). Sec18 is released from the membrane when PA is hydrolyzed to diacylglycerol (DAG) by the PA phosphatase Pah1. Although PA can inhibit SNARE priming, it binds other proteins and thus cannot be used as a specific tool to further probe Sec18 activity. Here, we report the discovery of a small-molecule compound, we call here IPA (Inhibitor of Priming Activity), that binds Sec18 with high affinity and blocks SNARE activation. We observed that IPA blocks SNARE priming and competes for PA binding to Sec18. Molecular dynamics simulations revealed that IPA induces a more rigid NSF/Sec18 conformation, which potentially disables the flexibility required for Sec18 to bind to PA or to activate SNAREs. We also show that IPA more potently and specifically inhibits NSF/Sec18 activity than does N-ethylmaleimide (NEM), requiring the administration of only low µM concentrations of IPA, demonstrating that this compound could help to further elucidate SNARE-priming dynamics.

INTRODUCTION

Eukaryotic life requires membrane fusion, which is integral to numerous processes involved in cellular homeostasis and distribution of biological molecules. The terminal catalysts of membrane fusion are SNARE (soluble NSF attachment protein receptor) proteins, adding specificity between membranes destined to fuse resulting through SNARE compatibility (1). SNAREs interact in trans (between two membranes) to form parallel four-helix bundles capable of generating the energy needed to merge two membranes into a continuous bilayer. After a fusion event, SNAREs remain as inactive cis-SNARE complexes on this newly formed single
bilayer. These complexes need to be disassembled so that a new cycle of fusion may occur. SNAPs are activated, or primed, by the AAA+ protein NSF/Sec18 (Fig. 1A). Sec18 is composed of three domains. The N-terminus forms the cap domain while the D1 and D2 nucleotide binding domains form the two rings of the hexameric complex. Both D1 and D2 domains contain ATP binding sites, however, the hydrolysis of ATP by D1 generates most of the energy necessary to disassemble inactive SNAP bundles, while the D2 ring binds ATP to stabilize the homo-hexamer (2).

During activation, Sec18/NSF associates with SNAP complexes through binding the adaptor protein Sec17/α-SNAP that aids in disrupting SNAP complexes into active individual proteins (3). Direct regulation of Sec18/NSF activity remains mostly unknown, although protein kinase C (PKC) has been implicated in negative regulation of NSF association with SNAP complexes (4). Currently, there are two methods of inhibiting priming in vitro. Sec18 can be blocked with an antibody or covalently modified with the alkylating agent N-ethylmaleimide (NEM), both of which inhibit the ATPase activity of Sec18/NSF (5). The exact mechanism by which alkylating with NEM affects priming is unclear and not specific. To illustrate, the inhibition of fusion by NEM requires a higher concentration than what is needed to inhibit NSF alone, suggesting the presence of multiple NEM-sensitive factors involved in fusion (6). This is due to the lack of specificity by NEM, as it only requires free thiol groups to function. Even though the dosage of NEM required to inhibit Sec18/NSF is in the millimolar range (5, 7), its promiscuity may have even been advantageous (8) to its use in adopting early models identifying the interaction of Sec18/NSF with Sec17/α-SNAP as being crucial to the continual cycle of fusion by membranes within the cell (9).

We previously demonstrated the importance of phosphatidic acid (PA) in regulating Sec18/NSF priming activity (10–12). Deletion of the PA phosphatase PAH1, the yeast orthologue of mammalian lipin1, leads to elevated concentrations of PA on the vacuole that we hypothesized sequesters Sec18 away from cis-SNAREs (10). Even when Pah1 is present, released Sec18 can be inhibited by adding soluble dioctanoyl PA (C8-PA). Conversely, deleting the diacylglycerol (DAG) kinase DGK1 elevates vacuolar DAG levels at the cost of lowering PA concentrations while resulting in augmented fusion (13). Thus, the temporal regulation of balancing PA and DAG concentrations has a direct effect on progression through the fusion pathway. Subsequent studies showed that PA binding by monomeric Sec18/NSF triggers large conformational changes that appear to be incompatible with the assembly of the active homohexamer needed to bind and prime SNAPs (12). The major site of conformational change, as shown by molecular dynamics, is the predominant PA binding site between the D1 and D2 domains of NSF.

Although PA serves as a natural regulator of Sec18 function, it has multiple limitations as a tool to further probe the mechanics of priming. The principal limitation with relying on PA as an inhibitor of Sec18 activity is due its insolubility, as it is part of the membrane bilayer, as well as its susceptibility to dephosphorylation by Pah1. Additionally, PA binds other proteins including the vacuolar SNARE Vam7 (14). Finally, PA is likely to serve both as an inhibitor of Sec18 activity while being a positive regulator through its interactions with Vam7. In fact, reconstituted proteoliposome fusion systems show that PA is essential for fusion to occur when the priming stage is eliminated (15).

Taken together, the lack of NEM specificity and the duality of PA in regulating vacuole fusion was the impetus for finding a specific soluble small molecule inhibitor of NSF/Sec18 function. We used structural data of NSF (16) to computationally screen for compounds that bound to the previously mapped PA binding site. Through this we discovered an uncharacterized molecule that we call IPA (Inhibitor of Priming Activity). IPA bound to Sec18 with high affinity and potently blocked SNAP priming and downstream vacuole fusion. Biochemical, biophysical and molecular dynamics examination of IPA-Sec18 complexes led us to conclude that IPA “locks” NSF/Sec18 into a rigid conformation that it incompatible with SNAP priming.
presumably by its ability to inhibit NSF/Sec18 binding to PA as shown below.

RESULTS
Identification of a small molecule inhibitor of Sec18 binding to PA – Because PA acts a potent inhibitor of Sec18 function we used computational modeling to search for small molecules that docked at the previously identified PA binding regions of Sec18 (12). To accomplish this, we used the cryo-EM guided resolution of the hexameric structure of NSF bound to SNAREs (17). Schrodinger’ SiteMap (18) was then performed on both hexameric and monomeric forms of NSF as well as a homology models of Sec18 hexameric and monomeric forms generated using Schrodinger Prime (19, 20). The top resulting binding sites for both NSF/Sec18 hexamer and monomer were docked using all compounds available from the Illinois High Throughput Facility initially using Glide HTVS, and the top hits were docked using Glide XP (19). Our screen included compounds from the Illinois high throughput screening facility (HTSF), NCI Open, NCI Diversity, and the Chembridge microformat libraries, which were prepared for docking using LigPrep (Schrödinger Release 2018-2: LigPrep, Schrödinger, LLC, New York, NY, 2018). Of the boxes examined, the 3rd and 4th highest lowest average gscore for binding to PA. Compounds with the best gscore, or lowest predicted ΔG for box 3 and 4 using Glide HTVS, and the top hits were docked using Glide XP (19). Our screen included compounds from the Illinois high throughput screening facility (HTSF), NCI Open, NCI Diversity, and the Chembridge microformat libraries, which were prepared for docking using LigPrep (Schrödinger Release 2018-2: LigPrep, Schrödinger, LLC, New York, NY, 2018). Of the boxes examined, the 3rd and 4th highest lowest average gscore for binding to PA. Compounds with the best gscore, or lowest predicted ΔG for box 3 and 4 using Glide HTVS, and the top hits were docked using Glide XP (19). Our screen included compounds from the Illinois high throughput screening facility (HTSF), NCI Open, NCI Diversity, and the Chembridge microformat libraries, which were prepared for docking using LigPrep (Schrödinger Release 2018-2: LigPrep, Schrödinger, LLC, New York, NY, 2018). Of the boxes examined, the 3rd and 4th highest lowest average gscore for binding to PA.

Sec18 monomer, D1, and D2 Domains bind IPA with high affinity – To further determine whether IPA could serve as a specific inhibitor of Sec18, we next measured its dissociation constant. To this aim we used Label-free microscale thermophoresis (LF-MST), labeled MST (MST), and surface plasmon resonance (SPR). All three techniques yielded a $K_D$ in the low µM range for IPA binding to monomeric Sec18 (mSec18). Labeled MST showed that mSec18 bound to IPA with a $K_D$ of 3.84 ± 1.3 µM (Fig. 3A and Table 1). Similarly, LF-MST showed that mSec18, labeled with Ni-NTA Atto 488, bound to IPA with a $K_D$ of 7.4 ± 3.7 µM (Fig. 3B). Discrepancies between the $K_D$ values of mSec18 to IPA using MST can be explained by the presence of the Atto 488 dye in the labeled experiment or as a result of IPA having measurable light absorbance (data not shown). We further verified these affinity measurements using surface plasmon resonance (SPR).
where mSec18-His8 was linked to an Ni-NTA Biacore chip, after which IPA was flowed and response units (RU) were measured. SPR measurements yielded a $K_D$ of $4.08 \pm 0.83 \mu M$ (Fig. 3C), which was consistent with the MST results. Together these results indicated that IPA bound Sec18 with high affinity.

Next, we determined which Sec18 domain contributed most to binding IPA. Using SPR, we tested the individual D1 and D2 domains, as well as a D1-D2 continuous polypeptide. These were added to separate channels of a Ni-NTA chip with fresh protein loaded between each injection. Capture of proteins per kinetic injection on the chip ranged from 2050-2900 RU for D1, 1500-2500 RU for D2, and 1400-1600 RU for D1-D2. The $K_D$ of IPA for D1 was determined from the sensorgrams as 320 nM (Fig. 3D). The $K_D$ of IPA for D2 was 1.1 µM (Fig. 3E), while the $K_D$ for D1-D2 was 958 nM (Fig 3F). We reasoned that the improved affinity for the domains was either the result of having measured the affinity via kinetic measurements as opposed to steady state measurements used above (Fig. 3A-C). Importantly, the on-rate for the D1-D2 construct was over 4-fold greater than for either D1 or D2 alone indicating that there may be some cooperativity between the D1 and D2 domains upon the initial recognition of PA. Together this data shows that both Sec18 domains participate in binding IPA similar to PA shown previously (12).

IPA inhibits Sec18 binding to PA Liposomes – In order to test whether IPA inhibits Sec18 binding to PA, we titrated IPA in the presence of PA liposomes and mSec18. Liposomes for floatation experiments (Fig. 4A-B) were prepared as previously described (12). IPA was added at the concentrations indicated in the presence of mSec18 at a final concentration of 500 nM. This showed that IPA blocked mSec18 binding to PA liposomes with an IC$_{50}$ of 3.2 µM, which was near the $K_D$ values IPA binding to mSec18 in Figure 3 (Fig. 4A and Table 2). This data was consistent with previous SPR competition experiments using PA containing nanodiscs (24). To examine if IPA blocked other PA binding proteins we used the DEP domain from the murine protein Dvl2 (25). Previously we used DEP to bind PA liposomes and vacuolar PA to displace Sec18 from membranes (11). Here we tested PA lipid binding DEP in the presence of 100 µM IPA. Unlike the competition we observed with Sec18, IPA was unable to compete at greater than 50% inhibition with DEP for PA binding at this IPA concentration (Fig. 4B). This suggested that IPA was specific for the Sec18-PA binding interface. We also tested IPA against the soluble SNARE Vam7, which binds PI3P and PA (14). To ensure that the PI3P binding domain was not contributing to PA binding we used the Y42A mutation in the PI3P binding site of the Vam7 PX domain (26). This experiment was performed by SPR where 800 nm extruded PA liposomes (close to the size of the yeast vacuole) were bound to an L1 Biacore chip, after which Vam7$^{Y42A}$ was injected and bound to saturation (Fig. 4C). When IPA was used at 100 nM, we observed no difference in Vam7$^{Y42A}$ binding to the PA liposomes. In comparison, IPA competed with Sec18 binding to the immobilized PA liposomes considerably at 100 nM (Fig. 4D).

In order to test whether IPA action was specific to anionic phospholipids we further checked whether it affected binding of mSec18 and Vam7$^{Y42A}$ to phosphatidylinositol (PI) containing liposomes. Both mSec18 and Vam7$^{Y42A}$ showed low overall binding to PI liposomes (Fig. 4D). Additionally, the inhibitory effect of IPA was absent when either Vam7$^{Y42A}$ or Sec18 were bound to PI liposomes. Together these data further verify the specificity of IPA for mSec18 to PA.

We then asked whether D1 or D2 domains of Sec18 were more or less important to IPA competition of PA as both domains were previously shown to bind PA (12). First, we measured the binding of each domain to immobilized 100 nm extruded PA liposomes. In Figure 4E, we show full length monomeric Sec18 and the D1 and D2 domains bound with similar affinities to the immobilized PA liposomes. Specifically, the $K_D$ was 781 ± 110 nM for D1, 866 ± 669 nM for D2, 505 ± 267 nM for mSec18. Next, IC$_{50}$ values for IPA and PA binding were determined using concentrations of mSec18, D1 and D2 near or above the $K_D$ values derived from the SPR experiments.
from liposome binding using constant concentration of 250 nM mSec18, 1000 nM D1, and 1000 nM D2 (Fig. 4F).

IPA inhibition of Sec18 binding to PA liposomes is not affected by membrane curvature – Next, we tested whether membrane curvature affected mSec18 binding and the effects of IPA. To this aim we used 3 different extrusion filters of 100, 400, and 800 nm containing 5% PA. The different sized liposomes bound to an L1 chip in HBS-N buffer for SPR analysis. First saturation was determined for each liposome type, which showed that mSec18 bound 800 nm liposomes with a KD of ~ 670 nM compared KD values of ~1600 nM and ~1000 nM to the 100 and 400 nm diameter liposomes (Fig. 5A). Although this showed that mSec18 bound more strongly to 800 nm liposomes, the binding affinities to smaller vesicles were still high. To determine whether membrane curvature affected how IPA blocked mSec18 from binding liposomes, we tested the competition of 100 nM IPA with 250 nM mSec18 for liposome binding. Reactions were incubated as before and liposomes were re-isolated by flotation. This showed that IPA reduced Sec18 (0.5 μM) binding by 90% to 800 nm liposomes, whereas IPA inhibited binding by 80 and 70% to 400 nm and 100 nm liposomes, respectively (Fig. 5B). While these results show differences in binding in the presence or absence of IPA, the fact remains, that IPA potently inhibits Sec18 from interacting with PA liposomes.

To determine whether IPA competition for Sec18 damages liposomes, we examined membrane integrity by using a Calcein dequenching assay. Here we used extruded 100 nm liposomes in the presence of 100 mM Calcein to capture the fluorophore at self-quenching concentrations (27, 28). Calcein loaded liposomes were treated with buffer, 0.2% Triton X100 or a dosage curve of IPA. As controls, we found that incubating with buffer had no effect on Calcein fluorescence, while Triton treatment led to increased fluorescence, indicating that the dye became diluted and de-quenched when the liposome was dissolved (Fig. 5C-D). When Calcein liposomes were incubated with IPA, there was no observable increase in fluorescence, demonstrating that IPA had no effect on the integrity of the liposomes.

While IPA did not damage liposome integrity, we next asked if this compound altered liposome diameter or dispersal. To address this, we used dynamic light scattering (DLS) to compare 80/20 PC/PE liposomes to 75/20/5 PC/PE/PA 100 nm extruded liposomes and found that there was no appreciable difference in size as between the two liposome types (Fig. S2A). To test if IPA affected the recovery of liposomes after flotation, we used DLS and measured the peak intensities (kilocounts per second) of 100 nm extruded liposomes listed above. In the absence of IPA, ~ 80% of the starting material was recovered, while ~90% was recovered in the presence of 100 μM IPA (Fig. S2B). Taken together, IPA had no effect on liposome size or flotation.

IPA Inhibits Fusion and Priming – The primary goal of finding a small molecule that specifically binds Sec18 was to use it to block SNARE priming and halt the fusion pathway. To test this, we used a vacuole fusion tester set where half of the vacuoles in a fusion reaction harbored inactive pro-Pho8 (alkaline phosphatase) and lacked the protease Pep4. The second set contained Pep4 but lacked pro-Pho8. Upon membrane fusion and content mixing Pep4 gained access to pro-Pho8 and cleaved the inhibitory pro-peptide to yield active Pho8 that can be tested as proxy for vacuole fusion efficiency. In Figure 6A, we show that IPA blocked fusion as measured by Pho8 activity with an IC50 of ~50 µM. The concentration needed to block fusion was notably higher that the dose needed to inhibit binding to PA liposomes. We attribute this to the accessibility of Sec18 interacting with the fusion machinery, and potentially the predominance of hexamer over monomer in this assay. Nevertheless, this is the first demonstration of a specific Sec18 ligand to inhibit membrane fusion. In comparison, inhibiting fusion with NEM requires millimolar concentrations (11).

To verify if IPA inhibited fusion during the priming stage, we performed multiple tests. First, we tested IPA in a gain of resistance assay. Here, individual fusion reactions were treated with buffer as a control, IPA, or antibody against Sec17 to directly block the priming machinery. The reagents were...
added at different time points to ask if fusion was still sensitive to the inhibitor. Thus, as a stage of fusion (e.g. priming) is passed, reagents that target the stage loses their efficacy. In Figure 6B we found that fusion reactions gained resistance to IPA with similar kinetics to those of anti-Sec17 antibody. In other words, as the bulk of fusion reactions passed the priming stage, both IPA and anti-Sec17 lost their ability to inhibit fusion. The similarity in the rate in which these reagents lost their inhibitory effect indicated that IPA only inhibited fusion at the priming stage. In parallel, untreated reactions were placed on ice to stop maximal fusion at each time point indicated, to establish a boundary for the final stage of fusion.

To test the effect of IPA directly on SNARE priming we used the release of Sec17 as a measure of Sec18 function (3, 11). As Sec18 disassembles cis-SNARE complexes, the co-chaperone Sec17 is released from the membrane and accumulates in the supernatant after membranes are pelleted by centrifugation. We tested IPA in comparison to NEM and C8-PA and found that IPA blocked Sec17 release as strongly as NEM and C8-PA (Fig. 6C-D). Both NEM and C8-PA inhibited priming as previously reported (11). However, both NEM and C8-PA lack specificity for Sec18, thus IPA is the first specific inhibitor of SNARE priming. Finally, we determined if the inhibitory concentration IPA was similar for both overall fusion inhibition and SNARE priming. A range of IPA concentrations was added to vacuoles and incubated for 30 min after which membrane were pelleted and the supernatants collected for Western blotting. Sec17 release was blocked by IPA with an IC$_{50}$ of ~50 µM (Fig. 6E-F), which matches the IC$_{50}$ for content mixing. To determine whether the effect of IPA was reversible, we added exogenous recombinant hexameric Sec18 to fusion reactions first incubated with IPA. In Figure 6G we show that IPA inhibited fusion as seen above. When 5.3 µM Sec18 was added, we found that vacuole fusion was restored. Adding Sec18 alone had no effect on vacuole fusion.

We next verified whether the effect of IPA on priming was not due to membrane damage. To this aim we incubated vacuoles in fusion reaction buffer alone or in the presence of 0.2% Triton X-100, 100 µM IPA or 100 µM Epirubicin. After incubating for 30 min at 27ºC, the reactions were fractionated by centrifugation to separate membranes from solubilized material. To monitor the release of luminal content, we probed for the soluble luminal protease Pep4 by Western blotting. Pep4 remained in the membrane fraction when vacuoles were treated with IPA, Epirubicin or buffer alone (Fig. 6H). As a control we used Triton X-100 to solubilize the vacuoles and release Pep4 into the supernatant. We also probed for the distribution of the Rab GTPase Ypt7 as a marker for membrane proteins. Similar to what we saw with Pep4, Ypt7 remained in the membrane fractions unless the vacuoles were treated with Triton X-100. Together this further demonstrates that IPA does not damage vacuoles.

To compare the effects IPA with another small molecule candidate we tested Epirubicin. While Epirubicin was predicted to bind Sec18, we found that it bound poorly with a $K_D$ of 677 ± 179 µM (Fig. 6I). We further tested Epirubicin by using a dosage curve in fusion reactions. We found that it reduced fusion, albeit with an IC$_{50}$ > 400 µM (Fig. 6J). Due to the poor binding to Sec18, we attributed the inhibition of fusion by Epirubicin to its ability to insert into membranes. This was evident by the bright pink coloring of liposomes or vacuoles when incubated with Epirubicin (not shown). Together, these data illustrate that even though Epirubicin was initially predicted to bind Sec18 at similar sites as IPA, it was ineffective in altering Sec18 function, thus bolstering the significance of discovering IPA.

IPA does not induce conformational changes in Sec18 – In order to determine how IPA operates, we asked if it could alter Sec18 conformation in a manner similar to what we previously observed with PA (12). In that work we showed that PA induced conformational changes in full length Sec18 allowing for increased proteolytic cleavage, whereas DAG and phosphatidylserine had no effect. Here we compared the conformational changes induced by PA binding Sec18 with any affects that IPA might have. We incubated Sec18 with a titration curve of Thrombin in the absence or presence of 100 µM IPA. As shown in Figure 7A IPA did not result in increased Thrombin cleavage. Instead, IPA protected Sec18 from cleavage relative to the buffer
control. This is the opposite to the effect of PA binding to Sec18, which results in enhanced cleavage. As a control, we incubated with Sec18 with Thrombin and PA as previously described. As predicted Thrombin cleaved Sec18 more efficiently when C8-PA was present (Fig. 7B). While IPA reduced Sec18 cleavage, the same major degradation products were found with buffer, IPA or PA. The cut sites for generating these products were determined by mass spectrometry. Thrombin cleaved Sec18 at R225 to give a C-terminal 60 kDa fragment (p1). K378 to give the C-terminal 30 kDa fragment (p2). The 25 kDa fragment was generated by cleaving at R225 and K455 (p3).

To further test the effects of IPA on Sec18 we next examined proteolytic cleavage of subdomains. In Figure 7C-D we show the effects of IPA and PA on Thrombin cleavage of N-D1. In reaction buffer alone Thrombin cleavage produced a 31 kDa band by cutting at K100 and R360 (p1). The 25 kDa band was a product of cutting at R225 and K455 (p2). As seen with full length Sec18, we found that IPA reduced the magnitude of cleavage (Fig. 7C). This suggests that IPA either blocks Thrombin sites or induces a distinct conformational change that masks cleavage sites. Interestingly, incubating with C8-PA had no effect on the level of proteolysis compared to the buffer control (Fig. 7D). This suggests that the absence of the D2 domain is needed for PA induced conformational changes that expose Thrombin sites. This does not exclude the possibility conformational changes that do not expose cleavage sites.

IPA binding blocks the effect of PA on thrombin cleavage – Because of the overlap in predicted binding sites for IPA and PA, we tested whether binding IPA first would block the effects of PA on Sec18 cleavage by thrombin. We indeed found that 100 µM IPA protected Sec18 from cleavage (Fig. 8). We interpret this as one of two possibilities. One is that IPA directly blocked PA binding. The second is that IPA induces a conformational change distinct from the one induced by PA in which key PA binding sites were obscured. Because IPA alone had no effect on Sec18 cleavage, it is more likely that the former scenario occurs.

To corroborate the limited proteolysis data, we performed ANS fluorescence assays. When ANS binds to exposed hydrophobic areas, it undergoes fluorescence dequenching, thus changes in ANS fluorescence between different conditions can serve as a reporter for conformational changes. (12). Previously we used ANS fluorescence to show that full length Sec18 underwent conformational changes when bound to PA, but not DAG or PS. The effects of PA binding on Sec18 were further examined using MD simulations on the D1-D2 domains. The N-domain was excluded due to its promiscuous binding to anionic surfaces. Nevertheless, it remained possible that PA binding would induce changes between the N and D1 domains. This is of particular importance as studies by others have shown that the N-domain undergoes conformational changes with respect to the D1-D2 domains when hexameric Sec18 catalyzes SNARE priming at the expenditure of ATP (16, 29–31).

Here we used ANS fluorescence assays to test whether the conformational changes were indeed limited to the D1 and D2 domains, or whether additional changes occurred between the N and D1 domains. First, we tested N-D1 by incubating it with a dose curve of C8-PA. We observed an increase in fluorescence as C8-PA concentrations increased, suggesting that there was some level of conformational change, however, the difference versus the control without PA was not statistically significant (Fig. 9A, E). We next tested the effect of C8-PI(4,5)P2 on N-D1 and found very little change compared to the control (Fig. 9B).
We continued with the D1-D2 construct and PA. Previously we mapped PA binding sites to the hinge region between D1 and D2, so we expected to see an increase in ANS fluorescence with the shortened protein when bound to PA (12). The showed a large statistically significant increase in ANS fluorescence when incubated with C8-PA (Fig. 9C, E). In contrast, incubating D1-D2 with C8-PI(4,5)P2 had no effect on ANS fluorescence (Fig. 9D).

The lack of a significant change in ANS fluorescence with N-D1 is consistent with the Thrombin cleavage patterns seen above. In both cases, the presence of PA had no apparent effect on conformational changes. The lack of ANS fluorescence changes in the presence of PI(4,5)P2 suggests that this lipid interacted poorly with the Sec18 constructs. This is in keeping with our previous report where we measured the $K_D$ for Sec18 to PI(4,5)P2 as $>400 \mu M$ (12). Together, these results indicated that the conformational changes seen in Sec18 upon PA binding primarily occur between the D1 and D2 domains and that changes were specific to PA binding.

IPA Binding and Molecular Dynamics Ensemble Docking – To further probe the interactions of Sec18 with PA and IPA, we performed ensemble molecular docking. We next analyzed scores of the poses from each cluster and selected poses for epirubicin, IPA, and PA from the interquartile region and the highest overall pose corresponding to the lowest $\Delta G$ representation for D1-D2 to run MD simulations for 100 ns. These results were further analyzed by determining the water exposure around the predicted thrombin cut residue for mSec18 of R638 using R628 on NSF (Fig. 10A). We then compared the average RMSD across the entire simulation trajectory for NSF D1-D2 using C8-PA (Fig. 10B), IPA (Fig. 10C), and epirubicin (Fig. 10D) over the course of the 100 ns simulations.

In these simulations it appears that IPA hindered initial conformational change in D1-D2 relative to PA, as there is a significant difference in overall RMSD starting at about 20 ns of the MD simulations, where PA has roughly a 15 Å average RMSD versus ~6 Å average RMSD for the IPA simulation (Fig. 10B-C). This conformational difference was explored by determining water accessibility as shown in Figure 10A, which could limit access for proteases explaining the results in Figure 10A-B. We postulate that IPA inhibits mSec18 binding PA by preventing the conformational change necessary for binding the lipid. Additionally, simulations on epirubicin demonstrate an average RMSD of approximately 5 Å, over the entire 100 ns simulations (Fig. 10D). The fact that IPA and epirubicin appear to lock the conformation of Sec18, whereas PA appears to stimulate conformational change leads to the conclusion that they have different modes of binding, even though IPA appears to competitively inhibit Sec18 binding to PA in biochemical assays.

DISCUSSION
Membrane fusion is required for vesicular trafficking and eukaryotic homeostasis. While most trafficking pathways have a unique signature of organelle-specific SNARE proteins, every set of SNAREs relies on Sec18/NSF, which is the only protein responsible for catalyzing the disassembly of SNARE complexes at the expense of ATP (32). Thus, it is important to develop tools to enable further study of this mechanism because of its ubiquitous operation on almost all organelle types. In this study we used structure based computational drug discovery to find a specific inhibitor of Sec18/NSF function that we call the Inhibitor of Priming Activity, or IPA. Although the structure of NSF was used to computationally dock chemical libraries, the candidate compounds were expected to bind Sec18, as these orthologs have been shown to be interchangeable (7, 33, 34).

Previous to the discovery of IPA, there was a complete lack of specific small molecule inhibitors to block SNARE priming. In fact, the only way to specifically inhibit NSF activity was to raise an antibody against it. As its name indicates, NSF (NEM sensitive factor) was discovered by its sensitivity to the alkylating agent $N$-Ethylmaleimide (NEM), which promiscuously modifies free thiols (35). Although NEM does inhibit SNARE priming in our system, it has downstream effects which are likely due to promiscuous alkylation, for example, NEM could additionally alkylate other proteins involved
in SNARE function such as the single Cys on the SNARE Vam7 (11).

In this study we report that IPA inhibits SNARE priming and subsequent vacuole fusion. Previously we found that priming could be inhibited by the PA phosphatase inhibitor propranolol (10). Although non-specific, the results from using propranolol led us to determine that Sec18 bound to PA reservoirs on the vacuole membrane that sequestered Sec18 away from cis-SNARE complexes (11). It is only after the PA phosphatase Pah1 converts enough PA to DAG that Sec18 is able to dissociate from the membrane and engage the SNARE complex. Through this we also found that C8-PA could be added to in vitro reactions and potently block priming by preventing Sec18 from attaching to SNAREs. The ability of C8-PA to inhibit SNARE priming was not entirely specific to priming, as it also inhibited downstream stages of the fusion pathway as indicated by gain of resistance assays. We attribute the later effects of C8-PA to interacting with the SNARE Vam7, which binds both PI3P and PA (14). While both propranolol and C8-PA helped to further understand Sec18 regulation, a more specific molecule such as IPA was in order to determine with more specificity the effect of PA on Sec18 priming, and not additional stages of fusion. Unlike propranolol and C8-PA, IPA does not appear to have downstream effects on the fusion machinery. This is due to the specificity of IPA for NSF at the PA-binding interface while the aforementioned propranolol and C8-PA do not specifically bind a single protein. Importantly, the lack of an effect after priming indicates that IPA does not inhibit the newly discovered priming-independent role of Sec18 in SNARE zippering at the docking stage (36).

Using molecular docking and MD simulations, we found that IPA bound to NSF at the hinge region of D1 linking to the D2 domain. This region of NSF overlaps with regions bound by the lipid PA (12). Consequently, IPA could compete for PA binding by Sec18 on liposomes. This was a specific competition as IPA failed to compete for PA binding against DEP and Vam7Y42A, or significantly interfere with interactions between Sec18 and PI liposomes. With regards to the two binding sites, IPA showed the best competition at the D1 hinge site, which coincides with the key regulatory site of PA binding, and the fact that PA bound D1 better than any other domain of Sec18 alone (10). When PA binds NSF it induces a conformational change at the hinge region causing the two domains to swing towards and away from each other, creating both closed and open forms of NSF. The “closed” conformation of NSF is incompatible with hexamerization and SNARE priming activity. We now show that IPA binds in the same hinge region and prevents its change to the closed conformation, presumably inhibiting the conformational change necessary for Sec18 to bind PA and potentially explaining the inhibition of Sec18 binding to soluble SNAREs shown previously (10). Instead, IPA appears to make the Sec18 protomers more rigid, which may in turn prevent interactions with Sec17 and cis-SNARE complexes.

In summary, we now report the discovery of a novel small molecule that inhibits SNARE priming through binding Sec18/NSF. Due to the lack of any such inhibitor, IPA will serve as a potent tool to examine the nature of Sec18/NSF function and SNARE priming. While IPA was potent in the inhibition of Sec18, we expect that it will have a lower $K_D$ and better activity towards NSF, as the molecule was found using the NSF structure. Thus far, only in vitro systems have been thoroughly tested with IPA, but preliminary in vivo tests with mammalian cells indicate that IPA is membrane permeable and active at nanomolar concentrations (Sparks & Fratti, unpublished). This suggests that IPA might not be limited to being an in vitro research tool.

### EXPERIMENTAL PROCEDURES

**Reagents** – All reagents were diluted in PS buffer (20 mM 1,4-piperazinediethane sulfonic acid (PIPES)-KOH pH 6.8 and 200 mM sorbitol) to a working concentration before use in an experiment. Antibody to Sec17 was described previously (37). POPA (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine), POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine), C8-PA (1,2-dioctanoyl-sn-glycero-3-phosphate)
were purchased from Avanti Polar Lipids as chloroform stock solutions and stored at -20°C. CM7, CM5, Ni-NTA (Standard and S series) and L1 sensor chips, and Regeneration buffers (Glycine pH 1-3) were procured from GE Healthcare. Ni-NTA Atto 488 dye, N-Ethylmaleimide (NEM) and Calcein were procured from MilliporeSigma. Monolith NT.115 standard treated capillaries for thermophoresis were purchased from Nanotemper (München Germany). Epirubicin was from Cayman Chemical. 7-Methyl-3-(4,5,6-trihydroxy-3-oxo-3H-xanthen-9-yl)bicyclo[2.2.1]hept-5-ene-2-carboxylic acid was procured via Ambinter under the identification numbers Amb16226271 and Amb4002159.

IPA purity was assessed using NMR and LC/MS. NMR data was recorded on a Bruker spectrophotometer equipped with a CryoProbe (500 MHz, 1H) using deuterated dimethyl sulfoxide as a solvent and internal reference (δ = 2.50 ppm). LC/MS data was performed by the University of Illinois Mass Spectrometry Laboratory using a 2.1 mm ID reverse phase C-18 column and a Waters Synapt G2-Si mass spectrometer (ESI).

Recombinant proteins – Recombinant expression of C-terminally His8 tagged full length Sec18, or its domains (N, D1, D2, D1-D1) were purified from Escherichia coli as previously described (12). Briefly, pSec18His8 (or one for a domain derivative) was transformed into Rosetta 2 (DE3) pLysS Competent Cells (Novagen) and Sec18-His8 expression was carried out using auto-inducing medium (AIM) (38). Cells were grown to stationary phase (37°C, 18 h, shaking) and harvested by centrifugation. Cells were resuspended in lysis buffer (20 mM HEPES pH 6.8, 300 mM NaCl, 1 mM 2-mercaptoethanol, 10% glycerol). Sec18-His8 elutes in two peaks corresponding to monomeric and hexameric pools (Fig. S1). Each pool was collected and concentrated before use. The DEP PA binding domain from murine Dvl2 was purified as a GST-fusion as described (25). Membrane scaffold protein 1D1 (MSP1D1-His) was prepared as described (39). GST-Vam7\textsuperscript{Y42A} was expressed and purified as described (40).

Calcein dequenching and membrane integrity – Liposomes were extruded in the presence of 100 mM Calcein to encapsulate the dye at self-quenching concentrations (27, 28). Liposomes were dialyzed in 4L of TBS pH 7.4 (10 mM Tris-Cl pH 7.5, 140 mM NaCl, 10 mM EDTA pH 8.0, 0.5% SDS) to remove excess un-encapsulated dye. Encapsulated Calcein liposomes were incubated with buffer, 0.2% Triton X-100, or a dose curve of IPA. Upon lysis, Calcein becomes diluted and fluorescence increases (ex. 494 nm, em. 515 nm).

Vacuole Isolation and in vitro vacuole fusion assay – Vacuoles were isolated from yeast strains by density gradient floatation as previously described (41). Fusion reactions (30 µL) contained 3 µg each of vacuoles from BJ3505 (pep4\textsuperscript{Δ} PHO8) and DK6281 (PEP4 pho8\textsuperscript{Δ}), fusion assay buffer (125 mM KCl, 5 mM MgCl\textsubscript{2}, 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 Pbi2p. Reactions were incubated at 27°C for 90 min and the Pho8 activity was measured in 250 mM Tris-Cl PH 8.5, 0.4% Triton X-100, 10 mM MgCl\textsubscript{2}, 1 mM \textit{p}-nitrophenyl phosphate. Fusion-dependent alkaline phosphatase maturation was measured by the amount of \textit{p}-nitrophenylate produced. \textit{p}-Nitrophenylate absorbance was measured at 400 nm.

Liposome Preparation and Co-Floatation Assay - Large unilamellar liposomes were prepared using an extrusion method (42). Extruded liposomes were prepared with 1 mM concentration of appropriate lipids 75% PC, 20% PE, and 5% PA, which were heated and passed through an Avanti mini-extruder.
equipped with filters of sizes 0.1, 0.4 and 0.8 µm filters over 10 times to assure homogeneous liposome sizes. Small unilamellar liposomes containing various lipid compositions were prepared using the sonication method (15). Briefly, stock lipids in chloroform were mixed to produce a lipid mixture with the desired lipid mole percentages of 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 min. The tubes were placed under vacuum in a desiccator for an additional 14 h. To the dried lipids, 2.6 mL of 1X PBS solution was added. Tubes were covered with parafilm and incubated at room temperature for 1 h. The lipids were resuspended with vortexing and disrupted in a water bath sonicator for 30 min.

To measure protein binding to liposomes we used a floatation assay as described (43). Briefly, 40 µL of lipid binding domain/PBS mixture was incubated with 150 µL of the 1 mM liposome suspension for 5 min at 30°C before 20 µg of recombinant Sec18-His8 was added to bring up the total volume to 200 µL and give a final concentration of 1.2 µM Sec18. Samples were incubated for an additional 10 min at 30°C and 630 µL of 1.65 M sucrose (PBS) was added. Samples were loaded into the bottom of a centrifuge tube and layered with 840 µL of 0.75 M sucrose (PBS), and 1X PBS to the top of the tube. Samples were centrifuged (200,000 x g, 90 min, 4°C) and 200 µL of floated liposomes were recovered from the top of the 0.75 M sucrose layer. The bottom 100 µL fraction was recovered and SDS sample buffer was added to sample unbound protein levels. Liposomes were resuspended in 1 mL of 1X PBS and isolated by centrifugation (16,000 x g, 10 min, 4°C). SDS sample buffer was added to the final liposome pellet and bound proteins were resolved by SDS-PAGE and probed by Western blotting. Images were acquired using a ChemiDoc MP Imaging System (Bio-Rad). Additionally, protein binding and inhibition of protein binding via inclusion of IPA to extruded liposomes was measured using SPR.

**Priming Assay** – Priming activity of Sec18 was assayed as previously described (11). Briefly, vacuoles were harvested from BJ3505. The equivalent of two standard fusion reactions was incubated at 27°C with buffer, 1 mM NEM, 300 µM C8-PA, or 100 µM IPA. At the indicated times, vacuoles were removed by centrifugation (16,000 x g, 5 min, 4°C) and SDS sample buffer was added to the supernatants. Samples were heated at 95°C for 5 min, resolved by SDS-PAGE, transferred to nitrocellulose, and probed by Western blot.

**Surface Plasmon Resonance** – Surface plasmon resonance (SPR) measurements were performed on a Biacore T200 instrument equipped with an Ni-NTA chip (24). Approximately 2000 RU of 5% PA nanodiscs were immobilized non-covalently using 100 mM NiSO₄ flowed at 10 µL/s followed by a blank buffer injection of HEPES pH 7.4, 150 mM NaCl (HBS Buffer). Injections were performed in HBS buffer at a flow rate of 30 µL/min with an association time of 90 sec, dissociation time of 300 sec., and binding was measured in relative response units (RU) as described (44). Regeneration with EDTA was performed at flow rate 30 µL/s for 120 s using 100 µM EDTA buffer. Proteins were injected using a series of 1:1 dilutions from highest concentration and steady state was obtained using GE BIAcore T200 evaluation software version 3.0 (BIAevaluate). Proteins were injected using a series of 1:1 dilutions for Sec18 monomer, D1, D2 and Sec18 hexamer with at least one concentration from each titration run in duplicate. Steady state data was exported using BiaEvaluate software into GraphPad Prism 7.00 for Windows, GraphPad Software (La Jolla, CA) and fit using either a one-site specific binding model or an IC₅₀ model generated using log[IPA] v. response (three parameters) equation.

For SPR using attached liposome an L-1 liposome chip was used with liposome attached to a sample flow cell and no liposome to the reference flow cell. Liposomes were attached after conditioning the chip with two injections of CHAPS (3-[(3-cholamidopropyl)-dimethylammonia]-1-propan-sulfonate over both flow cells for 30 sec at 30 µL/min. Each liposome capture was regenerated when a different protein was flowed using 30 sec injections of 20 mM CHAPS at 5 µL/min to clean the sensor chip. Proteins were attached freshly for each type of protein, where proteins were titrated in the pres-
ence and absence of IPA yielding IC$_{50}$ and $K_D$ values. $K_D$ values to liposomes were performed at 30 µL/min with association of 70 sec and disassociation of 300 sec for D1, 60 sec association and 300 sec dissociation for Sec18 monomer and hexamer, and 60 µL/min for D2 with association 75 and disassociation of 225 sec. Results were exported from BiaEvaluate into Graphpad and fit via one-site specific binding model for each saturation curve yielding $K_D$ values for different constructs.

**Microscale thermophoresis** – Thermophoresis measurements were performed using a Monolith NT.115 labeled thermophoresis machine (45). Sec18-His8 was labeled with Ni-NTA Atto 488 according to the manufacturer’s protocol mixing 200 nM protein with 100 nM dye and allowing to sit at room temperature for 30 min followed by centrifugation. M.O. Control software was used for operation of MST. Target protein concentrations were 50 nM for all His-tag labeled proteins Sec18 monomer, Sec18 hexamer, PA nanodiscs, and PC nanodiscs. LED excitation power was set to 90% and MST set to high allowing 3 s prior to MST on to check for initial fluorescence differences, 25 s for thermophoresis, and 3 s for regeneration after MST off. Analysis was performed using M.O. Affinity Analysis Software as the difference between initial fluorescence measure in the first 5 s as compared with thermophoresis at 15 s. All measurements were performed in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$, pH 7.4) without Tween and binding affinity was generated using Graphpad Sigmoidal 4PL fit from points exported from M.O. Affinity Analysis software using $K_D$ Model with target concentration fixed at 50 nM generating bound, unbound, and fraction bound for export to Graphpad in order to estimate final $K_D$.

**1,8-ANS Fluorescence Spectroscopy** – ANS binding experiments were carried out as described previously (12). Reactions were performed in fluorescence assay buffer with 5 µM 1- anilino-8-naphthalenesulfonate (ANS) (Cayman Chemical). Initial spectra were taken without protein to measure any background fluorescence from buffer or added lipids (ex. 350 nm, em. 390-620 nm). His8-tagged Sec18 truncations containing the N-domain and D1-domains (N-D1), or D1 and D2 domains (D1-D2) were diluted in assay conditions and then added to the assay to the indicated concentration and incubated at 25°C for 5 min before spectra were obtained. Initial background fluorescence spectra for each lipid concentration were subtracted from final measurements.

**Limited Proteolysis** – Cleavage reactions were carried out as described (12). Sec18-His8 (2 µM) was added to proteolysis buffer (20 mM HEPES pH 7.2, 150 mM NaCl, 2 mM ATP, 2 mM MgCl$_2$) and incubated with indicated lipid or IPA concentration on ice for 5 min. Thrombin diluted in 1X HBS was added to assay tubes at indicated concentrations and incubated at 25°C for 30 min. Cleavage reactions were stopped with the addition of SDS sample buffer containing 1 mM PMSF. Samples were resolved with SDS-PAGE and gels were stained using Coomassie Blue. Gels were de-stained with methanol/acetic acid solution (50%/7%) and imaged using a ChemiDoc MP Imaging System (BioRad).

**Mass Spectrometry** – Cleavage products from limited proteolysis experiments were excised from SDS-PAGE gels and submitted for LC-MS/MS performed and analyzed by Bioinformatics Solutions Inc. (Ontario, Canada).

**MD Simulations of Top Poses from Ensemble Scoring Function** – A previously reported model of D1-D2 by Starr, Sparks, Arango et al. was utilized for this study. The model was derived from a Cryo-EM structure of ATP-bound NSF complex (PDB 3J94 - chain A) containing residues 215-737 (31). Molecular dynamics simulations were done using NAMD 2.12 (46), with the CHARMM36m force field (47). To maintain a constant pressure of 1 atm and temperature of 310.15 K, Langevin dynamics and Langevin piston Nosé–Hoover methods were used respectively (48, 49). Particle mesh Ewald (PME) methods were used to calculate long-range electrostatic forces using 1 Å grid spacing (50, 51). Van der Waals interactions were evaluated with a cutoff of 12 Å, and after 10 Å used a force-based switching scheme. Integration time step was set at 2 fs with the SETTLE algorithm (52) applied. VMD 1.9.3 was used for MD trajectory visualization and
analysis (53). The D1-D2 monomer was equilibrated for 20ns using harmonic restraints on the Cα atoms (0.05 kcal/mol/Å²), barring the previously modelled loops. The simulation was continued without restraints to 200 ns.

Probing Binding Sites of IPA – To identify potential IPA, PA, and epirubicin interactions with D1-D2 monomer, molecular ensemble docking of was done on D1-D2 monomer using AutoDock Vina (54). The previously mentioned equilibrium simulation of D1-D2 was used to fully sample the dynamics of D1-D2 for molecular docking, where snapshots were taken every 1000 ps of the 200 ns trajectory. For each snapshot, an 80Å by 94Å by 108Å grid box was used to fully sample the entire structure. Each snapshot was docked with an exhaustiveness of 10, yielding a total of 2000 IPA and PA docked poses, with the affinities of each pose obtained from the resultant log files. These poses where then clustered using a hybrid K-centers and K-medoids clustering algorithm using an RMSD method (55, 56) with which four main clusters where identified. Poses from each cluster provided by the ensemble docking from AutoDock Vina with the highest interquartile scores were selected for simulation. Poses from each cluster provided by the ensemble docking from AutoDock Vina with the highest interquartile scores were selected for simulation. Selected poses were solvated and ionized to a NaCl concentration of 150 mM using the SOLVATE and AUTOIONIZE plugins within VMD respectively (53). These systems were simulated for 100 ns and were analyzed with VMD as well as MDAnalysis package (57, 58)

Dynamic light scattering – A Malvern Zetasizer Nano ZS was used to measure both 100 nm extruded liposomes and water bath sonicated liposomes to compare 80/20 PC/PE liposomes to 75/20/5 PC/PE/PA liposomes. Liposomes created for co-floatation and binding experiments were measured exporting a frequency plot for size distribution, taking 3 separate measurements, and choosing a representative measurement. Liposomes were diluted to 5 μM in TBS (50 mM TRIS-Cl pH 7.5, 150 mM NaCl) using a Zetasizer set with dispersant with a refractive index of 1.331 for TBS, material of DPPC liposomes, and disposable cuvetttes. The presence of PA had no effect on the diameter of extruded liposomes (Fig. S2A).

Liposome floatation was performed as described above with 100 nm PA extruded liposomes in the presence or absence of 100 μM IPA. Approximate concentrations of liposome were performed using DLS and measuring kilocounts per second (kcps) (59, 60). Scattering intensity Kcps values of liposomes before and after floatation were plotted on Y axis for each liposome type (Fig. S2B).

Data Analysis and Statistics – Results are expressed as the mean ± S.E. Experimental replicates (n) is defined as the number of separate experiments with different batches of protein, liposomes, and nanodiscs. Where appropriate, significant differences were calculated using two-tailed unpaired t-tests. P values ≤0.05 were considered significant.

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**FOOTNOTES**

The abbreviations used are – AIM, autoinducing medium; α-SNAP, soluble NSF adaptor protein; ANS, 8-anilino-1-naphthalenesulfonic acid; DAG, diaclylglycerol; DEP, Dishevelled, Egl-10, and pleckstein domain; diC8, dioctanoyl; C8-PA, 1,2-dioctanoyl-sn-glycerol-3-phosphate; GST, glutathione S-transferase; MD, molecular dynamics; MST, microscale thermophoresis; Ni-NTA, nickel-nitritriacetic acid; NEM, N-ethylmaleimide; NSF, NEM-sensitive factor; PA, phosphatidic acid; PC, phosphatidylcholine; PDB, Protein Data Bank; PE, phosphatidylethanolamine; POPA, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidyl-ethanolamine; PMSF, phenylmethylsulfonylfluoride; r.m.s. deviation, root mean square deviation; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SPR, surface plasmon resonance.
Table 1. Binding affinities for Sec18 and its domains

| Protein      | Analyte        | Method          | $K_D$  |
|--------------|----------------|-----------------|--------|
| mSec18       | IPA            | SPR             | 4 µM   |
| mSec18       | IPA            | Label Free MST  | 7.5 µM |
| mSec18       | IPA            | Labeled MST     | 3.8 µM |
| mSec18       | Epirubicin     | Labeled MST     | 677 µM |
| D1           | IPA            | SPR             | 320 nM |
| D2           | IPA            | SPR             | 1.1 µM |
| D1D2         | IPA            | SPR             | 1 µM   |
| mSec18       | 5% PA Lip .1 µm| SPR             | 1.6 µM |
| mSec18       | 5% PA Lip .4 µm| SPR             | 1 µM   |
| mSec18       | 5% PA Lip .8 µm| SPR             | 505 nM |
| D1           | 5% PA Lip .8 µm| SPR             | 784 nM |
| D2           | 5% PA Lip .8 µm| SPR             | 867 nM |

mSec18, monomeric Sec18; Lip., Liposomes

Table 2. IPA competition for Sec18 binding and function

| Analyte      | Ligand         | Competitor     | Method          | IC$_{50}$ |
|--------------|----------------|----------------|-----------------|-----------|
| mSec18       | 5% PA Lip .8 µm| IPA            | SPR             | 762 nM    |
| D1           | 5% PA Lip .8 µm| IPA            | SPR             | 220 nM    |
| D2           | 5% PA Lip .8 µm| IPA            | SPR             | 641 nM    |
| mSec18       | Sonicated Lip. | IPA            | Lip. Floatation | 3 µM      |
| N/A          | Isolated Vacuoles| IPA           | Fusion          | 50 µM     |
| N/A          | hSec18         | IPA            | Fusion Priming  | 50 µM     |

mSec18, monomeric Sec18; hSec18, hexameric Sec18; Lip., Liposomes
Figure 1. Small Molecule Candidates for Sec18 Binding and Priming Inhibition. (A). Left; Schematic of Sec18 with labeled domains (N, D1 and D2) in its monomeric (mSec18) and hexameric (hSec18) forms. Right; Schematic model of Sec18 mediated SNARE activation. Pah1 is a PA phosphatase. Sec17/α-SNAP is the adaptor protein linking Sec18 to inactive *cis*-SNARE complexes. (Adapted from (12)). (B) Structures of IPA (7-Methyl-3-(4,5,6-trihydroxy-3-oxo-3H-xanthen-9-yl)bicyclo[2.2.1]hept-5-ene-2-carboxylic acid) and other small molecule candidates.
Figure 2. IPA and Epirubicin binding to Sec18. (A) Ligand Interaction Diagram of IPA binding to homology model of mSec18 and receptor grid for Box 3 of homology model of Sec18 corresponding to Schrodinger Sitemap predicted site 3. Pi stacking indicated with red arrows H-bonding including salt bridge between Lys159 and D374 hydrogen bonding with IPA. (B) Ligand Interaction Diagram of IPA binding to mSec18 corresponding to Schrodinger Sitemap predicted site 4. Salt bridge with Ser378 and IPA with arrow. (C) Ligand Interaction Diagram of Epirubicin binding to receptor grid for Box 3. (D) Ligand Interaction Diagram of Epirubicin binding to receptor grid for Box 4. (E) Bar graph depicting gscore of best IPA and Epirubicin poses corresponding to (Fig 3b-e) to boxes 3 and 4 indicated with lowest -ΔG using Schrodinger Glide and exported into GraphPad. (F) IPA cluster analysis displayed and edited with VMD for IPA to D1-D2 of NSF with D1 indicated with purple and D2 green and top 4 most populated clusters represented with dark grey for cluster 1, orange for box, light blue for box 3 and light gray for box 4. (G) Epirubicin cluster analysis displayed and edited with VMD for Epirubicin to D1-D2 of NSF as in (Fig. 1F).
Figure 3. Sec18 Binding Affinities for IPA. (A) Fluorescence MST of IPA binding to Sec18-His8 labeled with Atto 488 Ni-NTA dye with fluorescence converted to fraction bound M.O. Affinity software and exported in Graphpad with $K_D$ of $3.84 \pm 1.3 \mu M$ using log-inhibitor vs. response 4 parameter equation and error using SEM (n=3). (B) Label-free MST of IPA binding to unlabeled Sec18-His8 converted to fraction bound as in (Fig. 1a) with $K_D$ of $7.4 \pm 3.7 \mu M$ using log-inhibitor vs. response 4 parameter equation error using SEM (n=3). (C) SPR of IPA to Sec18-His8 linked to a Ni-NTA biosensor chip at approximately 2000 RU with Response measured subtracting blank reference cell and relevant blank injections. Data exported from BiaEvaluate and into GraphPad and fit using a one-site specific binding model indicating a $K_D$ of $4 \mu M$. (D) SPR sensorgrams of D1 to IPA as in (Fig. 2D) with $K_D$ of $320 \text{ nM}$ and $k_a$ of about 1716 M s$^{-1}$. (E) SPR sensorgrams of D2 to IPA as in (Fig. 2D) with $K_D$ of $1.1 \mu M$ with $k_a$ of about 1900 M s$^{-1}$. (F) SPR sensorgrams of D1-D2 to IPA as in (Fig. 2D) with KD of $960 \text{ nM}$ and $k_a$ of about 8000 M s$^{-1}$. 
Figure 4. IPA competes for PA binding. (A) Sec18 was incubated with PA liposomes in the presence or absence IPA. After incubation the liposomes were re-isolated to detect bound and unbound Sec18 by immunoblotting with anti-Sec18 antibody. (B) GST-DEP was incubated with PA liposomes in the presence of buffer or IPA. After incubation the liposomes were re-isolated and bound and un-bound DEP was detected by immunoblotting with anti-GST antibody. (C) SPR using an L-1 chip captured with approximately ~3000 RU 5% PA (solid lines) or 2.5% PI (dotted lines) was titrated with GST-Vam7Y42A. Binding was performed by incubating a fixed protein concentration with a titration of IPA. (D) SPR of tagged mSec18-His8 bound to an L-1 Chip as in C. (E) His tagged mSec18, D1 and D2 constructs binding to 0.8 μm PA liposomes at about 4000 RU captured to an L1 biosensor chip exported yielding KD values for D1 (781 ± 110 nM), D2 (866 ± 669 nM), and mSec18 (505 ± 267 nM). (F) SPR competition of Sec18 constructs binding to PA liposomes titrated with IPA.
Figure 5. Liposome diameter affects IPA inhibition of Sec18 binding to PA. (A) Extruded PA liposomes of 0.1 µm, 0.4 µm and 0.8 µm were bound to L1 biosensor chips. Sec18 was titrated to give a with $K_D$ 1600 ± 933 nM, 1020 ± 549 nM and 673 ± 239 nM for 0.1, 0.4 and 0.8 µm diameter liposomes, respectively. Error bars representing SEM (n=3). (B) As in panel A, Sec18 was flowed over PA liposomes attached to L1 chips in the presence or absence of 100 µM IPA. The difference in RU of Sec18 binding to liposomes in the presence of IPA was calculated relative to the maximum Sec18 bound in the absence of IPA. The bar graph represents the Mean values of inhibition with individual points shown. The error bars represent SEM (n=3). (C-D) Liposome integrity in the presence of IPA was tested by Calcein release. PA Liposomes were extruded in the presence of 100 mM Calcein, a concentration that leads to fluorescence quenching. Calcein containing liposomes were incubated with buffer, Triton X-100 or a dosage curve of IPA. Liposome damage, as measured by content leakage was detected by the dilution of Calcein and gain in fluorescence.
Figure 6. IPA Inhibits Vacuole Fusion at the SNARE Priming Stage. (A) In vitro vacuole homotypic fusion incubated with a concentration curve of IPA and incubated for 90 min at 27°C. Fusion was tested by luminal mixing, proPho8 maturation and conversion of p-nitrophenylphosphate to p-nitrophenolate measured at 400 nm. (B) Gain of resistance kinetics assays were performed in the presence of 140 µg/mL α-Sec17p IgG, 100 µM IPA, 1 mM NEM, or PS buffer. Data was fit using first-order exponential decay with weights and errors. (C) Vacuoles from BJ3505 yeast were tested for priming activity as a measure of Sec17 release from the membrane fraction. Fusion reactions of were incubated in the presence of buffer, NEM, C8-PA, or IPA. 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. Densitometry values were normalized against input sample for each condition. (D) Quantitation of three repeats of panel C. (E) Dose response curve of IPA and the inhibition of priming as detailed in panel C. (F) Quantitation of three trials presented in panel E. (G) The inhibitor effects of IPA on fusion were reversed with the addition of 5.3 µM
recombinant hexameric Sec18-His8. (H) Vacuole damage caused by IPA was tested by the release of luminal content. Vacuole fusion reactions were incubated with buffer, 0.2% Triton X-100 (TX), 100 µM IPA or 100 µM Epirubicin (Epi) for 30 min at 27°C. After incubation, the reactions were fractionated by centrifugation to separate membranes from the supernatant. Membrane and Supernatant fractions were resolved by SDS-PAGE and probed for Pep4 and Ypt7 by Western blotting. (I) The affinity of Sec18 binding to Epirubicin determined by labeled MST using Ni-Atto 488 dye indicating a $K_D$ of 677 ± 179 µM. (J) Vacuole fusion reactions were incubated with a dosage curve of Epirubicin and processed as described above. Error bars represent SEM (n=3). ** p < 0.01
Figure 7. IPA and conformation changes in full length Sec18. Sec18-His8 (A-B), N-D1 (C-D) and D1-D2 (D-E) samples were incubated with buffer, IPA, or C8-PA before incubation with increasing concentrations of thrombin for 30 min. Reactions were stopped by adding SDS-PAGE loading buffer. Protein digests were resolved by SDS-PAGE and stained with One-Step Blue® Protein Gel Stain (Biotium). Quantitation was determined for the relative amounts of un-cleaved protein. n=3 for all data shown. Mr relative molecular mass markers. *, p<0.05; **, p<0.01; *** p<0.001; ****, p<0.0001. Schematics represent Sec18, N-D1 and D1-D2. Cleavage sites are depicted by the amino acid number. Major degradation products are labeled as p1, p2 and p3.
Figure 8. IPA blocks the effect of PA on Sec18 degradation by thrombin. Full length Sec18 was incubated with 100 μM C8-PA alone or 100 μM IPA followed by 100 μM C8-PA, after which the samples were incubated with thrombin for 30 min. Samples were mixed with SDS loading buffer and resolved by SDS-PAGE and visualized with One-Step Blue® Protein Gel Stain. Quantitation was determined for the relative amounts of un-cleaved protein. n=3 for data shown. M₇, relative molecular mass markers. **, p<0.01; ****, p<0.0001. Major degradation products are labeled as p1, p2 and p3.
Figure 9. ANS Fluorescence of Sec18 domains bound to lipids. N-D1 (0.5 µM) was incubated with dosage curves of C8-PA (A) or C8-PI(4,5)P₂ (B) and 5 µM ANS. Fluorescence was measured by exciting at 390 nm and scanning a range of emission wavelengths. (C-D) D1-D2 (0.5 µM) was incubated with C8-PA or C8-PI(4,5)P₂, and 5 µM ANS. Fluorescence was detected as above. (E) Quantitation of maximum fluorescence measurements at each concentration of lipid. Error bars represent SEM (n=3). * p < 0.05
Figure 10. Molecular Dynamics of PA, IPA, and Epirubicin. (A) Exposed waters within 3 Å of Arginine 638, the proposed cleavage residue for thrombin as shown in Figure 5A-B. (B) Average RMSD of PA (n=2) in 100 ns simulations. (C) Average RMSD of IPA (n=4) in 100 ns simulations. (D) Average RMSD of epirubicin (n=2) in 100 ns simulations.
A small-molecule competitive inhibitor of phosphatidic acid binding by the AAA+ protein NSF/Sec18 blocks the SNARE-priming stage of vacuole fusion
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