Ergosterol is required for the Sec18/ATP-dependent priming step of homotypic vacuole fusion

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***In vitro* homotypic fusion of yeast vacuoles occurs in three stages: priming, the Sec18 (NSF)-mediated changes that precede vacuole association; docking, the Ypt7 and SNARE-mediated pairing of vacuoles; and fusion, mediated by calmodulin/V0/t-SNARE interactions. Defects in catalysts of each stage result in fragmented (unfused) vacuoles. Strains with deletions in any of ERG genes 3–6, lacking normal ergosterol biosynthesis, have fragmented vacuoles. The ergosterol ligands filipin, nystatin and amphotericin B block the *in vitro* fusion of vacuoles from wild-type cells. Each of these inhibitors acts at the priming stage to inhibit Sec17p release from vacuoles. A reversible delay in Sec18p action prevents vacuoles from acquiring resistance to any of these three drugs, confirming that their action is on the normal fusion pathway. Ergosterol or cholesterol delivery to wild-type vacuoles stimulates their *in vitro* fusion, and the *in vitro* fusion of *ergΔ* vacuoles requires added sterol. The need for ergosterol for vacuole priming underscores the role of lipids in organizing the membrane elements of this complex reaction.**

Keywords: ergosterol/membrane fusion/Sec17p/Sec18p/vacuoles

Introduction

The compartmentalization of eukaryotic cells is achieved through selective protein translocation into organelles, followed by a dynamic sorting among organelles during vesicular traffic. The fidelity of this traffic, and the low copy number of organelles, depend on accurate docking between membranes, and regulated fusion. This complex process is of basic importance to organelle identity, cell growth, neural transmission and protein secretion. Studies of membrane fusion, employing many organelles and organisms, have revealed common catalytic factors. These include SNAREs (Gerst, 1999), which are integral membrane proteins that can associate with each other through coiled-coil domains; Sec1p and homologs, which associate with specific SNAREs (Misura *et al.*, 2000); Sec18p (NSF) and Sec17p (α -SNAP) chaperones, which disassemble SNARE complexes (Fasshauer *et al.*, 1997); Rab-family GTPases and their effectors (Chavrier and Goud, 1999), which include nucleotide exchange factors and GTPase activating proteins; regulated channels for Ca^{2+} flux (Bennett, 1997); calcium signal effector proteins such as

calmodulin (Peters and Mayer, 1998) and synaptotagmin (Desai *et al.*, 2000); and phosphoinositol polyphosphatides (Huijbregts *et al.*, 2000). The relationships between these factors, and how they work together to promote selective membrane fusion, are receiving intense study.

We are studying the homotypic fusion of yeast vacuoles, which requires all of the above elements (Wickner and Haas, 2000). The study of vacuole fusion is aided by the excellent state of knowledge of *Saccharomyces cerevisiae* genetics and genomics, by the genetic and biochemical definition of the Vps proteins (Rothman and Stevens, 1986; Banta *et al.*, 1988), which catalyze protein delivery to the vacuole and also, in some cases, vacuole homotypic fusion, and by a rapid and colorimetric *in vitro* assay of vacuole fusion (Haas *et al.*, 1994). Fusion occurs in three stages: (i) priming (the reactions that precede functional association of vacuoles), (ii) docking, which is productive vacuole association for (iii) fusion, which culminates in the mixing of luminal aqueous compartments and membrane constituents. During priming, salt, Sec18p, Sec17p and ATP disassemble complexes of SNAREs (Ungermann *et al.*, 1998a) *in cis* (on the same vacuole), and alter the associations of a multisubunit HOPS (homotypic fusion and vacuole protein sorting) complex (Price *et al.*, 2000; Sato *et al.*, 2000; Seals *et al.*, 2000). Priming and docking require phosphatidylinositol-4-phosphate and -4,5-bisphosphate (Mayer *et al.*, 2000). Docking is initiated through reversible tethering by a functional complex of HOPS and Vam7p (a homolog of the neural SNAP25 protein) with Ypt7p (Price *et al.*, 2000; Ungermann *et al.*, 2000). Tethering leads to stable docking and *trans*-association of a few percent of the SNAREs (Ungermann *et al.*, 1998b). This docking process requires the membrane electrochemical potential (Ungermann *et al.*, 1999) and Rho GTPases (G.Eitzen, N.Thorngren and W.Wickner, submitted) as well as phosphatidylinositol-4- and -4,5-phosphatides, although it is not yet clear how these docking elements are functionally related. Docking triggers a calcium efflux from the vacuole (Peters and Mayer, 1988), which is GTPase gated (Eitzen *et al.*, 2000). This calcium, in complex with calmodulin, binds to vacuole H^{+} -ATPase V0 domains (Peters *et al.*, 2001) and fosters their association *in trans* (on apposed vacuoles). Upon action of protein phosphatase 1 (Conradt *et al.*, 1994; Peters *et al.*, 1999), there is complete membrane fusion and luminal content mixing.

Yeast vacuoles normally undergo fission as well as fusion, and strains with deletions in genes encoding fusion catalysts are left with highly fragmented vacuoles (Banta *et al.*, 1988; Wada *et al.*, 1992). We have therefore searched for other essential catalysts of vacuole fusion by screening a library of yeast gene deletions (S.Seeley, M.Kato, W.Wickner and G.Eitzen, submitted) for those with fragmented vacuoles. We now report that deletion of

Fig. 1. Vacuole appearance in wild-type and *ergΔ* strains. Wild-type strain BY4742 (A) and isogenic *erg3–6Δ* strains (B–E) were grown in 0.2 ml of YPD with 200 μ g/ml G418 in 15 ml culture tubes at 30°C. After 16 h, 0.4 ml of YPD with 3 μ M FM4-64 (Vida and Emr, 1995) were added, incubation was continued for 4 h at 30°C, and cells were photographed as reported (L.Wang, W.Wickner and A.Merz, submitted).

genes encoding the enzymes of ergosterol biosynthesis causes striking vacuole fragmentation, and show that ergosterol is needed for the priming stage of the reaction.

Results

Many strains of *S.cerevisiae* have 1–3 vacuoles, but deletion of the *ERG3*, *ERG4*, *ERG5* or *ERG6* genes, encoding the enzymes of ergosterol biosynthesis (Lees *et al.*, 1995), causes striking vacuole fragmentation (Figure 1). While <5% of wild-type (BY4742) cells had highly fragmented vacuoles, >80% of cells from *erg6Δ*, *erg3Δ* or *erg5Δ* strains had complete vacuole fragmentation, suggesting that ergosterol has a role in vacuole fusion. Deletion of the *ERG4* gene, encoding the last step of ergosterol biosynthesis, caused only 40% vacuole fragmentation, indicating that the penultimate ergosterol precursor may be partially functional for vacuole fusion. This possible requirement for ergosterol was reflected by an *in vitro* fusion assay (Haas *et al.*, 1994). For this assay, we employed vacuoles purified from two strains: one that lacks proteinase A and accumulates catalytically inactive pro-alkaline phosphatase, and the other with normal vacuolar proteases but with a deletion of the *PHO8* gene, which encodes alkaline phosphatase. Fusion allows the protease from one vacuole to gain access to the pro-phosphatase of its fusion partner, converting it to the active form, which is assayed colorimetrically. The fusion seen with *ERG5/pep4Δ* and *ERG5/pho8Δ* vacuoles (Figure 2A, lanes 1 and 2) is diminished when one of the fusion partners is from an *erg5Δ* background (lanes 3 and 4), and is almost entirely lost when both vacuoles are from *erg5Δ* strains (lanes 5 and 6). These vacuoles had comparable levels of proteins such as Ypt7p and Vam3p, which are

Fig. 2. (A) Fusion activity of vacuoles from *ergΔ* cells. Standard fusion reactions were performed with vacuoles from *ERG5/pep4Δ*, *ERG5/Pho8Δ*, *erg5Δ/pep4Δ* and *erg5Δ/pho8Δ* strains. (B) Vacuoles (3.75 μ g) from each indicated strain were analyzed by immunoblotting.

Fig. 3. Inhibition of the fusion of wild-type vacuoles by nystatin, filipin and amphotericin B. Standard reactions of vacuoles from BJ3505 and DKY6281 strains contained the indicated concentrations of nystatin, filipin or amphotericin B.

required for fusion, as well as the 'reporter' proteins proPho8p and Pep4p (Figure 2B).

As a complementary test of the role of ergosterol in vacuole homotypic fusion, we assayed the effect of sterol-specific drugs on the *in vitro* fusion of vacuoles from wild-type (*ERG*) strains. With this assay, the addition of filipin, nystatin or amphotericin B, each known to be a specific sterol ligand (Kinsky, 1970), caused striking inhibition of the *in vitro* fusion reaction (Figure 3). Each of these drugs still allows ATP-driven acidification of vacuoles (data not

Fig. 4. Cholesterol promotes the fusion of vacuoles from *erg5Δ* strains. Vacuoles from *erg5Δ/pep4Δ* and *erg5Δ/pho8Δ* strains [5 μg each, 0.3 mg/ml in buffer A (20 mM PIPES–KOH pH 6.8, 200 mM sorbitol, 125 mM KCl, 5 mM MgCl₂)] were mixed, centrifuged (10 000 g, 4°C, 2 min), and resuspended in 30 μl of buffer A on ice in the presence or absence of methyl-β-cyclodextrin with 4 μg of cholesterol. After incubation on ice for 60 min, the samples were again centrifuged (10 000 g, 4°C, 2 min), resuspended in fusion reaction buffer with calmodulin (183.3 μg/ml), LMA1 (11.5 μg/ml) and Sec18p (26.6 μg/ml), incubated at 27°C for 90 min, and fusion activity was measured.

shown), as assayed by quantitative quinacrine uptake (Ungermann *et al.*, 1999), showing overall organelle integrity.

Added pure sterols can directly stimulate the *in vitro* fusion reaction. Vacuoles from *erg5Δ* strains, which are inactive for fusion (Figure 2A, lanes 5 and 6; Figure 4, lanes 1 and 2), are stimulated by the addition of sterol (Figure 4, lane 7). This stimulated fusion is on the normal pathway since it is sensitive to anti-Sec17p, anti-Vam3p and Gdi1p (lanes 8–10). Even the fusion of vacuoles from wild-type, ERG strains (Figure 5, lanes 1 and 2) can be stimulated by ergosterol (delivered to the vacuoles by liposomes; Figure 5, lanes 5–7) or cholesterol (delivered to the vacuole from a water-soluble complex with methyl-β-cyclodextrin; lanes 13 and 14), and the stimulated reaction is fully sensitive to normal reaction inhibitors (lanes 8–10 and 15–17). Without sterol, there was no effect on fusion of even the highest level of added liposomes (lane 4) or methyl-β-cyclodextrin (lane 12). Nystatin inhibition of vacuole fusion is also partially reversible by added sterol (data not shown). Taken together, the vacuole fragmentation seen *in vivo* in *ergΔ* strains, the sensitivity of *in vitro* fusion of vacuoles from ERG strains to ergosterol ligands, and the ability of added sterol to stimulate the fusion of

Fig. 5. Ergosterol or cholesterol can promote the fusion of wild-type vacuoles. Mixed vacuoles from BJ3505 and DKY6281 strains (5 μg each, 0.3 mg/ml in fusion reaction buffer) were centrifuged (10 000 g, 4°C, 2 min), and resuspended in fusion reaction buffer (33 μl) with anti-Sec18 antibody (33.3 μg/ml) in the presence or absence of 0.16–16 μg of liposomes with 0.04–4 μg of ergosterol, respectively, or of methyl-β-cyclodextrin (MβCD; 0.4–4 mM) with 0.4–4 μg of cholesterol, respectively. After incubation at 27°C for 30 min, the samples were centrifuged (10 000 g, 4°C, 2 min), pellets were resuspended in fusion reaction buffer with calmodulin (183.3 μg/ml), LMA1 (11.5 μg/ml) and Sec18p (26.6 μg/ml), incubated at 27°C for 90 min in the presence or absence of anti-Sec17 antibody (66.7 μg/ml, lanes 8 and 15), anti-Vam3 antibody (66.7 μg/ml, lanes 9 and 16) or GTPγS (6.7 mM, lanes 10 and 17), and fusion activity was measured.

ergΔ or wild-type vacuoles show that ergosterol is needed for this fusion reaction.

Priming requires ergosterol

The requirement of ergosterol for vacuole fusion might reflect a role in general membrane integrity or bilayer function, which would then extend throughout the complex membrane fusion pathway, or be specific to a defined subreaction. To test this, aliquots of a large vacuole fusion reaction were mixed with inhibitors at different times during the *in vitro* fusion incubation, and the effect on the eventual fusion yield was measured (Figure 6). As controls, the addition of aliquots to tubes with buffer (filled circles) has no effect, while the transfer of aliquots to an ice bath (×) stops the reaction and is a measure of the fusion that has occurred by the time of transfer. As previously reported (Nichols *et al.*, 1997), mixing an aliquot with antibody directed against the t-SNARE Vam3p (closed squares) from the start of the reaction causes complete fusion inhibition, but the reaction later becomes resistant to anti-Vam3p, reflecting completion of the docking step. The reaction becomes resistant to antibody directed against the priming co-chaperone Sec17p (open squares) earlier, reflecting the fact that priming precedes docking. The kinetics of the fusion reaction acquiring resistance to nystatin (open triangles), filipin (closed triangles) and amphotericin B (open circles)

Fig. 6. Nystatin, filipin and amphotericin B resistance is acquired with the same kinetics as resistance to anti-Sec18p antibody. Reactions (1350 μ l) contained vacuoles from BJ3505 and DKY6281 strains in fusion reaction buffer. Aliquots (30 μ l) were removed and placed on ice or added to inhibitors [anti-Sec17 antibody (66.7 μ g/ml), anti-Vam3 antibody (66.7 μ g/ml), nystatin (50 μ g/ml), filipin (50 μ g/ml) and amphotericin B (50 μ g/ml)] at the indicated times. After 90 min, fusion activity was measured.

paralleled that of anti-Sec17p, suggesting that these ligands largely block priming.

To determine whether the simultaneous resistance to anti-Sec17p and to sterol ligands is a kinetic coincidence of independent events, or whether the mechanisms that confer resistance to filipin, nystatin and amphotericin B are the same as those that catalyze fusion, we used antibody directed against Sec18p to specifically delay the onset of the priming cascade while incubating the vacuoles with ATP (Figure 7A). This first incubation period (30 min) otherwise suffices (Figures 6 and 7B, lanes 1–4) for the acquisition of drug resistance. Although antibody directed against Sec18p prevents fusion during this first incubation (Figure 7A, lane 5) or during a subsequent second incubation (lane 6), it is restored by the addition of excess Sec18p at the end of the first incubation (lane 4). During this first incubation, when priming was blocked by antibody directed against Sec18p, the vacuoles did not become resistant to nystatin, filipin or amphotericin B (lanes 1–3), showing that resistance is only acquired (Figures 6 and 7B) during the course of the Sec18p-triggered cascade of the normal fusion reaction. Thus, ergosterol is needed for priming and not merely for general membrane integrity.

One essential part of the priming reaction is the Sec18p-mediated release of Sec17p (Mayer *et al.*, 1996). This release, which is assayed by the Sec17p recovered after the vacuoles are removed by centrifugation (Figure 8A), requires ATP and Sec18p (Figure 8B). Even in the presence of excess Sec18p, Sec17p release is strongly inhibited by nystatin (Figure 8C), filipin (Figure 8D), or amphotericin B (Figure 8E). Ergosterol is thus specifically needed for this priming reaction, which triggers the vacuole fusion cascade.

Discussion

Ergosterol has an important role in vacuole homotypic fusion, both *in vivo* and *in vitro*. The fragmentation seen

Fig. 7. Resistance to nystatin, filipin or amphotericin B is not acquired until Sec18p has acted. Standard fusion reactions were started with (A) or without (B) 33.3 μ g/ml anti-Sec18p antibody. After incubation for 30 min at 27°C, the samples received nystatin (250 μ g/ml), filipin (50 μ g/ml), amphotericin B (25 μ g/ml) or buffer. After incubation for 5 min at 27°C, Sec18p (13.3 μ g/ml) was added to reactivate the Sec18p pathway. A control sample received only buffer. After further incubation for 90 min at 27°C or on ice, fusion activity was measured. A standard reaction, kept on ice throughout the incubations, indicates the background.

Fig. 8. Nystatin, filipin and amphotericin B inhibit Sec17p release from vacuoles. Samples equivalent to three standard fusion reactions were incubated at 27°C with Sec18p (13.3 μ g/ml), apyrase (0.033 U/ml), anti-Sec18 antibody (66.7 μ g/ml), nystatin (50 μ g/ml), filipin (200 μ g/ml) and amphotericin B (25 μ g/ml), where indicated. After the incubation times, vacuoles were removed by centrifugation and samples of the supernatant were analyzed by immunoblotting with anti-Sec17p antibody.

with *ergΔ* vacuoles (Figure 1) is a very reliable indicator of direct involvement in vacuole fusion. The first nine isolates in a screen for mutants with highly fragmented vacuoles (*vam* mutants) yielded the GTPase Ypt7p, the SNAREs Vam3p and Vam7p, and the six subunits of the HOPS complex, all directly involved in the fusion reaction. These *ergΔ* vacuoles have normal protein composition but are incapable of *in vitro* fusion (Figure 2). This is not simply due to the small size of the vacuoles, as small *vam3Δ* vacuoles fuse well with wild-type or *nyvΔ* vacuoles (Nichols *et al.*, 1977; Ungermann *et al.*, 1998b), and *bem4Δ* vacuoles, which are fragmented, show normal *in vitro* fusion (G.Eitzen and W.Wickner, unpublished). Most importantly, *ergΔ* vacuole fusion is at least partially restored by the re-addition of sterol (Figure 4), which even stimulates the fusion of wild-type vacuoles (Figure 5). Although the restoration of fusion to

erg Δ vacuoles is incomplete, the incorporation of added sterol into vacuoles may not fully mimic normal sterol content and asymmetry. Three drugs that are specific for sterols block the *in vitro* vacuole fusion reaction (Figure 3) at the priming stage (Figures 6 and 7) and prevent the release of Sec17p (Figure 8), a defined subreaction of priming (Mayer *et al.*, 1996). While these drugs may have other effects, their major effect on vacuole fusion occurs in the priming reaction (Figure 6). It is the later docking reaction that requires a membrane potential, a sensitive indicator of membrane integrity (Ungermann *et al.*, 1999). Although before now priming has been characterized by its protein rearrangements (Mayer *et al.*, 1996; Ungermann *et al.*, 1998a, 1999; Xu *et al.*, 1998), the need for both ergosterol and phosphatidylinositol-4,5-bisphosphate (Mayer *et al.*, 2000) for priming suggests that membrane lipids play a vital role as well.

It is surprising that ergosterol is important for the vacuole, as it represents only a minor portion of the membrane lipid. Yeast glycosphingolipids and ergosterol are progressively enriched along the secretory pathway, reaching their highest levels in the plasma membrane (Zinser *et al.*, 1991, 1993). Other organelles, such as the vacuole, exhibit a low ratio of ergosterol to phospholipids and have low levels of sphingolipids. The need for sphingolipids for the sorting of glycosylphosphatidylinositol-anchored Gas1p (but not for carboxypeptidase Y) from the endoplasmic reticulum to the Golgi (Hovarh *et al.*, 1994; Skrzypek *et al.*, 1997; Sutterlin *et al.*, 1997), and for ergosterol for vacuole fusion, as reported here, shows that lipids and proteins may often fulfill vital functions at cellular locations where they are not most abundant.

Lipids are thought to fulfill several roles in membrane trafficking. These include modulating the physical properties of membranes, forming a separate phase or 'raft', and serving as a scaffold for binding specific sets of proteins. Ergosterol and other sterols are known to alter membrane fluidity. If Sec18p-mediated Sec17p release is coupled to movement of the membrane anchors of Sec17p-associated SNAREs, then ergosterol might affect Sec17p release through governing SNARE mobility. Indeed, the Sec18p-mediated release of Sec17p from vacuole membranes is blocked by antibody directed against Vam3p, the vacuolar t-SNARE (Sato and Wickner, 1998), and it has recently been reported (Lang *et al.*, 2001) that SNAREs can be found concentrated in cholesterol-rich clusters, which are important for membrane fusion. Vacuolar sterol might also participate in forming a separate phase, or 'raft', in the vacuole membrane, enriched in proteins and lipids that partake in the fusion pathway. Triton X-100 extracts of vacuole membranes have a 65S particle, which bears several key proteins of the fusion cascade (Seals *et al.*, 2000), but almost no Sec17p or Sec18p (D.Seals, A.Merz and W.Wickner, unpublished). Cholesterol-rich protein rafts have been heavily studied in the secretory pathway, both in mammalian cells and in yeast (Ikonen and Simons, 1998; Bagnat *et al.*, 2000), but have not yet been implicated in Sec18p/NSF-mediated priming *per se*. A third possible role for lipids in trafficking is as a scaffold for membrane association of specific proteins. Just as proteins bearing FYVE motifs recognize phosphatidylinositol-3-phosphatides, and this recognition is essential to

allow these proteins to function (Burd and Emr, 1998), it is conceivable that ergosterol might provide a binding site for certain vacuolar peripheral membrane proteins.

Sterols have been reported to govern traffic and membrane fusion in other systems. Sterols are required for the formation of 'rafts' of distinct lipid and protein composition, which have well studied roles in the trafficking of these lipids and proteins to target membranes. In addition, *Mycoplasma* fusion with Sendai or influenza virus requires cholesterol (Citovsky *et al.*, 1988). This fusion reaction, however, begins on the extra-cytoplasmic faces of two membranes, and thus does not entail the actions of Sec18/NSF and Sec17p/ α -SNAP.

Sec18p is an ATP-driven chaperone that releases Sec17p from the vacuoles, causing disassembly of *cis*-SNARE complexes. These proteins, as well as the ternary SNARE complex, are of known structure (Sutton *et al.*, 1998; Rice and Brünger, 1999; Yu *et al.*, 1999), and it has been shown that pure Sec18p/NSF and Sec17p/ α -SNAP can disassemble the ternary complex of pure SNAREs (Fasshauer *et al.*, 1997). These model studies largely employ recombinant SNAREs without their membrane anchors, or full-length SNAREs with their membrane anchors, which were solubilized with the aid of detergents. *cis*-SNARE complexes may not be simply trimeric, but may also include Sec17p (Ungermann *et al.*, 1998a), the HOPS complex, which itself includes Vps33p (a Sec1p homolog with affinity for the t-SNARE Vam3p), and other components as well. The functional role of ergosterol in Sec18p-driven Sec17p release from the vacuole may become clearer as the complete molecular constituents and structure of the *cis*-SNARE complex and its bound Sec17p are studied further.

Materials and methods

The yeast strains BJ3505 and DKY were described (Haas *et al.*, 1994). Deletion mutants of ERG3, ERG4, ERG5 or ERG6 genes in BY4742 yeast were purchased from Research Genetics. Deletions of PEP4 and PHO8 were made in these strains as described (Peters and Mayer, 1998). Reagents were as described (Mayer *et al.*, 1996; Eitzen *et al.*, 2000). Nystatin, filipin III and amphotericin B were from Sigma, and were dissolved in dimethylsulfoxide at 150, 30 and 30 mg/ml, and diluted prior to addition to the assay with PS buffer (10 mM PIPES-KOH pH 6.8, 200 mM sorbitol). Ergosterol, cholesterol, L-*d*-phosphatidylcholine and methyl- β -cyclodextrin were purchased from Sigma. The assay for the release of Sec17p was described previously (Mayer *et al.*, 1996).

A standard fusion reaction contained 3 μ g of each vacuole type, isolated from strains BJ3505 and DKY6281, in a total volume of 30–35 μ l of reaction buffer (10 mM PIPES-KOH pH 6.8, 200 mM sorbitol, 125 mM KCl, 5 mM MgCl₂, 10 μ M coenzyme A, 1 mM Mg-ATP, 40 mM creatine phosphate, 0.5 mg/ml creatine kinase, 6.6 ng/ml leupeptin, 16.6 ng/ml pepstatin, 16.6 μ M *o*-phenanthroline, 3.3 μ M Pefabloc SC). After 90 min at 27°C, alkaline phosphatase activity was determined (Haas *et al.*, 1994).

Vacuoles were supplemented with ergosterol from liposomes (Cruz *et al.*, 2000) and with cholesterol from its complex with methyl- β -cyclodextrin as described (Christian *et al.*, 1997; Cruz *et al.*, 2000).

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