Improved reconstitution of yeast vacuole fusion with physiological SNARE concentrations reveals an asymmetric Rab(GTP) requirement

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ABSTRACT **In vitro reconstitution of homotypic yeast vacuole fusion from purified components enables detailed study of membrane fusion mechanisms. Current reconstitutions have yet to faithfully replicate the fusion process in at least three respects: 1) The density of SNARE proteins required for fusion in vitro is substantially higher than on the organelle. 2) Substantial lysis accompanies reconstituted fusion. 3) The Rab GTPase Ypt7 is essential in vivo but often dispensable in vitro. Here we report that changes in fatty acyl chain composition dramatically lower the density of SNAREs that are required for fusion. By providing more physiological lipids with a lower phase transition temperature, we achieved efficient fusion with SNARE concentrations as low as on the native organelle. Although fused proteoliposomes became unstable at elevated SNARE concentrations, releasing their content after fusion had occurred, reconstituted proteoliposomes with substantially reduced SNARE concentrations fused without concomitant lysis. The Rab GTPase Ypt7 is essential on both membranes for proteoliposome fusion to occur at these SNARE concentrations. Strikingly, it was only critical for Ypt7 to be GTP loaded on membranes bearing the R-SNARE Nyv1, whereas the bound nucleotide of Ypt7 was irrelevant on membranes bearing the Q-SNAREs Vam3 and Vti1.**

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INTRODUCTION

Intracellular membrane fusion is essential for endocytic and exocytic vesicular traffic. Fusion is regulated and catalyzed by families of proteins that are conserved among all eukaryotic cells. Organelle-specific Rab/Ypt-family GTPases bind effector proteins to tether membranes, allowing associations in-*trans* among soluble *N*–ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins anchored to apposed membranes (Grosshans *et al.*, 2006). SNAREs are defined by their heptad-repeat SNARE domains bearing either a central arginyl or glutaminyl residue and are categorized accordingly as R- or Qa/Qb/Qc-SNAREs (Fasshauer *et al.*, 1998; Kloepper *et al.*, 2007). R- and Qa-SNAREs bind to conserved regions of Sec1/Munc18-family proteins, which likely catalyze assembly of a complex of all four SNAREs (Baker *et al.*, 2015). Completion of such *trans*-SNARE associations, termed docking, brings the bilayers into close apposition. Membrane fusion converts *trans*-SNARE complexes into *cis*-SNARE complexes, which are then disassembled by the ATP-driven chaperone NSF/Sec18 and its cochaperone, α-SNAP/Sec17 (Mayer *et al.*, 1996). The regulated interactions among these fusion catalysts have been studied in systems ranging from baker's yeast to the neuronal synapse.

Homotypic membrane fusion among the vacuoles (lysosomes) of *Saccharomyces cerevisiae* requires each of these conserved proteins. Vacuoles are tethered for fusion by the Rab Ypt7 and by a heterohexameric protein complex termed the homotypic fusion and vacuole protein sorting (HOPS) complex (Hickey *et al.*, 2009). Two of the HOPS subunits (Vps39 and Vps41) have direct affinity for Ypt7 (Brett *et al.*, 2008; Plemel *et al.*, 2011), and the subunit Vps33 is a member of the Sec1/Munc18 family (Baker *et al.*, 2015). Vacuolar fusion uses four SNAREs: the R-SNARE Nyv1, the Qa-SNARE Vam3, the Qb-SNARE Vti1, and the Qc-SNARE Vam7. Three of these SNAREs are integrally membrane anchored at their C-terminus, whereas Vam7 is a peripheral membrane protein that is bound to membranes by its affinities for acidic lipids,

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Abbreviations used: DAG, diacylglycerol; FRET, fluorescence resonance energy transfer; HOPS, homotypic fusion and vacuole protein sorting; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PhycoE, R-phycoerythrin; PI, phosphatidylinositol; PS, phosphatidylserine; RPL, reconstituted proteoliposome; Sa-Cy5, Cy5-labeled streptavidin; SNARE, soluble *N*–ethylmaleimide–sensitive factor attachment protein receptor; VML, vacuolar mimic lipid.

A

	mol%	26:0	28:0	28:1	30:0	30:1	30:2	32:1	32:2	34:0	34:1	34:2	36:1	36:2	Saturated
PC	47.6			2.0		2.8	0.9	15.3	38.3		9.7	25.4	1.6	1.6	15.7%
PE	18					1.3		16.8	32.8		12.2	32.8	0.4	3.0	15.4%
PI	18	1.9	4.4		3.1	3.1	7.0	22.2	4.9	8.5	34.8	4.6	5.4		57.7%
PS	4.4							17.2	11.2		34.6	33.3		1.3	25.9%

B

PC	T_m (°C)
14:0	24
18:0 18:1	6
16:0 18:1	-2
18:1	-17
16:1	-36
18:2	-57

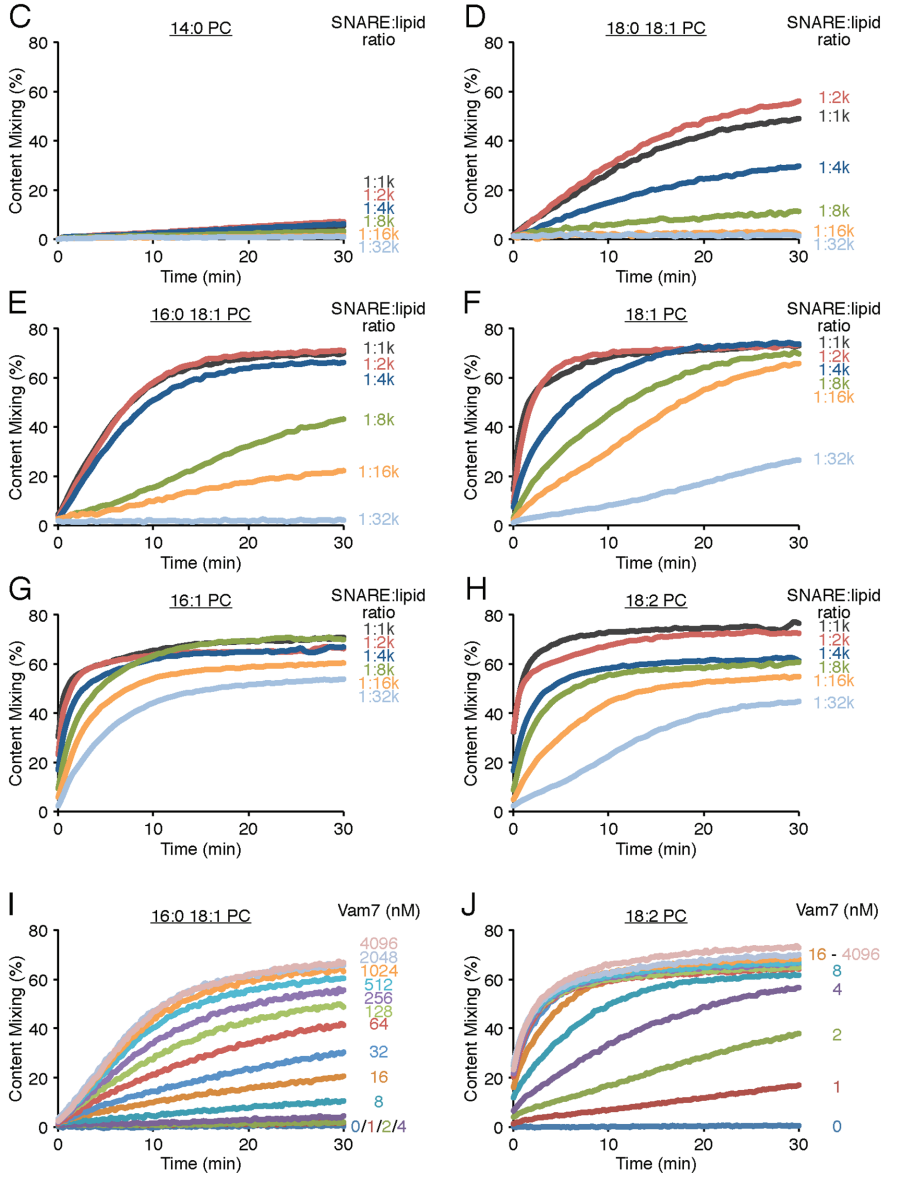

FIGURE 1: Increased membrane fluidity lowers the SNARE density that is required for RPL fusion. (A) Summary of fatty acyl chain compositions found in yeast vacuoles, as described in Schneiter *et al.* (1999). Fatty acid compositions of phospholipids are grouped by chain length and number of double bonds (e.g., 34:2, composed of palmitoleic acid [C16:1] and oleic acid [C18:1]). (B) Phase transition temperatures of multiple PC species (Marsh, 2013), which can be used as a surrogate measure of membrane fluidity. (C–H) Fusion reactions of RPLs composed of vacuolar mimic lipids (PC, PE, PI, PS, PA, ergosterol, DAG, PI(3)P) bearing Nyv1 (R) or Vam3/Vti1 (2Q) at various SNARE:lipid ratios (1:1000–1:32,000) and Ypt7(GTP) at a protein:lipid ratio of 1:2000 were incubated with 50 nM HOPS, 50 nM Sec18, 600 nM Sec17, and 100 nM Vam7 for 30 min at 27°C. The species of PC (47.6 mol%) was chosen as indicated, with all other lipids unaltered. (I, J) Fusion reactions of RPLs as in E and H with a SNARE:lipid ratio of 1:1000 (concentration of each membrane-embedded SNARE in reaction, 250 nM) were performed in the presence of indicated levels of Vam7 (1–4096 nM). Kinetic curves of content mixing assays in this and subsequent figures are representative of at least three experiments.

1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1-myo-inositol-3-phosphate) (PI(3)P), HOPS, and the other SNAREs (Lee *et al.*, 2006; Stroupe *et al.*, 2006; Karunakaran and Wickner, 2013). HOPS- and Ypt7-dependent tethering draws apposed membranes together, and a ring-shaped microdomain enriched in the SNAREs, Rab, HOPS, and certain lipids (phosphoinositides, ergosterol, and diacylglycerol [DAG]) forms around the docked, apposed membranes (Wang *et al.*, 2002, 2003; Fratti *et al.*, 2004). Vacuole fusion depends on Sec17/Sec18 to liberate SNAREs from *cis*-complexes (Mayer *et al.*, 1996) and on HOPS to promote the formation of SNARE complexes in-*trans* (Zick and Wickner, 2013; Baker *et al.*, 2015). Finally, the lipid rearrangements of fusion itself are strongly dependent on small–head group lipids, which can be accommodated in the presumed nonbilayer fusion intermediate structures (Zick *et al.*, 2014) while affecting membrane fluidity (Dawaliby *et al.*, 2016).

Vacuole fusion has been studied extensively in vivo (Wada *et al.*, 1992), in vitro with the purified organelle (reviewed in Wickner, 2010), and more recently with reconstituted proteoliposomes of defined lipids and purified proteins. Although it requires most of the same components, the fusion of proteoliposomes has differed from that of the native membrane in that it has required far higher molar ratios of SNAREs to lipid (Zick *et al.*, 2014, 2015b), has not shown as strict a dependence on Ypt7 (Orr *et al.*, 2015), and has exhibited far more lysis (Zucchi and Zick, 2011) than is seen with wild-type vacuoles (Starai *et al.*, 2007). We now report that each of these characteristics is dramatically affected by preparing the proteoliposomes with lipids of different fatty acyl chain compositions, creating a more fluid model membrane that better resembles the native organelle (Schneiter *et al.*, 1999). Fusion is now seen at physiological levels of SNAREs, lysis is reduced and occurs only well after fusion, and Ypt7 is essential for fusion as Ypt7(GTP) in-*cis* to the R-SNARE and either Ypt7(GTP) or Ypt7(GDP) in-*cis* to the Q-SNAREs. This asymmetry may orient HOPS for productive association with SNAREs.

RESULTS

The vacuolar membrane is composed of a complex mixture of lipids (Zinser and Daum, 1995). Lipid diversity is not limited to the polar head groups. There is also a striking variability among the membrane phospholipids regarding length and saturation of the fatty acyl chains (Schneiter *et al.*, 1999), with only a minor fraction of the fatty acyl chains being saturated (Figure 1A). Lipids within

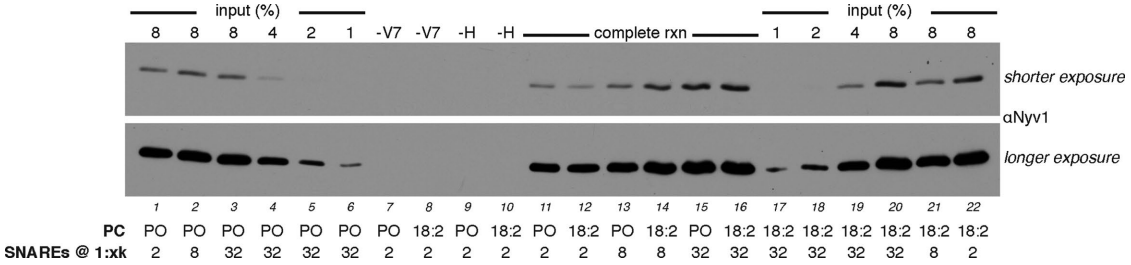

FIGURE 2: The lipid side-chain choice did not substantially affect the amount of *trans*-SNARE complex formation. Fusion reactions of R- and 2Q-RPLs with SNARE:lipid ratios of 1:2000, 1:8000, or 1:32,000 and PC lipid side-chain choice of 16:0 18:1 (palmitoyl-oleoyl [PO]) or 18:2 18:2 (18:2; compare with Figure 1, C and F) were incubated for 20 min at 27°C with 50 nM HOPS and 100 nM Vam7Δ3 (complete reaction), only 50 nM HOPS (–V7), or only 100 nM Vam7Δ3 (–H). Reactions (20 µl for SNARE:lipid ratios of 1:2000, 80 µl for SNARE:lipid ratios of 1:8000, 320 µl for SNARE:lipid ratios of 1:32,000) were diluted in β-octylglucoside buffer (1% wt/vol final) to a final volume of 0.5 ml and incubated with 20 µl of protein A magnetic beads and 20 µg of affinity-purified αVam3 antibody for 2 h at room temperature. The beads were washed three times with 1 ml of β-octylglucoside buffer, and the samples were eluted in 100 µl of SDS sample buffer at 95°C for 5 min. Samples were analyzed by SDS–PAGE and Western blot with αNyv1 antibody.

the membrane bilayer engage through van der Waals interactions among neighboring hydrophobic chains. Both chain length and the number of unsaturated double bonds, which kink the otherwise straight hydrocarbon chains, determine the strength of those intermolecular interactions and influence the biophysical properties of a membrane. The relative mobility of individual molecules within a lipid bilayer determines its fluidity and viscoelastic behavior in response to bilayer deformations (Wu *et al.*, 2015). The phase transition temperature (the temperature at which the phase behavior of the membrane transitions from a solid to a liquid state) is a surrogate measure of the degree of membrane fluidity and is intimately linked to acyl chain length and saturation (Figure 1B).

Membrane fluidity modulates fusion competence

To test what effect the fatty acyl chain composition of a bilayer has on the ability of SNAREs to drive membrane fusion, we prepared reconstituted proteoliposomes (RPLs) of vacuolar mimic lipid (VML) composition with SNAREs at a variety of protein:lipid ratios and with phosphatidylcholine (PC), the most abundant phospholipid, bearing different fatty acyl chains (Figure 1, C–H). Membranes containing the PC with the highest phase transition temperature that we tested (14:0-PC) showed almost no fusion activity (Figure 1C), even at a molar SNARE:lipid ratio of 1:1000. RPLs with a PC species of intermediate fluidity (Figure 1, D and E) showed considerable fusion activity as long as SNAREs were present at high concentrations. Reducing the SNARE concentration under these conditions, however, resulted in a gradual loss of fusion activity. At a molar SNARE:lipid ratio of 1:32,000, which is similar to the density of SNARE proteins that is found on the organelle (Zick *et al.*, 2014, 2015b), no detectable fusion activity remained. Membranes with PC species that contained a double bond in both fatty acyl chains retained at least some fusion activity even at the lowest SNARE densities (Figure 1, F–H). Membranes containing 16:1 PC, the most abundant form of PC found on native vacuolar membranes (Figure 1A), demonstrated good fusion activity even at low SNARE:lipid ratios (Figure 1G). Because RPLs that were made with 16:1 PC were unstable during storage at –80°C (the content marker proteins were released within days), we used another PC species with similar characteristics for this practical reason, even though it is not found in yeast, which does not have the ability to synthesize polyunsaturated fatty acids. RPLs containing 18:2 PC, which also has a low phase transition temperature and did not exhibit leakiness upon storage, had considerable fusion activity at reduced SNARE levels (Figure 1H). The influence of membrane fluidity on fusion activity can also be seen when the soluble Qc-SNARE Vam7 is added at limiting concentrations to RPLs bearing the other three SNAREs at molar protein:lipid ratios of 1:1000 (Figure 1, I and J). Membranes containing 16:0 18:1 PC require considerably higher concentrations of Vam7 for efficient fusion than membranes containing 18:2 PC (e.g., compare Figure 1, I, 256 nM Vam7, vs. J, 4 nM Vam7).

To determine whether membrane fluidity affects fusion capacity indirectly by modulating the propensity of SNAREs to engage in *trans* complexes or directly by allowing fusion to be catalyzed by fewer *trans*-SNARE complexes, we determined the amount of *trans*-SNARE complexes that formed when using RPLs with either 16:0 18:1 PC or 18:2 PC at various SNARE concentrations. For this, we performed fusion reactions with a form of Vam7 that had a C-terminal truncation (Vam7Δ3), which allows stable *trans*-SNARE complex formation but prevents fusion (Schwartz and Merz, 2009), and analyzed the association of Vam3 and Nyv1 by coimmunoprecipitation with antibody to the Qa SNARE Vam3 (Figure 2). To maintain comparable conditions during the immunoprecipitations regardless of the SNARE density on the membranes, we increased the scale of the initial reactions for RPLs with reduced SNARE density to compensate for the imbalance (i.e., SNAREs at 1:2000, 1× scale; SNAREs at 1:8000, 4× scale; SNAREs at 1:32,000, 16× scale). Proteoliposomes were incubated under fusion conditions, solubilized with detergent, and assayed for the Nyv1 bound to Vam3 by coimmunoprecipitation with antibody to Vam3. The amount of *trans* complex that formed during a 20-min incubation was not significantly altered by the species of PC that was present in the RPLs (compare lane 11 vs. 12; 13 vs. 14; and 15 vs. 16). When Vam7 or HOPS was omitted, no *trans*-SNARE complex was detectable (lanes 7–10). This indicates that membrane fluidity directly affects the capacity of lipid bilayers to fuse rather than modulating the efficiency of *trans*-SNARE complex formation.

Membranes with elevated SNARE levels are unstable after fusion

Having achieved fusion of reconstituted membranes with significantly reduced SNARE densities, we reexamined some of the earlier inconsistencies between our in vitro reconstitution model and fusion of the isolated organelle. It was shown previously (Starai *et al.*, 2007) that little lysis occurs during the fusion of vacuoles from wild-type

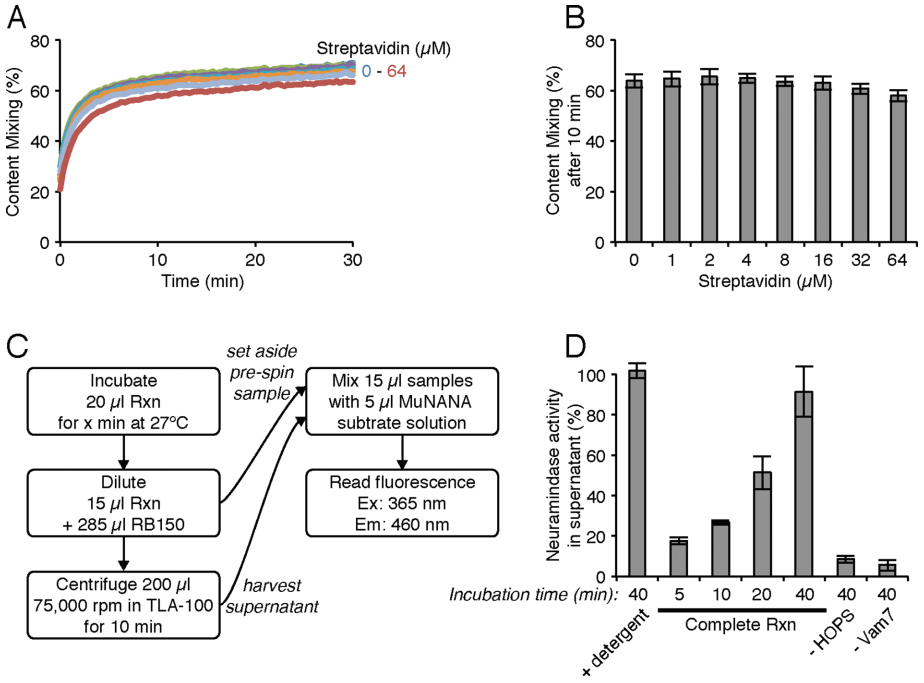

FIGURE 3: The mixing of lumenal contents was protected from an external competitor during fusion reactions, but the content did not remain intralumenal over time. (A, B) Fusion reactions of R and 2Q RPLs (molar SNARE:lipid ratio 1:2000, Ypt7(GTP):lipid ratio 1:8000) were performed in the presence of 0, 1, 2, 4, 8, 16, 32, or 64 μM unlabeled streptavidin. Reactions contained 50 nM HOPS, 50 nM Sec18, 600 nM Sec17, and 100 nM Vam7. (C, D) Fusion reactions as in A with RPLs that contained lumenal neuraminidase (20 μg/ml) were incubated for various times and then diluted and centrifuged to pellet the RPLs. Pre-spin and postspin supernatant samples were mixed with MuNANA substrate solution (containing 4-methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid [50 μM final] and Thesit [0.2% wt/vol final]) in a 384-well plate, and the fluorescence signal that resulted from substrate hydrolysis by neuraminidase was recorded for 20 min. (D) Neuraminidase activity that was recovered in the supernatant fraction is displayed as averages and SDs from three separate experiments. The pre-spin samples were defined as 100% activity.

yeast, but markedly enhanced lysis accompanies the fusion of vacuoles from strains that overexpressed the vacuolar SNAREs. This fusion had lost Ypt7(GTP) dependence as well. Lysis and loss of Ypt7(GTP) dependence also accompanied the fusion of proteoliposomes reconstituted with high SNARE levels (Zucchi and Zick, 2011; Orr *et al.*, 2015). To reexamine the lysis component of the reconstituted fusion reaction, we performed fusion reactions with SNAREs at high levels, as had been used in prior studies (R- and 2Q-RPLs with 1:2000 M SNARE:lipid ratios, 100 nM Vam7). Fusion reactions had increasing concentrations of external, unlabeled streptavidin to occupy any released biotinylated R-phycoerythrin (PhycoE) and therefore suppress any PhycoE:Cy5–fluorescence resonance energy transfer (FRET) signal due to lysis (Figure 3A). Even a large excess of external streptavidin gave little suppression of the FRET signal, confirming that the initial content mixing during fusion reactions occurs primarily intralumenally (Figure 3B). If fusion occurred entirely without lysis, we would expect lumenal markers to remain entrapped throughout the process. To test this idea, we coentrapped the enzyme neuraminidase (a soluble protein of 36 kDa) and analyzed its release during fusion incubations (Figure 3C). Even though the mixing of contents occurred primarily intralumenally (Figure 3, A and B) and was complete by 5 min, the content was not retained inside the vesicles during subsequent incubation and was released after fusion had occurred (Figure 3D). Thus, at high SNARE levels, proteoliposomes initially fuse but eventually lyse.

To test whether the lysis of fused proteoliposomes was invariant at each level of SNARE pairing and fusion, we introduced modified assays able to measure lysis directly (Figure 4). RPLs bearing the R-SNARE Nyv1, Marina-Blue-PE, and lumenal biotinylated PhycoE were incubated with RPLs bearing the Q-SNAREs Vam3 and Vti1 and nitrobenzoxadiazole (NBD)-PE and had one of three lumenal compositions: 1) Cy-labeled streptavidin (Sa-Cy5), 2) no content marker (empty), or 3) unlabeled streptavidin. The level of the Qc SNARE Vam7 was systematically varied from 0.25 to 16 nM, regulating the capacity for *trans*-SNARE pairing. All incubations were in the presence of external Sa-Cy5. To avoid the substantial background signal caused by the large quantity of external Sa-Cy5, we measured the interaction of PhycoE with Sa-Cy5 via PhycoE quenching rather than the routinely used PhycoE:Cy5-FRET. When the Q-SNARE RPLs contained Sa-Cy5, we would expect a signal (PhycoE quenching) for any event (fusion or lysis). When the Q-SNARE RPLs were empty, we would expect a signal for all lytic events, regardless of whether RPLs had previously fused. When the Q-SNARE RPLs contained unlabeled Sa, we would expect a signal only for lytic events that were not preceded by fusion. Conducting such experiments in the presence of increasing amounts of Vam7 revealed that almost all lysis occurred after fusion, as there was little detectable signal when unlabeled streptavidin was entrapped in 2Q-RPLs (Figure 4C). Of note, there was almost no lysis at 1 nM or less Vam7, which, however, displayed a considerable fusion signal (e.g., compare purple curves in Figure 4, A and B). At higher Vam7 levels, which induced more robust fusion, a higher level of lysis was also detected. The lipid mixing signals (Figure 4, bottom) showed that the different content markers did not adversely affect the overall reaction. Lysis was also exclusively seen at high SNARE levels when the molar ratio of Nyv1 to lipids was varied and the concentration of Vam7 was invariant (Supplemental Figure S1).

Ypt7(GTP) is essential for fusion at physiological SNARE concentrations

Because SNARE overexpression also enabled Δypt7 vacuoles to fuse (Starai *et al.*, 2007), we reevaluated the Ypt7 requirement in the reconstituted system and how it relates to the level of SNAREs and Ypt7 that are present on the RPLs (Figure 5). At high SNARE concentrations, fusion occurred regardless of the nucleotide state of Ypt7. This may reflect the direct affinity of HOPS for the vacuolar SNAREs (Stroupe *et al.*, 2006; Baker *et al.*, 2015), bypassing the physiological role of Ypt7 as a HOPS receptor. When the concentrations of SNAREs were reduced, fusion occurred only when Ypt7 was loaded with GTP (blue curves) and was lost when Ypt7 was present in its GDP-loaded state (red curves). The Rab GTPase Ypt7 was present in these reconstitutions at concentrations approximately fivefold higher than found on the organelle. When SNAREs are present at the physiological levels of 1:32,000 molar ratio to lipids, the capacity

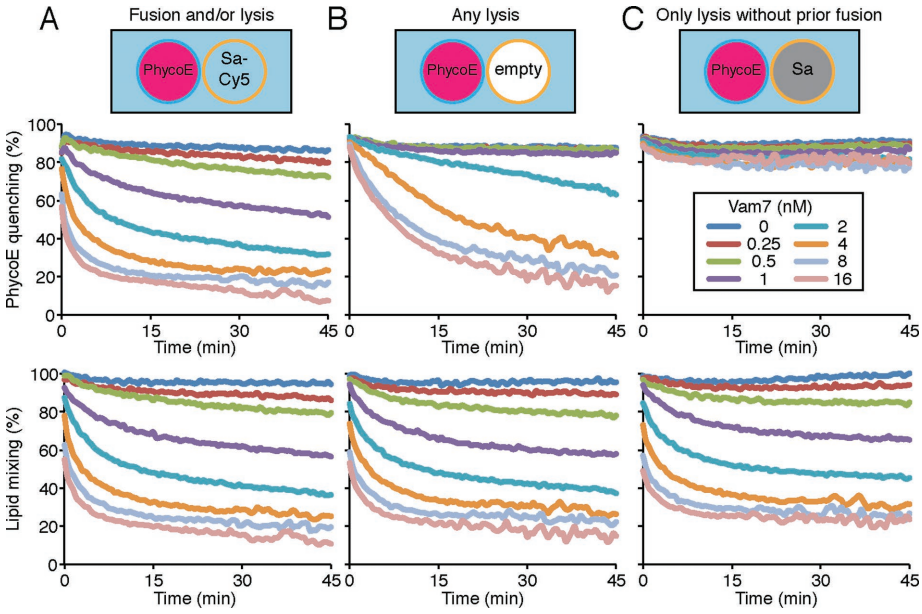

FIGURE 4: The current reconstitution of membrane fusion with RPLs leads to an unstable fusion product. (A–C) A modification of the PhycoE- and Sa-Cy5–based content mixing assay allows the discrimination of when lysis occurs. RPLs containing biotinylated PhycoE were subjected to fusion reactions with RPLs containing (A) Cy5-labeled streptavidin (lumenal concentration, 8 μM), (B) no content marker, or (C) unlabeled streptavidin (lumenal concentration, 32 μM), all in the presence of extralumenal Cy5-labeled streptavidin (4 μM). Fusion reactions of R and 2Q RPLs (molar SNARE:lipid ratio 1:2000, Ypt7(GTP):lipid ratio 1:8000) were performed in the presence of 0–16 nM Vam7, 50 nM HOPS, 50 nM Sec18, and 600 nM Sec17. Top, quenching data of PhycoE as a result of its binding interaction with Sa-Cy5. Bottom, corresponding lipid mixing data (quenching of Marina-Blue by NBD) from the same reactions.

to form *trans*-SNARE complexes can be finely regulated by adjusting the concentration of added Vam7. Under these conditions, there is no fusion without Ypt7 (Figure 6, a6 and b6). With high levels of both Vam7 (100 nM, purple curves) and Ypt7 (1:2000 molar ratio to lipids), fusion was supported by either Ypt7:GTP or Ypt7:GDP (Figure 6, a1 and b1). At reduced concentrations of Ypt7, fusion was strictly dependent on Ypt7 being loaded with GTP (Figure 6, b3 vs. a3). We further examined the Ypt7 requirement at the newly established standard of reconstitution with more physiological protein:lipid ratios for both SNAREs (1:32,000) and Ypt7 (1:8000). Strikingly, Ypt7, while strictly required on both fusion partners, only needed to be in its GTP-loaded form on membranes that bore the R-SNARE Nyv1 (Figure 7). Ypt7 also needed to be present on the membranes bearing the Q-SNAREs, but its nucleotide state (GTP vs. GDP) did not substantially alter the fusion activity.

DISCUSSION

Genetic studies identified 10 proteins that are specifically required for vacuole fusion: the six subunits of HOPS, three vacuolar SNAREs, and Ypt7. Sec17/18 and the vacuolar Qb SNARE function in other trafficking pathways and thus eluded the initial vacuole-specific genetic screen. Biochemical fusion studies showed that each of these proteins is recovered with the isolated organelle and is strictly required for fusion, as seen in vivo. Fusion of purified vacuoles was not accompanied by lysis unless the vacuoles were isolated from strains that were genetically modified to have elevated levels of all four vacuolar SNAREs or when excess Vam7 was added (Starai *et al.*, 2007). Purified vacuoles are still chemically complex, and fusion components that are integral cannot be removed and added at will. Thus molecular understanding of the fusion reaction requires that it be reproduced, in all its essential features, with pure and chemically defined proteins and lipids.

Achieving reconstituted vacuole fusion with pure components has been an iterative process. Initial assays relied on RPLs containing two fluorescent lipids, which would dequench when incubated with nonfluorescent RPLs (Fukuda *et al.*, 2000; Mima *et al.*, 2008). With unphysiologically high SNARE levels, spontaneous *trans*-SNARE interactions occurred. However, subsequent application of more rigorous fusion assays using lumenal compartment mixing showed that these *trans* interactions were accompanied by very little fusion (Zick and Wickner, 2014). At those high SNARE levels, the addition of HOPS supported true fusion, although this fusion was accompanied by substantial lysis (Zucchi and Zick, 2011), and the Rab Ypt7 was not strictly required (Orr *et al.*, 2015). Simply reducing the SNARE levels resulted in a rapid and progressive loss of fusion activity.

We now report that a fatty acyl composition providing more physiological membrane fluidity allows rapid fusion at more physiological concentrations of SNAREs and Rab. The initial, rapid interaction between RPLs yields protected lumenal compartment mixing—the rigorous definition of fusion—yet excessive SNAREs cause the content markers to be released over time. Although our study shows that almost all lysis occurs after fusion, we do not yet know whether lysis is mechanistically coupled to fusion. The observed lysis could either be the result of fused membranes becoming inherently unstable or reflect the cumulative collateral damage of many consecutive rounds of *trans*-SNARE interactions. It is noteworthy that fusion can occur without lysis when the ability to form *trans*-SNARE complex is restricted, for example, by limiting the concentration of the soluble SNARE Vam7. This reduction in *trans*-SNARE complexes may eliminate the existence of simultaneous, competing fusion sites at multiple membrane interfaces. Whether such competition could occur in vivo is unclear, but the regular occurrence of even minor amounts of lysis and the uncontrolled release of vacuolar enzymes into the cytosol would have devastating consequences. The formation of *trans*-SNARE complexes thus has to happen in a highly regulated manner, catalyzed by specific machinery such as HOPS, rather than being driven by mass effect among highly concentrated SNAREs. Reconstitutions of fusion need to consider the physiological levels of each protein, the lipid composition, and the possibility of lysis.

At the newly established standard conditions (molar ratios of SNAREs:lipid 1:32,000 and Ypt7:lipid 1:8000), fusion exhibits a strict dependence on Ypt7, as seen in vivo. Curiously, we discovered an asymmetry of required guanine nucleotide bound to the Ypt7 with respect to it being adjacent to R- versus Q-SNAREs. We speculate that this may reflect the reported difference in affinities of the two HOPS subunits that bind to Ypt7, which are Vps39 and Vps41 (Brett *et al.*, 2008; Plemel *et al.*, 2011). Vps41 selectively binds to Ypt7(GTP), whereas Vps39 interacts with Ypt7 regardless of its bound nucleotide (Plemel *et al.*, 2011). The simultaneous interaction

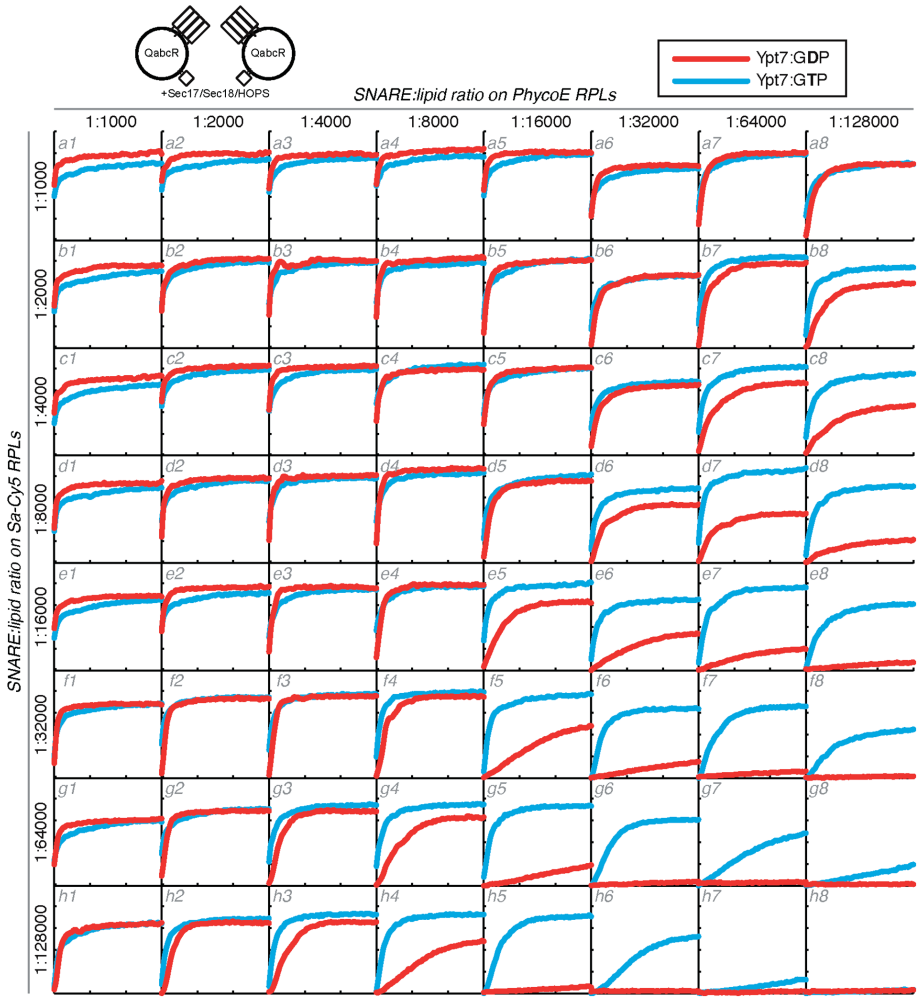

FIGURE 5: The nucleotide state of the Rab GTPase Ypt7 is critical for fusion at reduced SNARE densities. Fusion reactions of RPLs bearing all four SNAREs (molar SNARE:lipid ratio 1:1000–1:128,000, Ypt7:lipid ratio 1:2000) were performed in the presence of 50 nM HOPS, 50 nM Sec18, and 600 nM Sec17. Ypt7 was nucleotide exchanged into its GTP or GDP form on both RPLs before addition of Sec17, Sec18, and HOPS. RPLs with a molar SNARE:lipid ratio of 1:32,000 and an average RPL diameter of ~200 nm (Zick and Wickner, 2014) carry ~10 copies of each SNARE. The horizontal axis represents 0–30 min, and the vertical axis represents 0–100% content mixing.

of Vps39 and Vps41 with two Ypt7 molecules on two separate membranes might be of central importance for tethering but less so for HOPS catalysis of *trans*-SNARE complex assembly. Whether HOPS remains bound to two Ypt7 molecules throughout the fusion cycle is unclear. Specifically orienting the HOPS complex in a preferred conformation with respect to the R- and Q-SNAREs through selective interaction of Vps41, the HOPS subunit that is adjacent to the SM subunit Vps33 (Brocker *et al.*, 2012), with Ypt7(GTP) might aid its catalysis of *trans*-SNARE complex formation.

Several important lessons have emerged from reconstituted vacuolar fusion. The spontaneous assembly of the four-helical coiled-coil *trans*-SNARE complex is very inefficient, so that SNAREs alone give very slow fusion and only at unphysiologically high concentrations that induce substantial lysis. SNARE complex assembly is likely catalyzed by HOPS, with a central role of its Vps33 SM subunit binding the SNARE domains of Vam3 and Nyv1 (Baker *et al.*, 2015). Independent tethering is required (Zick and Wickner, 2014) and performed by HOPS based on the affinity of two of its subunits for Ypt7 (Brett *et al.*, 2008), with contributions of undetermined magnitude from the affinity of HOPS for phosphoinositides, other acidic lipids, and SNAREs (Stroupe *et al.*, 2006; Orr *et al.*, 2015). Small–head group lipids that are nonbilayer prone and fluid fatty acyl chains are also crucial to lowering the energy barrier for fusion (Figure 1; Zick *et al.*, 2014). We recently reported that Sec17 (α-SNAP) can associate with *trans*-SNARE complexes and trigger the fusion event via an N-terminal apolar loop (Zick *et al.*, 2015a).

Further iterations and refinements of the reconstituted fusion system are necessary to determine any roles of sphingolipids, lipid asymmetry, and membrane curvature in fusion. Additional components that stabilize membranes against postfusion lysis might still be missing. If lysis is due to an inability to adjust the ratio of inner leaflet to outer leaflet lipids, then it may be cured by the addition of a lipid flippase or by the budding of small vesicles, as occurs at the organelle to recycle components to the endosome. Finally, additional layers of regulation and their interplay should be reconstituted, such as GAP and GEF cycling of the guanine nucleotide on Ypt7 and regulation of HOPS by the vacuolar Yck3 kinase (LaGrassa and Ungermann, 2005; Brett *et al.*, 2008; Nordmann *et al.*, 2010; Lawrence *et al.*, 2014) and unidentified phosphatase(s).

Two other intracellular membrane fusion events have been reconstituted with purified components. Fusion has been reconstituted with neuronal SNAREs, NSF and SNAP, Munc18-1, Munc13, and synaptotagmin-1, the sensor for calcium (Ma *et al.*, 2013). Other essential components, such as complexin and Rab3a, are being tested in such reconstitutions. Elements of neuronal fusion, such as complexin, synaptotagmin, and calcium, are required only at the synapse, making this a special, albeit particularly important, case of intracellular fusion. The other example is the Rab- and SNARE-dependent reconstitution of endosomal fusion (Ohya *et al.*, 2009). Further studies are needed in this system to establish the role of each factor and which functions are shared, or distinct, from vacuolar or neuronal fusion. Embracing both the complexity of biological systems and the rigor of chemically defined reconstitution will be a fruitful guiding principle in studies of membrane fusion.

MATERIALS AND METHODS

Proteins and reagents

The purification of HOPS, prenylated Ypt7 (Zick and Wickner, 2013), Vam7, Sec17 (Schwartz and Merz, 2009), Sec18 (Haas and Wickner, 1996), and neuraminidase (Zucchi and Zick, 2011) were as described. Membrane-anchored vacuolar SNAREs (Vam3, Vti1, Nyv1) were isolated (Mima *et al.*, 2008) and exchanged into octylglucoside buffer (Zucchi and Zick, 2011). Proteins were frozen in aliquots in liquid nitrogen and stored at –80°C. Most lipids were obtained from Avanti Polar Lipids (Alabaster, AL), with the exception of ergosterol, which was from Sigma-Aldrich (St. Louis, MO), PI(3)P, which was from

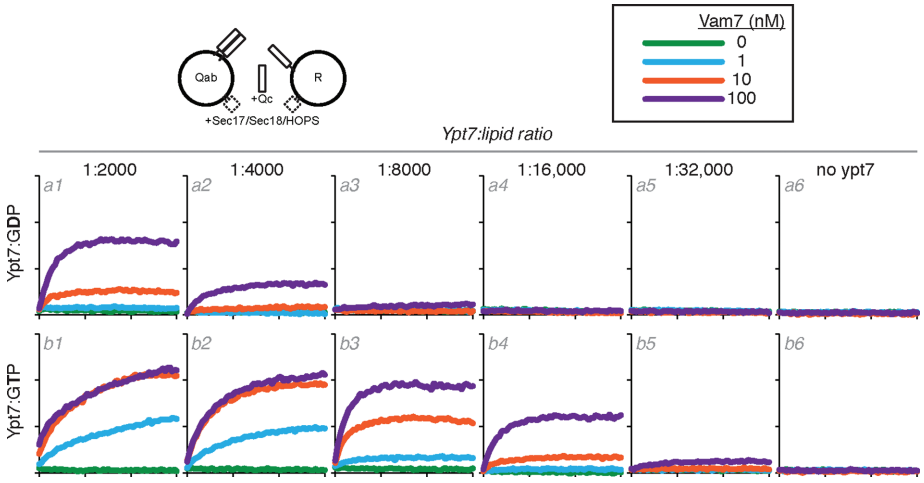

FIGURE 6: A titration of Ypt7 shows its critical importance for fusion at moderate SNARE concentrations. Fusion reactions of R and 2Q RPLs (molar SNARE:lipid ratio 1:32,000, Ypt7:lipid ratio 1:2000–1:32,000) were performed in the presence of 0–100 nM Vam7, 50 nM HOPS, 50 nM Sec18, and 600 nM Sec17. Ypt7, where present, was nucleotide exchanged into its GTP or GDP form on both RPLs before addition of Sec17, Sec18, and HOPS. The horizontal axis represents 0–30 min, and the vertical axis represents 0–60% content mixing.

Echelon Biosciences (Salt Lake City, UT), and the fluorescent lipids (Marina Blue–1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine [DHPE] and NBD-DHPE), which were from Thermo Fisher Scientific (Waltham, MA). Cy5-derivatized streptavidin was purchased from KPL (now SeraCare Life Sciences, Milford, MA) and biotinylated PhycoE and unlabeled streptavidin from Thermo Fisher Scientific. Protein concentrations were determined by Bio-Rad (Hercules, CA) protein assay, which is based on the Bradford dye-binding method (Bradford, 1976).

Proteoliposome preparation

Proteoliposomes were prepared (Zick *et al.*, 2014) from mixed micellar solutions (containing 50 mM β-octyl-glucoside) by detergent dialysis (20-kDa cutoff membrane) in RB150/Mg^{2+} (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid·NaOH, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 10% glycerol [vol/vol]) with individual SNAREs and prenylated Ypt7 at various molar protein:lipid ratios. Lipids dissolved in chloroform were mixed in vials containing β-octyl-glucoside at the following proportions. For vacuolar mixed lipids (16:0 18:1), 44.8–47.6 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 18 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 18 mol% soy L-α-phosphatidylinositol (PI), 4.4 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine, 2 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate, 1 mol% 16:0 1,2-dipalmitoyl-*sn*-glycerol, 8 mol% ergosterol, and 1 mol% PI(3)P. For VML (18:2) compositions, as for RPLs used in Figures 3–7, lipids were mixed in the same proportions as described but with the dilinoleoyl forms of PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidic acid (PA). All proteoliposomes throughout this study contained 0.2 mol% Marina-Blue-PE or 3 mol% NBD-PE.

Fusion assay

Fusion reactions were assembled in 20 µl. Fusion assay pairs of RPLs (each 250 µM lipid) in RB150, 5 µM streptavidin, 1 mM EDTA, and 10 µM GDP or GTP were preincubated for 10 min at 27°C before addition of 1.25 mM $MgCl_2$ to exchange Ypt7 to its GTP- or GDP-bound form. For reactions with asymmetric Ypt7 disposition, R- and 2Q-RPLs were nucleotide exchanged separately and mixed only immediately before reactions were started. Of the preincubated RPLs, 14 µl (or 2 × 7 µl) was transferred to wells of 384-well plates, and soluble components (6 µl; e.g., HOPS, Sec17, Sec18, ATP, Vam7) or their respective buffers were added to initiate reactions. All reactions contained 0.5% (wt/vol) defatted bovine serum albumin, 5 mM reduced glutathione, and 1 mM dithiothreitol. Plates were incubated at 27°C in a fluorescence plate reader, and content mixing signals (PhycoE–Cy5-FRET: excitation [ex], 565 nm; emission [em], 670 nm; cutoff, 630 nm), PhycoE quenching (ex, 560 nm; em, 590 nm; cutoff, 590 nm), or lipid mixing (Marina-Blue quenching) signals (ex, 370 nm; em, 465 nm; cutoff, 420 nm) were recorded at intervals of 10–30 s in a SpectraMax Gemini XPS (Molecular Devices, Sunnyvale, CA) fluorescence plate reader. Maximal content mixing values were determined after addition of 0.2% (wt/vol) Thesit to samples that had not received streptavidin.

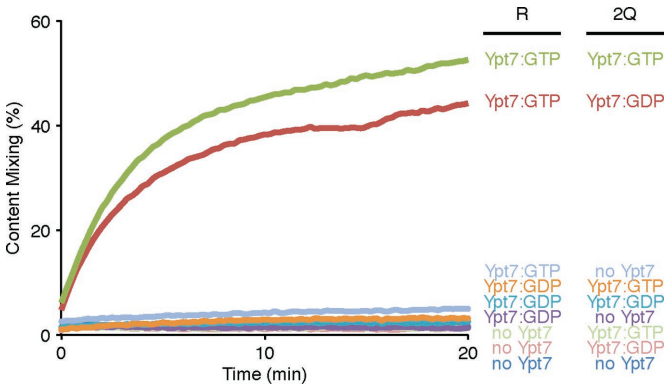

FIGURE 7: The requirement for Ypt7 to bear GTP is asymmetric. Fusion reactions of R and 2Q RPLs (molar SNARE:lipid ratio 1:32,000, Ypt7:lipid ratio 1:8000) were performed in the presence of 100 nM Vam7, 50 nM HOPS, 50 nM Sec18, and 600 nM Sec17. Ypt7, where present, was nucleotide exchanged into its GTP or GDP form before mixing of RPLs and immediate addition of Sec17, Sec18, and HOPS.

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