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Vacuole membrane fusion: V_0 functions after trans-SNARE pairing and is coupled to the Ca^{2+}-releasing channel

Martin J. Bayer, Christoph Reese, Susanne Bühler, Christopher Peters, and Andreas Mayer

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, 72076 Tübingen, Germany

Pore models of membrane fusion postulate that cylinders of integral membrane proteins can initiate a fusion pore after conformational rearrangement of pore subunits. In the fusion of yeast vacuoles, V-ATPase V_0 sectors, which contain a central cylinder of membrane integral proteolipid subunits, associate to form a transcomplex that might resemble an intermediate postulated in some pore models. We tested the role of V_0 sectors in vacuole fusion. V_0 functions in fusion and proton translocation could be experimentally separated via the differential effects of mutations and inhibitory antibodies. Inactivation of the V_0 subunit Vph1p blocked fusion in the terminal reaction stage that is independent of a proton gradient. Δvph1 mutants were capable of docking and trans-SNARE pairing and of subsequent release of lumenal Ca^{2+}, but they did not fuse. The Ca^{2+}-releasing channel appears to be tightly coupled to V_0 because inactivation of Vph1p by antibodies blocked Ca^{2+} release. Vph1 deletion on only one fusion partner sufficed to severely reduce fusion activity. The functional requirement for Vph1p correlates to V_0 transcomplex formation in that both occur after docking and Ca^{2+} release. These observations establish V_0 as a crucial factor in vacuole fusion acting downstream of trans-SNARE pairing.

Introduction

The fusion of intracellular membranes depends on a proteinaceous machinery which has been partially conserved in evolution. Homologous components act on different stations of membrane traffic in the secretory and endocytic pathways (Gotte et al., 2000; Chen and Scheller, 2001; Pelham, 2001). This led to the suggestion that all intracellular fusion reactions of eukaryotic cells might follow a universal reaction mechanism implemented by variations of the same core machinery, the SNARE proteins (Rothman, 1994). After activation by the ATPase Sec18p/NSF, cognate SNAREs from opposing membranes can form transcomplexes which are believed to force the membranes into close apposition and cause fusion. In line with this proposal, a basal fusion activity of SNAREs could be reconstituted from purified components inserted into liposomes (Weber et al., 1998).

The fusion of vacuoles from the yeast *Saccharomyces cerevisiae* shares many key features with other fusion reactions (Mayer, 2001). Thus, it can serve to test hypotheses about the fusion mechanism and about the role of specific conserved components. Vacuole fusion depends on the activation of t- and v-SNAREs by the ATPase Sec18p/NSF and its cofactor Sec17p/α-SNAP and on a Rab-GTPase, Ypt7p (Haas et al., 1995; Haas and Wickner, 1996; Mayer et al., 1996; Ungermann et al., 1999a). Ypt7p cooperates with the HOPS complex, an oligomeric assembly of tethering factors containing the class C Vps proteins (Price et al., 2000ab; Sato et al., 2000; Seals et al., 2000; Wurmser et al., 2000). During priming, ATP hydrolysis by Sec18p/NSF disrupts cis-SNARE complexes (Nichols et al., 1997; Ungermann et al., 1998a) and yields SNAREs in a labile, activated state which is stabilized by the LMA1 complex (Xu and Wickner, 1996; Slusarewicz et al., 1997; Xu et al., 1997, 1998). Priming also releases the armadillo repeat protein Vac8p from SNAREs and triggers its palmitoylation (Veit et al., 2001; Rohde et al., 2003), a modification that might be relevant to the function of Vac8p in later stages of fusion (Wang et al., 2000). Priming facilitates tethering, the initial and less stable attachment of the fusion partners that depends on Ypt7p and the HOPS complex (Mayer and Wickner, 1997; Ungermann et al., 1998b; Price et al., 2000a). Specific interactions between HOPS and SNAREs involve the NH_2-terminal domain of the SNARE Vam3p (Laage and Ungermann, 2001; Wang et al., 2001a). Tethering is a prerequisite for subsequent docking,

M.J. Bayer, C. Reese, and S. Bühler contributed equally to this paper.

Address correspondence to Andreas Mayer, Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstr. 37-39, 72076 Tübingen, Germany. Tel.: 49-7071-601850. Fax: 49-7071-601455.
E-mail: Andreas.Mayer@Tuebingen.mpg.de

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a tighter binding of vacuoles that requires SNAREs and might involve the formation of trans-SNARE complexes, i.e., complexes of cognate t- and v-SNAREs on the opposing membranes (Ungermann et al., 1998b; Laage and Ungermann, 2001). Tethering and docking are accompanied by a concentration of many fusion-relevant components around the contact zones between vacuoles (Wang et al., 2002). Trans-SNARE complexes accumulate to low abundance during the fusion reaction (Ungermann et al., 1998b; Rohde et al., 2003). An enormous advantage of the vacuole fusion system is that trans-SNARE pairing can be directly assayed as an intermediate which is well integrated into the reaction pathway, a property that distinguishes it from the other major systems used to study membrane fusion. Notably, trans-SNARE pairs between vacuoles can be disassembled after docking without blocking further progression of fusion (Ungermann et al., 1998b). This indicates that SNAREs are required at least up to the docking stage but that trans-SNARE pairing may be dispensable for completion of the reaction.

Priming and docking also show specific lipid requirements, in particular for phosphatidylinositol 4,5-bisphosphate (Mayer et al., 2000), ergosterol (Kato and Wickner, 2001), and phosphatidylinositol 3-phosphate (Cheever et al., 2001; Boeddinghaus et al., 2002). Like exocytosis (Adamo et al., 1999, 2001; Guo et al., 2001; Zhang et al., 2001), vacuole fusion requires more than one small GTPase. In addition to the Rab-GTPase Ypt7p, the Rho-GTPases Cdc42p and Rho1p are involved (Eitzen et al., 2001; Muller et al., 2001), probably by regulating the remodeling of vacuolar actin. Dynamic changes of vacuolar actin occur during fusion (Eitzen et al., 2002; Seeley et al., 2002).

Vacuole docking triggers an efflux of calcium from the lumen of the organelle which fosters the binding of calmodulin to the membranes (Peters and Mayer, 1998). Calmodulin binds to a high molecular weight complex which contains the protein phosphatase 1 Glc7p (Peters et al., 1999) and V_0 sectors, the membrane integral part of the vacuolar H^+-ATPase (V-ATPase). Calmodulin was also found in association with the membrane integral VTC complex (Peters et al., 2001). The VTC complex binds to the V-ATPase, is required for the priming activity of Sec18p/NSF, and influences the binding of LMA1 to the membrane (Muller et al., 2002). VTC proteins may, therefore, couple Sec18p/NSF to V_0, perhaps to activate it for a potential role in fusion. This speculation appears particularly attractive because a subset of V_0 sectors, which is enriched in calmodulin and in the vacuolar t-SNARE Vam3p, form transcomplexes. V_0 sectors from the opposing membranes bind to each other before fusion has been completed (Peters et al., 2001). A multimeric (probably hexameric) cylinder of proteolipids is a central part of the V_0 sector (Hirata et al., 1997; Powell et al., 2000). Thus, V_0 transcomplexes resemble the situation postulated in some pore models of fusion (Lindau and Almers, 1995; Mayer, 2001; Zimmerberg, 2001) which suggest that continuous proteinaceous pores composed of multiple subunits could mediate membrane fusion. This could occur, for example, by radial expansion of the cylinder and partial lateral separation of their subunits (Lindau and Almers, 1995) or by conformational changes of the pore subunits, resulting in rotation or the transient shifting of hydrophobic and hydrophilic surfaces (Zimmerberg, 2001). If vacuole fusion followed this or a similar pathway, proteolipids or entire V_0 sectors should act in the postdocking phase of this reaction. We have begun to test this hypothesis by manipulating different V-ATPase subunits and assaying the effects on vacuolar membrane fusion.

Results

Dissecting the role of V_0 in vacuolar membrane fusion poses the problem to separate the requirement for a proton motive force (pmf)* from a direct involvement of V_0 in bilayer fusion. Completion of vacuole docking and trans-SNARE pairing require a pmf (Ungermann et al., 1999b). However, full V-ATPase proton pump activity is not required because significant fusion activity remains after V-ATPase inactivation by chaotropic salts (Peters et al., 2001). This suggests that a comparably low pmf may suffice to drive fusion which could be satisfied by residual V-ATPase activity provided by a fraction of intact V-ATPases below the detection limit. More likely, however, alternative pathways create a pmf, for example, by combination of ATP-driven ion or metabolite transporters and proton-translocating antiporters. Many such transport systems exist in vacuolar membranes. In any case, the requirement for a basal pmf necessitates to test a direct involvement of V_0 in bilayer fusion under conditions in which proton translocation activity of the V-ATPase is not limiting for the reaction.

We pursued in vivo and in vitro approaches to address this question. V-ATPase–deficient mutants die on neutral media and survive with slow growth on acidic media (Stevens and Forgac, 1997; Kane, 1999). Due to altered physiological properties of the cells, this complicates an in vivo analysis of vacuole structure. All deletions of core V-ATPase subunits result in this "vma phenotype," with one important exception: The a-subunit of the V-ATPase exists in two isoforms, the vacuolar isoform Vph1p and the Golgi or endosomal isoform Stv1p (Manolson et al., 1994). Both isoforms can substitute for one another in proton translocation, although they differ with respect to regulated V_1/V_0 dissociation that occurs upon glucose depletion (Parra and Kane, 1998; Kawasaki-Nishi et al., 2001a,b). A single deletion of either Vph1 or Stv1 does not result in the vma phenotype and permits healthy growth of the cells, also on neutral media (Manolson et al., 1994).

Deletion of the V_0 subunit Vph1p results in vacuolar fusion defects

Absence of the vma phenotype makes vph1 mutants suitable for probing a role of V_0 in vacuolar fusion in vivo. Mutation of fusion-relevant components frequently results in a fragmentation of vacuoles into multiple small vesicles in vivo (Raymond et al., 1992; Wada et al., 1992b). In some mutants, fragmentation does not occur, for example, after mutation of the v-SNARE Nyv1, Vtc1, or Vtc4 (Nichols et al., 1997; Muller et al., 2002). We assayed the in vivo effects of

*Abbreviations used in this paper: BCECF, 2′7′-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein; FCCP, carbonylcyanide-4-trifluormethoxyphenylhydrazon; pmf, proton motive force.

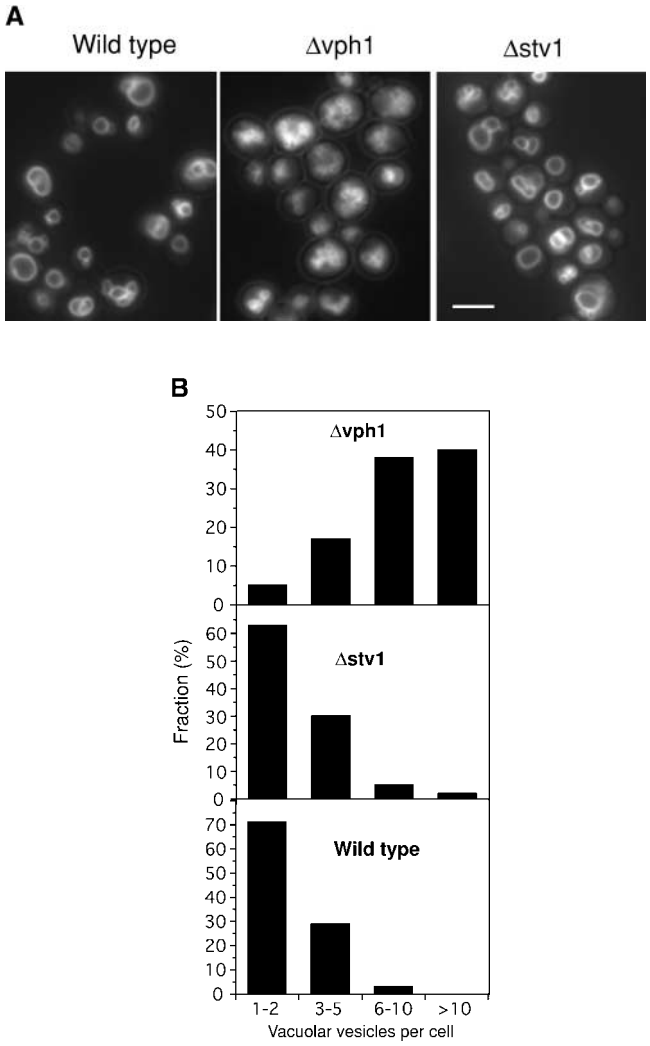

Figure 1. **Vacuolar structure in V-ATPase mutants.** (A) Yeast cells (BY4742) containing the indicated deletions of different V-ATPase subunits were grown logarithmically in liquid YPD medium, stained with FM4–64, and analyzed by fluorescence microscopy as described in Materials and methods. (B) Quantitation of vacuole morphology. The number of vacuolar vesicles (stained by FM4–64) per cell was determined for the strains shown in A. For each experiment, 200 cells per strain were analyzed and grouped into the indicated categories. Two experiments were averaged. Bar, 5 μm.

V-ATPase mutations by light microscopy after staining the vacuolar membranes with the red fluorescent vital dye FM4–64. Mutants lacking the vacuolar V_0 subunit Vph1p showed numerous small vacuolar fragments which formed clusters (Fig. 1). This phenotype was observed with high frequency and resembles that of mutants in other genes with a function in the postdocking phase of vacuole fusion, such as Vtc3, Glc7 (protein phosphatase 1), calmodulin, or Vac8 (Peters and Mayer, 1998; Peters et al., 1999, 2001; Wang et al., 2001b; Muller et al., 2002). In contrast, mutation of Stv1, the Golgi/endosomal isoform of Vph1, did not result in vacuolar fragmentation (Fig. 1), nor did deletion of the V_1 subunit Vma1p (unpublished data; with the strong caveat that this last mutant has the severe vma phenotype). Our results differ from those published in another study (Perzov et al., 2002) that reported vacuolar fragmentation for Δstv1 cells but not for Δvph1 cells. Due to this discrepancy, we generated Δvph1 mutants in three independent strain backgrounds and consistently observed vacuolar fragmentation. Currently, we have no explanation for this difference. However, our results are strongly corroborated by an unbiased microscopic screen for deletion mutants showing vacuolar fragmentation (Seeley et al., 2002). This genome wide screen identified mutants in four out of five V_o subunits as defective in vacuolar morphology, reporting a particularly strong phenotype for Δvph1 but none for Δstv1 or Δvma1.

In vivo experiments provide only indirect information about the proton uptake and fusion activities of the vacuoles. To overcome this limitation, we analyzed this aspect in an in vitro system reconstituting vacuolar fusion (Conradt et al., 1992). Vacuoles can be extracted from yeast spheroplasts after enzymatic digestion of the cell wall and gentle lysis by DEAE-dextrane, a treatment that leaves vacuoles intact and fusion competent. The isolated organelles fuse upon incubation with an ATP-regenerating system and cytosolic extracts. Since both V_1 and V_0 sectors are essential for proton translocation activity of the V-ATPase (Stevens and Forgac, 1997; Kane, 1999), we compared the fusion competence of vacuoles from V_1 and V_0 mutants, testing whether this V-ATPase activity was required for vacuole fusion. Vacuoles purified from the V_0 mutant Δvph1 were deficient for in vitro fusion. The fusion defect was even observed if only one of the fusion partners was a Δvph1 vacuole and the other fusion partner was a wild-type vacuole (Fig. 2 A). This suggests that Vph1p is required for fusion on both fusion partners. Vacuoles from the V_1 mutant Δvma1 fused with ~50% of wild-type activity (Fig. 2 A). Addition of a cytosolic extract increased the fusion activity of Δvma1 vacuoles to 70%. This

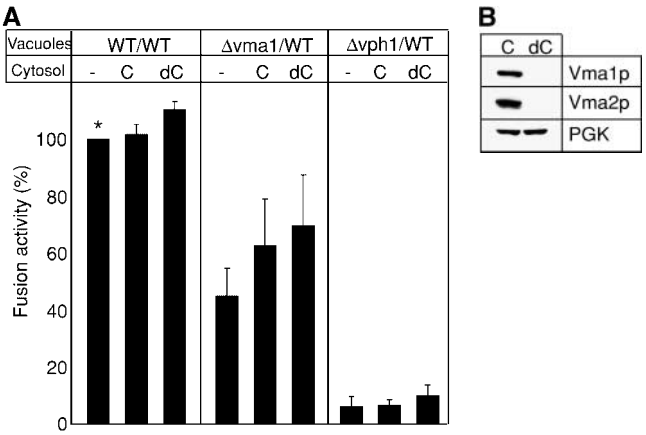

Figure 2. **Fusion activity of vacuoles from strains deficient in V_1 sector (Δvma1) or in V_0 sector (Δvph1).** (A) Vacuoles from the indicated mutants (DKY 6281 background) were fused with wild-type vacuoles (BJ3505). Incubations were performed in the presence or absence of untreated cytosol (C) or of cytosol immunodepleted for Vma1p and Vma2p (dC). Fusion was assayed after 70 min at 27°C. The fusion activities of control reactions (asterisk) were set to 100%. The activity of the samples on ice was set to 0% ($n = 5$). Fusion activities of the wild-type–wild-type combination ranged from 2.2 to 4.1 U. (B) Western blot of equal protein amounts of cytosol (C) and immunodepleted cytosol (dC) against Vma1p, Vma2p, and the cytosolic marker protein phosphoglycerate kinase (PGK), verifying the absence of V_1 sectors in the depleted cytosol.

activation was not due to readdition of intact V_1 subunits via the cytosolic extract because immunodepletion of V_1 from these extracts (Fig. 2 B) did not diminish their activities (Fig. 2 A). Δvph1 vacuoles remained fusion incompetent under all conditions. Since both deletions of the V_0 subunit Vph1 and deletion of the V_1 subunit Vma1 completely abolish the pump activity of the V-ATPase (Stevens and Forgac, 1997; Kane, 1999), the difference in fusion activity between these strains is best explained by a function of Vph1p for vacuolar fusion that is independent of proton translocation.

Vacuolar proton uptake is not limiting for fusion of Δvph1 vacuoles

Since vacuole fusion depends on a pmf across the vacuolar membrane (Conradt et al., 1994; Ungermann et al., 1999b) and the V-ATPase is the major vacuolar proton pump (Stevens and Forgac, 1997), we asked whether the fusion activity of Δvph1 vacuoles could be limited by lack of a pmf. We tested whether Δvph1 vacuoles are able to generate a basal pmf by a V-ATPase–independent pathway and whether this pmf would be above the threshold required for fusion. V-ATPase–independent antiport mechanisms can reconstitute a basal proton uptake activity by antiport of, for example, amino acids and protons (Ohsumi and Anraku, 1981, 1983; Sato et al., 1984; Klionsky et al., 1990; Wada et al., 1992a; Wada and Anraku, 1994) and/or by combination of an active vacuolar Ca^{2+} pump and a Ca^{2+}/H^+ antiporter (Ohsumi and Anraku, 1983; Cunningham and Fink, 1994; Forster and Kane, 2000). The pmf as such is important to drive fusion, but for the purpose of our experiments it is irrelevant whether proton uptake occurs via the V-ATPase or alternative mechanisms. We measured the apparent proton uptake activity of whole vacuoles under the conditions of our fusion reaction by following the change in absorbance of acridine orange at 491 and 540 nm, a common assay for the vacuolar pmf (Gluck et al., 1982). Since acridine orange is also fluorescent, we could monitor its distribution in the membrane preparation by fluorescence microscopy. At least 92% of the dye localized to structures that, by size and morphology, could be identified as vacuoles (unpublished data). Therefore, acridine orange will mostly report changes of the pmf on vacuoles. At standard dye to membrane ratios wild-type vacuoles showed a clear ATP-dependent decrease in the absorbance signal, indicating that a pmf had formed (Fig. 3 A, bottom panel). Addition of the protonophor carbonylcyanide-4-trifluormethoxyphenylhydrazon (FCCP) at the end of the assay period collapsed the pmf and restored the signal to the initial values. No signal was observed in the absence of ATP or after addition of even low concentrations (0.1 μM) of the V-ATPase inhibitor concanamycin A. Concanamycin A blocks V-ATPase–dependent H^+

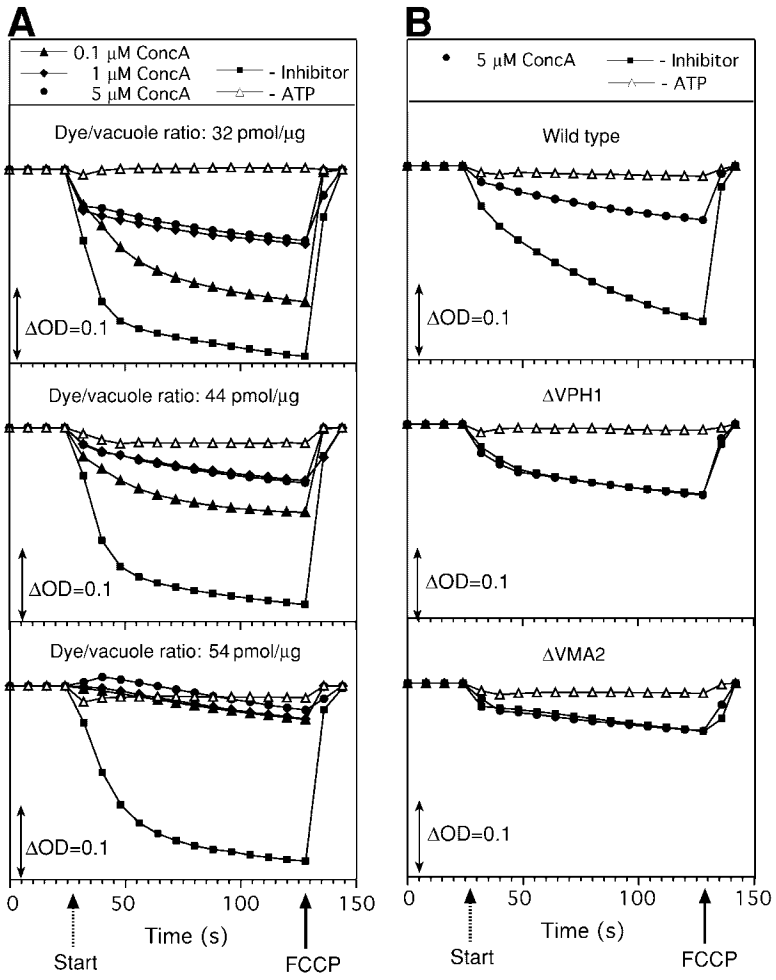

Figure 3. **Detection of V-ATPase–independent proton uptake activity by acridine orange.** (A) Concentration dependence of the assay. Proton uptake activity of the vacuoles was assayed by measuring acridine orange absorption. Fusion reactions were started in the presence of 15 μM acridine orange and different vacuole concentrations to vary the dye to vacuole ratio. Where indicated (start), the ATP regenerating system and concanamycin A had been added. At the end of the assay period, FCCP (30 μM) was added to dissipate the proton gradient. (B) Assay of mutant vacuoles. Vacuoles from wild-type, Δvph1, and Δvma2 cells were assayed for proton uptake activity as in A. 54 pmol acridine orange (final concentration 15 μM) were used per μg vacuoles.

translocation at nanomolar concentrations (Drose and Altendorf, 1997). Our observation is consistent with earlier results from various other groups using subvacuolar vesicles (Drose and Altendorf, 1997). These studies demonstrated that the V-ATPase was the main source of the vacuolar pmf and could be completely inhibited by bafilomycin A or concanamycin A. At lower dye to vacuole ratios (Fig. 3 A, top two panels), however, the assay was more sensitive and could still detect a pmf in the presence of 0.1 μM concanamycin A. 1 and 5 μM of the inhibitor reduced the signal further and yielded overlapping curves, suggesting that these concentrations had saturated the system and that V-ATPase had been completely inhibited. Nevertheless, significant ATP-dependent proton uptake activity remained even at these high concentrations. This concanamycin-insensitive activity was comparable to the levels of ATP-dependent proton uptake observable with vacuoles prepared from V_1 (Δvma2) or V_0 (Δvph1) mutant cells (Fig. 3 B). The signal in V-ATPase mutant vacuoles could not be further reduced by concanamycin A. Therefore, this signal appears to reflect the V-ATPase–independent part of vacuolar proton uptake.

Next, we asked whether the V-ATPase–independent proton uptake activity could suffice to drive fusion. We compared the fusion and proton translocation activities of Δvph1 vacuoles to those of wild-type vacuoles in which V-ATPase pump activity had been pharmacologically suppressed with concanamycin A. In this case, we measured apparent proton uptake activity by following alkalinization of the medium with the pH-sensitive dye 2′7′-bis-[2-carboxyethyl]-5-[and 6]-carboxyfluorescein (BCECF; Peters et al., 2001). Unlike acridine orange, which inserts into the vacuolar membrane (unpublished data), this dye is coupled to high molecular weight dextrane, does not interfere with vacuole fusion, and permits assay of fusion and proton translocation in the same samples. In this assay, the apparent proton translocation activity of Δvph1 vacuoles was equal to that of wild-type vacuoles treated with 1 μM concanamycin A (Fig. 4 B). As in the acridine orange assay, this signal was ATP dependent and FCCP sensitive, suggesting that it reflected the same V-ATPase–independent component of vacuolar proton uptake. Comparison of the fusion activities revealed that Δvph1 vacuoles did not fuse, but wild-type vacuoles with 1 μM concanamycin A retained full fusion activity (Fig. 4 A). FCCP dissipates the proton gradient across the vacuolar membrane, eliminating both its V-ATPase–dependent and V-ATPase–independent components. In agreement with earlier studies (Conradt et al., 1994; Mayer et al., 1996; Ungermann et al., 1999b), FCCP inhibited fusion. Together, vacuole fusion seems to depend on a pmf across the vacuolar membrane but not on V-ATPase proton pump activity. Hence, the fusion defect of Δvph1 vacuoles cannot be explained by a reduction in the apparent proton uptake activity, supporting a direct function of V_0 in vacuole fusion.

We sought to specifically interfere with the fusion relevant aspect of V_0. Available low molecular weight V-ATPase inhibitors affect only the proton translocation function of the V-ATPase, (e.g., concanamycin A) and/or are slowly reacting and of unsatisfactory specificity (e.g., dicyclohexylcarbodiimide). We raised antibodies to V_0 subunits in order to generate highly specific agents that might interfere with V_0 function and be suitable for analysis of the fusion reaction. We immunized goats with the recombinantly expressed and purified NH_2-terminal cytosolic part of Vph1p and prepared affinity-purified polyclonal antibodies from the sera. If titrated into in vitro fusion reactions, these antibodies inhibited fusion in a concentration-dependent manner (IC_{50}= 6 μM; Fig. 5 A). Nonimmune antibodies had no effect at comparable concentrations (Fig. 5 B). To exclude potential interference due to the divalent nature of whole IgG, we also prepared monovalent F_{ab} fragments from these antibodies. They also inhibited fusion at concentrations comparable to those observed for whole IgGs (Fig. 5 C), matching our general experience for many other fusion relevant components: the inhibitory potency of whole IgGs and F_{ab} fragments is not significantly different (Haas et al., 1995; Haas and Wickner, 1996; Mayer and Wickner, 1997; Nichols et al., 1997).

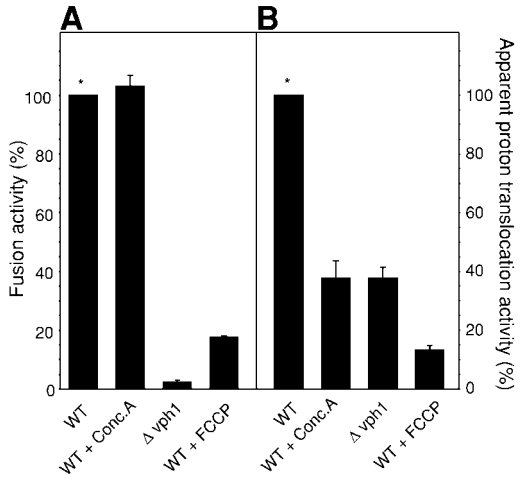

Figure 4. **Fusion and apparent proton translocation activity of vacuoles from vph1 deletion strains (Δvph1).** (A) Fusion and apparent proton translocation activity (B) were examined in parallel fusion reactions using wild-type or Δvph1 vacuoles in the presence or absence of concanamycin A (Conc.A; 1 μM) or FCCP (30 μM). Fusion and pump activities of wild-type vacuoles (asterisk) were set to 100% (n = 3). Fusion activities of these control samples ranged from 2.5 to 3.8 U.

As described for the Δvph1 mutants above, we tested whether antibodies to Vph1p would interfere with the apparent vacuolar proton uptake. This was not the case, although the antibodies blocked fusion (Fig. 6). The lack of effect of anti-Vph1p on proton uptake is probably due to the fact that the antibodies recognize only solitary V_0 sectors but not the assembled V_0/V_1 holoenzyme (unpublished data). The antibodies were also tested on vacuoles from which the V_1 sector had been removed by cold extraction with KSCN and ATP, a treatment that leaves the V_0 sector in the membranes (Arai et al., 1989; Moriyama and Nelson, 1989; Adachi et al., 1990; Peters et al., 2001) but inactivates V-ATPase–dependent proton translocation (Fig. 7 B). Fusion of such vacuoles was inhibited by anti-Vph1p as efficiently as by GTPγS or antibodies to Sec18p/NSF (Fig. 7 A), two established antagonists of vacuole fusion (Haas et

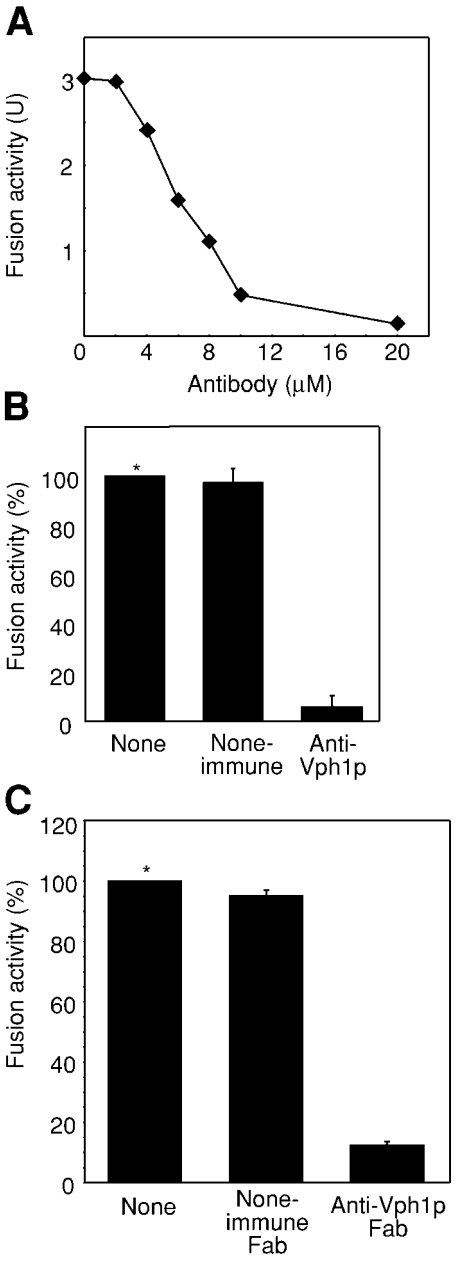

Figure 5. **Inhibition by antibodies to Vph1p.** (A) Different concentrations of affinity-purified antibodies to Vph1p (from goat) were added to a standard fusion reaction without cytosol. The reaction was started after preincubation for 10 min on ice by adding ATP. Fusion was assayed after 70 min at 27°C. (B) Nonimmune antibodies do not interfere with vacuole fusion. Standard fusion reactions were run with 20 μM IgG purified from nonimmune or anti-Vph1p sera from goats, or with control buffer only, and assayed after 70 min at 27°C ($n = 6$). (C) Inhibition of fusion by F_{ab} fragments of antibodies to Vph1p. Standard fusion reactions were incubated with F_{ab} fragments derived either from nonimmune antibodies or from antibodies to Vph1p. Fusion was assayed as in the legend to Fig. 2 ($n = 3$). Fusion activities of the control samples (asterisk) ranged from 3.2 to 5.1 U, and those of ice values ranged from 0.2 to 0.3 U. Inhibitor concentrations were 45 μM anti-Vph1p F_{ab} and 45 μM nonimmune F_{ab}.

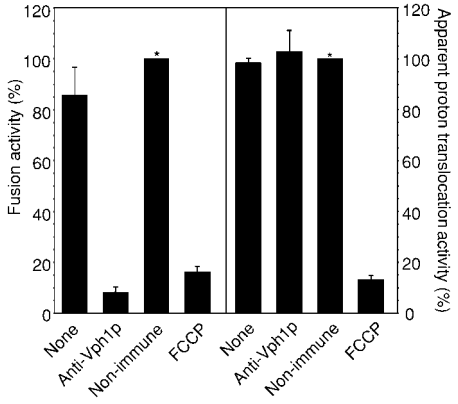

Figure 6. **Influence of antibodies to Vph1p on the apparent proton translocation and fusion activity of intact vacuoles.** Standard fusion reactions were run in the presence or absence of the indicated inhibitors. Pump activity was determined with the BCECF assay as described in Materials and methods. Fusion activities were assayed in parallel samples as in Fig. 2 ($n = 4$). Fusion activities of control samples ranged from 3.3 to 4.8 U. Inhibitors were FCCP (30 μM), anti-Vph1p (20 μM), and nonimmune antibodies (20 μM).

al., 1994; Mayer et al., 1996). Thus, antibodies to the cytosolic part of Vph1p provide an additional tool to separate the fusion function of V_0 from its role in proton pumping.

Vph1p acts in the postdocking phase of vacuole fusion

The antibodies to Vph1p were used for kinetic analyses in order to explore which stage of vacuole fusion required Vph1p. First, we tested when the reaction became resistant to anti-Vph1p (Fig. 8 A). The majority of vacuoles completes distinct reaction steps within defined intervals and becomes resistant to inhibitors of these steps (Conradt et al., 1994; Mayer et al., 1996), revealing the sequence of events. Inhibitors were added to an aliquot of an ongoing fusion reaction at different times after the start point. Then, the incubation was continued until the end of a standard fusion period (70 min). Another aliquot was chilled to stop fusion at the time of inhibitor addition. All inhibitors abolished fusion when added at the start of the reaction (Fig. 8 A). After 35 min, the reaction was resistant to antibodies to the t-SNARE Vam3p, indicating the completion of docking (Mayer and Wickner, 1997; Ungermann et al., 1998b). The inhibition curve for anti-Vph1p lagged significantly behind that for anti-Vam3p, suggesting that Vph1p was required past the docking stage. This behavior resembled the inhibition curves obtained previously with calmodulin antagonists or with the Ca^{2+} chelator BAPTA, which inhibit completion of fusion after the docking step (Peters and Mayer, 1998; Ungermann et al., 1999b).

Trans-SNARE pairing is believed to be a key event in docking membranes and committing them to fusion (Sollner et al., 1993; Weber et al., 1998). It can be assayed as a molecular indicator of docking (Ungermann et al., 1998b). To test whether deletion of Vph1 affected a step before or concomitant with docking, or rather an event required for subsequent fusion per se, we tested whether Δvph1 vacuoles were able to form trans-SNARE complexes. Yeast vacuoles offer the possibility to measure trans-SNARE pairing by use of a combination of vacuoles lacking either the vacuolar v-SNARE Nyv1p or the vacuolar t-SNARE Vam3p (Ungermann et al., 1998b). These vacuoles are prepared from separate strains and mixed in vitro. v/t-SNARE complexes can only

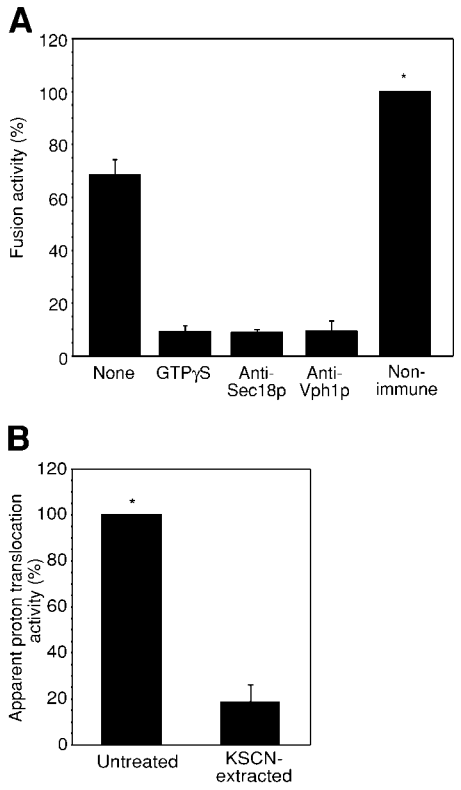

Figure 7. **Fusion of vacuoles without V_1 sector is inhibited by anti-Vph1p.** (A) Vacuoles were extracted with SCN^-/ATP to remove V_1. Fusion activity was assayed in the absence or presence of the indicated inhibitors as in the legend to Fig. 2 ($n = 3$). Fusion activities of the control samples (asterisk) ranged from 0.75 to 1.4 U. (B) Apparent proton pumping activity of untreated or stripped vacuoles was determined as described in Materials and methods. The activity of untreated vacuoles was set to 100%. Inhibitors were GTPγS (2 mM), anti-Vph1p (20 μM), nonimmune antibodies (20 μM), and anti-Sec18p (2 μM).

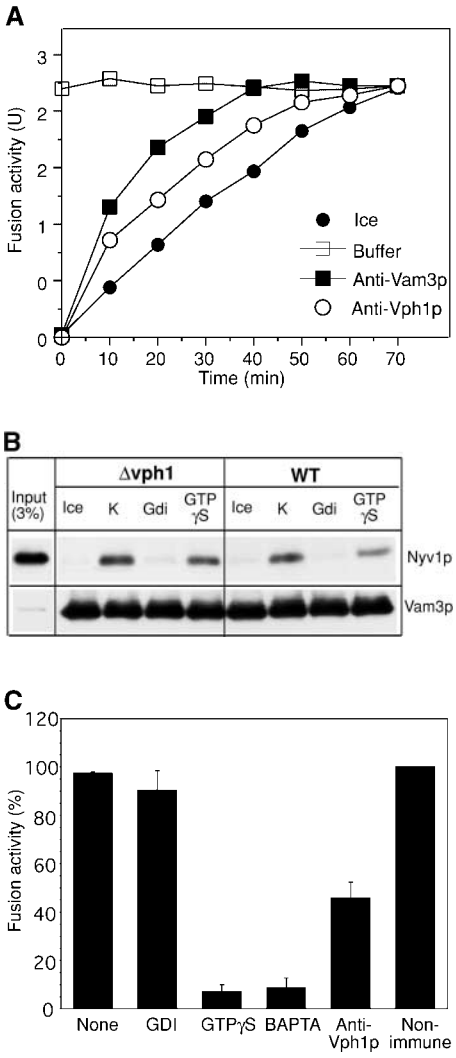

Figure 8. **Kinetic resolution of Vph1p requirement for fusion.** (A) Standard fusion reactions without cytosol were started at 27°C. At the indicated times, inhibitors or control buffer were added. The samples were left on ice for 10 min. Then, they were transferred to 27°C or left on ice for the remainder of the 70-min reaction period. After 70 min, fusion activity was assayed. (B) Trans-SNARE pairing. Fusion reactions with vacuoles from strains SBY521 (Δ*vam3* Δ*vph1*) or no. 418 (Δ*vam3* VPH1) and no. 120 (Δ*nyv1* VPH1) were incubated (50 min, 27°C) with the indicated inhibitors at 27°C or left on ice. Then, the membranes were reisolated, solubilized, and assayed for trans-SNARE complexes by determining the amounts of the v-SNARE Nyv1p coimmunoprecipitating with the t-SNARE Vam3p. (C) Standard fusion reactions without cytosol were incubated at 27°C in the presence of 1 mM $MgCl_2$ and 5 mM BAPTA for 30 min. The reactions were chilled on ice, and vacuoles were reisolated (10,000 *g*, 2 min, 2°C). Vacuoles were resuspended in fusion buffer with cytosol and 200 μM $CaCl_2$ but without ATP. Aliquots were preincubated for 5 min on ice with the indicated inhibitors and incubated further for 70 min at 27°C. Fusion activities were assayed and plotted as in the legend to Fig. 2 ($n = 3$). Activities of the control sample ranged from 1.1 to 1.9 U. Inhibitors were BAPTA (5 mM), GTPγS (2 mM), anti-Vam3p (2 μM), Gdi1p (5 μM), anti-Vph1p (20 μM), and FCCP (30 μM).

be formed if a v-SNARE–containing vacuole docks to a t-SNARE–containing vacuole. If this experiment is performed in the presence of a late acting fusion inhibitor, such as GTPγS, accumulating SNARE complexes represent trans-complexes. We generated a strain carrying deletions of Vam3 (t-SNARE) and Vph1. Vacuoles from this strain were fused with vacuoles from a Δnyv1 (v-SNARE) strain. In agreement with previous results (Ungermann et al., 1998b), SNARE complexes accumulated at 27°C but not on ice (Fig. 8 B). There was no significant difference in the yield of trans-SNARE pairing between VPH1–containing vacuoles and Δvph1 vacuoles. The docking inhibitor Gdi1p, which extracts the Rab-GTPase Ypt7p from the membranes, suppressed formation of trans-SNARE complexes, demonstrating that the signal from the coimmunoprecipitation assay was specific and a consequence of docking. GTPγS, which permits docking and the formation of V_0 transcomplexes (Peters et al., 2001) but not fusion, did not interfere with trans-SNARE pairing. Since trans-SNARE pairing depends on a pmf (Ungermann et al., 1999b), this experiment also indicates that the apparent proton uptake activity of Δvph1 vacuoles sufficed to drive trans-SNARE pairing. More importantly, however, it demonstrates that Δvph1 vacuoles do not fuse (Fig. 2), although they form trans-SNARE complexes (Fig. 8 B), indicating that a step subsequent to or independent of trans-SNARE pairing is blocked by deletion of Vph1.

Coupling of Vph1p to the Ca^{2+}-releasing channel

Docking triggers a Ca^{2+} release from the vacuolar lumen that strengthens the binding of calmodulin to the vacuolar membrane and triggers fusion (Peters and Mayer, 1998). We tested whether Vph1p function would even be required after the Ca^{2+}/calmodulin-dependent step by accumulating reaction intermediates in the presence of the Ca^{2+} chelator BAPTA. This reversible block can be relieved by removing BAPTA and replenishing Ca^{2+} (Ungermann et al., 1999b). Completion of the reaction from this stage still showed significant sensitivity to anti-Vph1p (Fig. 8 C), although the effect was not as pronounced as for the low molecular weight inhibitor GTPγS. This suggests that the antibody-sensitive stage might be tightly coupled to the Ca^{2+}-requiring step and/or be overcome rapidly after removal of BAPTA. The requirement for Vph1p can obviously not be completely satisfied before completion of the Ca^{2+}/calmodulin-dependent step. Together, the results from the kinetic analysis (Fig. 8 A), the direct assay of trans-SNARE pairing (Fig. 8 B), and the staging experiments (Fig. 8 C) all suggest that Vph1p functions after the completion of docking and trans-SNARE pairing, concomitant with or after the release of lumenal Ca^{2+} and close to the completion of fusion.

The relationship of Ca^{2+} release and Vph1p function in fusion was further characterized by monitoring Ca^{2+} release in the course of fusion. Fusion reactions were run in the presence of aequorin and coelenterazine (Muller et al., 2001). Aequorin is a protein that oxidizes its substrate coelenterazine in a Ca^{2+}-dependent fashion, emitting light (Shimomura et al., 1993). This permits continuous recording of Ca^{2+} levels in the buffer of ongoing fusion reactions. In agreement with previous results obtained with a different approach (Peters and Mayer, 1998), treatments preventing priming, such as antibodies to Sec18p/NSF or Sec17p/α-SNAP, suppressed the release of Ca^{2+} from the vacuolar lumen (Fig. 9 A). Docking inhibitors, such as antibodies to the t-SNAREs Vam3p and Vam7p had the same effect (not depicted). Surprisingly, antibodies to Vph1p also inhibited Ca^{2+} release, suggesting that either Vph1p or V_0 itself might be involved in Ca^{2+} release or that Vph1p might be coupled to the Ca^{2+} channel. In both cases, adding an antibody to Vph1p might block a conformational change required for activation of the channel. Only in the first case, however, should deletion of Vph1p, which eliminates the vacuolar V_0 sector as a whole (Stevens and Forgac, 1997; Kane and Parra, 2000), abolish Ca^{2+} release. This was not the case. *Δvph1* vacuoles showed Ca^{2+}efflux just as their wild-type counterparts (Fig. 9 B). Efflux was sensitive to the Rab-GTPase inhibitor Gdi1p, demonstrating that it depended on docking and was triggered by the authentic fusion pathway. This permits several conclusions. First, it suggests that the fusion defect of *Δvph1* vacuoles is not due to the inability to release calcium. Second, Vph1p itself is not part of the Ca^{2+}-releasing channel. Third, Vph1p may be coupled to the Ca^{2+} channel, leaving calcium release intact if Vph1p is absent but blocking it if Vph1p is inactivated by an antibody.

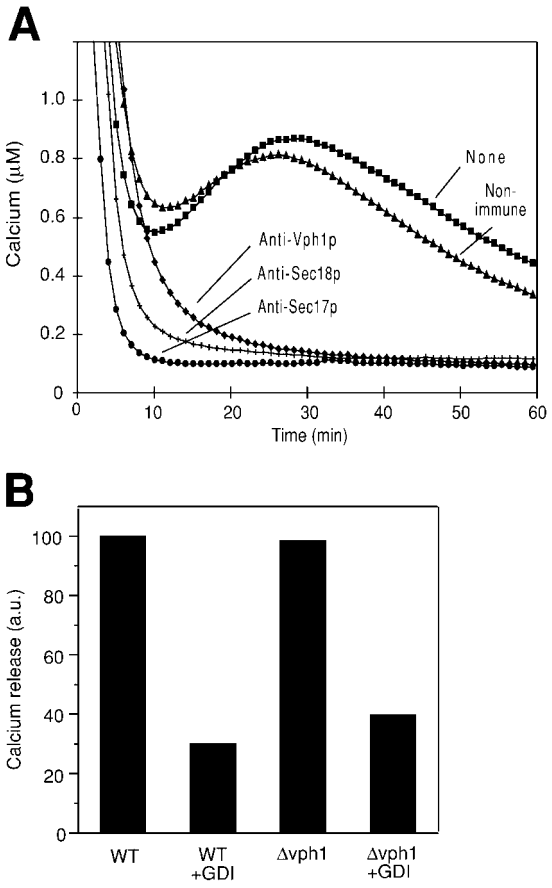

Figure 9. **Vacuolar Ca^{2+} release during fusion.** (A) Inhibition by antibodies to Vph1p. Standard fusion reactions were started, and Ca^{2+} was monitored continuously as described in the Materials and methods. Before the reactions were started, vacuoles had been preincubated with one of the indicated inhibitors, or with one of the following buffers only (3 min, 0°C): anti-Sec18p (2 μM), anti-Sec17p (2 μM), anti-Vph1p (20 μM), and nonimmune antibodies (20 μM). (B) Effect of vph1 deletion. Vacuoles were prepared from wild-type and Δvph1 cells and incubated under fusion conditions in the presence or absence of 5 μM Gdi1p. Ca^{2+} efflux was monitored as in A, and peak signals were plotted.

Discussion

A previous study demonstrated that V_0 sectors from opposing vacuolar membranes can associate into transcomplexes before fusion has occurred (Peters et al., 2001). Vacuolar V_0 sectors consist of a cylinder composed of (probably six) proteolipids, the large multispanning integral membrane protein Vph1p, and the peripheral subunit Vma6p (Kane et al., 1989; Hirata et al., 1997; Powell et al., 2000). V_0 transcomplexes might thus connect proteolipid cylinders from opposing membranes, creating a condition that might resemble the situation postulated in pore models of fusion (Lindau and Almers, 1995; Zimmerberg, 2001). Formation of V_0 transcomplexes requires ATP, the Rab-GTPase Ypt7p, and trans-SNARE pairing. Therefore, it depends on the early reaction steps of priming and docking (Peters et al., 2001). Maintenance of V_0 transcomplexes is independent of trans-SNARE pairing. These observations link V_0 transcomplex formation into the reaction sequence. It is important, however, to investigate whether V_0 is required in the final stage of fusion. The results presented here address this aspect.

First, we demonstrate that V_0 does not only form transcomplexes but is critically involved in vacuole fusion in vitro

and in vivo. The V_0 requirement kinetically mapped to the reaction phase that follows completion of docking and trans-SNARE pairing (Ungermann et al., 1998b) and is independent of a proton gradient (Mayer et al., 1996; Mayer and Wickner, 1997; Ungermann et al., 1999b). However, this terminal reaction phase depends on Ca^{2+} release from the vacuolar lumen which triggers bilayer fusion (Peters and Mayer, 1998). This is consistent with the observation that V_0 transcomplexes form after docking and depend on Ca^{2+} release (Peters et al., 2001). It establishes a correlation between the timing of V_0 function in fusion and V_0 transcomplex formation, strengthening the hypothesis that V_0 transcomplex formation is tied to V_0 function in vacuole fusion. Second, we demonstrate that Vph1p is required on both fusion partners. This symmetric requirement for Vph1p is consistent with a feature of some published pore models, namely that two membrane integral multisubunit cylinders connect the two membranes before fusion (Lindau and Almers, 1995; Zimmerberg, 2001).

Several lines of evidence suggest that trans-SNARE pairing is a necessary intermediate in vacuole fusion but that further events are required to drive the transition from docking to full fusion. First, quenching of the Ca^{2+} efflux by BAPTA reduces fusion activity to 10% of the control samples (Peters and Mayer, 1998), although BAPTA permits trans-SNARE pairing (Ungermann et al., 1999b). However, BAPTA blocks V_0 transcomplex formation completely (Peters et al., 2001). Second, deletion of Vtc3 permits docking of the vacuoles, Ca^{2+} release, and V_0 transcomplex formation but not fusion (Muller et al., 2002). Third, inactivation of V_0 by deletion of Vph1p reduced fusion activity to 10–15% of the control value, although trans-SNARE pairing was unaffected. This demonstrates that V_0 is crucial for most of the observable fusion activity of vacuoles. Fourth, trans-SNARE complexes appear to be largely dispensable after the BAPTA-sensitive stage (Ungermann et al., 1998a, 1999b) but V_0 is not. This correlates to the behavior of V_0 transcomplexes because docking and trans-SNARE pairing are required to establish V_0 transcomplexes. Once formed, however, V_0 transcomplexes persist in the absence of SNARE complexes (Peters et al., 2001).

The 10–15% of fusion activity observed after V_0 inactivation is probably caused by trans-SNARE complexes, reflecting a basal level of fusion that may result from forcing the membranes into close proximity. It is possible that this basal activity is what could be reconstituted with purified SNAREs in liposomes. Although these liposomes contained high concentrations of SNAREs, they yielded significantly lower fusion rates than the equivalent complete fusion system can show in situ (Weber et al., 1998; Parlati et al., 1999). Lowering the v-SNARE concentration in liposomes to roughly physiological levels abolished this fusion activity (Mahal et al., 2002). It made the system completely dependent on an additional factor, the exocytic Ca^{2+} sensor synaptotagmin. Synaptotagmin strongly stimulated fusion, though in this case in a Ca^{2+}-independent fashion.

It appears that both in triggered fusion events, such as regulated exocytosis, and in "constitutive" fusion events, such as vacuole fusion, the membranes require additional factors to efficiently progress from docking and trans-SNARE pairing to fusion. Such a function may be fulfilled by synaptotagmin in regulated exocytosis (Fernandez-Chacon et al., 2001; Chapman, 2002) or by V_0 and Vtc3p in vacuole fusion. It is possible that proteins with related properties participate in other membrane fusion reactions. Candidates exist, as exemplified by Got1p and Sft2p, tetraspanning membrane proteins with a genetic interaction with the t-SNARE Sed5p and some similarities to the V_0 proteolipids. Got1p is required in a postdocking stage of ER-Golgi trafficking and forms multimeric assemblies in the membrane (Conchon et al., 1999). SCAMP2 was identified as a factor required for endocytosis but also for a very late step in mast cell exocytosis. SCAMP2 is also a tetraspanning membrane protein that belongs to a protein family with members on various compartments, exists as a multimeric complex in the membrane, and binds to the t-SNARE subunit SNAP-23 (Singleton et al., 1997; Fernandez-Chacon et al., 2000; Fernandez-Chacon and Sudhof, 2000; Guo et al., 2002).

Ca^{2+} is released from the vacuolar lumen in a way that depends on priming and docking (Peters and Mayer, 1998). Now we have discovered that inactivation of the V_0 subunit Vph1p by antibodies prevents this release, but deletion of Vph1p does not. Since inactivation of Vph1p by deletion or antibodies interferes neither with priming nor with docking (Fig. 8 B; unpublished data), we propose that the effect of Vph1p antibodies on Ca^{2+} release may reflect a coupling of Vph1p to the putative Ca^{2+} channel. Whether this coupling would be direct or indirect via shared binding partners cannot be judged at the moment. But it is striking that V_0 is associated with the t-SNARE Vam3p (Peters et al., 2001) and that t-SNAREs in regulated exocytosis were shown to specifically bind to calcium channels (Leveque et al., 1994; Sheng et al., 1994). Our hypothesis is further supported by the differential effects of Ca^{2+} chelators on fusion (Peters and Mayer, 1998). Only BAPTA, a Ca^{2+} chelator with a rapid association rate, can block fusion. EGTA, which has a comparable K_d for Ca^{2+} but a much slower association rate, is not effective. This difference is best explained by assuming that the site of Ca^{2+} release and the Ca^{2+} effector proteins (in case of the vacuoles the effectors are most likely V_0 and calmodulin [Peters et al., 2001]) are tightly associated. Then, chelators must act rapidly to be able to quench Ca^{2+} before it diffuses to and binds its receptor site (Neher, 1998). An analogous situation exists in regulated exocytosis. The triggering stage of this reaction is only sensitive to fast Ca^{2+} chelators (Adler et al., 1991), and the Ca^{2+} channels are associated with syntaxin (t-SNARE) in the plasma membrane (Leveque et al., 1994; Sheng et al., 1994). They should hence be close to the fusion site. This provides a physical correlate for the differential sensitivity of exocytosis to BAPTA and EGTA.

The reconstituted proteolipid cylinder of the V-ATPase can form large pores in response to binding of Ca^{2+} and calmodulin, demonstrating a potential for significant conformational changes in the membrane (Peters et al., 2001). On the other hand, a fraction of V_0 in vacuolar membranes is associated with the VTC complex, a membrane integral complex that is required for the priming activity of Sec18p/NSF (Muller et al., 2002) and for membrane binding of LMA1, i.e., it is coupled to two components that are directly involved in generating (Sec18p; Mayer et al., 1996; Unger-

mann et al., 1998a) or maintaining (LMA1; Mayer and Wickner, 1997; Xu et al., 1997) an activated state of the fusion machinery. Therefore, we speculate that Vph1p, or the entire V_0 sector, might undergo significant conformational changes during fusion, perhaps driven by Sec18p/NSF. Antibodies to Vph1p may prevent such conformational changes and thereby also block Ca^{2+} release through a channel associated with V_0. Such coupling should be functionally relevant because Ca^{2+} release from the lumen of an organelle can only be transient. If the fusion system is not competent to respond to Ca^{2+} at the time of release, the reaction might be nonproductive. One might expect higher efficiency of the overall process if Ca^{2+} release and the Ca^{2+} controlled fusion system were activated in a coordinated fashion. This question can be answered with certainty only once the Ca^{2+}-releasing channel will have been identified and assays for conformational changes of V_0 during fusion will be available.

Materials and methods

Strains

BJ3505 and DKY6281 are standard fusion strains (Mayer et al., 1996). Strain no. 120 is BJ3505 (Δnyv1) (Nichols et al., 1997). Strain no. 418 is DKY 6281 Δvam3 provided by Christian Ungerman (Biochemiezentrum, Heidelberg, Germany). SBY521 (Δvam3, Δvph1) was derived from no. 418 by replacing VPH1 with a kanamycin resistance cassette as described below. BY4742 (Mat α his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and its Δvph1::kanMX4 or Δstv1::kanMX4 derivates were purchased from EUROSCARF.

Deletion strains of VPH1 were made by replacing the gene with a loxP-kanMX-loxP cassette from plasmid pUG6 (Guldener et al., 1996) using the following primers: Vph1 forward K.O., GCCTCTCAATATATCTATACTTGCTTAGAGGGCTACCTGTGTCAGCTGAAGCTTCGTACGC and Vph1 reverse K.O., CTGGTGGATTGGATTGCAAGTCTAACGTTTTCATGAGATAAGGCATAGGCCACTAGTGGATCTG. Vma1 and Vma2 were deleted by replacement with the HIS3 cassette from plasmid pRS 303 using the following primers: VMA1 forward, ATGGCTGGTGCAATTGAAAAGCTCGTAAGGAAATAAAAAGAATCAGATTGTACTGAGAGTGCAC, VMA1 reverse, CAGACAAGGCCGATTTACAACTAATTGAACAACTTGTTCCTGTGCGGTATTTCACACCG, VMA2 forward, GGTGTGAACGGTCCATTAGTCATTTGGAAAAGGTCAAGTTCCCAGATTGTACTGAGAGTGCAC, and VMA2 reverse, GAATCTTTGGGGAGATTCTATTCAACATCTCCTTAGGGTAGCTGTGCGGTATTTCACACCG. All deletions were verified by diagnostic colony PCR and by Western blotting of purified vacuoles from the mutants.

General procedures

Vacuole isolation, cytosol preparation, fusion, purification of antibodies or Gdi1p, inhibitors, and FM4–64 staining were as described (Peters and Mayer, 1998; Peters et al., 1999). PS buffer is 10 mM Pipes/KOH, pH 6.8, and 200 mM sorbitol. 1× PIC is a protease inhibitor cocktail: 100 μM pefabloc SC, 100 ng/ml leupeptin, 50 μM o-phenanthroline, and 500 ng/ml pepstatin A. Vacuoles without V_1 sector were prepared as described (Peters et al., 2001). In experiments with BAPTA, the alkaline phosphatase assay mixture contained 10 mM $CaCl_2$. All samples were centrifuged (1 min, 14,000 *g*) before spectrophotometry to remove precipitated Ca^{2+} salts.

Proton uptake activity of vacuoles

BCECF assay. For the determination of pump activity, 100-μl fusion reactions were incubated at 25°C with 0.5 μM BCECF-dextran (70 K; Molecular Probes). The change of fluorescence was measured at an excitation of 490 nm and an emission of 520 nm in a Perkin Elmer LS50B fluorometer or in a plate reader fluorometer (Spectra Max Gemini; Molecular Devices). Initial pump activity was determined as described (Peters et al., 2001). In brief, the pH shift in the medium was followed via the pH-dependent shift in fluorescence of BCECF. This shift could be calibrated by buffering the solution to different pH values using PS buffer with 150 mM KCl.

Acridine orange assay. Proton uptake of vacuoles was measured essentially as described. The absorbance changes of acridine orange at 491 and 540 nm were followed in a Beckman Coulter DU-600 spectrophotometer (Gluck et al., 1982). The difference between these values at any time point serves as a measure of the vacuolar pmf. The reaction mixture in a final volume of 100 μl contained 20 μg of vacuoles under standard fusion conditions with 15 μM acridine orange. The reaction was started by the addition of 5 μl of ATP regenerating system. At the end, 30 μM FCCP were added. Proton uptake activity was defined as the absorbance change in the first 20 s of the reaction.

Trans-SNARE assay

120-μl fusion reactions (containing 24-μg vacuoles) with cytosol (0.5 μg/μl) were incubated for 50 min. The reactions were stopped by chilling the samples on ice. 380 μl of precipitation buffer were added (150 mM KCl, 1.3% [wt/vol] Triton X-100, 1.3 mM PMSF, 1.2× PIC, 13 mM EDTA, 200 mM Sorbitol, 10 mM Pipes/KOH, pH 6.8). Samples were shaken (10 min, 4°C) and centrifuged (21,000 *g*, 10 min). 450 μl of the solubilisate were transferred to a new reaction tube. 3% of the volume was kept as load control. 14 μg of affinity-purified anti-Vam3p antibody and 100 μl of a 1:4 slurry of protein A–Sepharose CL-4B (Amersham Biosciences) preequilibrated in washing buffer (150 mM KCl, 1% [wt/vol] Triton X-100, 1 mM PMSF, 1× PIC, 10 mM EDTA, 200 mM Sorbitol, 10 mM Pipes/KOH, pH 6.8) were added to each sample. After 1 h of shaking at 4°C, the protein A–Sepharose was washed three times with washing buffer. Residual liquid was removed with a Hamilton pipette. 80 μl SDS sample buffer was added, and aliquots were analyzed by gel electrophoresis and Western blotting.

Generation of antibodies to Vph1p

Vph1 cytosolic domain (Vph1-Cyt) was cloned with an NH_2-terminal His_6 tag for expression in *Escherichia coli*. The coding region of this domain was amplified by PCR using primers cyt Vph1 forward, GCAGAGAAGGAGGAAGCGATTTTTC, cyt Vph1 reverse, TCACTGGACTTCAATTTGATCGGT, and genomic DNA as a template. The PCR product was cloned into pCR T7/NT-TOPO (Invitrogen). The resulting plasmid was transformed into *E. coli* BL21. Cells were grown in LB/Ampicillin medium (37°C, 225 rpm) to an OD_{600} of ~0.6. Expression was induced with 1 mM IPTG at 37°C for 4 h. Cells were harvested by centrifugation (10,000 *g*, 7 min), washed with twofold-concentrated PBS, and lysed by two freeze–thaw cycles and sonication in twofold-concentrated PBS, 8 M urea. The lysate (50 ml out of 8 liters culture) was centrifuged (20,000 *g*, 15 min, 4°C) and batch incubated with Ni-NTA resin (1 ml bed volume, 2 h, 4°C). The resin was washed with 20 ml of twofold-concentrated PBS, 0.05% SDS and with 20 ml twofold-concentrated PBS, 0.05% SDS, and 10 mM imidazole. Urea was omitted during washing to avoid interference in the following coupling reaction. 0.05% SDS was added to avoid precipitation during elution from the column. The protein was eluted with 150 mM imidazole. The eluate (containing 3 mg of protein) was directly coupled (1 h, RT) to 1.5 ml CH-activated Sepharose 4 B (Amersham Biosciences).

Antibodies to Vph1-Cyt were raised by injecting purified recombinant protein eluate (300–500 μg per boost) into a goat. Serum (10 ml) was affinity purified using the recombinant protein immobilized on activated CH–Sepharose 4B. The serum was diluted with 1 vol PBS and passed over the column at RT. The column was washed with 20–25 column volumes of PBS. Antibodies were eluted with 0.1 M glycine pH 2.75/150 mM NaCl, and the pH was immediately neutralized by adding 1 M Tris/HCl, pH 8.8. The antibodies were transferred into PS buffer with 150 mM KCl by repeated dilution and ultrafiltration in centricon-30 (Amicon) and stored at −20°C. Control antibodies were affinity purified with protein G from preimmune serum and treated as above.

Preparation of F_{ab} fragments

F_{ab} fragments were prepared from Vph1p antibodies essentially according to the manufacturer's instructions (ImmunoPure Fab Preparation kit; Pierce Chemical Co.). 20 mg of purified antibody were diluted with 1 vol of digestion buffer (as supplied). The solution (2 ml) was incubated overnight at 30°C with 0.5 ml immobilized papain. The sample was centrifuged, and the supernatant was passed over a protein G column to remove uncleaved antibodies and F_c Fragments. The flowthrough containing the F_{ab} fragments was repeatedly diluted and concentrated to exchange the buffer for PS with 150 mM KCl.

Ca^{2+} assay

Ca^{2+} release from vacuoles in the course of the fusion reaction was assayed similarly as described (Peters and Mayer, 1998) with the following modifications. Ca^{2+} was directly measured with aequorin in ongoing fusion reactions running in parallel in a microtiter plate luminometer (EG&G Berthold). Reactions (30 μl) contained 10 μg of vacuoles from strain BJ3505 in PS buffer with 150 mM KCl and 0.5 μl cytosol (30 mg/ml). Respective inhibitors were included. After 2 min of preincubation on ice, 2 μl of ATP regenerating system (same composition as in the fusion assay)

and 3 μl of a freshly prepared 2:2:1 mix of the following stock solutions were added: 2.5 μM aequorin (Aqualite; Molecular Probes), 1 mM EGTA/KOH, pH 7.5, and 500 μM native coelenterazine (Molecular Probes) in EtOH. Samples were pipetted into a microtiter plate, and light emission of aequorin was measured with the luminometer for 60 min at 27°C. The assay was calibrated with solutions of different calcium concentrations as described (Peters and Mayer, 1998).

FM4–64 staining

Cells were grown logarithmically in liquid YPD overnight ($OD_{600} < 2$) at 30°C. Cells were harvested (2 min, 3,000 *g*), resuspended in YPD with 10 μM FM4–64 at $OD_{600} = 1$, and shaken for 3 h at 30°C. Cells were harvested as above, resuspended in YPD at $OD_{600} = 0.2$, and shaken for 2–3 h at 30°C. For microscopy, individual samples were removed from the shaker. Cells were harvested (1 min, 3,000 *g*), resuspended in YPD at $OD_{600} = 10$, and immediately analyzed by fluorescence microscopy. For quantitation of vacuole morphology, photos of at least 20 random fields were taken. Vacuoles were counted in at least 200 cells per experiment and condition.

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