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Stringent 3Q·1R Composition of the SNARE 0-Layer Can Be Bypassed for Fusion by Compensatory SNARE Mutation or by Lipid Bilayer Modification*[S]

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SNARE proteins form bundles of four α-helical SNARE domains with conserved polar amino acids, 3Q and 1R, at the "0-layer" of the bundle. Previous studies have confirmed the importance of 3Q·1R for fusion but have not shown whether it regulates SNARE complex assembly or the downstream functions of assembled SNAREs. Yeast vacuole fusion requires regulatory lipids (ergosterol, phosphoinositides, and diacylglycerol), the Rab Ypt7p, the Rab-effector complex HOPS, and 4 SNAREs: the Q-SNAREs Vti1p, Vam3p, and Vam7p and the R-SNARE Nyv1p. We now report that alterations in the 0-layer Gln or Arg residues of Vam7p or Nyv1p, respectively, strongly inhibit fusion. Vacuoles with wild-type Nyv1p show exquisite discrimination for the wild-type Vam7p over Vam7^{Q283R}, yet Vam7^{Q283R} is preferred by vacuoles with Nyv1^{R191Q}. Rotation of the position of the arginine in the 0-layer increases the K_m for Vam7p but does not affect the maximal rate of fusion. Vam7^{Q283R} forms stable 2Q·2R complexes that do not promote fusion. However, fusion is restored by the lipophilic amphiphile chlorpromazine or by the phospholipase C inhibitor U73122, perturbants of the lipid phase of the membrane. Thus, SNARE function as regulated by the 0-layer is intimately coupled to the lipids, which must rearrange for fusion.

SNARE[4] proteins (1) are vital for membrane fusion. Initially discovered in neuronal tissues, SNAREs are found on all organelles of the exocytic and endocytic pathways, from yeast to humans. Their characteristic feature is a heptad-repeat "SNARE domain," flanked by varied N-domains and by C-terminal membrane anchors, either a single membrane-spanning apolar polypeptide or an acyl anchor. SNAREs form 4-helical complexes through their SNARE domains (2). Although the residues in each SNARE that face the others in a 4-helical complex are generally apolar, 3 glutamine (Q) and 1 arginine (R) near the center of the 4-complexed SNARE domains form a conserved and polar "0-layer" (3). Recombinant neuronal SNAREs spontaneously assemble into stable bundles, yet SNARE complex assembly *in vivo* requires SNARE-binding proteins of the Sec1-Munc18 "SM" family. SNARE complexes are disassembled by two chaperones: Sec17p/α-SNAP, which binds directly to SNARE complexes, and Sec18p/NSF, which binds to Sec17p/α-SNAP and couples the energy of ATP binding and hydrolysis to SNARE complex disassembly (4). SNARE complexes form in *cis*, with each SNARE anchored to the same membrane, or in *trans*, with SNAREs anchored to apposed, "tethered" membranes.

SNARE function has been studied *in vivo*, on isolated organelles, and through the reconstitution of recombinant SNAREs into proteoliposomes. When several SNAREs, which are found *in vivo* on a "target" membrane, are co-reconstituted into one population of liposomes, they form a t-SNARE complex. These liposomes can selectively interact with other liposomes bearing a reconstituted "vesicle," or v-, SNARE to allow selective lipid mixing (5), vesicle content mixing (6), or lysis (7, 8). This reconstituted reaction shows impressive specificity for cognate SNARE pairs (9) and can be directly promoted by other fusion-regulatory factors such as synaptotagmin and calcium (10) or Sec1p (11) under conditions that minimize lysis (12).

SNAREs are required for the homotypic fusion of yeast vacuoles (13). Vacuoles fission and fuse during cell division and organelle inheritance (14) and in response to growth medium osmolarity (15). Purified vacuoles undergo a multistep fusion pathway, which can be monitored by content-mixing assays (16). In the initial "priming" step, Sec18p/Sec17p disassemble *cis*-SNARE complexes (17). The following tethering stage of "docking" requires the Rab-family GTPase Ypt7p (18) and its effector complex, termed HOPS (homotypic fusion and vacuole protein sorting) (19), which has Vps11p, Vps16p, Vps18p, Vps33p, Vps39p, and Vps41p as subunits (20, 21). Tethered vacuoles are drawn against each other at closely apposed disc-shaped microdomains, termed "boundary membranes" to distinguish them from the "outside membranes," which are not in contact (22). The proteins (Ypt7p, HOPS, and SNAREs) and lipids (phosphoinositides, ergosterol, and diacylglycerol), which are required for fusion undergo striking, interdependent enrichment at a ring-shaped microdomain, termed the "vertex

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[4] The abbreviations used are: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors; HOPS, homotypic fusion and vacuole protein sorting complex; SNAP, soluble NSF attachment protein; NSF, *N*-ethylmaleimide-sensitive protein.

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ring," which surrounds the boundary membrane (22–25). Vacuole SNAREs form *trans*-complexes between the apposed organelles (17). Ypt7p and HOPS are needed for SNARE complex assembly, and HOPS is an integral part of the assembled SNARE complex (26). Fusion occurs around the vertex ring, joining the outside membrane from each vacuole to form the larger, fused organelle and joining the two apposed boundary membrane discs to yield a luminal vesicle (22, 27).

Vacuoles have four SNAREs; their roles have been studied in the organelle-based fusion reaction (26, 28) and in liposome-based studies (29). Vam3p, Vam7p, and Vit1p are Q-SNAREs, which constitute the t-SNARE, and the R-SNARE Nyv1p can serve as the v-SNARE. Three of these have C-terminal *trans*-membrane anchors, but Vam7p has no apolar membrane anchor. Whereas most purified SNAREs require detergent for their solubility, and thus cannot be directly added to purified organelles without causing lysis, recombinant Vam7p is water-soluble without detergent and supports vacuole fusion (30).

Although SNAREs are central to fusion, it remains unclear how they act. SNAREs may apply torque to membranes (31, 32), destabilize bilayers through poorly fitting TM domains (33, 34), gather fusogenic lipids (*e.g.* diacylglycerol) into fusion microdomains (25), form the walls of a fusion pore (35), or act by other means. Studies of integral membrane SNAREs in the context of their native membrane and organelle are limited to producing and characterizing mutants, which may have significant effects on organelle composition and cell growth. The vacuolar Vam7p SNARE affords a unique window into SNARE function, because its cloned recombinant form is functional for fusion *in vitro*, it can readily be expressed and purified in soluble recombinant form, and many subreaction assays of vacuole fusion are available. Vam7p is a Q-SNARE with an N-terminal PX (Phox homology) domain, which directly binds phosphatidylinositol 3-phosphate (36) and HOPS (19), and a C-terminal SNARE domain. We now compare the functions of wild-type Vam7p with Vam7p bearing a Q283R mutation, changing the 0-layer Gln to Arg, in the context of purified vacuoles with the wild-type R-SNARE Nyv1p or with its 0-layer Arg changed to Gln. We find that the 3Q·1R rule (37) is important but not inviolate. Substantial fusion is seen with SNARE complexes having "rotated" positions of the 3Q·1R or with 4Q complexes, each showing dramatically elevated K_m for Vam7p but similar maximal rates of fusion. 2Q·2R SNAREs form SNARE complex without fusion, but substantial fusion can be restored by the addition of chlorpromazine or U73122. Either of these agents modifies the lipid bilayer, suggesting that 0-layer function is directly coupled to the lipid bilayer properties.

EXPERIMENTAL PROCEDURES

Strains—The strains used for fusion assays were BJ3505 (*MATα pep4::HIS3 prb1-Δ1.6R his3–200 lys2–801 trp1Δ101 (gal3) ura3–52 gal2 can1*) (38) and DKY6281 (*MATα leu2–3 leu 2–112 ura3–52 his3-Δ200 trp1-Δ901 lys2–801*) (16). BJ3505 *nyv1Δ* and DKY6281 *nyv1Δ* were described previously (13). *Nyv1*^{R192Q} was introduced into BJ3505 *nyv1Δ* and DK6281 *nyv1Δ* by recombination. The promoter region of the *NYV1* gene was amplified by PCR from DKY6281 genomic DNA with the forward primer 5′-CGATATT**AAGCTT**GCCCTAATTA-

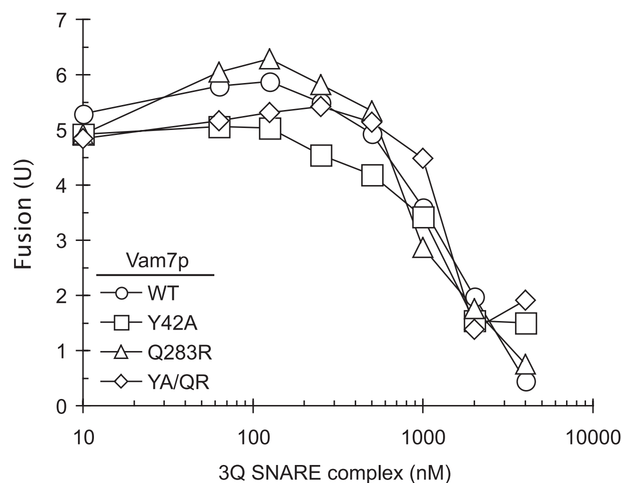

FIGURE 1. **Equivalent function of wild-type and mutant Vam7p in inhibitory 3sQ complexes.** Standard fusion reactions ("Experimental Procedures") were incubated for 90 min in the absence or presence of equimolar mixtures of soluble His6-Vam3p, MBP-Vti1p, and GST·Vam7p. Mixtures contained either wild-type Vam7p, Vam7p^{Y42A} (*Y42A*), Vam7p^{Q283R} (*Q283R*), or Vam7p$^{Y42A/Q283R}$ (*YA/QR*).

CTAGG-3′ containing a HindIII site (bold) and reverse primer 5′-GCTGTTAGAGCATTGGACTTTTATATTTTTACCA-A**GGATCC**GCG-3′ containing a BamHI site. The product was subcloned into pRS406 using HindIII and BamHI sites, generating the plasmid pRS406-p*NYV1*. The *NYV1* 3′-untranslated region containing the terminator was amplified from DKY6281 genomic DNA with forward primer 5′-TCCC**TCTAGA**ATG-AAACGCTTTAATGGTATGTAT-3′ containing an XbaI site and reverse primer 5′-GTGCCACTTGTGAGAAGTCTAA-GC**CCGCGG**GGA-3′ with a SacII site. The resulting PCR product was subcloned into pRS406-p*NYV1* using XbaI and SacII sites, generating pRS406-p*NYV1-NYV1*. QuikChange mutagenesis (39) was used to generate pRS406-*nyv1*^{R192Q} using the forward primer 5′-AACATCGACAAGTTCTTGGAG-CAACAAGAAAGAGTTTCTTTATTGTG-3′ and reverse primer 5′-CACCAATAAAGAAACTCTTTCTTG**TTG**CTC-CAAGAACTTGTCGATGTT-3′. RFY1 and RFY2 were generated by transforming BJ3505 *nyv1Δ* and DKY6281 *nyv1Δ*, respectively, with pRS406-*nyv1*^{R192Q} by standard lithium acetate methods and plated on complete synthetic media lacking uracil.

Reagents—Reagents were dissolved in PS buffer (20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol). Myristoylated alanine-rich C kinase substrate-effector domain (KKKKKRFSFKKSFK-LSGFSFKKNKK, Keck Center, Yale) was dissolved at 10 μM in PS buffer. Anti-Vam3p F_{ab} (24), anti-Vps33p (21), and anti-Sec17p (40) were previously described. His_6-Sec18p was prepared as described (40). His_6-Sec18p was further purified by gel filtration (30). Gdi1p (41), His_6-Gyp1–46p (24), and IB2 (42) were prepared as described.

Recombinant Vam7p—The *VAM7* open reading frame, excluding the first methionine, was amplified from BY4742 chromosomal DNA using forward primer 5′-GCG**GGATCC**-GCAGCTAATTCTGTAGGGAAA-3′ with a BamHI restriction site (bold), and reverse primer 5′-CGC**GAATTC**TCAAG-CACTGTTGTTAAAATG-3′ with an EcoRI site. Digested PCR product was ligated to EcoRI/BamHI-linearized

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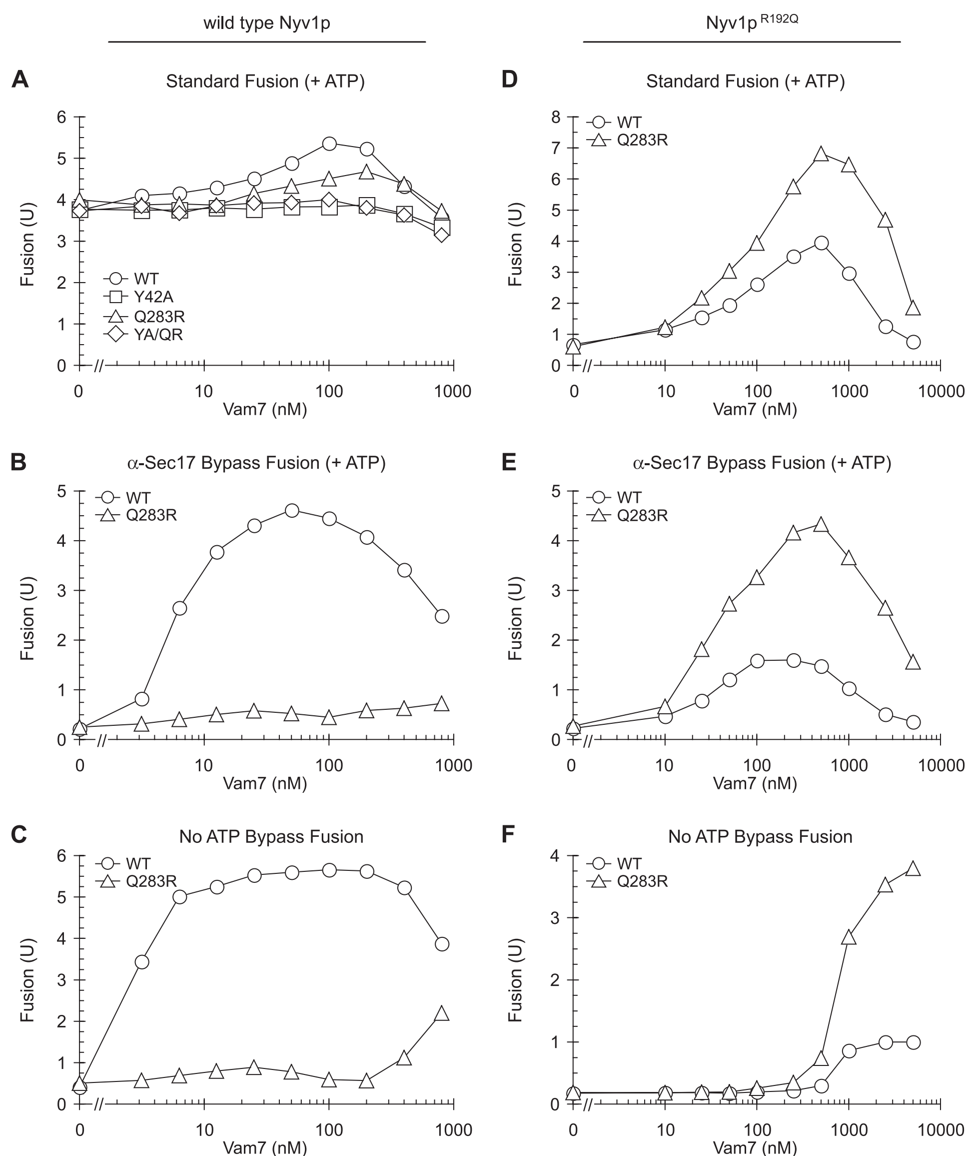

FIGURE 2. **Regulation of fusion by the 0-layer of Vam7p and Nyv1p.** Fusion assays were performed using vacuoles harvested from yeast strains BJ3505 and DKY6281 (*A–C*), which are wild-type for SNAREs but permit assay of fusion ("Experimental Procedures") or from their isogenic strains RFY1 and RFY2 harboring Nyv1p^{R192Q} (*D–F*). Vacuoles were incubated in the absence or presence of added recombinant wild-type or mutant Vam7p at the indicated concentrations. Vam7p was present from the start of the fusion incubation. Reactions were under standard conditions, as under "Experimental Procedures" (*A* and *D*) or α-Sec17 bypass (*B* and *E*) or no ATP bypass conditions (*C* and *F*) as described in (30). Fusion was measured by alkaline phosphatase activity and expressed in units.

pET42a(+) to generate pET42a-GST·Vam7p. Sequenced plasmid was transformed into *Escherichia coli* Rosetta-2 (DE3) pLysS (Novagen) and grown on Luria broth with kanamycin and chloramphenicol. Using pET42a-GST-*VAM7* as a template, QuikChange mutagenesis was used to create *VAM7*^{Y42A}, *VAM7*^{Q283R}, and *VAM7*$^{Y42A/Q283R}$. Forward primer 5′-AACAAGCGCCTTTACAAAAGG**GCA**TCCGAGTTTTGGAAACTGAAG-3′ and reverse primer 5′-CTTCAGTTTCCAAAACTCGGA**TGC**CCTTTTGTAAAGGCGCTTGTT-3′ were used to make *VAM7*^{Y42A}. To make *VAM7*^{Q283R} we used 5′-GAGATGAACGAGGAGCTGCAAACA**CGG**AATGAGCTACTTACAGCACTT-3′ and 5′-AAGTGCTGTAAGTAGCTCATT**CCG**TGTTTGCAGCTCCTCGTTCATCTC-3′.

To express GST-Vam7, cultures were grown in 1 liter of Terrific broth at 37 °C to A_{600} = 0.6. Cells were induced with 500 μM isopropyl 1-thio-β-D-galactopyranoside at 37 °C for 4 h, collected by centrifugation, and washed with lysis buffer (50 mM Tris-Cl, pH 8, 500 mM NaCl, 1 mM dithiothreitol, 2 mM EGTA, 1 mM EDTA) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μM Pefabloc-SC, 1 μM leupeptin, and 5 μM pepstatin). Cells were lysed by 3 passages through a French pressure cell. Lysates were clarified by centrifugation (60 min, 45,000 rpm, 4 °C, Beckman 60-Ti rotor). Supernatants were incubated with GSH-Sepharose 4B resin (Amersham Biosciences) equilibrated with lysis buffer (16 h, 4 °C, nutating). Resin was washed with 20 column volumes of lysis buffer. GST-Vam7p was eluted with lysis buffer containing 10 mM reduced glutathione. Proteins were dialyzed against PS buffer with 125 mM KCl and 5 mM $MgCl_2$.

Vacuole Isolation and in Vitro Fusion Assay—Vacuoles were isolated from the yeast strains BJ3505 (38) and DKY6281 (16). Fusion reactions (30 μl) contained 3 μg of BJ3505 vacuoles with inactive pro-Pho8p (pro-alkaline phosphatase) and lacking the protease Pep4p, 3 μg of DKY6281 vacuoles containing Pep4p but lacking Pho8p, standard fusion reaction buffer (125 mM KCl, 5 mM $MgCl_2$, 20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol), ATP regenerating system (1 mM ATP, 40 mM creatine phosphate, 0.1 mg/ml creatine kinase), 10 μM coenzyme A (18), and 930 nM I_2^B (inhibitor of protease B). After 90 min at 27 °C, Pho8p activity was assayed in 250 mM Tris-Cl, pH 8.5, 0.4% Triton X-100, 10 mM $MgCl_2$, 1 mM *p*-nitrophenyl phosphate. One unit of fusion is 1 μmol of *p*-nitrophenolate produced per min per μg of BJ3505 vacuoles. *p*-Nitrophenolate absorbance was measured at 400 nm.

RESULTS

Because Vam7p has no apolar membrane anchor, it can be produced in bacteria, purified in the absence of detergent, and added to *in vitro* fusion assays. To assess the role of the Vam7p C-terminal SNARE domain, we mutated its zero-layer glutamine to arginine (Q283R) to disrupt the 3Q·1R ratio. As a first test of this mutant Vam7p, we mixed bacterially expressed

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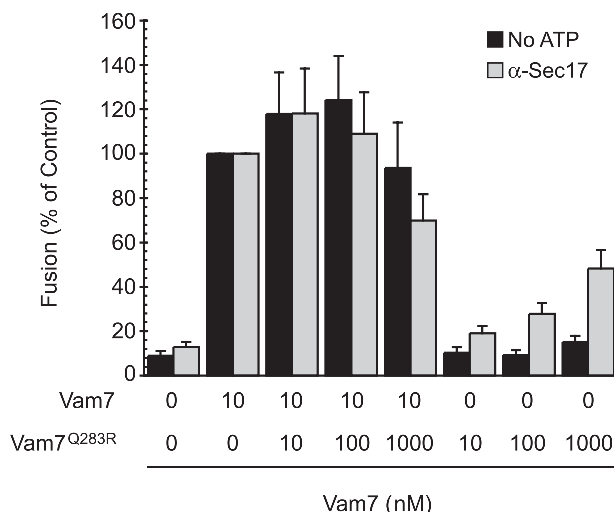

FIGURE 3. **Vacuoles discriminate between Vam7p and Vam7p^{Q283R} according to the 0-layer.** Vacuoles blocked for priming with α-Sec17p antibody or by ATP omission (30) were incubated with indicated amounts of premixed wild-type Vam7p and Vam7p^{Q283R}. Reactions were incubated for 90 min, and fusion was measured by alkaline phosphatase activity. Data represent mean fusion ± S.E. ($n = 3$).

recombinant Vam7p or Vam7p^{Q283R} with the recombinant soluble cytoplasmic domains of Vti1p and Vam3p prior to introducing vacuoles and assaying vacuole fusion. The mixed soluble domains of the three Q-SNAREs interact directly with Nyv1p to form nonfunctional SNARE bundles and block vacuole fusion (43). Although this inhibition requires all three of the Q-SNARE-soluble domains, it is simpler than the physiological SNARE complex assembly, because it bypasses regulation by Ypt7p (43). The inhibitory effect of the mixed soluble domains of the t-SNAREs was undiminished by violation of the 3Q·1R rule or by another mutation, which we characterize in detail elsewhere,[5] that disrupts the affinity of Vam7p for phosphatidylinositol 3-phosphate (Fig. 1). Because Vam7p^{Q283R} can still interact with other SNAREs to regulate fusion, it warranted further characterization.

Fusion Activity of Vam7^{Q283R} Protein—Although the other, integral-membrane SNAREs are present on isolated vacuoles in the unpaired state as well as in SNARE complexes, all the Vam7p on purified vacuoles appears to be in *cis*-SNARE complexes (26). In standard fusion reactions with ATP, the disassembly of these complexes by Sec18p and Sec17p provides the Vam7p for *trans*-SNARE complex formation. For this reason, the requirement for Sec18p/Sec17p and for ATP can be bypassed by added Vam7p (30). We first tested the effects of adding recombinant wild-type and mutant Vam7p to our standard fusion reaction, which does not required exogenously added Vam7p. The addition of either wild-type Vam7p, Vam7p^{Q283R}, or other mutants had little effect on fusion (Fig. 2*A*). Recombinant wild-type Vam7p can "bypass" the need for priming, the disassembly of *cis*-SNARE complexes, by forming complexes with the unpaired vacuolar SNAREs (30). In bypass fusion assays, priming is blocked by either the absence of ATP or, in the presence of ATP, by antibody to Sec17p. Although wild-type Vam7p supported bypass fusion (*circles*, Fig. 2, *B* and *C*), Vam7p^{Q283R} did not support the bypass fusion of wild-type vacuoles (*triangles*, Fig. 2, *B* and *C*). These assays allow direct determination of whether Vam7p^{Q283R} can compete with wild-type Vam7p. Even when Vam7p and Vam7^{Q283R} were premixed prior to addition to vacuoles, a 100-fold excess of Vam7p^{Q283R} only gave modest inhibition of Vam7p-supported fusion (Fig. 3). In these studies, Vam7p^{Q283R} was added at up to micromolar levels, a large excess over the endogenous Vam7p (30). Because Vam7p^{Q283R} binds to vacuoles as well as wild-type Vam7p (data not shown) but does not support fusion (Fig.

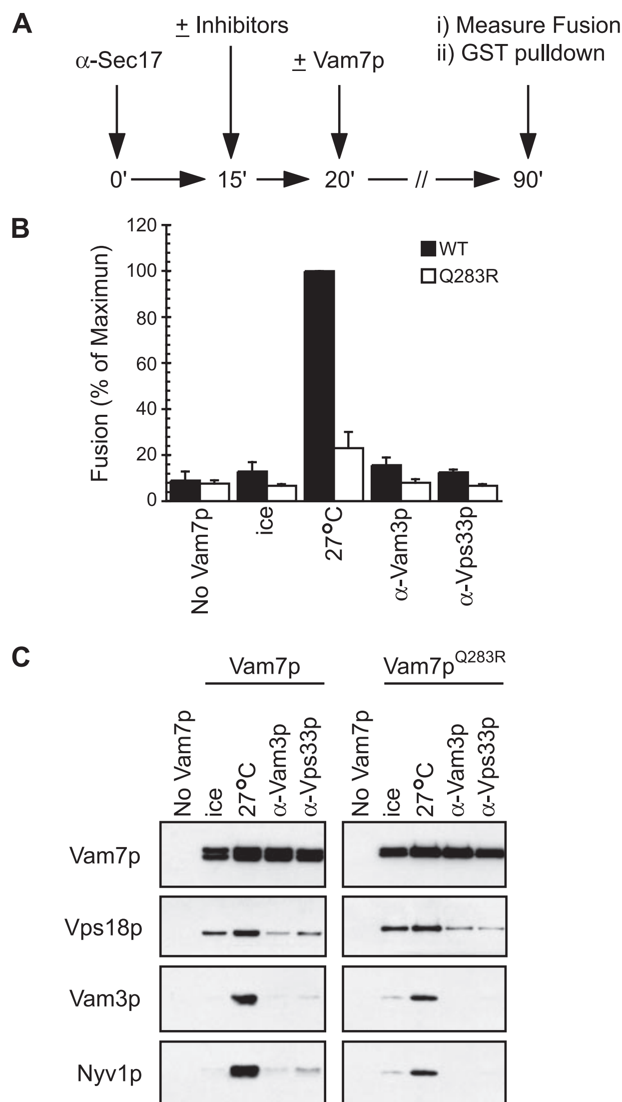

FIGURE 4. **Vam7p forms complexes with SNAREs and HOPS.** *A*, reaction scheme. *B* and *C*, large scale (180 μl) fusion reactions were incubated with α-Sec17 to block priming. After 15 min, inhibitors (353 nM α-Vam3p or 32 nM α-Vps33p) were added, and the mixture was incubated for 5 min before addition of 400 nM Vam7p or Vam7p^{Q283R}. After 90 min, reactions were placed on ice and 30-μl aliquots were used to measure fusion. The remaining vacuoles were sedimented (11,000 × *g*, 10 min, 4 °C), and pellets were extracted with solubilization buffer (20 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 2 mM EDTA, 20% glycerol, 0.5% Triton X-100, 1 mM dithiothreitol) with protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 10 μM Pefabloc-SC, 5 μM pepstatin A, and 1 μM leupeptin) on ice for 20 min. Insoluble debris was sedimented (16,000 × *g*, 10 min, 4 °C), and supernatants were incubated with glutathione-Sepharose 4B (15 h and 4 °C) while nutating. Beads were washed 5× with 1 ml of solubilization buffer, and bound material was eluted by heating with reducing SDS-PAGE sample buffer. GST-Vam7p complexes were examined by Western blotting. *Bars* represent mean fusion ± S.E. ($n = 3$).

[5] Fratti, R. A., and Wickner, W. (2007) *J. Biol. Chem.* **282,** 13133–13138.

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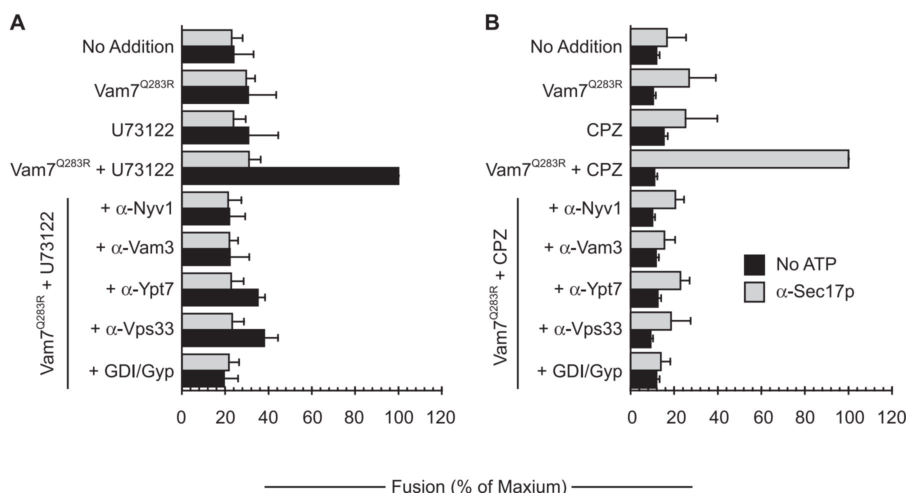

FIGURE 5. **Vacuole 2Q·2R fusion, with Vam7^{Q283R} and vacuoles bearing wild-type Nyv1p, can be restored by U73122 or chlorpromazine.** Bypass fusion (30) was assayed either without ATP or with ATP present but in the presence of antibody to Sec17p. Fusion reactions were incubated for 10 min, and inhibitors were added and incubated for 5 min before addition of 1.6 μM U73122 (*A*) or 150 μM chlorpromazine (CPZ) (*B*). U73122 and chlorpromazine were allowed to act for 5 min before addition of 100 nM Vam7p^{Q283R}. Reactions were incubated for an additional 70 min, and then assayed for fusion. Fusion inhibitors were 120 nM affinity-purified α-Nyv1p, 353 nM α-Vam3p IgG, 133 nM affinity-purified α-Ypt7p, 32 nM affinity-purified α-Vps33p, 2.8 μM GDP dissociation inhibitor protein (*GDI*), and 11.4 μM Gyp1–46. Data represent mean fusion ± S.E. (n = 3).

2, *B* and *C*), vacuoles show exquisite discrimination for Vam7p with the normal Gln residue at the 0-layer position.

Vam7p^{Q283R} Stimulates the Fusion of Vacuoles Bearing Nyv1p^{R192Q}—To test whether Vam7p^{Q283R} is merely denatured or could under some circumstances form functional SNARE complexes, we replaced the 0-layer arginine of Nyv1p with a glutamine and purified vacuoles from yeast harboring Nyv1p^{R192Q}. In a standard fusion reaction with ATP but without added Vam7p, vacuoles bearing Nyv1p^{R192Q} were unable to fuse (Fig. 2*D*), suggesting that four Q-SNAREs are not readily primed (44). In standard fusion reactions, the same Vam7p^{Q283R} that was inert with vacuoles bearing the wild-type Nyv1p supported the fusion of Nyv1^{R192Q} vacuoles with a K_m of ~50 nM (Fig. 2*D*). Surprisingly, wild-type Vam7p also promoted fusion of Nyv1^{R192Q} with a similar K_m, albeit only ~60% as well as Vam7^{Q283R}. Thus, as seen in other studies (45), the 3Q·1R rule is not inviolable; fusion can occur with four Q-SNAREs. When priming was blocked in the presence of ATP by the addition of anti-Sec17p, Vam7p^{Q283R} or wild-type Vam7p supported fusion (Fig. 2*E*), as seen under standard fusion conditions (Fig. 2*D*).

In the absence of ATP (Fig. 2*F*), Vam7^{Q283R} promoted fusion of Nyv1p^{R192Q} vacuoles whereas wild type Vam7p did not, just as vacuoles bearing wild type Nyv1p could only undergo bypass fusion with wild-type Vam7p (Fig. 2*C*). However, the concentration of Vam7^{Q283R} needed for fusion of Nyv1p^{R192Q} vacuoles was ~100 times greater than with wild-type Vam7p and Nyv1p (Fig. 2, *F versus C*). It may take far more energy to form SNARE bundles containing both Vam7p^{Q283R} and Nyv1p^{R192Q} than with wild-type Vam7p and Nyv1p, even though both have 3Q·1R 0-layers. This is consistent with energy being needed to distort the SNARE α-helical backbone at the 0-layer to accommodate the altered positions of bulky arginine *versus* the less bulky glutamine, or with "proofreading" by a SNARE-bound factor such as HOPS. Once formed, these 3Q·1R complexes have the same capacity to promote fusion. A detailed examination of the specific protein and lipid requirements for fusion supported by each form of Vam7p, with wild-type vacuoles or Nyv1^{R192Q} vacuoles, is presented in the supplemental Fig. S1.

Physical Interactions of Vam7p with SNAREs and HOPS—Because Vam7p mutant proteins bind to vacuoles in a manner indistinguishable from wild-type Vam7p yet differentially support fusion, we examined their capacities to form protein complexes with SNAREs and HOPS. For this study, vacuole fusion was blocked by antibody to Sec17p for 15min, secondary inhibitors were added, and, after 5 min, the priming block was rescued by the addition of Vam7p (Fig. 4*A*). As reported (26), wild-type Vam7p relieves the anti-Sec17p block to allow fusion (Fig. 4*B*, *filled bars*) and enters into complexes with SNARES and HOPS (Fig. 4*C*, *left panel*). These interactions were inhibited by antibodies to Vam3p or Vps33p. Strikingly, Vam7p^{Q283R}, although it does not support the fusion of wild-type vacuoles (Figs. 2*B*, 2*C*, and 4*B*, *open symbols*), forms stable and isolable HOPS·SNARE complexes (Fig. 4*C*) which contained Nyv1p and are thus 2Q·2R. The formation of this complex is also inhibited by antibody to Vam3p or to the HOPS subunit Vps33p, the SM protein of the vacuole.

Bypass of the 2Q·2R Block—Because Vam7p^{Q283R} binds to vacuoles and enters SNARE complexes as readily as wild-type Vam7p, but with little or no consequent fusion, we sought conditions that might activate fusion in this 2Q·2R system when priming was blocked by either antibody to Sec17p or by the omission of ATP. Bypass fusion with Vam7p^{Q283R} in the absence of ATP was restored by the addition of the phospholipase C inhibitor U73122, but restoration was not seen in the presence of ATP (Fig. 5*A*). Strikingly, bypass fusion with Vam7p^{Q283R} in the presence of ATP, when priming was blocked by antibody to Sec17p, was restored by the addition of chlorpromazine (Fig. 5*B*), and this restoration required ATP. In each case, the restored fusion required the normal fusion pathway, because it was blocked by characterized inhibitors that target SNAREs, Ypt7p, and HOPS. Even though bypass fusion with wild-type Vam7p is unaffected by ATP (30), ATP directly regulates the ability of U73122 or chlorpromazine to restore 2Q·2R fusion. To further examine this bypass, we characterized the effects of chlorpromazine on both the physical associations of added Vam7p^{Q283R} as well as on the functional restoration of fusion for the same samples (Fig. 6). Chlorpromazine had no measurable effect on the binding of Vam7p^{Q283R} to vacuoles (Fig. 6*B*, *top panel*). Chlorpromazine had only a small effect on the association of vacuole-bound Vam7p^{Q283R} with HOPS or with other SNAREs (Fig.

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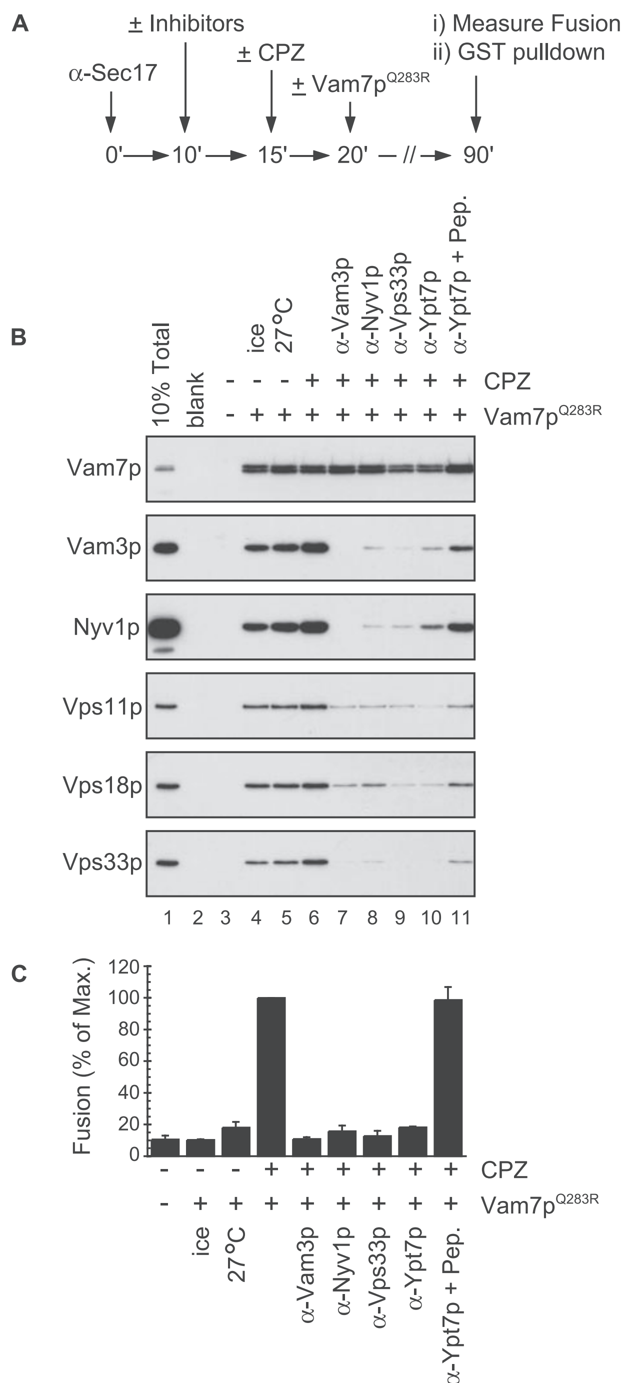

FIGURE 6. **Chlorpromazine permits Vam7p^{Q283R} to form normal complexes with SNAREs and HOPS.** *A*, reaction scheme. *B*, fusion reactions were incubated with α-Sec17 to block priming. After 10 min, inhibitors were added and the mixture was incubated for 5 min before addition of 150 μM chlorpromazine (*CPZ*). Chlorpromazine was allowed to act for 5 min before addition of 100 nM Vam7p^{Q283R}. Reactions were incubated for an additional 70 min, and then assayed for Vam7p^{Q283R}-associated proteins as described in Fig. 4. *C*, fusion was determined by alkaline phosphatase activity. *Bars* represent mean fusion ± S.E. ($n = 3$).

6*B*, *lane 4 versus 5*) but was absolutely required for fusion (Fig. 6*C*). In the presence of chlorpromazine, both the physical association of the Vam7p^{Q283R} with HOPS and with SNAREs and the function of restored fusion were still dependent on the R-SNARE Nyv1p (Figs. 5, 6*B* (*lane 8*), and 6*C*), showing that fusion occurred using a 2Q·2R SNARE complex, and on Vam3p, Ypt7p, and the SM Vps33p subunit of HOPS (Fig. 6, *B* (*lanes 7, 9–11*) and *C*).

DISCUSSION

With vacuoles bearing the wild-type R-SNARE Nyv1p, Vam7p^{Q283R} is inactive for fusion, yet forms an HOPS·SNARE complex of 2Q·2R composition. Vacuoles bearing Nyv1p^{R192Q} can fuse when given either Vam7p^{Q283R}, restoring the 3Q·1R composition of the 0-layer, or wild-type Vam7p, yielding a 4Q 0-layer. These findings are in accord with studies showing that 4Q SNARE complexes are functional for exocytosis (46) or endoplasmic reticulum to Golgi traffic (45), whereas 2Q·2R complexes are at least somewhat defective (45). Studies with recombinant neuronal SNAREs have shown that the syntaxin 0-layer Gln is essential for NSF factor and α-SNAP-mediated SNARE bundle disassembly (44), and this may account for some of the loss of fusion that accompanies mutation of the Vam3p 0-layer Gln to Arg in an earlier study (47). However, diminished disassembly of *cis*-SNARE complexes is not a factor in our current studies in which the action of Sec18p(NSF)/Sec17p(α-SNAP) is blocked, by antibody or by the absence of ATP.

What is the role of the 0-layer in vacuole fusion? Our studies show that the composition of glutamine and arginine residues in the 0-layer and their precise spatial distribution control the energy needed to assemble functional SNARE complexes, because normal bypass fusion has a K_m for Vam7p of only 3–8 nM, whereas far higher concentrations of mutant or wild-type Vam7p are needed for 4Q fusion (Fig. 2). Once formed, SNARE complexes with 2Q·2R are stable but inactive, yet fusion can be restored when the membrane is perturbed by the intercalating amphiphile chlorpromazine or by the phospholipase C inhibitor U73122. These may modulate bilayer properties such that a "weakened" 2Q·2R complex is still capable of driving fusion.

Chlorpromazine is an amphipathic molecule that partitions into the negatively curved inner leaflets of membrane bilayers. Once intercalated into membranes, chlorpromazine alters the physical properties of bilayers. Upon insertion into inner leaflets, chlorpromazine deforms membranes to induce cupping (48) and can alter lateral diffusion and membrane tension (48). In doing so, chlorpromazine reduces relaxation times of membrane tethers by lowering thresholds for remodeling. This was seen in force measurement experiments using optical tweezers to pull membranes (49). Membrane remodeling by chlorpromazine may also alter phosphoinositide metabolism by activating both phosphoinositide kinases and phospholipase C (50). Each of these actions of chlorpromazine may lower the threshold for the bilayer rearrangements of fusion, permitting 2Q·2R SNARE complexes to function. Similarly, U73122 inhibits vacuolar phospholipase C activities (51) and thereby alters the ratio of two vacuolar lipids that are crucial for fusion, phosphatidylinositol 4,5-bisphosphate and diacylglycerol. Restoration of 2Q·2R fusion by chlorpromazine or U73122 is regulated by the presence or absence of ATP; one of the effects of ATP in this restoration may be to support phosphoinositide synthesis.

SNAREs may promote membrane fusion by several means: 1) They may provide physical stress on the bilayer, which could lower the activation energy for lipid rearrangements for fusion

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(5). 2) SNAREs are needed to gather lipids such as diacylglycerol, which promote the bilayer rearrangements of fusion, to the fusion site (25). 3) The *trans*-membrane domain of certain SNAREs will destabilize bilayers (33, 52), and SNARE-driven bilayer stress and destabilization can lead to the lipid rearrangements of lysis as well as fusion (7, 8). 4) SNAREs may also serve as a binding platform to localize and even activate other fusion factors, such as surface-active and calcium-triggered synaptotagmin or lipid-binding HOPS. Assays of each of these four separate SNARE contributions to fusion will be required to determine the importance of each means of SNARE action and the role of the 0-layer in their regulation. Our current findings suggest that SNARE 0-layer function is intimately tied to the lipid bilayer composition and physical properties.

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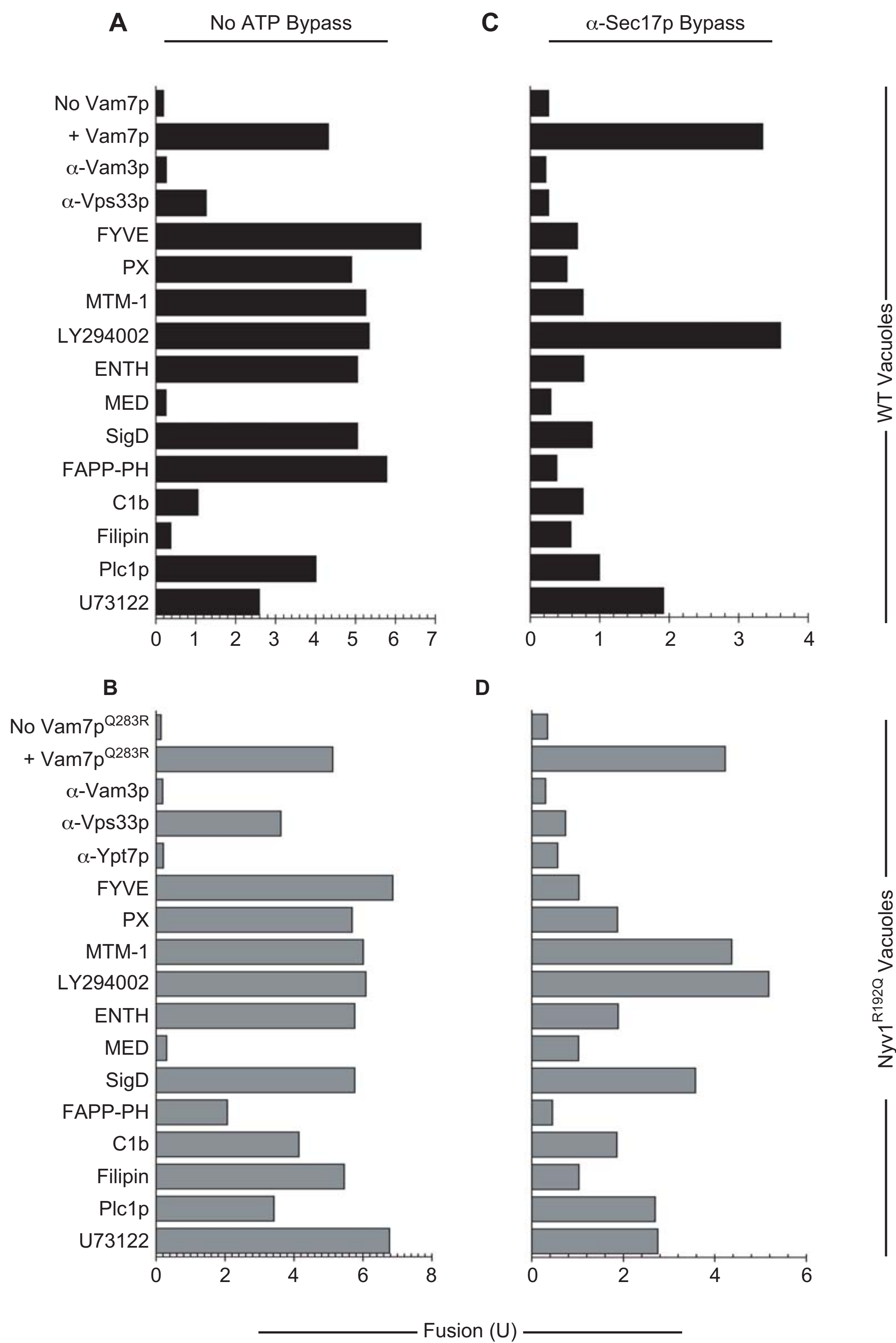
Supplementary Figure

Regulation of Vam7p bypass fusion.

To compare the regulation of fusion under the different conditions tested here and in the presence of mutant SNAREs, we examined the effects of well characterized inhibitors of fusion. Bypass fusion reaction had wild-type Vam7p or Vam7p^{Q283R} and were run under no-ATP conditions, or with ATP and antibody to Sec17p (see Methods). Bypass fusion of vacuoles bearing Nyv1p^{R192Q} (Fig. S1) followed similar patterns of regulation compared to its wild type Nyv1p counterparts. Fusion was sensitive to antibodies to Vam3p, Vps33p and Ypt7p. One notable exception is the relative sensitivity to 4-phosphoinositides concentrations. Fusion supported by wild-type Vam7p in the absence of ATP required free DAG and ergosterol but not PI(4,5)P_2. In contrast, Vam7p^{Q283R} bypass of Nyv1p^{R192Q} in the absence of ATP did not require free DAG or ergosterol but did required PI4P as shown by its sensitivity to FAPP-PH. Added Plc1p or inhibition of endogenous PLC activity by U73122 also blocked this fusion, further demonstrating that PI(4,5)P_2 levels are critical.

Figure S1. Nyv1p^{R192Q} vacuole fusion is on pathway. The sensitivity of fusion incubations with Vam7p or Vam7p^{Q283R} to inhibitors was tested under no ATP bypass **(A** and **B)** or anti-Sec17 bypass **(C** and **D)** conditions using vacuoles bearing wild-type Nyv1p (**A** and **C**) or Nyv1p^{R192Q} from RFY1 and RFY2 (**B** and **D**). Vacuoles were incubated with 1000 nM Vam7p or Vam7p^{Q283R} in the presence or absence of fusion inhibitors. For concentrations of fusion inhibitors, see Materials and Methods.


A
No ATP Bypass
C
α-Sec17p Bypass
No Vam7p
+ Vam7p
α-Vam3p
α-Vps33p
FYVE
PX
MTM-1
LY294002
ENTH
MED
SigD
FAPP-PH
C1b
Filipin
Plc1p
U73122
0 1 2 3 4 5 6 7
0 1 2 3 4
WT Vacuoles
B
D
No Vam7pQ283R
+ Vam7pQ283R
α-Vam3p
α-Vps33p
α-Ypt7p
FYVE
PX
MTM-1
LY294002
ENTH
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FAPP-PH
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0 2 4 6 8
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Nyv1R192Q Vacuoles
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Stringent 3Q·1R Composition of the SNARE 0-Layer Can Be Bypassed for Fusion by Compensatory SNARE Mutation or by Lipid Bilayer Modification

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