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NEW EMBO MEMBER'S REVIEW

Yeast vacuoles and membrane fusion pathways

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Selective membrane fusion underlies subcellular compartmentation, cell growth, neurotransmission and hormone secretion. Its fundamental mechanisms are conserved among organelles, tissues and organisms. As befits a conserved process, reductionism led to its study in microorganisms. Homotypic fusion of the vacuole of *Saccharomyces cerevisiae* is particularly accessible to study as vacuoles are readily visualized, there is a rapid and quantitative *in vitro* assay of vacuole fusion, and the genetics and genomics of this organism and of vacuole fusion are highly advanced. Recent progress is reviewed in the context of general questions in the membrane fusion field.

Keywords: GTPases/phosphoinositides/SNARES

Introduction

Regulated membrane fusion has received intensive study. For example, vesicles with neurotransmitters cluster at the active zone of synapses, awaiting a voltage-triggered calcium flux to fuse and release their contents into the synaptic cleft (Jahn and Sudhof, 1999). Study of this process has benefited from excellent electrophysiology and cytology as well as extensive pharmacology, including selective toxins. Biochemical definition of proteins in synaptic complexes has been complemented by their genetic study in mice, flies, worms and yeast. *In vitro* synaptic fusion has been elusive, though regulated secretion in semi-intact PC12 cells has many shared features with neurons, such as requirements for ATP, calcium, chaperones and phosphoinositides (Hay and Martin, 1992; Hay *et al*., 1995) and toxin sensitivities (Martin and Kowalchyk, 1997). Endosome fusion has yielded novel insights, and Golgi trafficking, the first *in vitro* membrane fusion reaction, defined the fundamental compartment-mixing assay of fusion (Balch *et al*., 1984) and led to the first isolation of functional fusion catalysts. The *SEC* (Novick and Schekman, 1979) and vacuole protein sorting (*VPS*; Rothman and Stevens, 1986; Banta *et al*., 1988) gene selections and development of *in vitro* assays for endoplasmic reticulum (ER) to Golgi traffic (Baker *et al*., 1988) have made yeast a premier organism for studying membrane fusion.

The shared features of these trafficking systems provide the basic outline of a conserved fusion mechanism. GTPases of the Rab/Ypt family are organelle-specific 'switches' which cycle between their active GTP and inactive GDP form (Novick and Zerial, 1997). Cycling is regulated by GTPase activating proteins (GAPs), promoting GTP hydrolysis, and guanine nucleotide exchange factors (GEFs), promoting exchange of GTP for GDP (see Table I for a glossary). GAPs and GEFs are themselves localized and regulated in their actions. Active (GTP)Rab/Ypt proteins bind organelle-specific 'effector' complexes, which allow them to initiate the docking of membranes prior to fusion (Stenmark *et al*., 1995; Horiuchi *et al*., 1997; Simonsen *et al*., 1998; Christoforidis *et al*., 1999a,b). SNAREs are a second conserved family of trafficking proteins. Their helical 'SNARE motif' segments can assemble into a stable four-helical 'coiled-coil' bundle (Sutton *et al*., 1998). SNAREs are anchored to membranes by a C-terminal apolar domain or by acyl derivatization. SNARE complexes form *in cis*, between SNAREs anchored to the same membrane, or *in trans*, between SNAREs anchored on the apposed membranes of docked organelles. SNARE complexes are disassembled by the ATPase NSF/Sec18p and its co-chaperone α-SNAP/Sec17p. Sec1 family proteins bind to SNAREs (Misura *et al*., 2000); their function, though essential, is not fully understood. For all membrane fusion reactions, localized transient calcium fluxes trigger fusion by calcium activation of target proteins (Burgoyne and Morgan, 1998). Where examined, membrane fusion also requires inositol phosphatides that function as signaling molecules (Sullivan *et al*., 1993) or bind proteins to the membrane (Burd and Emr, 1998).

Each fusion event occurs in three stages: (i) priming, the ATP-driven modification of the association status of SNAREs, Rabs and effector complexes, (ii) docking, initiated by Rab/Ypt and its effectors and leading to *trans*-SNARE pairing and (iii) membrane fusion, initiated by calcium flux and culminating in the fusion of membrane bilayers and mixing of lumenal contents. For different fusion reactions, priming may preceed docking, as for yeast vacuole fusion, or follow it, as for neural and PC12 cell exocytosis or trafficking from the ER to Golgi in yeast (Barlowe, 1997).

Despite the substantial progress in understanding membrane fusion, the catalysts of each reaction stage and the connections between their actions remain unclear. To paraphrase Efraim Racker, 'If you're not confused by this problem, you just don't understand it.' This review presents recent progress in dissecting one membrane fusion event, the homotypic fusion of vacuoles from *S.cerevisiae* and a perspective on how we may progress from here.

Homotypic fusion of yeast vacuoles

Yeast vacuoles undergo constant fission and fusion in the cell, with a steady-state of 1–5 vacuoles in many strains. Purified vacuoles will fuse when incubated with ATP.

Table I. Glossary

	Definition
Arp2/3 complex	regulated by Bee1/Las17/WASp and triggers actin polymerization
Bee1/Las17p	the yeast homolog of WASp; regulates actin polymerization
Cdc42p	a Rho-class GTPase that regulates actin polymerization and other processes; is needed for vacuole fusion
Gdi1p	extracts Ypt7p and other Ypt/Rab proteins from the membrane when they are bound to GDP
HOPS complex	homotypic fusion and vacuole protein sorting complex (also called Class C VPS complex) includes Vps 11, 16, 18, 33, 39 and 41p. Vps33 is a Sec1p-like SNARE binding protein, Vps39p is a guanine nucleotide exchange factor for Ypt7p
LMA1 and 2	low M_r activities which interact with several catalysts of vacuole fusion
Nyv1p	a vacuolar *v*-SNARE
Rho1p	a Rho class GTPase required for vacuole fusion.
SNAREs	form four helix coiled coils, *in cis* (on the same membrane) or *in trans* (on opposed, docked membranes)
SEC genes	encode proteins of the secretory pathway
Sec17p (yeast α-SNAP)	the co-chaperone of Sec18p
Sec18p (yeast NSF)	an ATP-driven chaperone that disassembles *cis*-SNARE complexes
V_1V_0	the vacuolar ATP-driven proton pump, consisting of V_1 (peripheral) domain and V_0 (integral) domain
VAC genes	required for vacuole inheritance. Vac8p is also needed for fusion.
VAM genes	required for low copy number of vacuoles
Vam3p	the vacuolar *t*-SNARE
Vam7p	the vacuolar homolog of neuronal SNAREs SNAP23 and SNAP 25; it is a peripheral membrane protein
VPS genes	encode proteins needed for sorting between the Golgi, endosome and vacuole
VTC	vacuolar transporter chaperone, a four-membered complex (Vtc1-4) that links the stages of the vacuole fusion reaction
Vti1p	a *v*-SNARE of the vacuole and other organelles
Ykt6p	a *v*-SNARE of the vacuole and other organelles
Ypt/Rab GTPases	regulate trafficking as they cycle between GTP and GDP bound forms. Ypt7p is vacuolar

Vacuoles are advantageous for studying membrane fusion: (i) they are readily visualized in cells and can be isolated in high purity; (ii) the genetics of trafficking to vacuoles (*VPS* genes; Rothman and Stevens, 1986; Banta *et al*., 1988; Wendland *et al*., 1998) and maintaining low vacuole copy number (vacuole morphology, *VAM* genes; Wada *et al*., 1992) is well-developed, with considerable overlap between the processes of heterotypic trafficking from endosome to vacuole and homotypic vacuole fusion; and (iii) vacuoles can be purified in 100 mg lots, stored frozen and used in rapid, colorimetric fusion assays. This assay has been particularly amenable to staging and biochemical dissection.

To assay fusion (Haas *et al*., 1994), vacuoles are isolated from two yeast strains, one with normal vacuole proteases but deleted for the PHO8 gene encoding the major phosphatase and the other deleted for vacuolar proteases and hence bearing the catalytically-inactive pro-Pho8p (Figure 1). Neither population of purified vacuoles has phosphatase activity. Upon fusion *in vitro*, the proteases gain access to the pro-Pho8p and convert it to the catalytically active form that can be assayed colorimetrically. Microscope observation of aliquots removed at various times from one fusion reaction shows the formation of vacuole clusters. Large, fused vacuoles form (Figure 2A) as immunoblots of the same aliquots reveals the processing of pro-Pho8p to Pho8p (Figure 2B) and a catalytic assay shows increased phosphatase activity (Figure 2C). Freshly prepared vacuoles only need ATP to fuse, though fusion needs many peripheral membrane proteins which may be lost with time or exposure to salt solutions. Biochemical and genetic studies have revealed that this is a complex and highly ordered reaction (Figure 3).

Although isolated vacuoles just look like round bubbles (Figure 3A), appearances of simplicity are deceiving! Some of the proteins needed for fusion are present on the

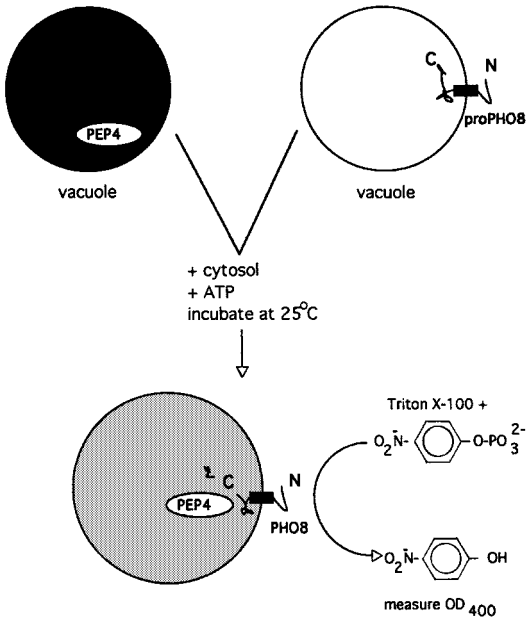

Fig. 1. Assay of homotypic vacuole fusion. Reproduced with permission from *J. Cell Biol.* (1994), **126**, 87–97.

vacuole surface in oligomeric complexes. For example, the SNARE proteins Vam3p (*t*-SNARE), Vam7p (SNAP-25 homolog), and Nyv1p, Vti1p, and Ykt6p (*v*-SNAREs) are present in *cis*-complexes along with Sec17p, yeast α-SNAP (Ungermann and Wickner, 1998; Ungermann *et al*., 1998a, 1999a). The hexameric HOPS (homotypic vacuole fusion and protein sorting)/Class C VPS complex (Price *et al*., 2000b; Sato *et al*., 2000; Seals *et al*., 2000), consisting of Vps 11, 16, 18, 33, 39 and 41 proteins, is part of a 65S complex on the vacuole surface (Nakamura *et al*., 1997; Price *et al*., 2000b); the other members of this 65S complex are unknown. The vacuole transporter chaperone

(Vtc) tetrameric complex (Cohen *et al*., 1999; Murray and Johnson, 2000) regulates V_1V_0 association and is intimately involved in each stage of vacuole fusion (Muller *et al*., 2002; O.Muller, M.J.Bayer and A.Mayer, submitted). The initial associations of other proteins of the vacuole fusion reaction, such as Ypt7p, remain to be explored.

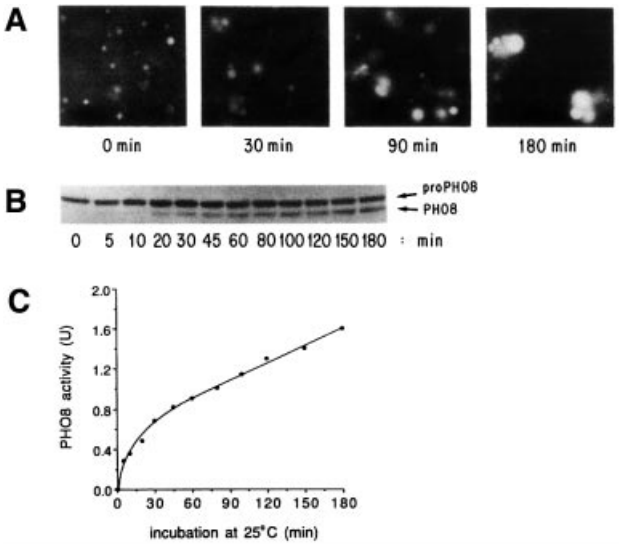

Fig. 2. Three assays of the kinetics of vacuole docking and fusion. All samples, withdrawn at times shown, were from one *in vitro* vacuole fusion reaction. Reproduced with permission from *J. Cell Biol.* (1994), **126**, 87–97.

The initial priming stage of the reaction (Figure 3A and B) needs ATP and physiological ionic strength and temperature (Conradt *et al*., 1994). It is a prerequisite for productive docking (Mayer and Wickner, 1997). Sec18p hydrolyzes ATP, driving Sec17p release (Mayer *et al*., 1996) and the disassembly of the *cis*-SNARE complex (Ungermann *et al*., 1998a). This reaction requires both phosphatidylinositol (4,5) bisphosphate [PI(4,5)P2] (Mayer *et al*., 2000) and ergosterol (Kato and Wickner, 2001); since Sec18p can catalyze Sec17p release from lecithin-liposomes (Sato and Wickner, 1998), ergosterol and PI(4,5)P2 may be needed for their interactions with other proteins such as the *cis*-SNARE complex or might form an essential lipid phase. LMA1 (low M_r activity 1), a co-chaperone that is initially bound to Sec18p, transfers to the SNARE Vam3p, which it stabilizes (Xu *et al*., 1997, 1998). The HOPS complex activates Ypt7p (Wurmser *et al*., 2000) and remains bound as a Ypt7p effector (Seals *et al*., 2000). It is not known how HOPS is bound to the vacuole before it reaches Ypt7p nor do we know what triggers its transfer to Ypt7p. Vam7p also depends on Ypt7p for its continued vacuole association (Ungermann *et al*., 2000) and may interact directly with this GTPase, as

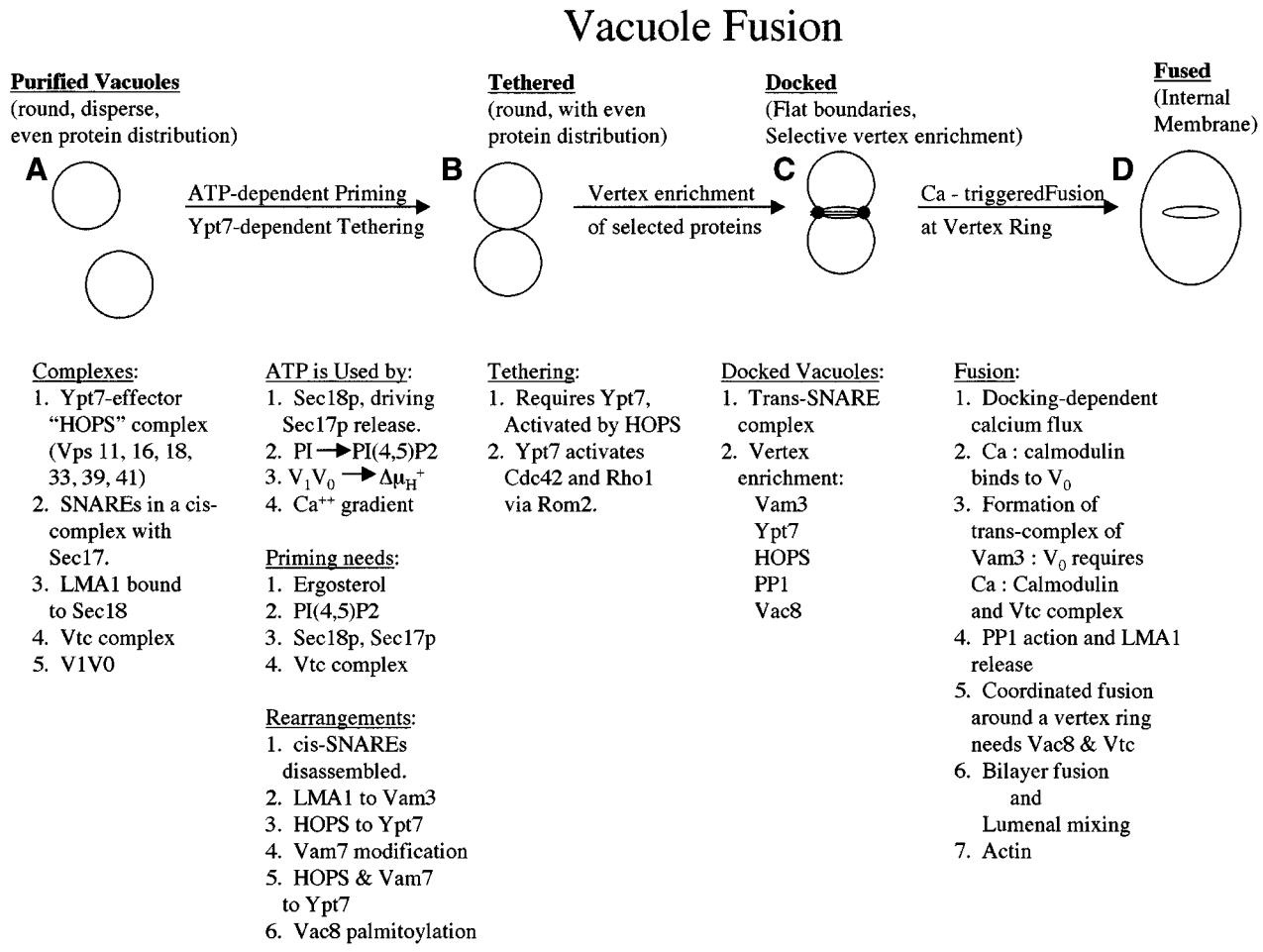

Fig. 3. Vacuole homotypic fusion. See text for details.

suggested by two-hybrid analysis (Uetz *et al*., 2000). Both Ypt7p (Ungermann *et al*., 2000) and phosphatidylinositol 3-phosphate (Cheever *et al*., 2001) are needed to keep Vam7p on the vacuole, though the relationship between these is not known. Priming also triggers acylation of Vac8p (Veit *et al*., 2001), which is later required in the reaction for fusion (Y.-X.Wang *et al*., 2001).

Primed vacuoles associate reversibly in a 'tethering' reaction (Figure 3B; Ungermann *et al*., 1998b), followed by stable docking (Figure 3C). Tethering requires activated Ypt7p and HOPS (Price *et al*., 2000a,b), though other factors may be necessary. Ypt7p activation is needed for two subsequent docking steps, the activation of two Rho GTPases, Rho1p and Cdc42p (Muller *et al*., 2001; Eitzen *et al*., 2001), and the formation of *trans*-SNARE pairs (Ungermann *et al*., 1998b). *Trans*-pairing of SNAREs has been demonstrated for docked vacuoles (Ungermann *et al*., 1998b) and only involves a small percent of the vacuole SNARE proteins (Ungermann *et al*., 1998b; Wang *et al*., 2002). The composition of the *trans*-SNARE complex, its locale on docked vacuoles (see below), the catalysis of its formation and its subsequent actions all await further studies. Docking requires vacuole acidification (Ungermann *et al*., 1999b) and PI(4,5)P2 (Mayer *et al*., 2000; also see below), though the molecular functions of each are unclear.

Docking causes a measurable release of calcium from the vacuole (Peters and Mayer, 1998). Calcium activates calmodulin to bind to V_0, the integral domain of the vacuolar H^+-ATPase, triggering *trans*-complex formation between V_0 complexes on apposed vacuoles (Peters *et al*., 2001). This *trans*-V_0 complex contains the *t*-SNARE Vam3p but not the *v*-SNARE Nyv1p. Fusion is triggered by the action of protein phosphatase 1 (Conradt *et al*., 1994; Peters *et al*., 1999), though its phosphoprotein target and the opposing kinase, are unknown. Protein phosphatase 1 action triggers the release of LMA1 from the vacuoles prior to membrane fusion and contents mixing (Xu *et al*., 1998). The relative roles of *trans*-SNARE pairs and *trans*-V_0 complexes in catalyzing the final steps of fusion are the subject of intense experimentation and debate.

Recent advances

Genomics

Our knowledge of this, or other, membrane fusion pathways seems less like the coupled, sequential steps of a metabolic pathway than the fragmented understanding of a play where only some of the actors are visible on the stage, yet each speaks their lines. One path towards a more complete compilation of the actors in the fusion play was pioneered by Wada *et al*. (1992), who screened a collection of mutagenized yeast for those with abnormal vacuole morphology (*vam* phenotype), specifically those with highly fragmented vacuoles. Vacuole fragmentation suggests a defect in fusion and indeed, each of the nine genes identified in their non-saturated screen was directly involved in the fusion reaction. We screened a commercially available collection of 4828 yeast strains, each bearing a deletion in a known non-essential gene, for the *vam* phenotype of fragmented vacuoles. In addition to known catalysts of the vacuole fusion reaction, new genes were identified where deletion yields a *vam* phenotype (Seeley *et al*., 2002). These included open reading frames of utterly unknown function, but also genes of lipid metabolism, GTPases and effectors, cytoskeletal proteins, kinases and phosphatases, and other trafficking proteins. Though each of the nine VAM genes of Wada *et al*. (1992) were also VPS genes (needed for biosynthetic sorting of proteins to the vacuoles), our deletion screen only uncovered a few new VPS genes, showing that the sets of proteins catalyzing trafficking to the vacuole and vacuole fusion have only partial overlap. In this *vam* screen, we discovered that ergosterol is needed for vacuole priming (Kato and Wickner, 2001). We also find that the turnover of vacuolar PI(4,5)P2, by phosphatases and phospholipase C, is essential for the fusion reaction. Inhibitor studies suggest that inositol tris-phosphate or a derivative might function to activate a calcium channel, though yeast has no obvious homolog to the mammalian IP3-activated calcium transporters.

Evaluation of each new VAM gene will permit an evaluation of whether it has a direct role in fusion. Other proteins, which are essential for cell growth and which participate in vacuole fusion, may be revealed by their genetic, two-hybrid and proteomic relationships to the non-essential VAM genes as well as by biochemical fractionation of the *in vitro* reaction.

Vac8p

Originally found as a protein needed for vacuole inheritance (Pan and Goldfarb, 1998; Wang *et al*., 1998), Vac8p has been found to cluster at docking sites between vacuoles, and between vacuoles and the nucleus (Pan *et al*., 2000). It has recently been shown that a portion of Vac8p is palmitoylated during priming (Veit *et al*., 2001) and functions during membrane fusion *per se* (Y.-X.Wang *et al*., 2001). Its functional role is unknown, but its many armadillo repeats suggest homo- or hetero-oligomerization.

Rho GTPases and actin

In addition to the Ypt7p Rab-GTPase, two Rho GTPases, Rho1p and Cdc42p, are required for the docking stage of fusion (Eitzen *et al*., 2001; Muller *et al*., 2001). Rho GTPases regulate actin cytoskeleton, yet the *in vitro* fusion of purified vacuoles does not require cytosol. Nevertheless, purified vacuoles have bound G actin, which undergoes polymerization to F actin during the fusion reaction. The fusion reaction is sensitive to latrunculin B and jasplakinolide, two actin-directed drugs, and fusion is defective with vacuoles from actin mutant strains (G.Eitzen, unpublished observations). The deletion screen and two-hybrid analysis trace the relationships between Cdc42p, Cla4p, Vrp1p, PI(4,5)P2, the Arp 2/3 complex, and actin, but biochemical experiments will be needed to fully establish this pathway. The function of vacuolar actin is unknown, but might regulate protein localization on the vacuole during docking (below). While PI(4,5)P2 regulates the activity of the Bee1p–Vrp1p complex (Higgs and Pollard, 2001), it is unclear whether Rho GTPases might regulate phosphoinositide metabolism, which would connect a pathway from priming to the docking-dependent calcium flux.

The VTC complex

What factors connect the proteins at each stage of the reaction? Four VTC genes encode proteins that are found in the vacuole membrane. The VTC proteins are in direct physical association with V_0 and Nyv1p and have genetic associations with V_1V_0 ATPase (Cohen *et al*., 1999; Murray and Johnson, 2000; Muller *et al*., 2002; O.Muller, M.J.Bayer and A.Mayer, submitted). Vtc1p and Vtc4p are needed for each aspect of priming, while Vtc3p is needed for LMA1 release and *trans*-V_0 complex disassembly in the very terminal phases of the membrane fusion reactions (Muller *et al*., 2002; O.Muller, M.J.Bayer and A.Mayer, submitted).

Vam3p N-domain

Though Vam3p is homologous to other syntaxins, recent structural studies have shown that its N-terminal domain does not form a 'closed conformation' with its SNARE domain (Dulubova *et al*., 2001). Indeed, though deletion of this N-terminal domain has no obvious effect on vacuole structure *in vivo* or on *in vitro* rates of vacuole fusion, the SNARE domain of Vam3p has a crucial role in membrane fusion (Y.Wang *et al*., 2001).

Sub-organellar localization

Proteins involved in the fusion process have been derivatized with green fluorescent protein (GFP), creating strains in which each GFP fusion protein is expressed at its normal level (Wang *et al*., 2002). This allows measurement of the relative molar abundance of each protein and its spatial distribution on docked vacuole clusters. For this purpose, the regions of vacuole membrane in a docked vacuole cluster are defined as outside edges (O), boundary membranes (B), where two vacuoles touch, and vertices (V), where boundary and outside regions end and meet (Figure 4A). Strikingly, ratiometric fluorescence microscopy, in which the ratio of a GFP-tagged protein to lipid is measured at each pixel of the image, reveals that Ypt7p and its effector HOPS complex accumulate at vertices (Figure 4B). Vac8p, the *t*-SNARE Vam3p and protein phosphatase 1 also accumulate at vertices. SNAREs must be released from their *cis*-associations for vertex enrichment to occur, showing that vertex enrichment is on the normal pathway to fusion. Since Ypt7p is almost 10 times as abundant as Vam3p, it may have a primary role in establishing these docking structures. Once docking has been established, extraction of Ypt7p leaves HOPS and Vam3p at the vertices, in accordance with biochemical findings that Ypt7p is no longer needed once docking is complete. The enrichment of Vac8p, Vam3p and protein phosphatase 1 at the vertices, three proteins involved in fusion *per se*, suggests that this is where fusion is initiated. Indeed, fluorescence microscopy of vacuoles in the *in vitro* reaction or in living cells (Wang *et al*., 2002) shows that fusion occurs at vertices, leaving a small fragment of membrane inside the fused vacuole. Further characterization of this vertex complex should shed light on its role in docking and fusion.

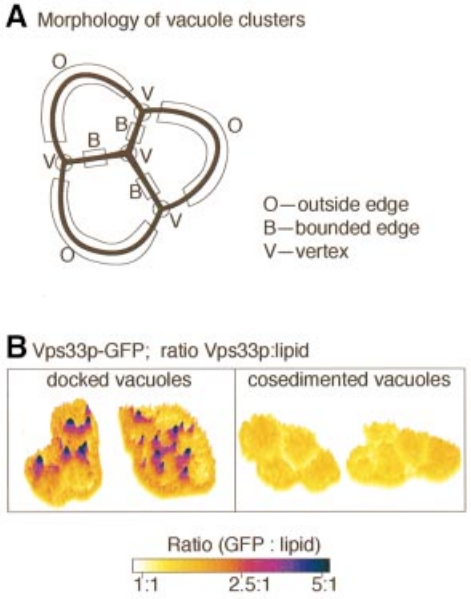

Fig. 4. Vertex enrichment of selected proteins during docking. (**A**) Membrane microdomains of docked vacuoles are defined as 'outside edges', which are not in contact with other vacuoles, 'boundary edges', which are opposed to other vacuoles in the cluster, and 'vertices', where boundary edge and outside edge membranes meet. (**B**) Docking-dependent enrichment of Vps33p at vertices. Left panel: purified vacuoles from cells with GFP-tagged Vps33p were stained with FM4-64, incubated in a standard fusion reaction for 30 min to allow docking and examined by ratiometric fluorescence microscopy to determine regions of membrane where tagged Vps33p is enriched. Right panel: vacuoles were not incubated under docking and fusion conditons with ATP, but rather were clustered by co-sedimentation. Reprinted with permission from Elsevier Science from L.Wang *et al*. (2002).

Current 'controversies'

It remains unclear which proteins actually catalyze the last stage of the reaction, the fusion of membranes and contents mixing; there are a surfeit of candidates! Sec18p/NSF and Sec17p/α-SNAP can drive the fusion of lipsomes (Otter-Nilsson *et al*., 1999; Brugger *et al*., 2000), but act on purified vacuoles well before fusion, to separate *cis*-SNARE complexes (Mayer *et al*., 1996). Calcium can trigger the fusion of lipid bilayers directly (Wilschut *et al*., 1980) and indeed triggers biological membrane fusion reactions, yet its physiological effects are clearly protein-mediated. Might the proteins serve to deliver calcium to an active site where it could be a crucial and catalytic element, much as zinc functions for DNA polymerases? SNAREs can catalyze liposome fusion (Weber *et al*., 1998), yet their pairing *in trans* seems dispensible for fusion *per se* (Ungermann *et al*., 1998b). The V_0 domain of the vacuolar ATPase serves as the membrane receptor for calcium/calmodulin (Peters *et al*., 2001), which triggers the formation of *trans*-complexes containing V_0 and the *t*-SNARE Vam3p (but not the other SNAREs). V_0 exhibits altered permeability when calcium/calmodulin binds and this may correspond to the expansion of a fusion pore (Peters *et al*., 2001).

How will the 'real' fusion machinery be established? Membrane fusion requires proximity (docking) and the imposition of strain on the bilayer. Though tightly regulated in the cell to prevent compartment mixing, liposome association and bilayer strain are all too readily achieved in model reactions. It will be essential to complete the task of identifying the proteins and lipids

that catalyze each stage of vacuole fusion, place them in their cascades of association and order of catalytic actions, and gain mechanistic understanding of their relationships to each other.

Prospects

The stage is set for evaluating each protein of the *vam* deletion screen and for a systematic search for vacuole fusion catalysts that are essential for cell viability and thus were not in that screen. The connections of each subreaction will be sought: what are the initial associations of Ypt7p, HOPS and Vac8p, what are the constituents of the *cis*-SNARE complex, does the ATPase domain of Vps33p participate in priming, what are the roles of inositol phosphatides and ergosterol and are they enriched at vertices, what are the cascades of physical interaction during docking, what assembles proteins at vertices, how are actin filaments participating in the reaction, what is the docking-dependent calcium channel, and what are the actions of *trans*-SNARE pairs and *trans*-V_0Vam3–V_0Vam3 pairs? The ease of combining enzymology, genetics, genomics and cytology in the study of vacuole fusion make it a promising avenue towards finally understanding regulated membrane fusion in all its glorious complexity.

Acknowledgements

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