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

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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\(_{\Delta}\) and vtc4\(_{\Delta}\) 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 Δvtc3 cells. In accordance with that, Δvtc3 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. Δvtc3 vacuoles carried significantly more V\(_{1}\) subunits, whereas Δvtc1, Δvtc2 and Δvtc4 had significantly less. The proteolytic sensitivity of the V\(_{0}\) subunit Vph1p was different in Δvtc 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.
Proton uptake activity of vacuoles. In wild-type (a), a control strain with normal vacuolar proteolytic activity (Fig. 1B, upper panel (c.f. Cohen et al., 1999)), the fusion protein stained the vacuolar rim, while in the deletion mutant (data not shown). In a protease-deficient strain (pep4Δ), the fusion protein was not detected (data not shown). 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 is 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 vacuole fusion defect of a vtc1 deletion mutant (data not shown). In a protease-deficient strain (pep4Δ), 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 pep4Δ 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 hydrodases, GFP stained the vacuolar membrane.

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 520-540 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 MnCl₂, 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 μl of FCCP were added. Proton uptake activity was defined as the absorbance change during the first 20 seconds of the reaction.
Vtc proteins influence the V-ATPase 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. 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 Na2CO3. 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.

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 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 luminal 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 proenzyme (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 35S-methionine/35S-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...
were stained in wild-type and transport (Vida and Emr, 1995). After incubating cells with endocytosed and then transferred to the vacuole by vesicular transport, all pulse-chase pattern was indistinguishable from wildtype and grew like the wildtype.

VTC1

Conflicting reports exist describing the effect of deletion of VTC1 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 V0 conformation

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 Ca2+ pump and a Ca2+/H+ antiporter that may drive 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.

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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 ∆vtc2 vacuoles were fusion competent, ∆vtc3 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. ∆vtc4 and ∆vtc1 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 ∆vtc1) or 50% (as observed with ∆vtc4; 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 ∆vtc1 and ∆vtc4 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, ∆vtc1 and ∆vtc3 cells expressing Vph1p with chromosomally encoded tags on the C-terminus, either a His6-HA3 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 ∆vtc1 and ∆vtc3 vacuoles. By contrast, the His6-HA3 tag was stable in all strains. The strains used were depleted of vacuolar proteases (∆ape4, ∆apr1), 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 V0 sector and a peripheral V1 sector (Stevens and Forgac, 1997). The major peripheral subunits Vma1p and Vma2p were significantly reduced on ∆vtc1 vacuoles (Fig. 6A). By contrast, Vma4p, which forms part of the interface to the V0 sector, was barely affected (Fig. 6A). Vma1p and Vma2p were also reduced on ∆vtc4 and on ∆vtc2 vacuoles (Fig. 5A). However, ∆vtc3 vacuoles carried more Vma1p, Vma2p and Vma4p than

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### Table A

| genotype | vacuoles |
|----------|----------|
|          | genotype |
| wt       | ∆vtc1    | ∆vtc3 |
| wt       | ∆vtc1    | ∆vtc3 |
| wt       | ∆vtc1    | ∆vtc3 |

### Table B

| genotype | whole cell extracts |
|----------|---------------------|
|          | genotype |
| wt       | ∆vtc1    | ∆vtc3 |
| wt       | ∆vtc1    | ∆vtc3 |
| wt       | ∆vtc1    | ∆vtc3 |

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 (∆ape4, ∆apr1). (B) Same analysis as in A, but with whole cell extracts from 10^7 cells.
Vtc proteins influence the V-ATPase

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 V0 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 V0 subunit Vph1p (Fig. 5). Presence of the Vtc proteins alters the proteolytic sensitivity of Vph1p and the interaction of V0 and V1 subunits. We detected significant differences in the levels of V1 subunits Vma1p and Vma2p on Δvtc1, Δvtc2, and Δvtc4 vacuoles. Previously, point mutations in Vph1p were shown to influence the assembly state and/or stability of the V1/V0 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 luminal side of the membrane (Leng et al., 1999), influences the assembly of V1 onto the cytosolic side could only be explained by significant conformational changes and/or molecular interactions of the V0 subunits.

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 V0 subunit Vph1p (Fig. 5). Presence of the Vtc proteins alters the proteolytic sensitivity of Vph1p and the interaction of V0 and V1 subunits. We detected significant differences in the levels of V1 subunits Vma1p and Vma2p on Δvtc1, Δvtc2, and Δvtc4 vacuoles. Previously, point mutations in Vph1p were shown to influence the assembly state and/or stability of the V1/V0 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 luminal side of the membrane (Leng et al., 1999), influences the assembly of V1 onto the cytosolic side could only be explained by significant conformational changes and/or molecular interactions of the V0 subunits.

As abundant as they are in wild-type extracts (Fig. 6B). Thus, the association between V0 and V1 subunits appears to be influenced by Vtc proteins. This association is labile on Δvtc1, Δvtc2 and Δvtc4 vacuoles. The V1 and V0 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 V1 subunits from the mutant vacuoles might therefore be caused by enhanced disassembly or by a block in reassembly. We assayed V1/V0 dissociation and reassociation in living cells according to published procedures (Parra and Kane, 1998) using coprecipitation of V1 and V0 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 V1/V0 holoenzyme is compromised in Δvtc1, Δvtc2 and Δvtc4 mutants, leading to partial loss of V1 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 V0 and the stability of the holoenzyme.
rearrangements of V0. 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 V0 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 V0 trans-complexes. Conformational changes of V0 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 polypolysynthetic synthases (Ogawa et al., 2000) because vtc mutations reduce the formation of vacuolar polysphosphate 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 polysynthetic synthesis or whether polysynthetic activity 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 polysynthetic synthase very unlikely. All parts of the Vtc proteins except their transmembrane domains face the cytosol. An enzyme-synthesizing polysynthetic inside the vacuole would be expected to face the vacuolar lumen.

Several observations indicate that Vtc proteins control membrane fusion independently of polysynthetic levels (Muller et al., 2002): first, Δvtc1 and Δvtc4 mutants have no polysyntheses (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 polysyntheses should not be possible. Second, antibodies to Vtc4p blocked SNARE priming and fusion on wild-type vacuoles – which should have polysyntheses. Third, Δvtc3 mutants show a less severe reduction in polysyntheses than Δvtc1 and Δvtc4 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 polysynthetic levels. This demonstrates that Vtc proteins do not influence fusion via polysynthetic 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.

References
Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P. and Boeke, J. D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14, 115-132.
Cohen, A., Perzov, N., Nelson, H. and Nelson, N. (1999). A novel family of yeast chaperons involved in the distribution of V-ATPase and other membrane proteins. J. Biol. Chem. 274, 26885-26893.
Conradt, B., Haas, A. and Wickner, W. (1994). Determination of four biochemically distinct, sequential stages during vacuole inheritance in vitro. J. Cell Biol. 126, 99-110.
Drose, S. and Altdorf, K. (1997). Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. J. Exp. Biol. 200, 1-8.
Elzen, G., Thorngren, N. and Wickner, W. (2001). RhO1p and Cdc42p act after Ypt7p to regulate vacuole docking. EMBO J. 20, 5650-5656.
Klionsky, D. J. and Emr, S. D. (1989). Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. EMBO J. 8, 2241-2250.
Leng, X. H., Manolson, M. F. and Forgac, M. (1998). Function of the COOH-terminal domain of Vph1p in activity and assembly of the yeast V-ATPase. J. Biol. Chem. 273, 6717-6723.
Leng, X. H., Nishi, T. and Forgac, M. (1999). Transmembrane topography of the 100-kDa subunit (Vph1p) of the yeast vacuolar proton-translocating ATPase. J. Biol. Chem. 274, 14655-14661.
Mayer, A., Wickner, W. and Haas, A. (1996). Sec18p (NSF)-driven release of Sec17p (alpha-SNAP) can precede docking and fusion of yeast vacuoles. Cell 85, 83-94.
Muller, O., Bayer, M. J., Peters, C., Andersen, J. S., Mann, M. and Mayer, A. (2001). The Vtc proteins in vacuole fusion: coupling NSF activity to V0 regulators Nrf1p and Scd1p. J. Biol. Chem. 276, 259-269.
Muller, O., Johnson, D. I. and Mayer, A. (2001). Cdc42p functions at the docking stage of yeast vacuole membrane fusion. EMBO J. 20, 5657-5665.
Murray, J. M. and Johnson, D. I. (2000). Isolation and characterization of Nfr1p, a novel negative regulator of the Cdc42p GTAPase in Schizosaccharomyces pombe. Genetics 154, 155-165.
Murray, J. M. and Johnson, D. I. (2001). The Cdc42p GTAPase and its regulators Nfr1p and Scd1p are involved in endocytic trafficking in the fission yeast Schizosaccharomyces pombe. J. Biol. Chem. 276, 3004-3009.
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Nelson, N., Perzov, N., Cohen, A., Hagai, K., Padler, V. and Nelson, H. (2000). The cellular biology of proton-motive force generation by V-ATPases. J. Exp. Biol. 203, 89-95.

Niedenthal, R. K., Riles, L., Johnston, M. and Hegemann, J. H. (1996). Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. Yeast 12, 773-786.

Novick, P., Field, C. and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell 21, 205-215.

Ohgawa, N., DeRisi, J. and Brown, P. O. (2000). New components of a system for phosphate accumulation and polyphosphate metabolism in Saccharomyces cerevisiae revealed by genomic expression analysis. Mol. Biol. Cell 11, 4309-4321.

Ohsumi, Y. and Anraku, Y. (1981). Active transport of basic amino acids driven by a proton motive force in vacuolar membrane vesicles of Saccharomyces cerevisiae. J. Biol. Chem. 256, 2079-2082.

Ohsumi, Y. and Anraku, Y. (1983). Calcium transport driven by a proton motive force in vacuolar membrane vesicles of Saccharomyces cerevisiae. J. Biol. Chem. 258, 5614-5617.

Parra, K. J. and Kane, P. M. (1998). Reversible association between the V1 and V0 domains of yeast vacuolar H+-ATPase is an unconventional glucose-induced effect. Mol. Cell. Biol. 18, 7064-7074.

Peters, C., Andrews, P. D., Stark, M. J., Cesaro-Tadic, S., Glatz, A., Podtelejnikov, A., Mann, M. and Mayer, A. (1999). Control of the terminal step of intracellular membrane fusion by protein phosphatase 1. Science 285, 1084-1087.

Peters, C., Bayer, M. J., Buhler, S., Andersen, J. S., Mann, M. and Mayer, A. (2001). Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. Nature 409, 581-588.

Stack, J. H., Horazdovsky, B. and Emr, S. D. (1995). Receptor-mediated protein sorting to the vacuole in yeast: roles for a protein kinase, a lipid kinase and GTP-binding proteins. Annu. Rev. Cell Dev. Biol. 11, 1-33.

Stevens, T. H. and Forgac, M. (1997). Structure, function and regulation of the vacuolar (H+)-ATPase. Annu. Rev. Cell Dev. Biol. 13, 779-808.

Ungermann, C., Wickner, W. and Xu, Z. (1999). Vacuole acidification is required for trans-SNARE pairing, LMA1 release, and homotypic fusion. Proc. Natl. Acad. Sci. USA 96, 11194-11199.

Vida, T. A. and Emr, S. D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J. Cell Biol. 128, 779-792.

Wada, Y. and Anraku, Y. (1994). Chemiosmotic coupling of ion transport in the yeast vacuole: its role in acidification inside organelles. J. Bioenerg. Biomembr. 26, 631-637.

Wada, Y., Ohsumi, Y. and Anraku, Y. (1992). Chloride transport of yeast vacuolar membrane vesicles: a study of in vitro vacuolar acidification. Biochim. Biophys. Acta 1101, 296-302.

Wickner, W. (2002). New EMBO member’s review: Yeast vacuoles and membrane fusion pathways. EMBO J. 21, 1241-1247.

Yabe, I., Horinchi, K., Nakahara, K., Hiyama, T., Yamanaka, T., Wang, P. C., Toda, K., Hirata, A., Ohsumi, Y., Hirata, R. et al. (1999). Patch clamp studies on V-type ATPase of vacuolar membrane of haploid Saccharomyces cerevisiae. Preparation and utilization of a giant cell containing a giant vacuole. J. Biol. Chem. 274, 34903-34910.