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Lipid molecules influence early stages of yeast SNARE mediated membrane fusion

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Abstract

Lipid molecules, structural components of biomembranes, have been proposed for an important role in membrane fusion. Through various techniques based on protein-reconstituted vesicle-vesicle fusion system, we investigated the influence of several lipid molecules on different stages of yeast SNARE mediated membrane fusion process. Lipid compositions played a significant role in early stages, docking and lipid mixing, while only exhibited a minor effect on fusion pore formation and dilation phases indicated by both small and large content mixing.

In eukaryotic vesicular trafficking, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) have been widely accepted as the central components for membrane fusion which is regulated by several other accessory proteins. Besides proteins, lipid molecules also function as important active regulators in the SNARE-mediated membrane fusion from two aspects: membrane curvature and protein assembly (van den Bogaart and Jahn, 2011). Regarding membrane curvature modulated by lipid molecules, previous studies at the lipid mixing level showed that lipid molecules, such as cholesterol (Chol) and phosphatidylethanolamine (PE) which stabilize negative curvature significantly influencing membrane fusion (Chang et al., 2009; Domanska et al., 2010; Tong et al., 2009). For the role of lipid molecules in modulating protein assembly, recent studies showed that cholesterol and phosphatidylinositol-4,5-bisphosphate (PIP₂) could induce SNARE protein clustering (Murray and Tamm, 2009, 2011), which may be a critical early step for SNARE-mediated membrane fusion. It is also reported that lipid molecules might regulate trans-SNARE complex formation (Mima and Wickner, 2009).

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To study SNARE-mediated membrane fusion, the *in vitro* protein-reconstituted vesicle-vesicle fusion assay using ensemble fluorescence resonance energy transfer (FRET) has been developed since the 1990's (Weber et al., 1998). In a typical experimental scheme, v- and t-SNARE proteins are reconstituted into two independent populations of vesicles (diameter: 40~100 nm, Figure 1A) in which one population of vesicles contains both donor and acceptor (7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)/rhodamine) fluorophore- labeled lipid molecules and the other contains no fluorophores. Upon vesicle-vesicle fusion (Figure 1B), lipid mixing results in an increase of the average distance between donors and acceptors, and the extent of fusion can be quantified from the recovery of the donor fluorescence signal.

Thus far, the ensemble vesicle-vesicle fusion assay based on lipid mixing has been widely used to study molecular mechanisms whereby SNAREs mediate membrane fusion and has achieved great successes. Here, we investigated the effect of cholesterol and that of PE as well in the fusion of vesicles composed of phosphocholine (PC) and phenylserine (PS). In traditional ensemble lipid mixing, as shown in the Figure 1C, cholesterol and PE molecules both dramatically stimulate lipid mixing.

However, it has been shown that the rate of ensemble lipid mixing is largely limited by early stages of membrane fusion, such as docking (Diao et al., 2013a; Diao et al., 2012b; Diao et al., 2013b; Lai et al., 2014). On the other hand, content-mixing through the fusion pore has been considered as a gold standard to access true membrane fusion. To this end, several content mixing assays have been developed at the single-vesicle level. Recently, a single-vesicle content-mixing assay based on dequenching of a small content indicator, sulforhodamine B, has been shown to monitor opening of the nascent fusion pores in Ca^{2+} -triggered fast fusion (Diao et al., 2012a; Diao et al., 2013c; Kyoung et al., 2011; Kyoung et al., 2013; Lai et al., 2014). Through a single vesicle assay involving mixing of small content which can delineate intermediates of the fusion pathway, we found that the effect of lipid compositions on vesicle docking (Figure 1D) is much larger than that on fusion pore opening (Figure 2A). We have also developed a single-vesicle content-mixing assay employing large content mixing indicators intended to report the expansion of fusion pores (Diao et al., 2012b; Diao et al., 2010; Lai et al., 2013). Through this new assay, we also found a minor influence of lipid composition on fusion pore expansion in our vesicle-vesicle fusion. As shown in the Figure 2B, even at the lipid composition that shows the highest lipid mixing in Figure 1C and the best docking in Figure 1D, 45 mol% PC, 40 mol% Chol, and 15 mol% PS, the deletion of cholesterol has only negligible effect on pore expansion; while the addition of PE molecules only slightly reduces content mixing, which is not significant. Through molecular dynamics (MD) simulation, we also confirmed that all three different lipid compositions have similar transition times from the hemifusion state to the full- fused state (Figure 3A). We also noticed an increase in fusion time for cholesterol-containing membrane. This could be explained by the faster formation of syntaxin clusters and SNARE complexes in the cholesterol-containing membrane (Murray and Tamm, 2009, 2011) resulting in a stronger pulling tension generated by the SNAREs. Meanwhile, SNARE-reconstitution efficiency was not dependent upon lipid compositions as confirmed by the sodium dodecyl sulfate (SDS) Page analysis (Figure 3B), which excludes the possible influence of the variation of the protein density in vesicles.

We knew that in neuronal SNARE mediated membrane fusion system, other than SNARE proteins, many other factors like C2AB and BAR domain (Diao et al., 2009; Peter et al., 2004; Sutton et al., 1995) could affect membrane curvature and therefor affect the fusion process. Also in neuronal SNARE system, it was reported that different lipid components could affect syntaxin-1A oligomerization and SNARE assembly (Lam et al., 2008; van den Bogaart et al., 2011), thereby regulate vesicle docking and lipid mixing. Here, we also have shown that PE, a lipid molecule with a small head group favoring the negatively curved monolayer, and cholesterol with the intrinsic negative curvature, promote ensemble lipid mixing substantially. Interestingly, however, we found, using single vesicle assays that vesicle docking is affected significantly by the lipid composition. These results might suggest that vesicle docking, which results from yeast SNARE complex formation, is strongly coupled with lipid mixing that accompanies bilayer remodeling involving curvature formation. Thus, our results suggest that trans-SNARE assembly occurs concurrently or in the similar time frame with some lipid mixing, perhaps hemifusion as has been previously proposed (Yang et al PNAS 2009).

On the other hand, it was reported that fusion pore opening and expansion in neuronal SNARE mediated membrane fusion required specific lipid molecules like cholesterol and acidic lipid PIP₂ (Cho et al., 2007; Lewis et al., 2014; Shin et al., 2010). This could be caused by certain protein regulators, such as C2AB which could significantly change membrane curvature upon binding to specific lipid molecules (Hui et al., 2009; Martens et al., 2007). In stark contrast, we found that fusion pore opening and expansion are less affected by the lipid composition in a simplified yeast system. For late fusion steps after hemifusion or outer leaflet mixing, transmembrane domains (TMDs) of both Q- and R-SNAREs are co-located in the same membrane to be able to interact with each other, which might ease curvature stress and line tension for the small nascent fusion pore (Risselada et al., 2011). Alternatively, the highly curved membrane in the vesicle-vesicle (100 nm in diameter) fusion system might experience less energy barrier towards full fusion. Therefore, it is reasonable to expect a mild effect of the lipid composition on the later steps of membrane fusion, warranting further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Negatively stained electron microscopy (EM) image of vesicles used in the study. Scale bar: 100 nm. (B) Illustration of vesicle-vesicle fusion system. (C) Ensemble lipid-mixing measurements of vesicle-vesicle fusion with various lipid compositions: 1.5 mol% each of NBD and rhodamine were added into yeast v-SNARE Snc2-reconstituted vesicles by replacing the equal amount of PC. The fluorescence change of NBD for lipid mixing was normalized with respect to the maximum fluorescence intensity obtained by adding 0.1%

Triton X-100. **(D)** Single-vesicle docking results of various lipid compositions. 1 μ M Sec9 was used for experiments in **(C)** and **(D)**.

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Figure 2.

Results of the single vesicle fusion assay for various lipid compositions; **(A)** mixing of sulforhodamine B after 1 min of the reaction at room temperature and **(B)** mixing of the larger DNA probe after 30 min of the reaction at 37 °C. The gray areas in **(B)** highlight the peaks representing content mixing. For yeast t-SNAREs, Syntaxin 1a-analog Sso1p was reconstituted into vesicles but SNAP-25-analog Sec9 was directly injected into the solution. 1 μ M Sec9 was used for all single vesicle experiments in **(A)** and **(B)** to initiate the fusion reaction.

Figure 3.

(**A**) The times it takes to go from the hemifused state to the fully- fused state (Supplementary Figure S1) for various lipid compositions and with various lateral pulling tensions to mimic SNARE-pulling in MD simulations. (**B**) Sodium dodecyl sulfate (SDS) gel image of Sso1p and Snc2 proteins before and after reconstitution at different lipid compositions.