# Role of the Vtc proteins in V-ATPase stability and membrane trafficking

**Oliver Müller, Heinz Neumann, Martin J. Bayer and Andreas Mayer***

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstr. 37-39, 72076 Tübingen, Germany

*Author for correspondence (e-mail: Andreas.Mayer@Tuebingen.mpg.de)

*Accepted 17 December 2002*
*Journal of Cell Science 116, 1107-1115 © 2003 The Company of Biologists Ltd*
*doi:10.1242/jcs.00328*

## Summary

**Vtc proteins have genetic and physical relations with the vacuolar $H^+$-ATPase (V-ATPase), influence vacuolar $H^+$ uptake and, like the V-ATPase $V_0$ sectors, are important factors in vacuolar membrane fusion. Vacuoles from *vtc1*Δ and *vtc4*Δ mutants had slightly reduced $H^+$-uptake activity. These defects could be separated from Vtc function in vacuole fusion, demonstrating that Vtc proteins have a direct role in membrane fusion. We analyzed their involvement in other membrane trafficking steps and in V-ATPase dynamics. Deletion of *VTC* genes did not impede endocytic trafficking to the vacuole. However, ER to Golgi trafficking and further transport to the vacuole was delayed in Δ*vtc3* cells. In accordance with that, Δ*vtc3* cells showed a reduced growth rate. Vtc mutations did not interfere with regulated assembly and disassembly of the V-ATPase, but they affected the number of peripheral $V_1$ subunits associated with the vacuoles. Δ*vtc3* vacuoles carried significantly more $V_1$ subunits, whereas Δ*vtc1*, Δ*vtc2* and Δ*vtc4* had significantly less. The proteolytic sensitivity of the $V_0$ subunit Vph1p was different in Δ*vtc* and wild-type cells in vivo, corroborating the physical interaction of Vtc proteins with the V-ATPase observed in vitro. We suggest that Vtc proteins affect the conformation of $V_0$. They might thereby influence the stability of the V-ATPase holoenzyme and support the function of its $V_0$ sector in vacuolar membrane fusion.**

Key words: Membrane fusion, NSF, *Saccharomyces cerevisiae*, SNARE, Vacuole, Yeast

## Introduction

Using yeast vacuoles as a model system (Wickner, 2002), we have identified a complex of Vtc proteins with a crucial role in vacuolar membrane fusion (Muller et al., 2002). The proteins of this complex may be an important interface connecting the early events of N-ethylmaleimide-sensitive factor (NSF)-dependent SNARE (SNAP receptor) activation and membrane attachment with the late events of $V_0$ trans-complex formation (Peters et al., 2001) close to bilayer mixing (Muller et al., 2002). The Vtc proteins form a heterotetrameric complex that associates with the vacuolar SNARE Nyv1p as well as with the $V_0$ sector of the V-ATPase (Muller et al., 2002). Two of its subunits, Vtc1p and Vtc4p, control Sec18p/NSF-dependent priming of SNAREs and HOPS, and membrane association of LMA1. A third subunit, Vtc3p, is neither required for priming nor for $V_0$ trans-complex formation, but it is necessary for fusion and for LMA1 release in the terminal phase of the reaction. Thus, Vtc3p could mediate a very late, post-docking function of the Vtc complex.

Other studies have identified the *VTC1* homologue *NRF1* in a screen for negative regulators of the Rho GTPase Cdc42p in *S. pombe* (negative regulator of Cdc forty-two, *NRF1*) (Murray and Johnson, 2000) or as a hypothetical polyphosphate synthase in the *S. cerevisiae* vacuole (Ogawa et al., 2000). Cdc42p is involved in yeast vacuole fusion (Eitzen et al., 2001; Muller et al., 2001), further supporting the role of Vtc proteins in this process.

Vtc proteins have also been suggested to be vacuolar transporter chaperons (VTC), a novel family of chaperons involved in the distribution of V-ATPase and other membrane proteins in *S. cerevisiae* (Cohen et al., 1999). In this study, *VTC1* was found to be a suppressor of V-ATPase function (*svf*). Null mutations in genes encoding V-ATPase subunits result in a phenotype that is unable to grow at high pH. Deletion of *VTC1* could suppress this phenotype, suggesting a relationship between Vtc1p and V-ATPase. The other members of the *VTC* family, that is, *VTC2*, *VTC3* and *VTC4*, were identified by sequence similarity. Vacuoles from a *Δvtc1* strain showed a reduction of some V-ATPase subunits and reduced proton uptake activity. Some proton uptake activity of the V-ATPase is needed for establishing a membrane potential (Stevens and Forgac, 1997; Wada and Anraku, 1994; Yabe et al., 1999). A proton motive force is required for proper vacuole membrane fusion, as the proton uncoupler p-(trifluoromethoxy)-phenylhydrazone (FCCP) inhibits the in vitro fusion reaction (Conradt et al., 1994; Mayer et al., 1996; Ungermann et al., 1999). Therefore, we carried out studies on the properties of Vtc proteins with special regard to discovering the relationships between V-ATPase activity, V-ATPase stability and membrane fusion.

## Materials and Methods

General procedures, vacuole isolation and fusion, and antibodies have been described previously (Muller et al., 2002). Monoclonal antibodies to Vph1p and alkaline phosphatase (Pho8p) were from Molecular Probes, Netherlands. Polyclonal antibodies to GFP were from Torrey Pines Biolabs, San Diego. Concanamycin A was from Alexis, San Diego, USA. PS buffer is 10 mM PIPES/KOH pH 6.8, 200 mM sorbitol.

### Strains

BJ3505, DKY6281, OMY1 through OMY13, SBY82, SBY83, SBY85 and SBY86 have been described previously (Muller et al., 2002). For the construction of strains with single, double and triple knock outs of *VTC1*, *VTC2* and *VTC3* genes, strain BY4727 (*MATα his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0*) (Brachmann et al., 1998) was used as a parent. The *VTC* genes were replaced by *HIS3*, *URA3* and *LEU2* markers using PCR-generated cassettes from plasmids pRS303 (*HIS3*), pRS306 (*URA3*) and pRS305 (*LEU2*), respectively (Brachmann et al., 1998). The oligonucleotides used for generation of deletion cassettes and for control PCR have been described elsewhere (Muller et al., 2002). The resulting strains were OMY20 (*Δvtc1::HIS3*), OMY21 (*Δvtc2::HIS3*), OMY22 (*Δvtc3::HIS3*), OMY23 (*Δvtc1::HIS3 Δvtc2::URA3*), OMY24 (*Δvtc1::HIS3 Δvtc3::URA3*), OMY25 (*Δvtc2::HIS3 Δvtc3::URA3*) and OMY26 (*Δvtc1::HIS3 Δvtc2::URA3 Δvtc3::LEU2*).

### Construction of GFP fusion proteins

The Vtc1p-GFP construct (pYER-GFP) was generated as follows: 500 bp upstream of the last codon before the stop codon of the *VTC1* ORF were amplified from genomic DNA using the primers 5′-CGG GCG GCC GCT TCT TAT TTC AAT CTG CAT ACT CAT TTT-3′ and 5′-CCT TCT AGA GCT AAC TTA GTG TTA GCG TCA TTG-3′, which introduced a 5′ *Not*I site and a 3′ *Xba*I site. Using these restriction sites, the PCR fragment was cloned into pRS416-GFP (T. Vida), resulting in C-terminal Vtc1-GFP with a seven amino acid spacer between the last codon of *VTC1* and the start codon of GFP. The construct was verified by sequencing. The plasmid was transformed into yeast strains BJ3505 and DKY6281 using the *URA3* marker. For the construction of Vtc3-GFP, GFP was chromosomally integrated at the 3′ end of the *VTC3* ORF in strain BJ3505 by homologous recombination. A PCR product was generated with primers (forward) 5′-CA CTA AAA CCA ATT CAA GAT TTT ATC TTC AAT TTG GTT GGG GAA ATG TCT AAA GGT GAA GAA TTA TTC AC-3′ and (reverse) 5′-GA TCT GGG TTT AAC TAT CAC ACA CAT CTT CTC ATT ATG TGC ATT GCA TAG GCC ACT AGT GGA TCT G-3′ and plasmid pUG24 (Niedenthal et al., 1996) as a template. The last codon of *VTC3* and the start codon of GFP are underlined. Integration was verified by PCR using primer (Vtc3 con fw) 5′-GAG GCC GCT AGG AGG GAA AGA GG-3′ binding inside *VTC3* and primer (kan con rev) 5′-CGA TAG ATT GTC GCA CCT GAT TGC C-3′ binding inside the kanamycin resistance marker box and by western analysis. The resulting strain was SBY593.

### Assay for proton uptake activity

Proton uptake of vacuoles was measured by the method described (Cohen et al., 1999). The absorbance changes of acridine orange at 491-540 nm were followed by a Beckman DU-600 spectrophotometer. The reaction mixture in a final volume of 100 μl contained 20 μg of vacuoles (mixture of fusion tester strains) at the fusion concentration and condition (PS buffer, 150 mM KCl, 500 μM $MnCl_2$, 27°C) with 15 μM acridine orange. The reaction was started by the addition of 5 μl of an ATP regenerating system. At the end, 10 μM of FCCP were added. Proton uptake activity was defined as the absorbance change during the first 20 seconds of the reaction.

### Preparation of whole cell extracts

$10^7$ cells from a logarithmically growing culture in YPD medium were harvested in a microfuge (6000 ***g***, 2 minutes at 4°C), washed with 1 ml of cold buffer C (50 mM Tris/HCl pH 7.5, 10 mM $NaN_3$) and resuspended in 30 μl of SDS sample buffer with protease inhibitors [2% SDS, 60 mM Tris/HCl pH 6.8, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.005% (w/v) bromphenol blue, 100 μM pefabloc SC, 100 ng/ml leupeptin, 50 μM o-phenanthroline, 500 ng/ml pepstatin A, 1 mM PMSF]. Glass beads were added and the samples vortexed for 2 minutes. Another 70 μl of sample buffer were added. For Vph1p-analysis, samples were not boiled because this hydrophobic protein aggregates when heated.

## Results

### Localization and topology of Vtc proteins

We studied the properties and localization of Vtc proteins because they are important factors in vacuole fusion. We used specific antisera (Muller et al., 2002) to analyze the membrane association of Vtc1p and Vtc4p. Vtc1p and Vtc4p resisted extraction with 4 M urea, 1.6 M KCl and 0.1 M $Na_2CO_3$ (Fig. 1A) and instead pelleted with membranes such as the vacuolar membrane marker alkaline phosphatase (Pho8p). All of these treatments removed the peripheral membrane protein Sec17p/α-SNAP (soluble NSF attachment protein) from the vacuoles (Fig. 1A). After detergent extraction of the membranes, Vtc1p and Vtc4p remained in the supernatant (data not shown). Thus, both Vtc1p and Vtc4p behave as integral membrane proteins. The published sequence in the databases did not predict transmembrane domains for Vtc4p. Therefore, we resequenced the Vtc4 locus from genomic yeast DNA. This revealed a frameshift of the published sequence. It misses a G after nucleotide 1826 of the coding sequence, leading to erroneous truncation of the predicted protein after 648 amino acids. Our sequencing data predicts Vtc4p as a protein of 721 amino acids containing three C-terminal transmembrane domains.

Vtc1p is homologous to the C-termini of Vtc2p and Vtc3p, which, like Vtc1p, contain three potential transmembrane segments (Cohen et al., 1999). The N-terminus of Vtc2p is homologous to Vtc4p and to the N-terminus of Vtc3p. All are predicted to form hydrophilic domains. Vtc4p may interact with Vtc1p. This interaction is supported by the observation that Vtc4p was completely absent in vacuoles from *vtc1* deletion mutants and that the level of Vtc1p was significantly reduced in *vtc4* deletion strains [Fig. 6A (c.f. Cohen et al., 1999)]. On the basis of the new sequence information, however, this effect can no longer be explained by Vtc1p functioning as a transmembrane anchor for Vtc4p, as originally proposed (Cohen et al., 1999).

A previous study showed diffuse staining of the vacuolar lumen by a Vtc3-GFP fusion protein (Ogawa et al., 2000). This pattern is typical for soluble vacuolar proteins but not for vacuolar membrane proteins – which stain only the vacuolar rim. In order to reanalyze Vtc localization, we constructed a Vtc1p fusion protein carrying GFP at the C-terminus. The fusion protein behaved as an integral membrane protein and was functional because it rescued the vacuolar fusion defect of a *vtc1* deletion mutant (data not shown). In a protease-deficient strain (*pep4*$^-$), the fusion protein stained the vacuolar rim, indicating localization at the vacuole membrane [Fig. 1B, upper panel (c.f. Murray and Johnson, 2001)]. Weak fluorescence signals could also be detected around the nucleus, in the periphery of the cell, and in dot-like structures that may be endosomes or Golgi elements. Vacuolar membrane staining by Vtc1p-GFP was only observed in *pep4*$^-$ cells, that is, in cells with reduced vacuolar proteolytic activity (Fig. 1B, upper panels). In wild-type (*PEP4*$^+$) cells, which have a full complement of vacuolar hydrolases, GFP stained the vacuolar

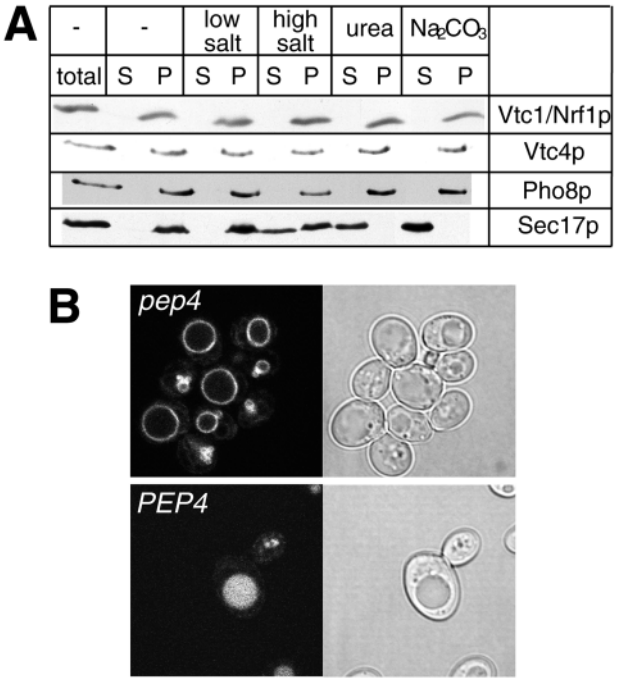


**Fig. 1.** Membrane association of Vtc proteins. (A**)** Vtc1p and Vtc4p behave as integral membrane proteins. 60 µg of vacuoles from strain OMY1 in 1 ml of PS were centrifuged (10,000 ***g*** for 5 minutes at 4°C) and resuspended in 0.2 ml PS with one of the following additions: 100 mM KCl, 50 mM KOAc (low salt); 1.6 M KCl (high salt); 4 M urea; or 0.1 M $Na_2CO_3$. After 10 minutes at 30°C (or 30 minutes on ice for carbonate extraction), the samples were centrifuged (125,000 ***g***, 20 minutes, 4°C). Pellets (P) were resuspended in 1 ml of PS and the supernatants (S) supplemented with PS ad 1 ml. All samples were TCA precipitated and analyzed by SDS-PAGE and western blotting. (B) Strains BJ3505 (*pep4*$^-$) and DKY6281 (*PEP4*$^+$) expressing a Vtc1p-GFP fusion were grown logarithmically in YPD and viewed under a confocal fluorescence microscope. Left panel: GFP fluorescence; right panel: Nomarski optics.

lumen (Fig. 1B, lower panels). As Vtc1p itself still behaves as an integral membrane protein in PEP4$^+$ cells (data not shown), a vacuolar protease probably cleaved the fusion protein between the membrane-embedded Vtc1p C-terminus and the hydrophilic GFP domain, releasing GFP into the vacuolar lumen. The same clipping must have occurred in the earlier study on Vtc3p-GFP (Ogawa et al., 2000) in which a PEP4$^+$ strain had been used. The Vtc1p-GFP fusion was resistant to proteinase K digestion from the cytosolic side (Fig. 2A), but it was degraded into two major fragments if proteolysis was performed in the presence of 0.5% Triton X-100 to lyse the vacuoles. The smaller fragment corresponds to the molecular weight of GFP alone, which, in its correctly folded form, is protease resistant (see also Fig. 1B, lower panels). This indicates that the C-terminus of Vtc1p faces the vacuolar lumen.

The N-termini of the Vtc proteins are thought to face the cytosol on the basis of the following observations: an approximately 80 kDa N-terminal piece of a Vtc3p-GFP* fusion protein (total size of Vtc3p-GFP*: 125 kDa) could be digested with protease from the cytosolic side (Fig. 2A). This is almost the entire Vtc3p portion of the fusion protein (Vtc3p

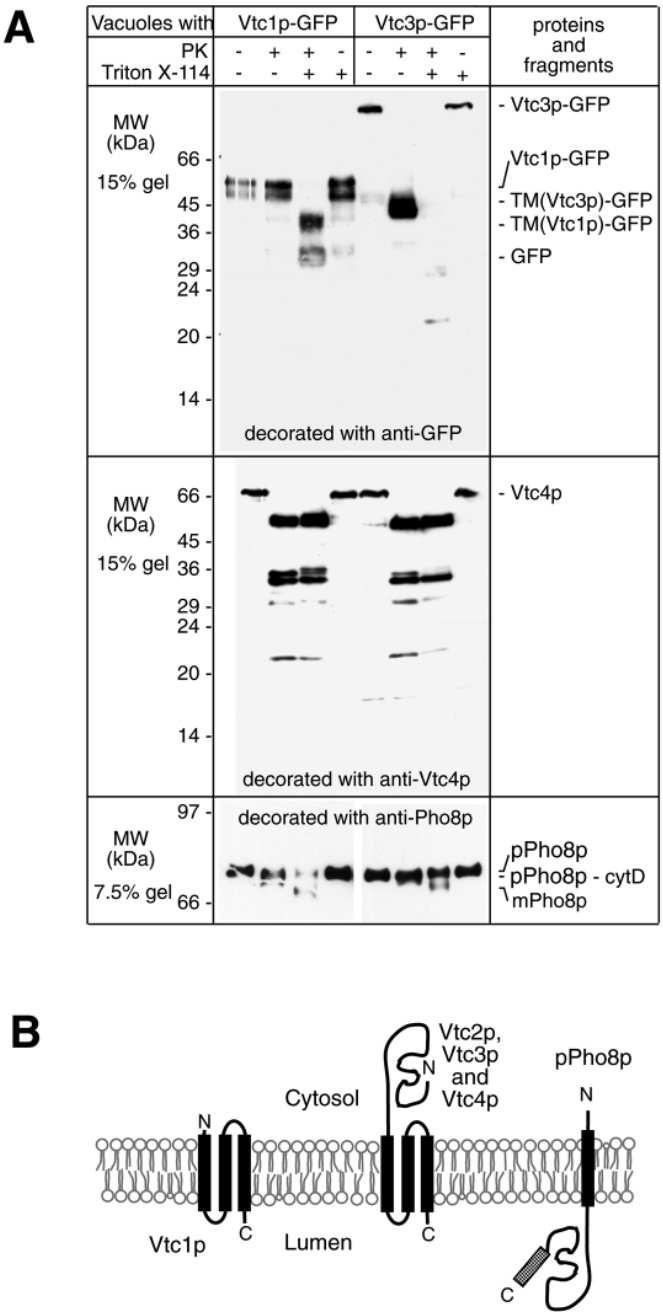


**Fig. 2.** Topology of Vtc proteins. (A) Protease digestion of vacuoles carrying Vtc1p-GFP or Vtc3p-GFP*. Vacuoles were isolated from BJ3505 cells expressing Vtc1p-GFP (from plasmid pYER-GFP) or from SBY593 cells expressing chromosomally encoded Vtc3p-GFP*. 20 µg vacuoles (0.1 mg/ml in PS buffer) were incubated with 10 µg/ml proteinase K in the presence or absence of 0.5% (w/v) Triton X-100 (5 minutes, 0°C). Digestion was stopped by adding one volume of 2 mM PMSF in PS buffer. Proteins were TCA precipitated, washed with acetone and solubilized in 100 µl non-reducing SDS-sample buffer. Coprecipitated Triton X-100 that can interfere with SDS-PAGE was extracted with chloroform/methanol (water:chloroform:methanol 2:1:2). Pellets were resolubilized in 50 µl reducing SDS-sample buffer, split and analyzed by 15% and 7.5% gels and western blotting with rabbit anti-GFP, goat anti-Vtc4p or mouse anti-Pho8p. TM, transmembrane fragment; pPho8p, pro-Pho8p; mPho8p, mature Pho8p; pPho8p-cytD, pPho8p fragment lacking the cytosolic tail. (B) Topology of the Vtc proteins and of Pho8p.

itself is 95 kDa). Note that, in contrast to Vtc1p-GFP, the GFP in Vtc3p-GFP* was not protease resistant (see below) and was not fluorescent (data not shown). We indicate this by the asterisk. Although Vtc3p-GFP* functionally substituted for Vtc3p (data not shown), its GFP domain could not reach the fully folded state. This is probably due to the fact that GFP was directly fused to the C-terminus of Vtc3p, whereas the Vtc1p-GFP fusion contained a seven amino acid spacer between the two parts.

We took advantage of the protease sensitivity of the C-terminal GFP domain in Vtc3p-GFP* to monitor its resistance to proteases. The C-terminal GFP in Vtc3p-GFP* was protease resistant when whole vacuoles were used, producing a 45 kDa fragment that corresponded in size to a fusion of the C-terminal three transmembrane domains of Vtc3p plus the GFP* (Fig. 2A). This fragment was digested when the vacuoles were lysed by Triton X-100, giving the protease access to the vacuolar lumen. The C-terminus of Vtc3p must therefore be exposed to the vacuolar lumen where it is protected from proteinase K.

The intactness of the vacuoles could be independently checked by proteolytic fragmentation of pro-alkaline phosphatase (pro-Pho8p), a vacuolar membrane protein oriented towards the vacuolar lumen (Klionsky and Emr, 1989). Pro-Pho8p has one transmembrane domain, a short cytosolic N-terminal tail and a large hydrophilic domain in the vacuolar lumen that carries a protease-sensitive pro-peptide (Fig. 2B). Proteinase K digested only the small N-terminal cytoplasmic tail when the vacuoles were intact (Fig. 2A). The lumenal propeptide became accessible to partial proteolysis after lysing the vacuolar membrane with Triton X-100.

Similarly to Vtc3p, Vtc4p could be degraded into fragments as small as ~22 kDa by low amounts of proteinase K added to intact vacuoles (Fig. 2A). Thus, not only the large hydrophilic N-terminal domain of Vtc3p but also that of Vtc4p must be exposed to the cytosol. Our data support the topology shown in Fig. 2B, that is, an arrangement in which the large hydrophilic parts of the Vtc complex face the cytosol and the C-termini face the vacuolar lumen. This experimental evidence matches previous speculations about Vtc topology (Cohen et al., 1999; Nelson et al., 2000).

## Role of Vtc proteins in membrane trafficking

Since Vtc proteins are involved in vacuole fusion (Muller et al., 2002), we also wanted to test whether other membrane trafficking processes depended on these factors. We assayed ER to Golgi trafficking of carboxypeptidase Y (CPY). CPY is a vacuolar protease that is translocated into the ER as a pro-enzyme (p1 form), travels to the Golgi and becomes glycosylated (p2 form). CPY is further transported through the prevacuolar endosomal compartment to the vacuole where the pro-peptide is cleaved off, resulting in the active vacuolar form (m). Cells were pulse labeled (Stack et al., 1995) with $^{35}$S-methionine/$^{35}$S-cysteine and chased in non-radioactive medium for different time periods before CPY was immunoprecipitated from the cell lysates (Fig. 3A). In wild-type cells, CPY was rapidly transported from the ER (0 minutes) to the Golgi (5 minutes) and finally to the vacuole (20 minutes) (Fig. 3A). Transition from p1 to p2, as well as from p2 to m, was delayed in *Δvtc3* cells. By contrast, *Δvtc1* [lacking also Vtc4p (Muller et al., 2002)] and *Δvtc2* cells behaved like the wildtype (Fig. 2A). Deletion of all four *VTC* genes did not result in a stronger phenotype than deletion of *VTC3* alone (data not shown). In line with ER-Golgi transport being a process essential for growth (Novick et al., 1980), *Δvtc3* cells also showed an increased generation time (107 minutes) when

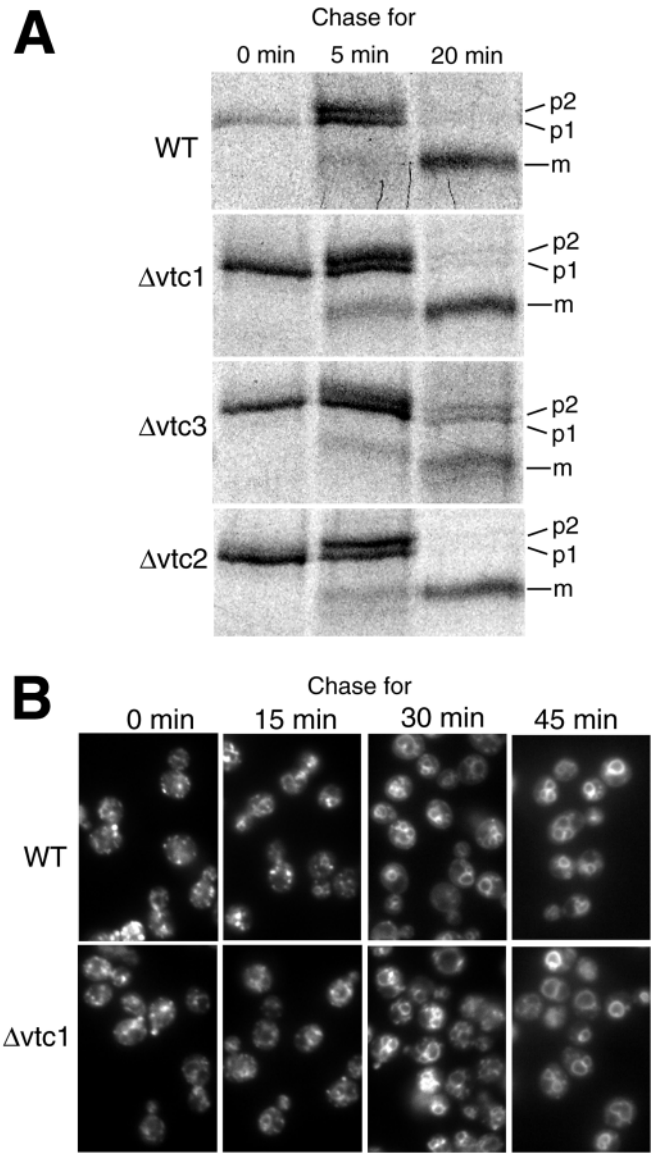


**Fig. 3.** Biosynthetic and endocytic trafficking to the vacuole. (A) CPY transport was assayed by pulse chase as described previously (Peters et al., 1999), except that the pulse and chase were performed at 30°C. The strains used were OMY20 (*Δvtc1::HIS3*), OMY21 (*Δvtc2::HIS3*), OMY22 (*Δvtc3::HIS3*) and BY4727 (wt). The growth medium was supplemented with methionine (20 μg/ml). (B) Pulse-labeling with FM4-64: Cells were grown in YPD medium (12 hours, 25°C), labeled with 200 μM FM4-64 (2 minutes, 25°C), reisolated (30 seconds, 3000 ***g***, 20°C), washed with YPD and reisolated as before. The cells were resuspended in YPD at $OD_{600}$=1 and chased at 25°C for various times. The cells were reisolated (30 seconds, 3000 ***g***, 20°C) and resuspended in the supernatant at $OD_{600}$=10. 5 μl of the suspension were transferred to a microscopy slide and were quickly analyzed in a fluorescence microscope under minimal excitation.

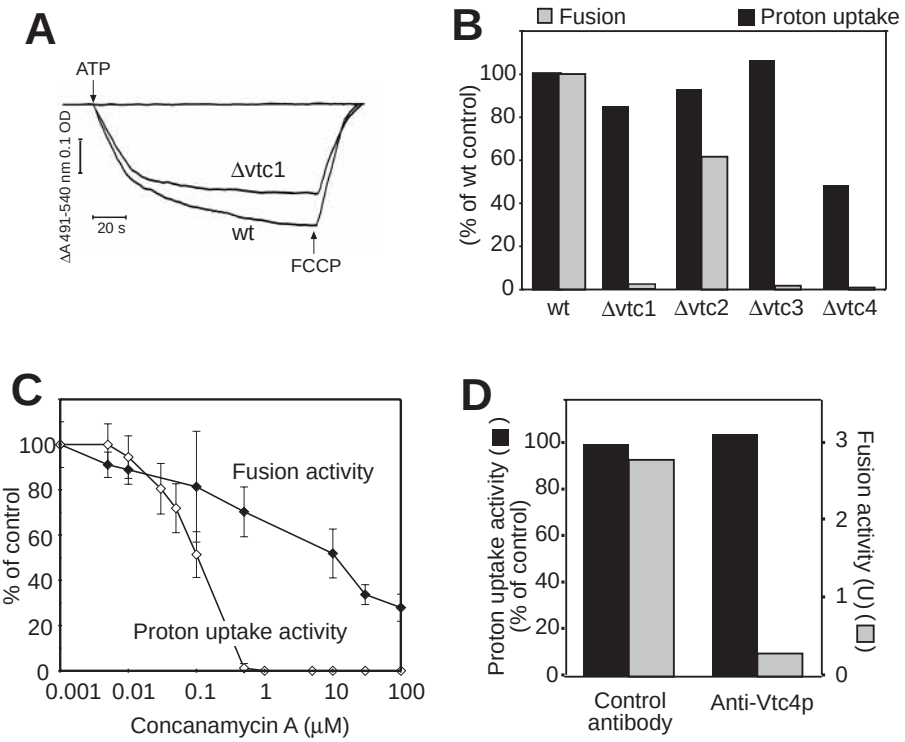


**Fig. 4.** Proton uptake and fusion of vacuoles. (A) Proton uptake of wild-type vacuoles (mixture of the fusion tester strains OMY1 and DKY6281) and vacuoles from *Δvtc1* (OMY2/OMY5) was measured. (B) Proton uptake and fusion activity of wild-type vacuoles (OMY1/DKY6281 or SBY86/SBY85, respectively) were compared with those of vacuoles from *Δvtc1* (OMY2/OMY5), *Δvtc2* (OMY4/OMY7), *Δvtc3* (OMY3/OMY6) and *Δvtc4* (SBY83/SBY82). Proton uptake activity of the wild-type vacuoles was set to 100%. 100% wild-type control fusion was 3.85 U (OMY1/DKY6281) and 3.08 U (SBY86/SBY85), respectively. (C) Comparison of proton uptake activity and fusion activity of wild-type vacuoles (OMY1/DKY6281) in the presence of different concentrations of concanamycin A. Values were plotted as a percentage of the control (vacuoles without concanamycin A). *n*=3. Control fusions were 2.93 U, 2.40 U and 4.11 U. (D) Proton uptake and fusion activity of wild-type vacuoles (OMY1/DKY6281) with either control antibody or antibodies to Vtc4p. The antibody concentration was 60 μM (c.f. Muller et al., 2002). Proton uptake activity of the sample with control antibodies was set to 100%.

compared to wildtype (84 minutes). *Δvtc1*, *Δvtc2* and *Δvtc4* grew like the wildtype.

Endocytosis was followed via the fluorescent styryl dye FM4-64. FM4-64 inserts into the plasma membrane, becomes endocytosed and then transferred to the vacuole by vesicular transport (Vida and Emr, 1995). After incubating cells with FM4-64 for 2 minutes, small intracellular vesicular structures were stained in wild-type and *Δvtc1* mutants (Fig. 3B). During a 45 minute chase in medium without dye, staining of the small vesicles in wild-type cells was gradually lost and the vacuolar membrane became increasingly fluorescent. Transiently, slightly larger punctate structures (three to five per cell) were also stained that might correspond to endosomes. After 45 minutes, all stain had been transferred to the vacuoles. The pulse-chase pattern was indistinguishable from wildtype and all *vtc* deletion mutants, even when all *VTC* genes or pairwise combinations thereof had been deleted (data not shown). This result differs from that obtained using a similar approach in *S. pombe*, where a *Δvtc1/nrf1* mutant was reported to have a severe endocytosis defect (Murray and Johnson, 2000; Murray and Johnson, 2001). The reason for this different behaviour is unclear. In summary, endocytic trafficking to the vacuole is independent of Vtc proteins in *S. cerevisiae*. By contrast, ER-Golgi transport and Golgi to vacuole transport appears to be facilitated by Vtc3p, although it does not absolutely depend on it.

### Proton uptake activity, V-ATPase assembly and $V_0$ conformation

Conflicting reports exist describing the effect of deletion of *VTC1* on the proton translocation activity of vacuolar membranes. In vitro assays with isolated subvacuolar vesicles indicated a reduction in proton translocation activity by 70% in subvacuolar vesicles prepared from *Δvtc1* mutants, as determined via the pH-dependent absorbance change of acridine orange in the vesicles (Cohen et al., 1999; Nelson et al., 2000). By contrast, qualitative in vivo assays using the ΔpH-dependent accumulation of quinacrin in vacuoles detected no changes to the wildtype (Ogawa et al., 2000). Since a proton motive force (pmf) across the membrane is required for vacuolar fusion (Conradt et al., 1994; Mayer et al., 1996; Ungermann et al., 1999), we tested whether the fusion defects of *vtc* mutants (Muller et al., 2002) could be explained by reduced proton translocation.

We measured the apparent proton uptake activity of vacuoles from different *vtc* deletion mutants using acridine orange (Cohen et al., 1999). In contrast to Cohen et al., we used intact vacuoles instead of subvacuolar vesicles. Our vacuoles are prepared by a rapid and gentle procedure that preserves the soluble contents of this compartment. Therefore, the apparent proton translocation activity we measure may comprise not only V-ATPase pump activity but also $H^+$ uptake via other mechanisms, such as import of protons by antiporting amino acids or ions. Vacuoles also contain an ATP-driven $Ca^{2+}$ pump and a $Ca^{2+}/H^+$ antiporter that may drive proton uptake and partially substitute for V-ATPase activity (Ohsumi and Anraku, 1981; Ohsumi and Anraku, 1983; Wada et al., 1992). Apparent proton translocation activity is the relevant parameter for our purpose, that is, for analyzing the correlation with vacuole fusion. Under the conditions of our in vitro fusion assay, the apparent proton translocation activity of *Δvtc1* and *Δvtc4* vacuoles was reduced to 85% and 50% of wild-type activity, respectively (Figs. 4A,B). Activities of *Δvtc2* and *Δvtc3*

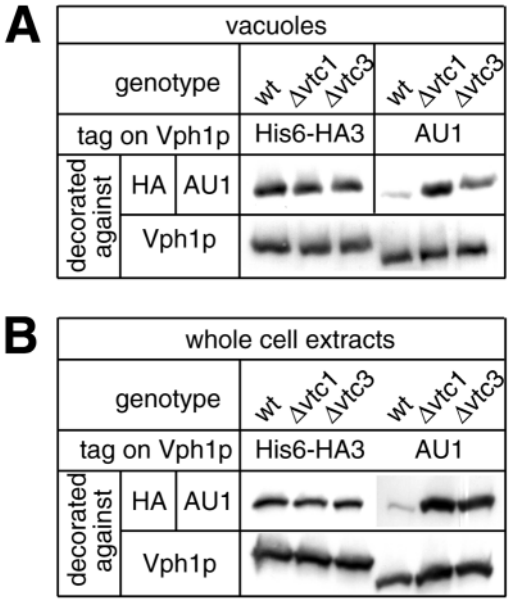


**Fig. 5.** Proteolytic sensitivity of Vph1p depends on Vtc proteins. (A) 25 μg of vacuoles from the indicated strains expressing Vph1p with chromosomally encoded C-terminal tags were precipitated with TCA and analyzed by SDS-PAGE and western blotting with the indicated antibodies. All strains were derived from OMY1, that is, deficient for vacuolar proteases (Δpep4, Δprb1). (B) Same analysis as in A, but with whole cell extracts from $10^7$ cells.

vacuoles were equal to or even slightly higher than those of wild-type vacuoles. The apparent proton uptake activities of *vtc* mutant vacuoles did not correlate with their fusion activities (Fig. 4B). Whereas *Δvtc2* vacuoles were fusion competent, *Δvtc3* vacuoles, despite their wild-type-like apparent proton uptake activity, did not fuse at all. Vtc3p must therefore have a direct role in fusion that is independent of proton uptake.

*Δvtc4* and *Δvtc1* vacuoles were unable to fuse, however, they showed significantly reduced apparent proton translocation activities. Therefore, we determined the level of translocation activity that would become limiting to fusion. We measured apparent proton translocation of wild-type vacuoles with different concentrations of the $H^+$-ATPase inhibitor concanamycin A (Drose and Altendorf, 1997) and in parallel determined the fusion activities. Concanamycin A reduced the apparent proton uptake activity in a concentration-dependent manner (Fig. 4C), abolishing the signal in the acridine orange assay completely at concentrations above 0.5 μM. Even at these concentrations fusion proceeded with an efficiency of ~70%. We attribute this to limitations in the sensitivity of the proton uptake assay. Below the levels of proton uptake detectable in this assay a basal pmf obviously remains that is sufficient to drive fusion. The fusion signal observed with >0.5 μM of concanamycin A was still sensitive to the proton uncoupler FCCP, demonstrating that it depended upon a basal proton motive force (data not shown). As we worked with intact vacuoles containing high concentrations of amino acids and other solutes, a basal pmf (below the detection level of the acridine orange assay) could be regenerated by efflux of these solutes via proton antiporters (Ohsumi and Anraku, 1981; Ohsumi and Anraku, 1983; Wada et al., 1992). This may enable intact isolated vacuoles to retain a basal V-ATPase-independent proton uptake activity. Subvacuolar vesicles that are commonly used for V-ATPase assays would not show such an activity (Cohen et al., 1999; Nelson et al., 2000).

Pharmacological reduction of the apparent proton uptake activity to 85% (the level observed with *Δvtc1*) or 50% (as observed with *Δvtc4*; Fig. 4B) of the control levels reduced the fusion activity of wild-type vacuoles only moderately, to 87% and 83% of the untreated control, respectively (Fig. 4C). This is in striking contrast to the profound fusion defect of *Δvtc1* and *Δvtc4* vacuoles (Fig. 4B) and thus separates these two phenomena. We could test this aspect with an independent second approach, using affinity-purified antibodies to Vtc4p that can inactivate the protein on wild-type vacuoles (Muller et al., 2002). This approach avoids potential secondary effects owing to deletion of genes. Antibodies to Vtc4p had no effect on the proton uptake activity of wild-type vacuoles, but they inhibited vacuole fusion (Fig. 4D). Taken together, Vtc proteins have a direct role in vacuolar membrane fusion (Muller et al., 2002) that is separable from their potential influence on vacuolar proton translocation activity (Cohen et al., 1999; Nelson et al., 2000).

The involvement of Vtc proteins in both vacuolar proton translocation and membrane fusion could be due to conformational changes of the V-ATPase caused by physical interactions of Vtc proteins with this enzyme. A physical interaction between V-ATPase and Vtc proteins was shown by cofractionation (Cohen et al., 1999) and coimmunoprecipitation (Muller et al., 2002). We tested whether Vtc mutations affect V-ATPase conformation or stability. Differences in proteolytic susceptibility are a well established indicator of altered conformations or associations of a protein. We discovered that the stability of an AU1 peptide tag on the C-terminus of Vph1p strongly depended on the presence of Vtc proteins. Vacuoles were isolated from wild-type, *Δvtc1* and *Δvtc3* cells expressing Vph1p with chromosomally encoded tags on the C-terminus, either a $His_6$-$HA_3$ tag or an AU1 tag (Fig. 5A). The amount of Vph1p was equal in all strains, as checked by decoration with a monoclonal antibody to Vph1p itself. However, antibodies against the tags revealed that the AU1 tag was largely degraded in wild-type vacuoles, whereas it was stable in *Δvtc1* and *Δvtc3* vacuoles. By contrast, the $His_6$-$HA_3$ tag was stable in all strains. The strains used were depleted of vacuolar proteases (Δ*pep4*, Δ*prb1*), making post-lysis effects by altered levels of vacuolar proteases unlikely. The picture was essentially the same in whole cell extracts of living yeast cells (Fig. 5B), suggesting that the tag was already degraded inside the cell and not during vacuole isolation. Therefore, the Vtc complex appears to modify the conformation of V-ATPase so that the C-terminus of Vph1p becomes more accessible to proteases.

This conclusion is supported by changes in the assembly state of the V-ATPase on isolated *vtc* mutant vacuoles. The fully assembled V-ATPase consists of a membrane-integral $V_0$ sector and a peripheral $V_1$ sector (Stevens and Forgac, 1997). The major peripheral subunits Vma1p and Vma2p were significantly reduced on *Δvtc1* vacuoles (Fig. 6A). By contrast, Vma4p, which forms part of the interface to the $V_0$ sector, was barely affected (Fig. 6A). Vma1p and Vma2p were also reduced on *Δvtc4* and on *Δvtc2* vacuoles (Fig. 5A). However, *Δvtc3* vacuoles carried more Vma1p, Vma2p and Vma4p than

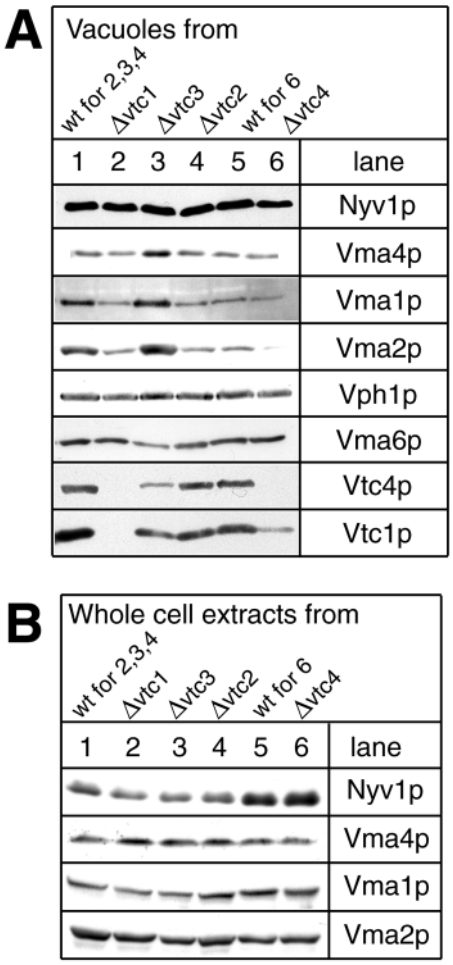


**Fig. 6.** Levels of V-ATPase subunits in different deletion mutants. (A) 30 μg each of vacuoles from the indicated strains were precipitated with TCA and analyzed by SDS-PAGE and western blotting with the indicated antibodies. All strains were deficient for vacuolar proteases (Δ*pep4*). Strain backgrounds were OMY1 (lanes 1-4) and SBY86 (lanes 5-6). (B) Same as in A, but with whole cell extracts.

wild-type vacuoles. The $V_0$ subunits Vma6p and Vph1p, and the vacuolar SNARE Nyv1p were present in equal amounts on the vacuoles of all strains, indicating equal loading of the lanes (Fig. 6A). Therefore, only some peripheral subunits of the $V_1$ sector differ in abundance on the membranes of *Δvtc* vacuoles, but not the membrane-integral $V_0$ sectors. Cohen et al. reported that only deletion of *VTC1* led to a reduction of $V_1$ association (Cohen et al., 1999), but that other *vtc* deletions had no effect (detected via the $V_1$ subunits Vma5p and Vma8p). Integral vacuolar membrane proteins were not included as internal reference, raising the possibility that different levels of vacuolar membranes had been analyzed. Alternatively, this could indicate that only some $V_1$ subunits are affected rather than the entire $V_1$ sector. This notion is supported by the fact that the differences we detected for the $V_1$ subunit Vma4p were less pronounced than those for Vma2p and that Cohen et al. (Cohen et al., 1999) also reported less significant differences for Vma4p.

In whole cell extracts, $V_1$ subunits of deletion mutants were as abundant as they are in wild-type extracts (Fig. 6B). Thus, the association between $V_0$ and $V_1$ subunits appears to be influenced by Vtc proteins. This association is labile on *Δvtc1*, *Δvtc2* and *Δvtc4* vacuoles. The $V_1$ and $V_0$ subunits can undergo regulated cycles of dissociation and reassociation in response to depletion or replenishment of glucose in the growth medium (Parra and Kane, 1998). Loss of $V_1$ subunits from the mutant vacuoles might therefore be caused by enhanced disassembly or by a block in reassembly. We assayed $V_1/V_0$ dissociation and reassociation in living cells according to published procedures (Parra and Kane, 1998) using coprecipitation of $V_1$ and $V_0$ from whole cell lysates as an assay. This did not reveal significant differences in any of the *vtc* mutants (data not shown). Thus, we prefer the interpretation that the stability of the $V_1/V_0$ holoenzyme is compromised in *Δvtc1*, *Δvtc2* and *Δvtc4* mutants, leading to partial loss of $V_1$ subunits. In combination with the altered proteolytic sensitivity of Vph1p-AU1, this provides in vivo evidence for an interaction of Vtc proteins with the V-ATPase, which affects the conformation of $V_0$ and the stability of the holoenzyme.

## Discussion

Vtc proteins were suggested to be vacuolar transporter chaperons controlling the distribution of membrane proteins over different compartments (Cohen et al., 1999; Nelson et al., 2000). Membranes containing plasma membrane ATPase (Pma1p) floated at slightly different densities in a *vtc1* mutant (Cohen et al., 1999). However, compartment markers had not been analyzed, and it was not resolved whether Pma1p had shifted to other compartments or whether the plasma membrane floated at a different density in the mutant. Furthermore, V-ATPase mutations had a much greater effect on flotation of Pma1p-containing membranes than a *vtc1* mutation did (Cohen et al., 1999). As far as the vacuole is concerned, our data do not argue in favor of Vtc proteins regulating the distribution of other integral membrane proteins. The level of the membrane-integral $V_0$ sector in *vtc* mutant vacuoles was normal (Fig. 6), and the steady-state levels of four other vacuolar integral membrane proteins we tested so far were not altered by *vtc* mutations (Muller et al., 2002).

A Vtc protein copurified with V-ATPase components upon chromatographic fractionation (Cohen et al., 1999), and we could coimmunoprecipitate V-ATPase and Vtc proteins, indicating a physical interaction (Muller et al., 2002). The data presented here suggest that Vtc proteins influence the conformation and/or molecular interactions of the $V_0$ subunit Vph1p (Fig. 5). Presence of the Vtc proteins alters the proteolytic sensitivity of Vph1p and the interaction of $V_0$ and $V_1$ subunits. We detected significant differences in the levels of $V_1$ subunits Vma1p and Vma2p on Δ*vtc1*, Δ*vtc2* and Δ*vtc4* vacuoles. Previously, point mutations in Vph1p were shown to influence the assembly state and/or stability of the $V_1/V_0$ holoenzyme (Leng et al., 1998; Leng et al., 1999). These point mutations mapped to the C-terminal 50 amino acids of Vph1p, that is, to the same region for which our tagged version of Vph1p indicated Vtc-dependent alterations of proteolytic sensitivity. The fact that this C-terminal stretch, which was suggested to be at the lumenal side of the membrane (Leng et al., 1999), influences the assembly of $V_1$ onto the cytosolic side could only be explained by significant conformational

rearrangements of $V_0$. When combined, the results of Leng et al. and our findings suggest that the C-terminus of Vph1p exhibits significant flexibility in its conformation and associations. This, together with the observation of genetic and physical interactions between V-ATPase and Vtc proteins, suggests that the Vtc–V-ATPase association is chaperon-like in the genuine sense, that is, that of a protein affecting the folding state of another polypeptide. We propose that such an influence on $V_0$ conformation may form one basis of Vtc protein function in vacuole fusion. Vtc proteins affect two stages of vacuole fusion. Vtc1p and Vtc4p regulate the activation of vacuolar SNAREs by Sec18p/NSF (Muller et al., 2002). Vtc3p is involved in a later step, probably subsequent to docking and the formation of $V_0$ trans-complexes. Conformational changes of $V_0$ would be strongly expected to play a role in this late stage, and it is conceivable that Vtc3p might regulate them.

Vtc proteins were proposed to be polyphosphate synthases (Ogawa et al., 2000) because *vtc* mutations reduce the formation of vacuolar polyphosphate to various degrees. This reduction becomes apparent only when yeast cells are shifted from phosphate-depleted media to high phosphate media (Ogawa et al., 2000). It remained unclear whether Vtc proteins play a direct role in polyphosphate synthesis or whether polyphosphate deficiency in *Δvtc1* and *Δvtc4* mutants arises as a secondary effect, perhaps from problems in membrane trafficking. The topology of the Vtc complex, which we have experimentally determined now, makes the possibility of it having a function as a polyphosphate synthase very unlikely. All parts of the Vtc proteins except their transmembrane domains face the cytosol. An enzyme-synthesizing polyphosphate inside the vacuole would be expected to face the vacuolar lumen.

Several observations indicate that Vtc proteins control membrane fusion independently of polyphosphate levels (Muller et al., 2002): first, Δ*vtc1* and Δ*vtc4* mutants have no polyphosphates (Ogawa et al., 2000) and are deficient in priming of SNARE proteins. SNARE priming and fusion can partially be rescued by exogenous Sec18p in vitro (Muller et al., 2002), where regeneration of vacuolar polyphosphates should not be possible. Second, antibodies to Vtc4p blocked SNARE priming and fusion on wild-type vacuoles – which should have polyphosphates. Third, Δ*vtc3* mutants show a less severe reduction in polyphosphates than Δ*vtc1* and Δ*vtc4* cells do (Ogawa et al., 2000). They do not fuse and cannot be rescued by Sec18p (Muller et al., 2002). Thus, the fusion activity of vacuoles does not correlate with polyphosphate levels. This demonstrates that Vtc proteins do not influence fusion via polyphosphate but perform a direct role in vacuole fusion.

Vacuoles from some of the *vtc* deletion mutants show altered proton uptake activity. The effects seen in our study are qualitatively similar to those observed in the previous studies (Cohen et al., 1999; Nelson et al., 2000). Cohen et al. reported that V-ATPase activity of *Δvtc1* vacuoles was reduced to ~10-30% of the wild-type signal (Cohen et al., 1999). We observed only a minor reduction in apparent proton translocation activity to ~85%, which is consistent with the observations by Ogawa et al.; they observed no vacuolar acidification defects in a qualitative in vivo assay (Ogawa et al., 2000). The difference in our results and those of Cohen et al. might be due to the use of different strains, incubation conditions and methods for vacuole isolation. For example, Cohen et al. used protease-competent cells and a slow method of membrane isolation that produces subvacuolar vesicles and should release vacuolar hydrolases (Cohen et al., 1999). This might explain the significant proteolytic degradation of the V-ATPase subunit Vma5p, which is visible in this study, and the stronger reduction of proton translocation activity observed. A further important aspect to be considered has already been outlined above: the apparent proton-translocation activity of intact vacuoles that we assay may comprise several different $H^+$-translocating processes, whereas $H^+$ translocation in subvacuolar vesicles depends solely on V-ATPase. For example, proton translocating antiporters could drive proton uptake by efflux of amino acids or ions from intact vacuoles but not from subvacuolar vesicles that have lost their soluble contents in the course of preparation.

We assume that the influence of Vtc proteins on V-ATPase conformation and stability is central to the effects of these proteins in vacuole fusion and possibly also in proton translocation. A major task in the functional analysis of these proteins will therefore be to characterize this interaction and its dynamics, particularly in the course of vacuole fusion.

We thank Johannes Hegemann and Tom Vida for plasmids and Susanne Bühler and Christa Baradoy for assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB446, A.M.), by the Boehringer-Ingelheim-Foundation (A.M.) and the Boehringer-Ingelheim-Fonds (O.M.) and by the HFSPO (A.M.).

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