Cell, Vol. 93, 1125–1134, June 26, 1998, Copyright ©1998 by Cell Press

LMA1 Binds to Vacuoles at Sec18p (NSF), Transfers upon ATP Hydrolysis to a t-SNARE (Vam3p) Complex, and Is Released during Fusion

Zuoyu Xu, Ken Sato, and William Wickner*
Department of Biochemistry
Dartmouth Medical School
Hanover, New Hampshire 03755

Summary

Vacuole fusion requires Sec18p (NSF), Sec17p (α-SNAP), Ypt7p (GTP binding protein), Vam3p (t-SNARE), Nyv1p (v-SNARE), and LMA1 (*l*ow *M*$_r$ *a*ctivity 1, a heterodimer of thioredoxin and I$^B_2$). LMA1 requires Sec18p for saturable, high-affinity binding to vacuoles, and Sec18p "priming" ATPase requires both Sec17p and LMA1. Either the *sec18-1* mutation and deletion of I$^B_2$, or deletion of both I$^B_2$ and p13 (an I$^B_2$ homolog) causes a striking synthetic vacuole fragmentation phenotype. Upon Sec18p ATP hydrolysis, LMA1 transfers to (and stabilizes) a Vam3p complex. LMA1 is released from vacuoles in a phosphatase-regulated reaction. This LMA1 cycle explains how priming by Sec18p is coupled to t-SNARE stabilization and to fusion.

Introduction

Subcellular organelles are inherited rather than synthesized de novo during cell division (Warren and Wickner, 1996). We have studied the inheritance of the vacuoles (lysosomes) of *Saccharomyces cerevisiae*. Early in S phase, the vacuole projects a tubular-vesicular "segregation" structure into the newly formed bud (Weisman and Wickner, 1988; Gomes de Mesquita et al., 1991; Raymond et al., 1992). Through this segregation structure, the bud receives maternal vacuolar vesicles, which fuse to establish the daughter vacuoles. In *vac* mutants, many large buds lack a vacuole entirely or have only an unusually small organelle (Weisman et al., 1990; Shaw and Wickner, 1991; Nicolson et al., 1995; Wang et al., 1996). Vacuole inheritance can also be studied in vitro (Conradt et al., 1992, 1994; Haas et al., 1994). Isolated vacuoles, when incubated with ATP and cytosol, form segregation structures and undergo fusion. This reaction is abolished when its components are prepared from *vac* mutants, establishing its authenticity (Haas et al., 1994; Nicolson et al., 1995).

The final step of the vacuole inheritance reaction is homotypic vacuole fusion. Like many membrane trafficking and fusion processes, this requires Sec18p/NSF (*N*-ethylmaleimide *s*ensitive *f*usion protein), Sec17p/α-SNAP (*s*oluble *N*SF *a*ttachment *p*rotein), and the SNAREs (*SNAP re*ceptors) Vam3p (t-SNARE) and Nyv1p (v-SNARE) (Rothman, 1994; Haas and Wickner, 1996; Nichols et al., 1997). NSF is an oligomeric ATPase (Whiteheart et al., 1994) that requires SNAPs to bind to the SNAREs (Söllner et al., 1993). SNAREs are integral membrane proteins that exist on both vesicle (v-SNARE) and target (t-SNARE) membranes (Söllner et al., 1993; Rothman, 1994). Though NSF and SNAPs are structurally and functionally conserved between organisms and among the different compartments within each cell (Ferro-Novick and Jahn, 1994), the specificity of membrane targeting has been ascribed to the pairing of v/t-SNAREs (Rothman, 1994; Hay and Scheller, 1997). A number of small Ras-like GTPases are also essential for most fusion reactions (Pfeffer, 1996). Sec18p, Sec17p, Vam3p, and Nyv1p are members of a SNARE complex on the purified vacuoles (Haas et al., 1995; Nichols et al., 1997; Ungermann et al., 1998). The hydrolysis of ATP by Sec18p triggers dissociation of this SNARE complex and the release of Sec17p from the vacuole membrane (Mayer et al., 1996). This Sec18p/Sec17p action activates the t-SNARE (Ungermann et al., 1998) and thereby primes the vacuoles for docking (Mayer and Wickner, 1997). The primed state, which is labile, is stabilized by LMA1 (Xu et al., 1997). Ypt7p (Haas et al., 1995) catalyzes docking, which in turn leads to fusion (Mayer and Wickner, 1997). The fusion reaction is regulated by the phosphorylation state of an unidentified phosphoprotein, as shown by its sensitivity to phosphatase inhibitors and ATP (Conradt et al., 1994; Haas et al., 1994). We have known little of how the priming, docking, and fusion steps are coupled in the overall vacuole fusion reaction; current studies of LMA1 (below) are providing insights into this mechanism.

LMA1 (*l*ow *M*r *a*ctivity #1), a novel trafficking factor, is a heterodimer of thioredoxin and proteinase B inhibitor 2 (I$^B_2$) (Xu and Wickner, 1996; Xu et al., 1997). These two cytosolic proteins (Maier et al., 1979) were not previously known to be associated. Both subunits are needed for efficient vacuole inheritance in vivo and for the LMA1 activity in cell extracts. In vivo studies showed that deletion of both yeast thioredoxin genes caused 47% of cells with large buds to have no bud vacuole (versus only 2.4% in wild type; Xu and Wickner, 1996), and deletion of I$^B_2$ caused 35% of cells with large buds to have no bud vacuole (versus 2.7% in wild type; Xu et al., 1997). Even those thioredoxin-deficient yeast that had a bud vacuole showed unusually small bud vacuole diameters (Xu and Wickner, 1996). Though we have only assayed the role of LMA1 in the final, fusion step of vacuole inheritance, its absence clearly affects the overall inheritance process, suggesting a coupling between the delivery of vacuolar material to the bud and fusion in the bud. Each LMA1 subunit acts via a novel mechanism, as the thioredoxin subunit is not acting through redox chemistry, and the protease B inhibitor activity of I$^B_2$ is not related to its ability to promote vacuole fusion (Slusarewicz et al., 1997; Xu et al., 1997). Using salt-washed vacuoles, the in vitro fusion reaction requires the addition of only two soluble proteins, Sec18p and LMA1 (Xu et al., 1997). Both Sec18p and LMA1 act early in the in vitro reaction. The action of Sec18p leads to Sec17p release from vacuoles and renders them labile for fusion. In this state, vacuoles have undergone no obvious morphological change but can not fuse without LMA1. This labile state of Sec18p-primed vacuoles is stabilized by LMA1 (Xu

*To whom correspondence should be addressed.

et al., 1997). We now report that LMA1 undergoes a biochemically defined cycle on vacuoles during the in vitro reaction. LMA1 employs Sec18p as its receptor for nucleotide-independent binding to vacuole membranes; the combination of the *sec18-1* mutation and deletion of the I^B_2 subunit of LMA1 causes a striking synthetic phenotype of vacuole fragmentation. After Sec18p hydrolyzes ATP, the bound LMA1 is transferred to a complex that includes the activated Vam3p (t-SNARE) to stabilize its labile, primed state. Although some reagents that block docking or fusion do not block the release of LMA1 from vacuoles, Microsystin-LR, a Ser/Thr protein phosphatase inhibitor, blocks both fusion and LMA1 release in an ATP-dependent manner. Thus, LMA1 links the action of Sec18p/Sec17p to the activation of Vam3p and finally to fusion.

Results

In vitro vacuole fusion is assayed with purified vacuoles from two different strains. Vacuoles isolated from BJ3505 lack proteases A and B and therefore contain a catalytically inactive form of proalkaline phosphatase, whereas vacuoles from DKY6281 contain normal vacuolar proteases but have no alkaline phosphatase due to a deletion in the *PHO8* gene. Upon fusion of vacuoles isolated from these two strains, the vacuolar lumenal contents are mixed, and the proteases from one fusion partner cleave the proalkaline phosphatase from the other to yield the active enzyme, allowing quantitative assay of fusion.

LMA1 Binds to Vacuoles and Is Released during a Normal Incubation

Homotypic fusion between salt-washed vacuoles requires the addition of only two purified soluble proteins, Sec18p and LMA1 (Xu et al., 1997). ATP hydrolysis by Sec18p triggers the release of Sec17p from the vacuoles (Mayer et al., 1996), creating an activated, labile state of vacuoles that is stabilized by LMA1 (Xu et al., 1997). To explore LMA1 function, salt-washed vacuoles were incubated with Sec18p, LMA1, and ATP at 25°C. At different times, aliquots were either transferred to ice and assayed for the fusion that had occurred or centrifuged to assay the bound LMA1 by SDS-PAGE and immunoblotting with anti-Trx1p antibodies. Fusion continued for 90 min (Figure 1A). Though salt-washed vacuoles initially had no LMA1 (Figure 1B, lane "vac"), LMA1 binds rapidly and is released after about 40 min (Figure 1B). LMA1 binding can even occur on ice (see Figures 2A and 5). The I^B_2 subunit of LMA1 behaved the same as Trx1p when the same blot was decorated with anti-I^B_2 antibodies (data not shown). LMA1 binds to salt-washed vacuoles at saturable high-affinity sites with a $K_d = 1.3 \times 10^{-8}$ M (Figure 1C).

To study LMA1 binding and release, we incubated vacuoles with Sec18p, LMA1, and ATP on ice. An excess of I^B_2 was added to the reaction either at 0 min (before LMA1 binding to vacuoles) or at 10 min (after LMA1 had bound to vacuoles), and the reactions were transferred to 25°C for a second incubation. When excess I^B_2 was added at the beginning of an incubation, it fully competed for LMA1 binding sites (Figure 2A, open squares).

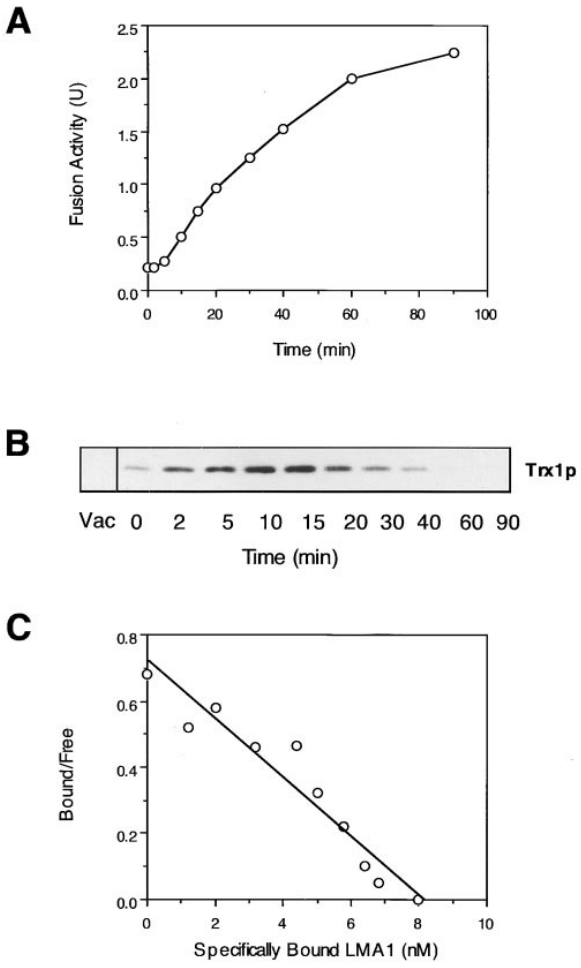


Figure 1. LMA1 Binding and Release

(A and B) For each timepoint, a fusion reaction corresponding to six standard reactions was incubated at 25°C. Aliquots equivalent to one standard reaction were used to measure fusion by alkaline phosphatase assay (A), and others, equivalent to five standard reactions, were used to measure bound LMA1 (B).

(C) Nonradioactive LMA1 (0–682 nM) was added to tubes containing a standard reaction and a constant amount of iodinated LMA1 (11 nM) prior to addition of vacuoles. After 15 min on ice, vacuoles were centrifuged (10,000 × g, 10 min, 4°C) and washed twice with 500 μl of 0% Ficoll containing 1× PIC, and the pellet radioactivity was determined. Scatchard analysis was as described in Hartl et al. (1990).

However, excess I^B_2 was unable to compete once LMA1 had bound to the vacuoles (Figure 2A, open circles). Thioredoxin did not compete with LMA1 for binding to vacuoles (data not shown). These data suggest that once LMA1 binds to saturable, high-affinity receptor(s) on vacuoles, it undergoes a specific reaction that regulates its release.

Since Vam3p (t-SNARE) and Nyv1p (v-SNARE) are required for vacuole inheritance and fusion (Nichols et al., 1997), we tested whether they are needed for LMA1 binding to vacuoles. Vacuoles were isolated from Δ*vam3*, Δ*nyv1*, Δ*vam3*Δ*nyv1*, and wild-type strains and the vacuolar components were analyzed by immunoblotting. The vacuolar protein Vma1p was used as a

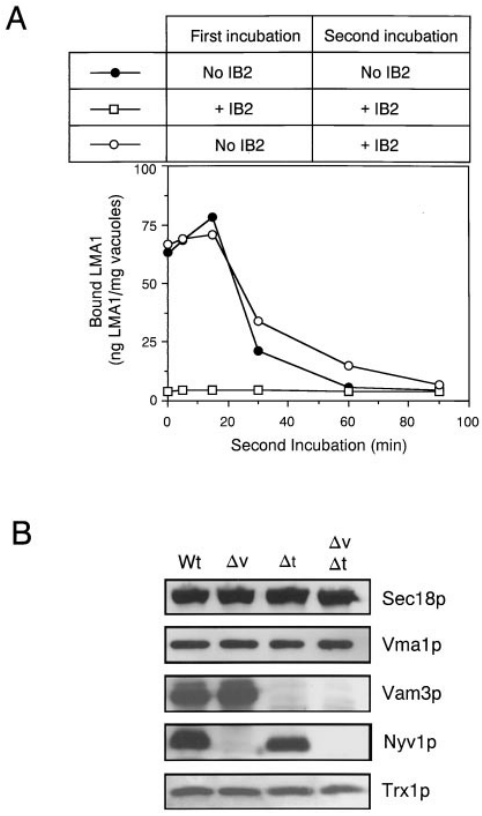


Figure 2. Characteristics of LMA1 Binding

(A) I^B_2 competes for initial LMA1 binding. For each timepoint, incubations equivalent to five standard fusion reactions were begun with 3 μg I^B_2 (open squares) or without (closed circles). After 10 min on ice, one group (open circles) was mixed with 3 μg I^B_2, and all were transferred to 25°C for a second incubation. At different times, vacuoles were recovered by centrifugation (10,000 × g, 10 min, 4°C), and the pellets were washed with 300 μl of 0% Ficoll. Bound LMA1 was assayed.

(B) Binding of LMA1 to vacuoles does not need Nyv1p or Vam3p. Vacuoles from BJ3505 (wt) and this strain with Δ*nyv1* (Δv), Δ*vam3p* (Δt), or Δ*nyv1*Δ*vam3* (Δv, Δt) were isolated as described in the Experimental Procedures. Vacuole proteins (60 μg) were analyzed by SDS-PAGE and immunoblotting.

marker to normalize the amount of vacuolar material. Both LMA1 and Sec18p appeared on wild-type, Δ*vam3*, Δ*nyv1*, or Δ*vam3*Δ*nyv1* vacuoles in comparable amounts (Figure 2B). This suggests that both Sec18p and LMA1 can employ receptors other than Vam3p/Nyv1p for binding to the vacuoles.

Sec18p Is the Vacuole Receptor for LMA1

Both Sec18p and LMA1 act at an early stage of in vitro vacuole fusion (Xu et al., 1997), and both are present on Δ*vam3* Δ*nyv1* vacuolar membranes (Figure 2B). To test whether Sec18p itself is the receptor for LMA1, we incubated vacuoles at 25°C with Sec18p, LMA1, and ATP, with no Sec18p or with anti-Sec18p antibodies. At different times, aliquots were centrifuged and the vacuole-bound LMA1 was assayed. LMA1 binding requires Sec18p and is blocked by anti-Sec18p antibodies (Figure 3A). This block is not due to nonspecific shielding of Sec18p/Sec17p sites by the antibodies, since anti-Sec17p (see Figure 6, below) and anti-Ypt7p antibodies (see Figure 7, below) do not inhibit LMA1 binding. These data indicate that Sec18p is the receptor for LMA1 binding to vacuoles. As a further test, we incubated ^{125}I-LMA1 with His_6-tagged Sec18p without ATP, precipitated the Sec18p with Ni-beads, and quantified the radioactivity on the beads by γ counting. ^{125}I-LMA1 bound to the beads via His_6-tagged Sec18p (Figure 3B, lanes 1 and 2), and this binding was sensitive to imidazole (lanes 3 and 4) but not to NaCl (lanes 5 and 6). Thus, Sec18p and LMA1 interact directly. This interaction is also seen through anti-Trx1p coimmunoprecipitation of Sec18p from detergent-solubilized vacuolar membranes (Figure 6, below). This binding serves to regulate Sec18p action, as both Sec17p and LMA1 are needed for activation of the ATPase activity of Sec18p (Figure 3C). Thus, Sec18p is the major receptor for LMA1 binding to vacuolar membranes, and the catalytic activity of Sec18p is regulated by its cochaperones, Sec17p and LMA1.

To test whether the physical (Figure 3B) and functional (Figure 3C) interactions between Sec18p and LMA1 seen in vitro are important for vacuole fusion in the intact cell, we examined the morphology of vacuoles in wild-type cells and isogenic strains with either the *sec18-1* temperature-sensitive mutation, with deletion of *PBI2*, the gene that encodes the small subunit of LMA1, or with both *sec18-1* and Δ*pbi2*. Deletion of I^B_2 abolishes LMA1 activity in vitro (Xu et al., 1997) and confers a partial vacuole inheritance defect. At 25°C, the vacuoles of Δ*pbi2* cells are indistinguishable from wild-type (Figures 4A and 4B). While occasional vacuole fragmentation is seen with the *sec18-1* mutation, suggesting diminished vacuole fusion, the *sec18-1,* Δ*pbi2* double mutant shows a striking synthetic phenotype of fragmented vacuoles (Figures 4A and 4B), confirming that these proteins function together in vacuole fusion in vivo.

Since deletion of *PBI2* alone had little effect on vacuole fusion in vivo, it seemed likely that there might be a homologous yeast protein with an overlapping function. Computer search revealed that p13, a 13 kDa protein encoded by yeast gene *YHR138c*, has 40% identity and 65% similarity with I^B_2 over the entire sequence of I^B_2 (Figure 4C). We constructed four isogenic strains: wild type, Δ*pbi2*, Δ*yhr138c*, and Δ*pbi2*Δ*yhr138c*. Neither deletion of PBI2 nor of YHR138c alone affected vacuole morphology, but the double deletion showed a striking synthetic phenotype of highly fragmented vacuoles (Figures 4D and 4E), indicating faulty vacuole fusion. While further studies of p13 are needed to establish its roles in Sec18p catalysis and organelle trafficking, the redundancies of the two yeast thioredoxins and of I^B_2 and p13 explains why recessive mutants in LMA1 were not discovered in the initial *sec* screens.

Bound LMA1 Is Transferred to a Complex with Vam3p (t-SNARE) upon ATP Hydrolysis by Sec18p

Though LMA1 binding to vacuoles does not require nucleotides (see Figures 5A and 6A, below), the release of bound LMA1 requires ATP hydrolysis (Figure 3A). To study this ATP requirement, vacuoles were incubated

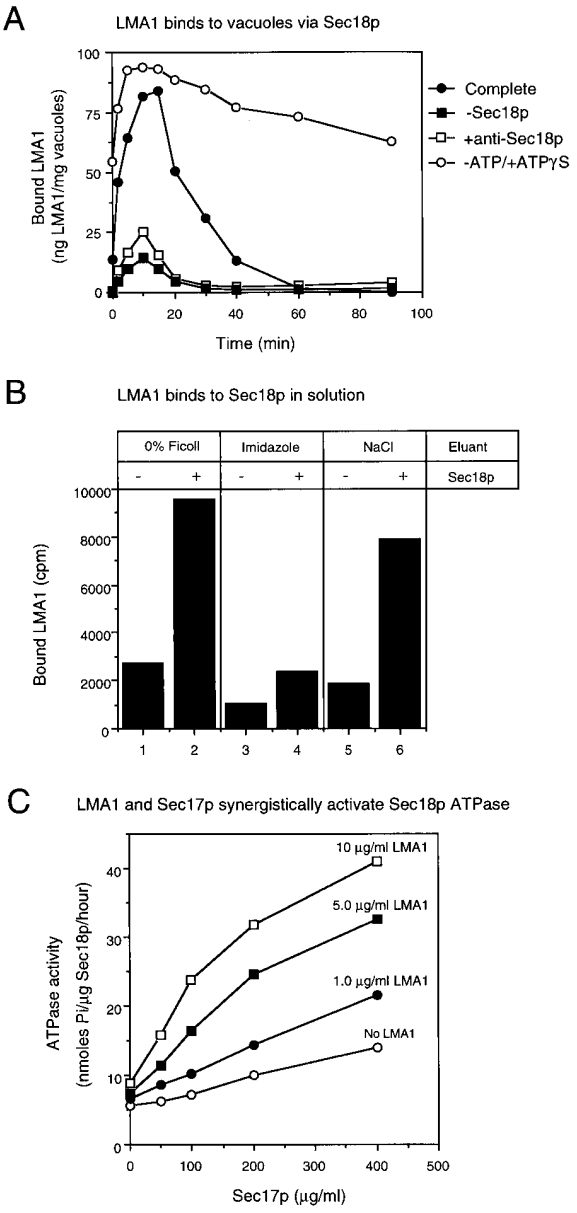


Figure 3. Sec18p Is the Membrane Receptor for LMA1

(A) For each timepoint, samples equivalent to five standard fusion reactions (closed circles), reactions without Sec18p (closed squares), reactions with 3.2 μg anti-Sec18p affinity-purified IgG (open squares), and reactions minus ATP but plus 1 mM ATPγS (open circles) were incubated at 25°C. At each time, samples were centrifuged and the bound LMA1 was assayed.

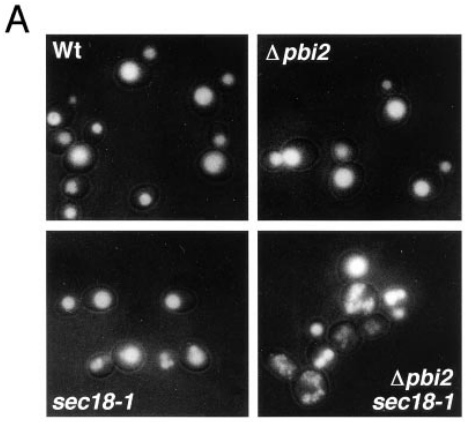
(B) ^{125}I-labeled LMA1 (10 ng) was mixed (lanes 2, 4, and 6) with 10 ng His_6-tagged Sec18p in 0% Ficoll buffer (10 mM Pipes-KOH [pH 6.8], 0.2 M sorbitol) on ice for 15 min. Ni-beads (20 μl) were then added and mixed on ice for 30 min. The beads were washed 3 times with 0% Ficoll and then once with 20 μl of either 0% Ficoll, 80 mM imidazole-Cl [pH7.5], or 80 mM NaCl, and twice with 500 μl of either 0% Ficoll, 40 mM imidazole-Cl [pH 7.5], or 40 mM NaCl. The beads were transferred to fresh tubes, and the bound radioactivity was determined by γ counting.

(C) ATPase activity was measured in 50 μl of reaction buffer (10 mM PIPES-KOH [pH 6.8], 200 mM sorbitol, 150 mM KCl, 0.5 mM $MnCl_2$, 1 mM ATP, 2 mM DTT, 0.1× PIC) in the presence of 2 μg/ml of purified His_6-Sec18p and the indicated concentrations of LMA1 and with Sec18p, LMA1, and with or without ATP at 25°C. LMA1 binding and release occured normally in the incubations with ATP (Figure 5A, lanes 1–3), but the LMA1 bound to vacuoles and was not released without ATP (lanes 4–6). In lane 7, the initial mixture containing vacuoles, Sec18p, LMA1, and ATP was incubated at 25°C for 10 min, then mixed with apyrase to hydrolyze the free ATP, and the reaction was continued at 25°C for a total incubation period of 45 min. The LMA1 that was bound at 10 min (lane 2) was released in the second incubation in the absence of free ATP (lane 7; compare to lanes 3 and 6). Thus, the ATP requirement for LMA1 release is fulfilled in the first 10 min of the incubation, well before the LMA1 is actually released from the vacuoles.

The in vitro vacuole fusion reaction becomes resistant to anti-Sec18p antibodies after only 10 min of incubation (Mayer et al., 1996). To determine whether Sec18p action is required for LMA1 release, vacuoles were incubated with Sec18p, LMA1, and ATP on ice or at 25°C for 10 min, and aliquots were centrifuged to measure the bound LMA1. Comparable amounts of LMA1 were bound to vacuoles after 10 min on ice or at 25°C (Figure 5B, lanes 1 and 4). The remaining portions received anti-Sec18p antibodies or the antibody buffer and were further incubated for a total time at 25°C of 45 min. Without anti-Sec18p antibodies, the bound LMA1 was released in the second incubation (Figure 5B, lanes 2 and 5). When vacuoles were first incubated on ice (Figure 5B, lanes 1–3) to allow only LMA1 binding (lane 1), LMA1 release during subsequent incubation at 25°C (lane 2) was blocked by anti-Sec18p antibodies (lane 3). However, if the vacuoles had first been incubated for 10 min at 25°C (lanes 4–6), the bound LMA1 (lane 4) had reached a reaction stage in which its release (lane 5) was not affected by antibody addition (lane 6). These data indicate that both Sec18p and ATP are required for the release of bound LMA1 from the vacuoles but that neither the ATP (Figure 5A) nor Sec18p (Figure 5B) is required after a 10 min incubation at 25°C for this release. Since ATP is required in the fusion reaction for hydrolysis by Sec18p (Mayer et al., 1996), we conclude that ATP hydrolysis transfers LMA1 from its Sec18p receptor to a second binding site on the vacuoles.

Since LMA1 is bound to Sec18p then undergoes Sec18 and ATP-dependent transfer in the first 10 min whereas LMA1 release takes 30–40 min, LMA1 may play a functional role during this interval. We have reported that LMA1 stabilizes primed vacuoles against a lability that is induced by Sec18p (Xu et al., 1997). Furthermore, Sec18p and ATP disassemble and activate the v/t-SNARE complex (Ungermann et al., 1998). We therefore asked whether LMA1 interacts with a SNARE complex component. Vacuoles were incubated with Sec18p, LMA1, and anti-Vam3p (t-SNARE) antibodies in the presence or absence of ATP and assayed for bound LMA1. As controls, one incubation was performed without antibodies and the other with anti-Sec17p antibodies. LMA1

His_6-Sec17p. Samples were incubated for 2 hr at 30°C and ATPase activity was determined as described (Lill et al., 1990). The background signal from reactions incubated with Sec18p and ATP on ice was subtracted.



B

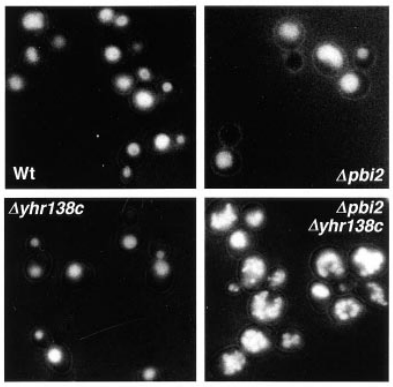
Strain	Vacuoles/cell or bud			
	0	1	2-5	Fragmented
	Per cent			
wild-type	1.2	97	1.5	0
Δ*pbi2*	6	90	4	0
sec18-1	0	57	36	7
Δ*pbi2 sec18-1*	2	9	7.8	81

C

```
IB2   1 MTKN-----FIVTLKKN---TPDVEAKKELDS--VHHAGGSILHEF 36
p13  16 MAQASSLSSYIVTFPKTDNMATDQNSI I EDVKKYVVD I GGKITHEY 61
          *::      :***: *.     :.* :: *  .  * . **.*.**:

IB2  37 D I IKGYT I KVPDVL H-LNKLKEKHNDVI------ENVEEDKEVHT-N 75
p13  62 SLIKGFTVDLPDSDQILDGLKERLSY IESEYGAKCNLEKDSEVHALN 108
          .:***:*:.:**   :*: ***: . :          *:*:*.***: *
```

D



E

Strain	Vacuoles/cell or bud			
	0	1	2-5	Fragmented
	Per cent			
wild-type	2	97	1	0
Δ*pbi2*	31	67	2	0
Δ*yhr138c*	13	87	0	0
Δ*pbi2* Δ*yhr138c*	15	2	0	83

Figure 4. Synthetic Phenotypes of Vacuole Fragmentation

(A) Wild-type, *sec18-1*, Δ*pbi2*, or *sec18-1*,Δ*pbi2* yeast (RSY 255α *SEC18*, *ura*3–52, *his*4–619 and RSY 271 α *sec18-1*, *ura*3–52, *his*4–619; also, each of these transformed to be Δ*pbi2*::URA3) were grown at 25°C in YPD medium to early log phase. Cells from 1 ml of each culture were harvested (3000 g, 5 min, 4°C), resuspended in 1 ml of YCM medium, and stained with FITC (Molecular Probes Inc., Eugene, OR) for fluorescence microscopy (Xu and Wickner, 1996). These strains were kindly provided by Dr. Charles Barlowe.

(B) Cells were grown and harvested, and vacuoles were stained and visualized as in (A). 334 wild-type cells, 314 Δ*pbi2* cells, 411 *sec18-1* cells, and 266 *sec18-1*,Δ*pbi2* cells were examined.

(C) The p13 protein encoded by YHR138c was found to be similar to I^{B}_{2} by scan of the yeast genome with the Blast program and alignment by ClustalW. The alignment of the protein sequence of I^{B}_{2} with residues 16–108 of p13 is shown.

(D) A synthetic phenotype of vacuole fragmentation in Δ*pbi2,* Δ*yhr138c* double mutant cells. Wild-type, Δ*pbi2*, Δ*yhr138c*, or Δ*pbi2,* Δ*yhr138c* yeast (XUY101 α *ura*3–5, *his*3–11, *leu*2–3, *can*^{R}, and transformants Δ*pbi2*::*URA*3, Δ*yhr*138c::*HIS*3, or both) were grown at 30°C in YCM to early log phase. Cells were harvested, and vacuoles were stained and visualized as in (A). Deletion of *YHR 138c* was by replacement with *HIS3* by standard methods (Ausubel et al., 1997). Primers (GIBCO/BRL) for PCR amplification of *HIS3* contained either 50 bases prior to the initiation codon of *YHR 138c* or 50 bases after the stop codon. The PCR products were transformed into either the wild-type or PBI2 deletion strain (Schu et al., 1991). Transformants were selected for growth on SD minus His plates, and the deletion of *YHR138c* was verified by PCR using primers 5′-GTGTTATTTGCAT ATCATATATAA-3′ (upstream of *YHR138c*), 5′-CGATGGAGAGAACA AATGGA-3′ (downstream of *YHR138c*), and 5′-GCCAGTAGGGCCT CTTTA-3′ (middle of *HIS3*).

(E) Cells were grown and harvested, and vacuoles were stained and visualized as in (A). 360 wild-type cells, 242 Δ*pbi2* cells, 238 Δ*p14 cells, and 288* Δ*pbi2*/Δ*p14* cells were examined.

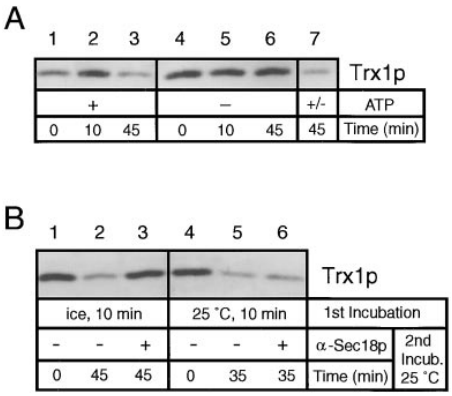


Figure 5. Sec18p and ATP Act Early in the LMA1 Cycle

(A) Fusion reactions equivalent to five standard reactions were incubated with ATP (lanes 1–3) or without ATP (lanes 4–6) at 25°C. In lane 7, vacuoles were incubated with ATP for 10 min at 25°C (equivalent to lane 2), then mixed with 10 U of apyrase (Sigma), and the incubation was continued at 25°C for 35 min (45 min total). At indicated times, vacuoles were assayed for bound LMA1.

(B) Samples equivalent to five standard fusion reactions were incubated on ice (lanes 1–3) or at 25°C (lanes 4–6) for 10 min. Reactions then received either 3.2 μg affinity-purified anti-Sec18p IgG (lanes 3 and 6), or buffer (lanes 1, 2, 4, and 5). Incubations were continued at 25°C for either 0 min (lanes 1 and 4), 35 min (lanes 5 and 6), or 45 min (lanes 2 and 3), followed by measuring bound LMA1.

was bound to and released from vacuoles in the complete incubation (Figure 6A, open circles) and in the incubation with anti-Sec17p antibodies (Figure 6A, closed circles). With anti-Vam3p antibodies in the absence of ATP, LMA1 bound to the vacuoles but was not released

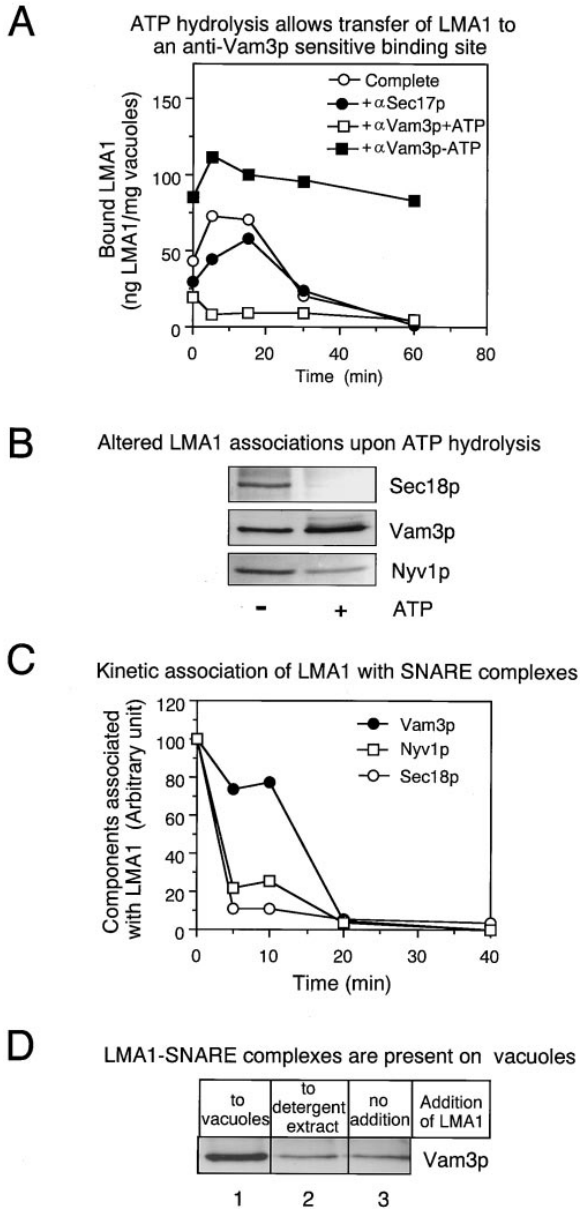


Figure 6. LMA1 Is Transferred to the t-SNARE Vam3p after ATP Hydrolysis by Sec18p

(A) ATP-dependent transfer of LMA1 to an anti-Vam3p-sensitive binding site. Samples equivalent to five standard fusion reactions were incubated at 25°C with either no addition (open circles), 3.2 μg of affinity-purified α-Sec17p IgG (closed circles), 2.4 μg α-Vam3p IgG (open squares), or 2.4 μg α-Vam3p IgG without ATP (closed squares). At the indicated times, vacuole-bound LMA1 was assayed.

(B) Coimmunoprecipitation of SNARE complexes with anti-Trx1p antibodies. Freshly isolated vacuoles (140 μg) that had not been salt-washed were incubated in the fusion reaction buffer with, or without, ATP at 25°C for 10 min. Vacuoles were then reisolated (10,000 × g, 10 min, 4°C) and washed with 500 μl of 0% Ficoll with 1× PIC. Vacuolar proteins were detergent extracted, immunoprecipitated with immobilized anti-Trx1p antibodies, separated by SDS-PAGE, and immunoblotted with anti-Sec18p, anti-Vam3p, and anti-Nyv1p.

(C) Kinetics of association of LMA1 with the SNARE complex components. Freshly isolated, non-salt-washed vacuoles (140 μg) were incubated in the fusion reaction buffer with ATP at 25°C. At indicated times, vacuoles were reisolated and analyzed as in (B). Vacuolar proteins were detergent extracted, immunoprecipitated with immobilized anti-Trx1p antibodies, separated by SDS-PAGE, and immunoblotted with anti-Sec18p, anti-Vam3p, and anti-Nyv1p.

(D) LMA1–Vam3p complexes are present on vacuoles. Salt-washed vacuoles (140 μg) were incubated in the fusion reaction buffer with ATP and Sec18p at 25°C for 10 min. The vacuoles were then reisolated, washed, and detergent extracted as in (B). Lane 1, LMA1 was added to vacuoles before the incubation. Lane 2, LMA1 was added to the detergent extract. Lane 3, no LMA1 was added. Detergent extracts were mixed with immobilized anti-Trx1p antibodies, and bound proteins were separated by SDS-PAGE and immunoblotted with anti-Vam3p antibodies.

(Figure 6A, closed squares), demonstrating that the anti-Vam3p antibodies do not block the initial binding of LMA1. This is consistent with the observation that normal levels of LMA1 are bound to vacuoles that lack one or both SNAREs (Figure 2B). Strikingly, the anti-Vam3p antibodies completely block the stable association of LMA1 with the vacuoles when ATP is present (Figure 6A, open squares). This suggests that LMA1 initially binds to vacuoles at Sec18p (Figure 3) and is transferred by Sec18p and ATP action (Figure 5) to a complex that includes the t-SNARE Vam3p. When Vam3p is complexed with antibodies (Figure 6A), there is no second binding site for LMA1 on the vacuoles, and it is released into the supernatant after the Sec18p and ATP reaction.

Since Sec18p activates Vam3p (Ungermann et al., 1998), LMA1 may stabilize labile vacuoles through its ATP-dependent release from Sec18p and transfer to a complex that includes this vacuolar t-SNARE. This working model was tested by coimmunoprecipitation. Vacuoles were incubated with or without ATP at 25°C for 15 min, extracted with detergent, and immunoprecipitated with immobilized anti-Trx1p antibodies. Without exposure to ATP, the anti-Trx1p antibodies coimmunoprecipitated Sec18p, the v-SNARE Nyv1p, and the t-SNARE Vam3p (Figure 6B). This confirms that LMA1 physically interacts with Sec18p (Figure 3B) and suggests that Sec18p, Nyv1p, Vam3p, and LMA1 are together in a complex prior to the action of Sec18p and ATP. When the vacuoles had been incubated with ATP, anti-Trx1p antibodies coimmunoprecipitated the t-SNARE Vam3p from detergent extracts (Figure 6B). However, there was no detectable Sec18p in the immunoprecipitate, and a reduced amount of Nyv1p remained in the LMA1–Vam3p complex (Figure 6B). Thus, as Sec18p hydrolyzes ATP and disassembles the SNARE complex (Ungermann et al., 1998), Sec18p-bound LMA1 is transferred to a complex that includes Vam3p. A quantitative kinetic analysis showed that LMA1 remained in a complex that includes Vam3p after the initial SNARE complex was disrupted (Figure 6C). After 20 min, as the LMA1 is released from the vacuoles, its association with Vam3p is perforce lost as well.

To establish that these LMA1–SNARE complexes had formed on the vacuoles and not simply in the detergent extract, vacuoles were either incubated with LMA1 and ATP for 10 min prior to detergent extraction and assay of LMA1–Vam3p coprecipitation (Figure 6D, lane 1), incubated for 10 min and only mixed with LMA1 after the vacuoles were detergent-solubilized (lane 2), or not provided exogenous LMA1 (lane 3). LMA1 clearly associates with the Vam3p complex on the vacuole and not

merely in detergent solution. We conclude that LMA1 is transferred from Sec18p in the SNARE complex to a subcomplex that contains Vam3p and suggest that it functions to stabilize the labile primed vacuoles (Xu et al., 1997) via its interaction with this Vam3p complex.

The Release of LMA1 and Vacuole Fusion Are Mediated by an Unknown Phosphoprotein

The release of LMA1 from the vacuoles (Figure 1) is not affected by antibodies to Sec17p (Figure 6), though these antibodies block docking and fusion (Mayer et al., 1996; Mayer and Wickner, 1997). Furthermore, neither Gdi1p nor antibodies to Ypt7p, which block docking, nor GTPγS, which blocks fusion (Mayer and Wickner, 1997), affects the binding and release cycle of LMA1 (Figure 7A). These data suggest that neither docking nor fusion per se causes LMA1 release. Rather, LMA1 release may be regulated by a factor which itself governs the docking or fusion reaction. In line with this concept, we find that Microcystin-LR, a potent inhibitor of protein phosphatases 1 and 2A (Honkanen et al., 1990), which blocks vacuole fusion (Conradt et al., 1994; Mayer et al., 1996; Mayer and Wickner, 1997), also inhibits the release of LMA1 (Figure 7A).

To investigate how Microcystin-LR inhibits LMA1 release, vacuoles were incubated with Sec18p, LMA1, and ATP at 25°C for 20 min to allow priming and docking, then reisolated and resuspended for a second incubation at 25°C for 70 min. After the second incubation, each reaction was divided into two portions. One was assayed for the fusion that had occurred (Figure 7B) and the other for LMA1 release (Figure 7C). The lanes a and b in Figure 7B show an ice control and a complete vacuole fusion reaction. After the first incubation (25°C, 20 min), ~25% of total fusion had occurred (Figure 7B, lane 1 versus lane b) and LMA1 had bound to the vacuoles (Figure 7C, lane 1). In the second incubation, neither ATP nor apyrase affects either the fusion reaction (Figure 7B, lanes 2, 4, and 6) or LMA1 release (Figure 7C, lanes 2, 4, and 6), in agreement with earlier observations (Figure 5; Conradt et al., 1994; Xu et al., 1997). When ATP was added, ensuring a high ATP concentration, Microcystin-LR blocked both fusion (Figure 7B, lane 5) and LMA1 release (Figure 7C, lane 5). Microcystin-LR partially inhibited both fusion (Figure 7B, lane 3) and LMA1 release (Figure 7C, lane 3) under conditions of low ATP concentration. Microcystin-LR inhibition of fusion and LMA1 release was totally lost upon addition of apyrase (Figure 7B and 7C, lane 7) to hydrolyze all the free ATP. These data suggest that both LMA1 release and fusion are regulated by an unknown phosphoprotein(s) and require an ATP-dependent phosphatase activity to keep this target in a dephosphorylated state. The observation that GTPγS does not block LMA1 release (Figure 7A), though it does block fusion (Haas et al., 1994; Mayer et al., 1996), suggests that LMA1 release occurs before or in parallel with a reaction that is sensitive to GTPγS. Since, as shown in Figure 1, fusion continues for 90 min or more while the LMA1 cycle is completed within 30–40 min, the release of LMA1 may occur earlier than fusion per se and LMA1 release may even trigger fusion.

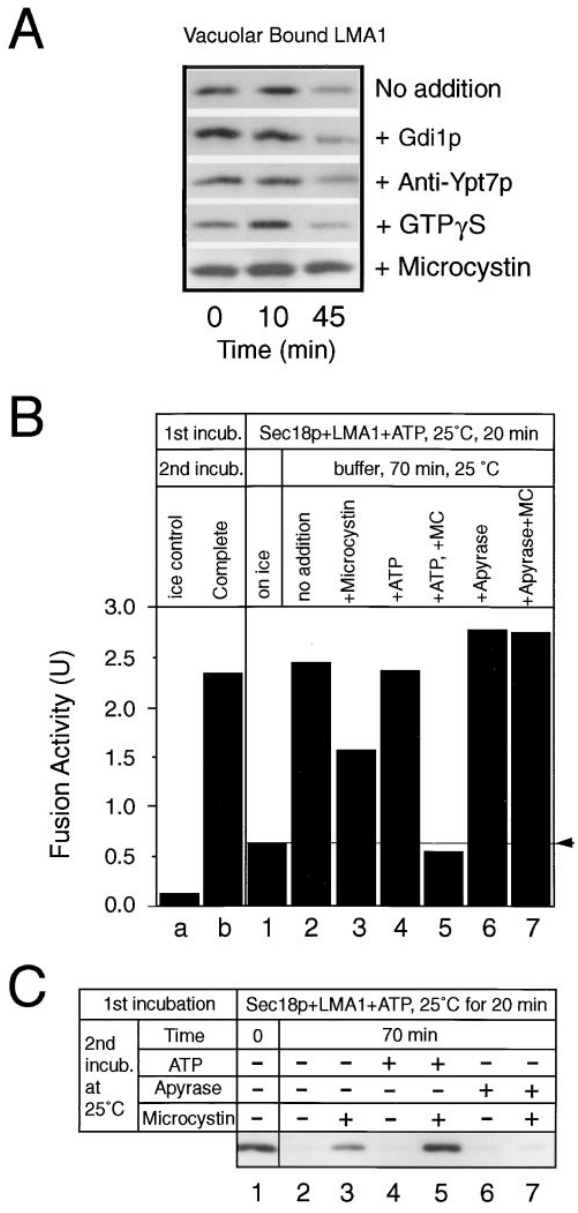


Figure 7. Regulation of Vacuole Fusion and LMA1 Release

(A) Samples equivalent to five standard fusion reactions were incubated at 25°C with Gdi1p (120 μg/ml), anti-Ypt7p (60 μg/ml), GTPγS (2 mM), or Microcystin-LR (10 μM) where indicated. At the indicated times, vacuoles were assayed for bound LMA1.

(B and C) A standard fusion reaction was incubated on ice (lane a) or at 25°C (lane b) for 90 min and the fusion activity was measured. Samples (lanes 1–7) equivalent to six standard fusion reactions were incubated at 25°C for 20 min (first incubation). The vacuoles were then reisolated (10,000× g, 5 min, 4°C), resuspended as indicated, and divided into two groups. In one group (B), each sample contained vacuoles equivalent to one standard fusion reaction. In the other group (C), each sample contained vacuoles equivalent to five standard fusion reactions. The reactions were incubated at 25°C for 70 min (second incubation) and then assayed for fusion activity (B) or for bound LMA1 (C). The fusion that had occurred after the first incubation is indicated in lane 1 of panel B, and the bound LMA1 after the first incubation is shown in lane 1 of (C). Conditions for lanes 2–7 are: Microcystin-LR (MC, 10 μM), ATP (1 mM with an ATP regenerating system), or apyrase (33.3 U/ml). All reactions contained 0.1 × PIC.

Discussion

LMA1 is a novel trafficking factor that is required for vacuole inheritance and fusion, both in vivo and in vitro (Xu and Wickner, 1996; Xu et al., 1997). To fulfill its function, LMA1 binds to vacuoles and is released in a defined cycle. LMA1, using Sec18p as its receptor, binds to the vacuole membrane at high-affinity sites comprised of Sec18p, Sec17p, Vam3p, and Nyv1p, the vacuole "SNARE complex." Upon ATP hydrolysis by Sec18p, promoted by Sec17p and LMA1, this complex is disassembled (Ungermann et al., 1998), Sec17p is released (Mayer et al., 1996), and LMA1 is transferred to its second binding site, a complex that includes the activated t-SNARE Vam3p, to stabilize the primed and labile vacuoles (Xu et al., 1997). We suggest that the lability of primed vacuoles for fusion may largely be a lability of the activated t-SNARE and that a major function of LMA1 is to stabilize this SNARE. After v/t-SNARE pairing and prior to fusion, an unidentified phosphoprotein is dephosphorylated by a (Microcystin-LR sensitive) phosphatase, allowing the release of LMA1 from the vacuoles, completing its cycle. Release may even initiate the fusion of vacuoles, though further studies are required to test this concept. Clearly the catalytic cycle of LMA1 explains how the priming action of Sec18p, Sec17p, and ATP is coupled to the stabilization of the activated t-SNARE.

The LMA1 cycle is intimately linked to the action of Sec18p, as revealed by the sensitivity of successive reaction stages to antibodies to Sec18p. Since Sec18p is the membrane receptor for LMA1, the initial binding of LMA1 can be blocked by anti-Sec18p antibodies. After LMA1 has bound to the vacuoles, anti-Sec18p antibodies block the ATP-dependent LMA1 transfer and Sec17p release (Mayer et al., 1996). After Sec18p hydrolyzes ATP and transfers LMA1 to Vam3p, the rest of the LMA1 cycle becomes resistant to anti-Sec18p antibodies and no longer needs ATP. Thus, the Sec18p functions include: (1) Sec17p release from the vacuoles, (2) disassembly of v/t-SNARE complexes, (3) activation of the t-SNARE, and (4) transfer of LMA1 to its second binding site, the t-SNARE Vam3p complex.

The activity of LMA1 for vacuole fusion depends on neither the redox activity of its thioredoxin subunit nor the proteinase B inhibitory activity of its I^{B}_{2} subunit (Slusarewicz et al., 1997; Xu et al., 1997). Working together with Sec17p, LMA1 stimulates Sec18p ATPase activity (Figure 3C) and rapidly stabilizes the Sec18p-primed, labile vacuoles at an early reaction stage (Xu et al., 1997). The data presented here further demonstrate that LMA1 functions through interactions with the activated t-SNARE Vam3p (Ungermann et al., 1998). Recent studies have shown that LMA1 is required for the Sec18p and ATP-mediated disassembly of a purified, functional SNARE complex (K. S. and W. W., unpublished data). This activation event probably involves a conformational change of the t-SNARE (Hanson et al., 1995, 1997) after its dissociation from other components such as Sec18p, Sec17p, and Nyv1p. The lability of primed vacuoles suggests that this activated form of the t-SNARE is very transient. However, it can be "captured" by LMA1 at or just after Sec18p/Sec17p action (Xu et al., 1997). How does LMA1 act to stabilize the activated t-SNARE? Yeast protein disulfide isomerase (PDI) has both thioredoxin-like redox activity and a chaperone-like activity (Freedman et al., 1994). Interestingly, the function of PDI that is essential for cell viability does not reside in its isomerase activity but rather depends on its chaperone-like peptide-binding domain (LaMantia and Lennarz, 1993). However, the chaperone activity of PDI is independent of its thioredoxin-like active site (Quan et al., 1995; Yao et al., 1997). Thioredoxin binds to coliphage T7 polymerase like a "cap," conferring processivity to T7 replication without redox chemistry (Huber et al., 1986). Since the redox activity of the thioredoxin subunit of LMA1 is also not required for vacuole fusion and since LMA1 binds to and stabilizes the activated t-SNARE, we suggest that LMA1 may also act in a chaperone-like fashion, preserving the active t-SNARE conformation.

LMA1 is released from vacuoles at fusion. Though little is known of the proteins that catalyze the fusion of docked vacuoles, fusion is inhibited by GTPγS (Haas et al., 1994; Mayer et al., 1996), suggesting regulation by a G protein other than Ypt7p. Though LMA1 is released from the vacuoles at a late stage of the reaction (Figure 1), release is not blocked by GTPγS, nor even by anti-Sec17p or Gdi1p (Figures 6 and 7), agents that block both docking and fusion (Mayer et al., 1996; Mayer and Wickner, 1997). Nevertheless, LMA1 release is regulated, as it is blocked by Microcystin-LR, and this block is only seen with ATP (Figure 7). The block to fusion by Microcystin-LR shows a similar ATP requirement (Figure 7). Fusion is not needed for LMA1 release; rather, we suggest that LMA1 release regulates fusion and that this release requires the dephosphorylated state of a (unidentified) phosphoprotein. Microcystin-LR then blocks the responsible phosphatase; recent studies (G. Eitzen and W. W., unpublished data) indicate that this phosphatase may belong to a class (Vogel et al., 1993) that is activated by ATP and a kinase through its own phosphorylation.

Is LMA1 a general factor of vesicular trafficking or specific for the vacuole? Since LMA1 interacts with both Sec18p and a t-SNARE and since a similar NSF/SNAP/SNARE system is required for many membrane trafficking pathways, LMA1 or its functional equivalents such as LMA2 (Slusarewicz et al., 1997) and p13 may also have a necessary role in other intracellular trafficking events. Recent studies have shown that LMA1 is also required for ER-to-Golgi trafficking (Barlowe, 1997). Using a reconstituted system, Barlowe has found that LMA1 plays a functional role in the fusion of ER-derived vesicles to Golgi membranes after Sec18p action, the same order as is seen in vacuole fusion (Barlowe, 1997). LMA1, a novel trafficking factor, is therefore required both for homotypic vacuole fusion as well as for heterotypic ER-to-Golgi membrane traffic. LMA1 or its functional homologs may be general factors in intracellular membrane trafficking.

Experimental Procedures

Strains and Reagents

Saccharomyces cerevisiae strains were BJ3505 (MAT**a** *pep4::HIS3 prb1-Δ1.6R HIS3 lys2–208 trp1-Δ101 ura3–52 gal2 can*) and

DKY6281 (MATα *leu2–3 leu2–112 ura3–52 his3-Δ200 trp1-Δ901 lys2–801 suc2-Δ9 pho8::TRP1*) from Dr. D. Klionsky, University of California, Davis, CA. BJ-Δvam3, BJ-Δnyv1, and BJ-Δvam3/Δnyv1 were described in Nichols et al. (1997).

Reagents were as described (Xu and Wickner, 1996; Xu et al., 1997). LMA1, I^B_2, Gdi1p, and His-tagged Sec17p and Sec18p were purified as described (Haas et al., 1995; Haas and Wickner, 1996; Xu and Wickner, 1996; Slusarewicz et al., 1997).

Recombinant LMA1 was purified from *E. coli*. Yeast *TRX1* and *PBI2* genes were isolated by PCR. Primers (GIBCO/BRL) for PCR amplification of *TRX1* were 5′-ATAGAAGACTAGACACCTCGATAC-3′ and 5′-CAAACACAGTATAGAAACACAATATATC-3′. Primers for PCR amplification of *PBI2* were 5′-CATCGGGAACTTCTTTCC-3′ and 5′-GTGTTTGCGTTTATCTCAGG-3′. PCR products were subcloned into pBAD22 (Guzman et al., 1995) and transformed into *E. coli* BL21 (DE3). *E. coli* cells expressing yeast *TRX1* and *PBI2* were grown in LB medium with 100 μg/ml ampicillin to OD = 0.3, then arabinose was added to 2% and the culture continued for 2 hr. Cells were harvested (5000 × g, 4°C, 10 min), resuspended in 50 mM Tris-Cl (pH 7.5), lysed by French Press (5000 psi, three passages), and centrifuged (260,000 × g, 2 hr, 2°C). Clarified lysate (100 ml) was applied to a 10 cm × 54 cm (4 l) Sephacryl S100HR column equilibrated with buffer C (20 mM Tris-OAc [pH 8.3], 5 mM $MgCl_2$, 0.2 mM $MnCl_2$) and the column was eluted with buffer C. Fractions (20 ml) were analyzed by SDS-PAGE and LMA1 activity assay. Fractions containing LMA1 were pooled (230 ml), adsorbed to Mono Q 10/10 (Pharmacia), and eluted with a KOAc gradient (0–500 mM; 120 ml) in buffer C at 4°C.

Antibody Production and Purification

Antibodies against Trx1p or I^B_2 were raised in rabbits using His_6-tagged Trx1p and I^B_2 purified from *E. coli* (Muller, 1995; Slusarewicz et al., 1997). Affinity purification of these antibodies and antibodies to Sec17p, Sec18p, Vam3p, Nyv1p, and Ypt7p was described in Haas and Wickner (1996).

Vacuole Preparation

Vacuoles were isolated from BJ3505 and DKY6281 (Xu and Wickner, 1996). To prepare salt-washed vacuoles, vacuoles were adjusted to 0.3 mg/ml with 0% Ficoll buffer (10 mM Pipes-KOH, [pH 6.8], 200 mM sorbitol). Equal amounts of vacuoles from BJ3505 and DKY6281 were mixed, and 3 M KCl and 3 M KOAc were added to final concentrations of 167 mM and 333 mM, respectively. Aliquots of 200 μl were incubated at 30°C for 10 min, chilled on ice for 1 min, and centrifuged (10,000 rpm, 90 s, 4°C, microfuge). The pellets were covered with 160 μl of 0% Ficoll buffer and stored at −20°C for 24 hr, then transferred to −80°C.

Vacuole Fusion Assay

For in vitro vacuole fusion (Haas et al., 1994; Xu et al., 1997), standard 30 μl reactions contained 3 μg vacuoles from BJ3505, 3 μg vacuoles from DKY 6281, 200 mM sorbitol, 20 mM Pipes-KOH (pH 6.8), 150 mM KOAc, 5 mM $MgCl_2$, an ATP regenerating system (1 mM ATP, 40 mM creatine phosphate, and 0.2 mg/ml creatine phosphokinase), 0.1× PIC (proteinase inhibitor cocktail; Xu and Wickner, 1996), 10 ng His_6-tagged Sec18p, and 10 ng LMA1 and were incubated at 25°C for 90–120 min. One unit fusion activity is 1 μmol *p*-nitrophenol produced at 30°C/min/μg of BJ3505 vacuolar protein.

Assay of Bound LMA1

A fusion reaction equivalent to five standard reactions was chilled on ice after an incubation at indicated conditions and times. Vacuoles were reisolated (5 min, 10,000 g, 4°C), resuspended in 300 μl of "washing buffer" (0% Ficoll buffer with 1× PIC and 0.5 mM PMSF), reisolated, resuspended in 25 μl of washing buffer, and transferred to a fresh tube. 5× SDS-PAGE sample buffer (10 μl) was added, and the sample was heated at 95°C for 3 min followed by "High Tris" SDS-PAGE and immunoblot (Xu and Wickner, 1996). Bound LMA1 was quantified by densitometry and normalized to a standard of pure LMA1.

Immunoprecipitation

Protein A-Sepharose CL-4B (Pharmacia) was equilibrated with 1× PBS. Antiserum (1 ml) was incubated with 0.5 ml of protein A beads on a rocker for 2 hr at room temperature (RT). The beads were collected (10,000 g, 30 s) and twice suspended in 10 vol of 0.2 M sodium borate (pH 9.0), then resuspended in 10 vol of 0.2 M sodium borate (pH 9.0), and dimethylpimelimidate (solid) was added to 20 mM. After incubation on the rocker for 30 min at RT, the beads were sedimented and suspended in 10 vol of 0.2 M ethanolamine–Cl (pH 8.0) and incubated for 2 hr at RT with gentle mixing. Beads were washed once with 1× PBS, once with PBS containing 500 mM NaCl, twice with 1× PBS, once with 2% SDS in 100 mM Tris-Cl (pH 8.3), twice with 1% Triton-100 in 100 mM Tris-Cl (pH 8.3), and three times with 50 mM Tris-Cl (pH 7.5), 50 mM NaCl.

Vacuoles were reisolated (10 min, 10,000 g, 4°C) from a fusion reaction corresponding to 140 μg vacuolar protein, resuspended in 700 μl of 0% Ficoll buffer, resedimented, resuspended in 1 ml of solubilization buffer (10 mM HEPES [pH 7.2], 50 mM NaCl, 5 mM EDTA, 1× PIC, 0.5 mM PMSF, 10% glycerol, 0.2 M sorbitol, and 0.5% digitonin), and incubated on ice for 20 min. Insoluble material was removed by centrifugation (14,000 g, 15 min, 4°C), and 10% of the extract was saved as starting material. To the remainder, 40 μl of a suspension (1:1) of the bead-immobilized antibody (above) was added and mixed on a rocker at 4°C overnight. The beads were washed five times with 20 mM Tris-Cl (pH 7.5), 50 mM NaCl. Bound material was eluted with 40 μl of 2% SDS at 95°C for 5 min, mixed with 15 μl of 5× SDS sample buffer and analyzed by 12% SDS-PAGE and immunoblotting.

Other Methods

Iodination of LMA1 was performed as described in Economou and Wickner (1994) and assayed by γ counting. Protein was measured with Bradford reagent (Bio-Rad) with a BSA standard. SDS-PAGE, High-Tris SDS-PAGE, and immunoblot analysis were described in Xu and Wickner (1996).

Acknowledgments

Z. X. dedicates this paper to Drs. Shinan Feng and Shusheng Xu. We thank Dr. Charles Barlowe for the generous gift of unpublished strains; Drs. Paul Slusarewicz, Albert Haas, Dan Klionsky, Ben Nichols, Hugh Pelham, Eric Muller, and Dieter Wolf for reagents, plasmids, strains, and antibodies; Drs. Christian Ungermann, Dan Klionsky, Charles Barlowe, Jerry Eichler, Franck Duong, and Gary Eitzen for discussions; Albert Price for ^{125}I-LMA1 labeling; Kim Seefeld for LMA1 plasmid construction; Duane Compton for photography guidance; and Marilyn Rice Leonard for excellent technical assistance. K. S. is supported by a JSPS fellowship. Z. X. was supported in part by the Autoimmunity and Connective Tissue Biology Program (T32AR07576). This work was supported by a grant from NIGMS.

Received September 12, 1997; revised April 23, 1998.

References

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K. (1997). Short Protocols in Molecular Biology, 3rd Edition (New York: John Wiley and Sons).

Barlowe, C. (1997). Coupled ER to Golgi transport reconstituted with purified cytosolic components. J. Cell Biol. *139*, 1097–1108.

Conradt, B., Shaw, J., Vida, T., Emr, S., and Wickner, W. (1992). *In vitro* reactions of vacuole inheritance in *Saccharomyces cerevisiae*. J. Cell Biol. *119*, 1469–1479.

Conradt, B., Haas, A., and Wickner, W. (1994). Determination of four biochemically distinct, sequential stages during vacuole inheritance in vitro. J. Cell Biol. *126*, 99–110.

Economou, A., and Wickner, W. (1994). SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. Cell *78*, 835–843.

Ferro-Novick, S., and Jahn, R. (1994). Vesicle fusion from yeast to man. Nature *370*, 191–193.

Freedman, R.B., Hirst, T.R., and Tuit, M.F. (1994). Protein disulfide

isomerase: building bridge in protein folding. Trends Biochem. Sci. *19*, 331–336.

Gomes de Mesquita, D.S., ten Hoopen, R., and Woldringh, C.L. (1991). Vacuolar segregation to the bud of *Saccharomyces cerevisiae*: an analysis of morphology and timing in the cell cycle. J. Gen. Microbiol. *137*, 2447–2454.

Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose *PBAD* promoter. J. Bacteriol. *177*, 4121–4130.

Haas, A., and Wickner, W. (1996). Homotypic vacuolar fusion requires Sec17p (yeast α-SNAP) and Sec18p (yeast NSF). EMBO J. *15*, 3296–3305.

Haas, A., Conradt, B., and Wickner, W. (1994). G-protein ligands inhibit in vitro reactions of vacuole inheritance. J. Cell Biol. *126*, 87–97.

Haas, A., Scheglmann, D., Lazar, T., Gallwitz, D., and Wickner, W. (1995). The GTPase Ypt7p is required on both partner vacuoles for the homotypic fusion step of vacuole inheritance. EMBO J. *14*, 5258–5270.

Hanson, P.I., Otto, H., Barton, N., and Jahn, R. (1995). The N-ethylmaleimide-sensitive fusion protein and α-SNAP induce a conformational change in syntaxin. J. Biol. Chem. *270*, 16955–16961.

Hanson, P.I., Roth, R., Morisaki, H., Jahn, R., and Heuser, J.E. (1997). Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. Cell *90*, 523–535.

Hartl, F.-U., Lecker, S., Schiebel, E., Hendrick, J.P., and Wickner, W. (1990). The binding of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* membrane. Cell *63*, 269–279.

Hay, J.C., and Scheller, R. (1997). SNAREs and NSF in targeted membrane fusion. Current Biol. *9*, 505–512.

Honkanen, R.E., Zwiller, J., Moore, R.E., Daily, L., Khatra, B.S., Dukelow, M., and Boynton, A.L. (1990). Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatase. J. Biol. Chem. *265*, 19401–19404.

Huber, H.E., Russel, M., Model, P., and Richardson, C.C. (1986). Interaction of mutant thioredoxins of *Escherichia coli* with Gene 5 protein of Phage T7. The redox capacity of thioredoxin is not required for stimulation of DNA polymerase activity. J. Biol. Chem. *261*, 15006–15012.

LaMantia, M., and Lennarz, W. (1993). The essential function of yeast protein disulfide isomerase does not reside in its isomerase activity. Cell. *74*, 899–908.

Lill, R., Downhan, W., and Wickner, W. (1990). The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. Cell *60*, 271–280.

Maier, K., Muller, H., and Holzer, H. (1979). Purification and molecular characterization of two inhibitors of yeast proteinase B. J. Biol. Chem. *254*, 8491–8497.

Mayer, A., and Wickner, W. (1997). Docking of yeast vacuoles is catalyzed by the ras-like GTPase Ypt7p after symmetric priming by Sec18p (NSF). J. Cell Biol. *136*, 307–317.

Mayer, A., Wickner, W., and Haas, A. (1996). Sec18p (NSF)-driven release of Sec17p (α-SNAP) precedes docking and fusion of yeast vacuoles. Cell *85*, 83–94.

Muller, E.G.D. (1995). A redox-dependent function of thioredoxin is necessary to sustain a rapid rate of DNA synthesis. Arch. Biochem. Biophys. *318*, 356–361.

Nichols, B.J., Ungermann, C., Pelham, H.R.B., Wickner, W., and Haas, A. (1997). Homotypic vacuolar fusion mediated by v- and t-SNAREs. Nature *387*, 199–202.

Nicolson, T.A., Weisman, L.S., Payne, G.S., and Wickner, W. (1995). A truncated form of the Pho80 cyclin redirects the Pho85 kinase to disrupt vacuole inheritance in *S. cerevisiae*. J. Cell. Biol. *130*, 835–845.

Pfeffer, S.R. (1996). Transport vesicle docking: SNARE and associates. Annu. Rev. Cell Biol. Dev. Biol. *12*, 441–461.

Quan, H., Fan, G., and Wang, C. (1995). Independence of the chaperone activity of protein disulfide isomerase from its thioredoxin-like active site. J. Biol. Chem. *270*, 17078–17080.

Raymond, C.K., Roberts, C.J., Moore, K.E., Howald, I., and Stevens, T.H. (1992). Biogenesis of the vacuole in *Saccharomyces cerevisiae*. Int. Rev. Cytol. *139*, 59–120.

Rothman, J.E. (1994). Mechanisms of intracellular membrane fusion. Nature *372*, 55–63.

Schu, P., Suarez Rendueles, P., and Wolf, D.H. (1991). The proteinase yscB inhibitor (*PBI2*) gene of yeast and studies on the function of its protein product. Eur. J. Biochem. *197*, 1–7.

Shaw, J., and Wickner, W. (1991). *vac2*: a yeast mutant which distinguishes vacuole segregation from Golgi-to-vacuole protein targeting. EMBO J. *10*, 1741–1748.

Slusarewicz, P., Xu, Z., Seefeld, K., Haas, A., and Wickner, W. (1997). I^B_2 is a small cytosolic protein that participates in vacuole fusion. Proc. Natl. Acad. Sci. USA *94*, 5582–5587.

Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993). SNAP receptors implicated in vesicle targeting and fusion. Nature *362*, 318–324.

Ungermann, C., Nichols, B.J., Pelham, H.R.B., and Wickner, W. (1998). A vacuolar v-t-SNARE complex, the predominant form in vivo and on isolated vacuoles, is disassembled and activated for docking and fusion. J. Cell Biol. *140*, 61–69.

Vogel, W., Lammers, R., Huang, J., and Ullrich, A. (1993). Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. Science *259*, 1611–1614.

Wang, Y.-X., Zhao, H., Harding, T.M., Gomes de Mesquita, D.S., Woldringh, C.L., Klionsky, D.J., Munn, A.L., and Weisman, L.S. (1996). Multiple classes of yeast mutants are defective in vacuole partitioning yet target vacuole proteins correctly. Mol. Biol. Cell. *7*, 1375–1389.

Warren, G., and Wickner, W. (1996). Organelle inheritance. Cell *84*, 395–400.

Weisman, L.S., and Wickner, W.T. (1988). Intervacuole exchange in the yeast zygote: a new pathway in organelle communication. Science *241*, 289–591.

Weisman, L.S., Emr, S.D., and Wickner, W. (1990). Mutants of *Saccharomyces cerevisiae* that block intervacuole vesicular traffic and vacuole division and segregation. Proc. Natl. Acad. Sci. USA *87*, 1076–1080.

Whiteheart, S.W., Rossnagel, K., Buhrow, S.A., Brunner, M., Jaenicke, R., and Rothman, J.E. (1994). *N*-ethylmaleimide-sensitive fusion protein: a trimeric ATPase whose hydrolysis is required for membrane fusion. J. Cell Biol. *126*, 945–954.

Xu, Z., and Wickner, W. (1996). Thioredoxin is required for vacuole inheritance in *S. cerevisiae*. J. Cell Biol. *132*, 787–794.

Xu, Z., Mayer, A., Muller, E., and Wickner, W. (1997). A heterodimer of thioredoxin and I^B_2 cooperates with Sec18p(NSF) to promote yeast vacuole inheritance. J. Cell Biol. *136*, 299–306.

Yao, Y., Zhou, Y., and Wang, C. (1997). Both the isomerase and chaperone activities of protein disulfide isomerase are required for the reactivation of reduced and denatured acidic phospholipase A2. EMBO J. *16*, 651–658.