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A Novel Family of Yeast Chaperons Involved in the Distribution of V-ATPase and Other Membrane Proteins*

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Null mutations in genes encoding V-ATPase subunits in *Saccharomyces cerevisiae* result in a phenotype that is unable to grow at high pH and is sensitive to high and low metal-ion concentrations. Treatment of these null mutants with ethylmethanesulfonate causes mutations that suppress the V-ATPase null phenotype, and the mutant cells are able to grow at pH 7.5. The suppressor mutants were denoted as *svf* (suppressor of V-ATPase function). The frequency of *svf* is relatively high, suggesting a large target containing several genes for the ethylmethanesulfonate mutagenesis. The suppressors' frequency is dependent on the individual genes that were inactivated to manifest the V-ATPase null mutation. The *svf* mutations are recessive, because crossing the *svf* mutants with their corresponding V-ATPase null mutants resulted in diploid strains that are unable to grow at pH 7.5. A novel gene family in which null mutations cause pleiotropic effects on metal-ion resistance or sensitivity and distribution of membrane proteins in different targets was discovered. The family was defined as *VTC* (Vacuolar Transporter Chaperon) and it contains four genes in the *S. cerevisiae* genome. Inactivation of one of them, *VTC1*, in the background of V-ATPase null mutations resulted in *svf* phenotype manifested by growth at pH 7.5. Deletion of the *VTC1* gene (Δ*VTC1*) results in a reduced amount of V-ATPase in the vacuolar membrane. These mutant cells fail to accumulate quinacrine into their vacuoles, but they are able to grow at pH 7.5. The *VTC1* null mutant also results in a reduced amount of the plasma membrane H^+-ATPase (Pma1p) in membrane preparations and possibly mistargeting. This observation may provide an explanation for the *svf* phenotype in the double disruptant mutants of Δ*VTC1* and Δ*VMA* subunits.

Null mutations in genes encoding vacuolar H^+-ATPase (V-ATPase)[1] subunits are likely to be lethal for most eukaryotic cells, because energization of the vacuolar system by this enzyme drives vital secondary transport processes across membranes of vacuolar-derived organelles (1, 2). Disruption of genes encoding V-ATPase subunits in *Neurospora* and *Drosophila melanogaster* caused lethality (3, 4). On the other hand, mutant *Saccharomyces cerevisiae* (yeast) cells can survive the lack of acidification that results from disruption of genes encoding V-ATPase subunits (5). With the exception of *VPH1* and *STV1*, which encode homologous proteins (6, 7), all genes encoding subunits of the V-ATPase are present as a single copy in the yeast genome (1, 8). Disruption of each of the single-copy genes yields a similar phenotype in which cells cannot grow at a pH higher than 7 and are sensitive to low and high calcium or metal ion concentrations in the medium (5, 9–11). Mutant *S. cerevisiae* (yeast) cells can survive the lack of acidification that results from disruption of genes encoding V-ATPase subunits by taking up acidic external fluid via endocytosis (5, 12). However the precise metabolic junction that prevents growth of V-ATPase null mutants at high pH is not known. Moreover the location of the vital acidic compartment in the vacuolar system is not apparent. Indirect evidence indicates that the vital acidic compartment is not the yeast vacuole (13). We use suppressor mutants to pinpoint the cellular structures and metabolic pathways that are involved in the expression of the V-ATPase null mutation phenotype.

Initial studies with null mutants showed very clearly that each of the V-ATPase subunits is required for the proper assembly of the holoenzyme (14, 15). In general, all the V_o subunits are required for assembly of the V_1 sector onto the membrane. The proteolipid (Vma3p) plays a central role in V-ATPase assembly, and none of the remaining V_o subunits assemble in its absence (5, 15, 16). The only exception is subunit F (Vma7p), which is considered to be a V_1 constituent, but a null mutation in its gene disrupts not only the assembly of V_1 but also the assembly of V_o (17). Biogenesis of V-ATPase in *S. cerevisiae* cells involves several parallel steps starting in the endoplasmic reticulum (ER) and the cytoplasm (11, 18). Partial complexes of V_1 subunits can be formed in the cytoplasm in the absence of an assembled V_o domain (16, 19). Using a native gel electrophoresis system that allows a fine resolution of cytosolic V-ATPase complexes, a major cytosolic V_1 complex (complex II, 576 kDa) was detected in wild type as well as in Δ*VMA3* and Δ*VMA5* strains. Strains having mutations in genes encoding the Vmap, Vma2p, Vma4p, Vma7p, or Vma8p V_1 subunits fail to assemble this complex, although large, intermediate-sized complexes were sometimes detected (20, 21).

Several newly discovered genes encode proteins that are not a part of the V_o complex but affect its assembly (18, 22–24). Vma21p is a particularly interesting integral membrane protein of 9.5 kDa (23) that resides in the endoplasmic reticulum where it is required for the assembly of the V_0 sector. Moreover, the unassembled Vph1p is rapidly degraded in the mutant lacking Vma21p. The 21-kDa Vma22 protein is also an ER-localized protein required for V-ATPase assembly (18). As with Vma21p, the absence of Vma22p results in degradation of Vphlp and prevents the assembly of V_1 onto the membrane. The association of Vma22p with the ER is itself dependent on

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[1] The abbreviations used are: V-ATPase, vacuolar H^+-ATPase; ER, endoplasmic reticulum; Mes, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PCR, polymerase chain reaction; EMS, ethylmethanesulfonate.

another assembly factor Vmal2p (25 kDa). Again, mutants defective in Vmal2p have low levels of V_o subunits in the vacuole membrane and fail to assemble the peripheral sector (18, 23). These results suggest that the V_o assembles in the ER and is subsequently moved to other locations within the cell. In the absence of correct assembly, the integral membrane domain may be degraded at the ER level.

So far most of the assembly factors of V-ATPase were shown to be necessary for its assembly. It is likely that additional factors will function in the correct distribution of V-ATPase and other membrane proteins. Inactivation of such factors will not give the phenotype of the V-ATPase null mutations but should impair the physiological function of the membrane complexes governed by these assembly factors. In this paper, we report on a novel gene family that function in the distribution of membrane proteins. The genes were named vacuolar transporter chaperons (*VTC*).

MATERIALS AND METHODS

Strains, Media, and Reagents—The "wild-type" that was used is *S. cerevisiae* W303 (*MATα α trp1 ade2 his3 leu2 ura3*). The other strains used in this work are: Δ*VTC1 (MATα ade2 his3 leu2 trp1 VTC1::URA3);* Δ*VTC2 (MATα ade2 his3 trp1 ura3 VTC2::LEU2);* Δ*VTC2 (MATα ade2 leu2 trp1 ura3 VTC2::HIS3);* Δ*VTC3 (MATα ade2 his3 trp1 ura3 VTC3::LEU2);* Δ*VTC4 (MATα ade2 his3 leu2 ura3 VTC4::TRP1);* Δ*VTC2+3 (MATα ade2 trp1 ura3 VTC2::HIS3 VTC3::LEU2);* Δ*VMA1 (MATα ade2 his3 trp1 ura3 VMA1::LEU2);* Δ*VMA3 (MATα ade2 trp1 ura3 his3 VMA3::LEU2);* Δ*VMA3 (MATα ade2 trp1 ura3 his3 VMA3::LEU2);* Δ*VMA3 (MATα ade2 trp1 leu2 his3 VMA3::URA3);* Δ*VMA3 (MATα ade2 trp1 leu2 his3 VMA3::URA3);* Δ*VMA4 (MATα ade2 trp1 ura3 his3 VMA4::LEU2);* Δ*VMA5 (MATα ade2 his3 leu2 VMA5::LEU2);* Δ*VMA7 (MATα ade2 trp1 leu2 his3 VMA7::URA3);* Δ*VMA8 (MATα ade2 his3 leu2 trp1 VMA8::URA3);* Δ*VMA10 (MATα ade2 his3 leu2 trp1 VMA10::URA3);* Δ*VMA11 (MATα ade2 trp1 his3 leu2 VMA11::URA3);* and Δ*VMA16 (MATα ade2 trp1 his3 leu2 VMA16::URA3).*

The cells were grown in a YPD medium containing 1% yeast extract, 2% Bactopeptone, and 2% dextrose. The medium was buffered by 50 mM Mes and 50 mM MOPS, and the pH was adjusted by NaOH (5, 10). Agar plates were prepared by adding 2% agar to the YPD buffer medium at the given pH. Yeast transformation was performed as described previously (25), and the transformed cells were grown on minimal plates containing a 0.67% yeast nitrogen base, 2% dextrose, 2% agar, and the appropriate nutritional requirements. In most experiments, 0.1% casamino acids were added to the minimal plates. Mating of cells was performed with MATα and MATa strains that were grown overnight in YPD. 0.1 ml of each strain were incubated together overnight, spread on selective plates, and the grown colony cells were checked by mating type-specific PCR probes for their ploidy. The diploid cells were washed with water, and the cells were grown in 10 ml of SPM medium containing 3 g/liter potassium acetate and 0.2 g/liter raffinose. Tetrads were dissected in a dissection microscope as described previously (26).

Chemical Mutagenesis and Analysis of svf Complementation Groups—An *S. cerevisiae* mutant, bearing disruption or deletion mutation in genes encoding different V-ATPase subunits, were grown on 1.2 OD at 600 nm in buffered YPD medium (pH 5.5). The cells were harvested, suspended in water at a cell density of 2 OD at 600 nm, and treated at 30 °C with 10 μl/ml ethylmethanesulfonate (EMS) for 60 min. The treated cells were washed with 5% sodium thiosulfate, followed by a washing in water, and resuspended in a minimal medium at a cell density of 2 OD at 600 nm. The treated cells were plated on buffered YPD medium (pH 7.5). The treatment resulted in 50–80% viability as judged by growing diluted samples before and after the treatment on YPD plates (pH 5.5). The number of *svf* colonies grown at pH 7.5 *versus* pH 5.5 was recorded as the mutation rate for V-ATPase-independent growth at the high pH. The minimal amount of complementation groups involved in the *svf* phenotype was estimated as follows: 10 *svf* mutants of *VMA8::URA3* Mat α were crossed with 7 *svf* mutants of *VMA8::LEU2* Mat a to give 70 diploid strains that grew on minimal plates without uracil and leucine. The diploid strains were analyzed for growth on YPD plates buffered at pH 7.5. Similarly, 9 *svf* mutants *VMA10::URA3* Mat α and 9 *svf* mutants *VMA10::LEU2* Mat a were crossed, and the resulting diploid strains were analyzed for growth at pH 7.5.

Gene Disruption—The gene knockout of the new strains was performed as follows: Whole or part of the target gene was replaced by a selectable marker (*URA3*, *TRP1*, *LEU2*, or *HIS4*), leaving flanking DNA sequences of about 0.3 kilobase pairs. When PCR was used for the construct, the DNA fragments were cloned into the TA plasmid of pGEM-T Easy (Promega). For the disruption, we cut the plasmid with the appropriate restriction enzymes and transformed the yeast strains with the plasmid DNA as described previously (25, 27). The yeast strains were grown on minimal medium in the absence of the auxotrophic marker. Colonies that grew on the selective medium were selected, checked by PCR for homologous recombination, and analyzed for their phenotype. *VTC1* containing 0.3-kilobase pair-flanking sequences was cloned by PCR into YPN2 plasmid (10). Δ*VTC1* was obtained by the introduction of *URA3* to replace most of the reading frame starting at amino acid 1 and ending at 129. Similarly *VTC2*, *VTC3*, and *VTC4* were cloned by PCR and introduced to the respective YPN2 plasmid. Disruptant mutants were obtained as described for Δ*VTC1* except that *VTC2* was interrupted by *LEU2* or *HIS4*, *VTC3* by *LEU2*, and *VTC4* by *TRP1*.

Yeast Transformation—Yeast transformation was performed either by the method of Ito *et al.* (25) or by a bench-top method according to Elble (27). Yeast cells were grown overnight in 5 ml of YPD medium (pH 5.5) to stationary phase. The cells were centrifuged for 10 s in an Eppendorf centrifuge at 13,000 rpm. 10 μl of salmon sperm (10 mg/ml) were added to the pellet as a DNA carrier. Then about 1 μg of the plasmid or the DNA construct was added. Finally, the pellet was suspended in 0.5 ml of PLATE medium containing 10 mM Tris, pH 7.5, 1 mM EDTA, 40% polyethylene glycol 4000, and 0.1 M lithium acetate. The suspension was incubated overnight at room temperature and plated on the appropriate plates (10).

DNA Isolation from Yeast—Yeast cells were grown in a selective medium or YPD to stationary phase. The cells were harvested by centrifugation for 2 min at 2,500 rpm. The pellet was suspended in 100 μl of STET solution containing 50 mM Tris (pH 8), 50 mM EDTA, 5% Triton X-100, and 8% sucrose. Glass beads (about 0.2 g) were added, and the suspension was vortexed for 20 min. Then, an additional 100 μl of STET were added, and the mixture was boiled for 3 min, cooled for 1 min on ice, and centrifuged for 10 min at 13,000 rpm. 100 μl were removed from the supernatant and 50 μl of 7.5 M ammonium acetate were added. The mixture was incubated for 1 h in −20 °C and centrifuged for 10 min at 13,000 rpm. 100 μl of the supernatant was removed to a fresh tube, 200 μl of cold ethanol were added, and the mixture was centrifuged for 30 min at 13,000 rpm. The pellet was washed with 70% ethanol and dissolved in 20 μl of 10 mM Tris and 1 mM EDTA (pH 8).

Preparation of yeast vacuoles—For preparation of vacuoles, cells were grown in YPD medium adjusted to pH 5.5 by HCl and harvested at cell density of about 0.8 OD units at 600 nm. Vacuolar membranes were prepared according to Uchida *et al.* (28), except that the homogenization buffer contained no magnesium, and the vacuoles were washed only once with the EDTA buffer. ATP-dependent proton uptake activity was assayed by following the absorbency changes of acridine orange at 490–540 nm as described previously (29). The 1-ml reaction mixture contained 20 mM MOPS-Tris (pH 7), 150 mM KCl, and 15 μM acridine orange. Isolated yeast vacuoles containing 10 to 30 μg were added to the reaction mixture followed by 10 μl of 0.1 M MgATP. The reaction was terminated by the addition of 1 μl of 1 mM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

Antibody Preparation and Western Analysis—A polyclonal antibody against Vtc4p antibodies was obtained by the injection into rabbits of a chimeric protein containing the maltose-binding protein and the hydrophilic sequence of amino acids 53–220 of Vtc4p. The DNA fragment coding to these amino acids was amplified by PCR with introduced *Eco*RI and *Hin*dIII restriction sites. The DNA was cloned in frame to the maltose-binding protein in the plasmid PMAL-C (New England Biolab). Following transformation and sequence verification, 500 ml of bacterial culture was grown to OD 0.5 at 600 nm, induced with isopropyl-1-thio-β-D-galactopyranoside for 3 h, and harvested by centrifugation at 5,000 × *g*. The cells were broken by French press, and the protein was purified by using a column containing maltose-agarose. The fractions containing the chimeric protein were dissociated by SDS, loaded on preparative gel, and electrophoresed. The gel was briefly stained by Coomassie Blue, the identified protein band was cut out, and the fusion protein was electroeluted. About 0.25 mg of the fusion protein was injected into rabbits as described previously. Antibody to Pma1p was also raised in rabbits using the purified protein that was electroeluted from polyacrylamide gels (30, 31). The monospecificity of the antibody was verified by V-8 partial digestion of Pma1p followed by SDS gel electrophoresis and immunoblotting as described previously

(30). This antibody was previously used in various laboratories (see Refs. 32 and 33).

The antibody detection system (ECL) was from Amersham Pharmacia Biotech. Western blots were performed according to the protocol of the ECL antibody detection system from Amersham. Samples were denatured by SDS sample buffer and electrophoresed on 12% polyacrylamide mini gels (Bio-Rad) as described previously (34). Following electrotransfer at 0.5 Ampere for 15 min, the nitrocellulose filters were blocked for 1 h in a solution containing 100 mM NaCl, 100 mM sodium phosphate (pH 7.5), 0.1% Tween 20, and 5% nonfat dry milk. Antibodies were incubated for 30 min at room temperature at a dilution of 1 to 1,000 in a similar solution containing dry milk at only 2%. Following five washes in the same solution, peroxidase-conjugated second antibody or protein A was added to the filters. After 30 min of incubation and five washes with the same solution, the nitrocellulose filters were subjected to the ECL amplification procedure. The filters were exposed to Kodak X-Omat AR film for 5–30 s.

Membrane Preparations—Yeast cells were grown in 500 ml of YPD medium (pH 5.5) to OD 1 at 600 nm. The suspension was centrifuged at 3,000 × *g* for 5 min, and the pellet was washed with 200 ml of water, and again with 1 M sorbitol. The cell wall was digested by 2.5 units of zymolyase in a 10-ml solution containing 10 mM Hepes pH 7.5 and 1 M sorbitol. After 30 min of incubation at 30 °C, the suspension was centrifuged in 15-ml Corex tubes at 3,000 × *g* for 5 min. 1 ml of glass beads were added to the pellet as well as 1 ml solution containing 30 mM MOPS pH 7, 1:100 protease inhibitor mixture (Sigma), 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 mM EGTA. The suspension was vortexed five times for 30 s with incubation on ice for 30 s in between. The solution was removed from the glass beads and placed in a new Corex tube. An additional 2 ml of the above solution was added to the tube with the glass beads and the tube was vortexed briefly. The suspension was added to the previous one and centrifuged at 3,000 rpm for 5 min to give a pellet containing the cell debris and nuclei. The supernatant was centrifuged at 10,000 × *g* for 10 min, and the pellet was suspended in 0.3 to 0.5 ml of a solution containing 10 mM Hepes, pH 7.5, and 0.5 M sorbitol and stored as the mitochondrial fraction. The supernatant was centrifuged at 40,000 rpm for 30 min and the pellet was suspended in 0.3–0.5 ml of a solution containing 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 2 mM dithiothreitol, and 25% glycerol, and stored as the membrane fraction at −80 °C. The supernatant was stored as the fraction containing soluble proteins.

Sucrose gradients were also used to estimate the relative density of various membrane fractions. The gradients was made as described in Lupashin *et al.* (35) except that gradients of 20% to 60% sucrose were used and the centrifugation was for 14 h.

Detection of Vacuole Acidification by Quinacrine Fluorescence—Yeast cells were grown in 5 ml of YPD to OD 0.8 at 600 nm. The cells where cooled on ice for 5 min, and 1 ml of the cells sedimented by centrifugation and resuspended in 100 μl YPD containing 100 mM Hepes (pH 7.6) and 200 μM freshly prepared quinacrine. The suspension was incubated 5–10 min at 30 °C and cooled on ice for 5 min. The cells were sedimented by centrifugation and resuspended in 1 ml 100 mM of Hepes (7.6), 2% glucose. The cells were washed twice with the same cold buffer, and resuspended in 0.1 ml of the same solution. 4 μl of the cell suspension was mixed on the microscope slide with 4 μl of 0.5% low melting agarose that is kept at about 45 °C and covered with a glass coverslip. Accumulation of quinacrine into the vacuoles was followed by fluorescence microscopy with excitation at 423 nm and emission through a filter of 503 nm maximal transmission.

RESULTS

Negative-Dominant (Recessive) Mutations That Induce Growth at pH 7.5 in V-ATPase Null Mutants—Preliminary experiments showed a sporadic growth of V-ATPase null mutants on YPD plates buffered at pH 7.5 (36). Following EMS treatment, many more colonies were able to grow under the same conditions. Because the V-ATPase null mutants are constructed by the deletion of most of the reading frames of their genes, it is unlikely that the EMS treatment revived the V-ATPase and proton pumping activity of the enzyme (36). Moreover, experiments with quinacrine uptake by these mutants and ATP-dependent proton uptake into isolated vacuoles demonstrated that their V-ATPase is not active (36). The mutants were denoted as *svf* mutants (suppressor of V-ATPase function). The large number of colonies that grew at pH 7.5 after

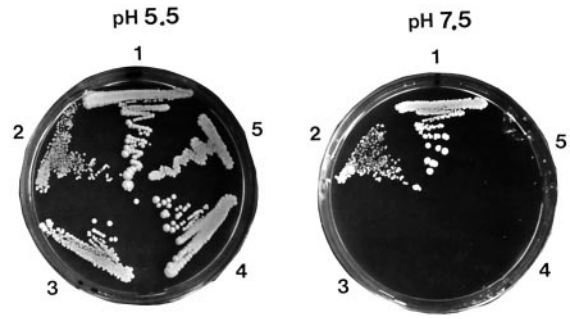

FIG. 1. **The suppressor of V-ATPase function (*svf*) is a recessive mutation.** *1*, wild-type W303 (MATα). *2*, *svf3–1* mutant was generated by EMS treatment of V-ATPase null mutant Δ*VMA3* (MATα) by selection for growth at pH 7.5. *3* and *4*, the mutant *svf3–1* was crossed with Δ*VMA3* (MATa) to give the diploid strain *svf3–1*/Δ*VMA3*. *5*, the V-ATPase null mutant Δ*VMA3* (MATa). Although *svf3–1* strain grew on YPD buffered at pH 7.5, the diploid strain failed to grow on medium buffered at pH 7.5.

EMS treatment suggests a big complex with multiple subunits as the target for mutagenesis. Fig. 1 shows that *svf* are recessive mutations. A *VMA3::URA3 (svf3)* mutant that can grow at pH 7.5 was crossed with the original *VMA3::URA3* null mutant that could not grow at pH 7.5. Diploid strains of V-ATPase null mutants that carried the wild-type and *svf* alleles were not able to grow on buffered plates at pH 7.5. It is likely, but not necessary, that the mutagenesis results in the inactivation of a protein that is part of a large complex. The minimal number of complementation groups was estimated by crossing the two mating types of *svf* mutants of Δ*VMA8* as well as Δ*VMA10*. Six of 62 diploid strains of Δ*VMA8* grew at pH 7.5, and 3 of 81 diploid strains of Δ*VMA10* grew at pH 7.5. This experiment indicates that several complementation groups are involved in the generation of *svf* mutants, suggesting the involvement of several gene products in rendering the V-ATPase null mutants insensitive to high pH. Therefore, the mutation obtained by the EMS treatment inactivated a protein complex, which resulted in growth at pH 7.5 even when the V-ATPase was totally inactive. The presence of one intact copy of the same gene in diploid cells reversed the phenomenon and resulted in growth arrest on a medium buffered at pH 7.5.

Because V-ATPase is composed of several subunits, we examined the frequency of *svf* in various V-ATPase null mutants in which different genes encoding subunits of the enzyme were interrupted. As depicted in Table I, EMS treatment of V-ATPase null mutants in which genes encoding subunits A, D, and E of the catalytic sector were interrupted gave raise to higher numbers of *svf* mutants. Similar treatment of V-ATPase null mutants in membrane sector subunits resulted in much lower frequency of *svf*. This also includes a V_1 (catalytic sector) subunit (Vma7p) that was shown to be necessary for the assembly of the membrane sector V_o (8, 17, 34). It is interesting that null mutants in Vma11p and Vma16p, which are part of the membrane sector, gave intermediate frequency of *svf* mutants, and the same goes for Vma13p, which does not affect the assembly of the rest of the subunits (23, 37). The results also suggest that neither the mating type nor the selection marker used for the interruption of the various genes have any effect on this phenomenon. There is no apparent explanation for the influence of the subunit-specific V-ATPase null mutants on the frequency of the *svf* mutations. It may suggest an interaction between unassembled subunits and the mutated proteins that their inactivation cause the *svf* phenotype.

Null Mutation in Vtc1p Reduce the Amount of V-ATPase in the Yeast Vacuole—In the course of purification of Vma10p and sequence determination following trypsin treatment (38), two

TABLE I
Frequency of growth at high pH of EMS-treated null mutants in the various V-ATPase subunits

Yeast strains, in which genes encoding V-ATPase subunits were interrupted, were treated with EMS as described under "Materials and Methods." About 10^7 viable cells at pH 5.5 were spread on YPD plates containing 50 mM MOPS (pH 7.5). Colonies that grew at pH 7.5 were scored. The experiment was repeated at least twice, and the results were varied by no more than 3-fold.

Strain	Colonies grew at pH 7.5 per 10^6
Δ*vma3*::*LEU2* (α)	100
Δ*vma3*::*LEU2* (a)	83
Δ*vma3*::*URA3* (α)	114
Δ*vma3*::*URA3* (a)	37
Δ*vma7*::*URA3* (α)	47
Δ*vma10*::*URA3* (α)	72
Δ*vma11*::*URA3* (a)	253
Δ*vma16*::*HIS3* (a)	276
Δ*vma13*::*URA3* (α)	355
Δ*vma1*::*LEU2* (α)	1211
Δ*vma4*::*LEU2* (α)	1433
Δ*vma8*::*URA3* (α)	1088

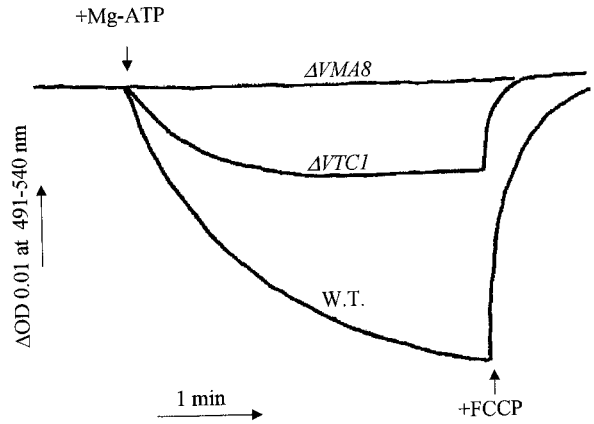

FIG. 2. **Disruption of the *VTC1* gene results in a reduced V-ATPase activity in isolated vacuoles.** Yeast vacuoles were isolated as described under "Material and Methods" and adjusted to protein concentration of 5 mg/ml. The equal amounts of proteins in the various preparations were verified by comparing the staining intensity of major proteins in SDS-polyacrylamide gels. ATP-dependent proton uptake was measured by following acridine orange absorption changes at 491–540 nm as described under "Material and Methods." Vacuoles containing 50 μg of protein were assayed in each sample. Where indicated, 10 μl of 0.1 M MgATP or 1 μl of 1 mM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (*FCCP*) were added.

additional polypeptides with the sequences KIALPTR and VFFANER were detected. Their relative amounts were about one-third of the Vma10p peptides, which is approximately one copy per V-ATPase (38). A search in the GenBank™ revealed a complete match with only one open reading frame in the yeast genome. The identified gene was named *VTC1* and the protein Vtc1p (Vacuolar Transporter Chaperon, see below). The open reading frame (YER072w) encodes a hydrophobic protein of 14,380 Da with three potential transmembrane helices.

A null mutation (Δ*VTC1*) was constructed in a and α haploid strains (see "Material and Methods"). The *VTC1* null mutants were able to grow on plates buffered at pH 7.5, suggesting that the V-ATPase is still active in these mutants. However, several of the individual colonies of the Δ*VTC1* failed to accumulate quinacrine into their vacuoles indicating that their V-ATPase activity is lower than the wild-type strains. Fig. 2 shows the ATP-dependent proton uptake activity of vacuoles isolated from wild-type and Δ*VTC1* strains. The vacuoles isolated from the null strains exhibited a reduced ATP-dependent proton uptake activity in comparison with wild-type strains. The relative activity of V-ATPase was variable and ranged between 10 and 30% of the wild-type. This explains the observation that quinacrine fluorescence was only occasionally observed in Δ*VTC1* strains.

*Null Mutation in Vtc1p on Δ*VMA* Background Results in Growth at pH 7.5*—Because most of the colonies of *VTC1* null mutants fail to accumulate quinacrine in their vacuoles, we examined the growth of Δ*VTC1* in wild-type and Δ*VMA* background. Fig. 3*A* shows the growth of the different strains on YPD plates buffered at pH 5.5 or 7.5. As expected, all the strains were able to grow at pH 5.5 and the *VMA* null mutant (Δ*VMA8* in this particular case) failed to grow at pH 7.5 (39, 40). It was surprising to observe that the double mutant Δ*VMA8* + Δ*VTC1* grew well at pH 7.5. To verify this observation, a diploid strain containing one allele of Δ*VMA8* and one allele of Δ*VTC1* was constructed. This diploid strain exhibited a wild-type phenotype. Fig. 3*B* shows that the properties of the four haploid strains resulted from tetrad dissection of one ascus. The intact and interrupted genes were assayed by PCR, and as expected, two of the spores (1 and 4) gave rise to MATa and the other two to MATα (2 and 3). Spore 1 was wild-type for *VMA8* but Δ*VTC1*. Cells that were grown from this spore grew at pH 7.5 but failed to accumulate quinacrine into their vacuoles. Spore 2 was wild-type for *VTC1* but Δ*VMA8,* and accordingly its cells failed to grow at pH 7.5 and to accumulate quinacrine. Spore 3 gave rise to a double disruptant mutant in both genes *VMA8* and *VTC1*. The cells that were grown from this double mutant were able to grow at pH 7.5 and as expected failed to accumulate quinacrine into their vacuoles. Spore 4 exhibited a wild-type phenotype in all the assays. The observations that interruption of *VTC1* in haploid strains, which carry interrupted genes encoding V-ATPase subunits, were able to grow at pH 7.5 suggest that *VTC1* is one candidate for *svf* mutants (Suppressor of V-ATPase Function). Moreover, a diploid strain containing two alleles of Δ*VMA8* and one allele of Δ*VTC1* failed to grow at pH 7.5 (not shown), indicating that the inactivation of *VTC1* resulted in a recessive phenotype. Finally, eleven haploid *svf* strains of Δ*VMA8*, which were used for the determination of complementation groups, were transformed with YPN2 plasmid carrying the *VTC1* gene. The transformed colonies were analyzed for growth at pH 7.5. One transformed *svf* strain failed to grow on plates buffered at pH 7.5, suggesting that this *svf* strain resulted from inactivation of its *VTC1* gene.

The VTC Genes Encode for a Family of Membrane Proteins—VTC1 encode a hydrophobic protein of 14,380 Da with potential three transmembrane domains. A search in the yeast genome data base with the Vtc1p sequence revealed two homologous genes that were named *VTC2* and *VTC3*. These genes encode proteins of 95,435 and 96,551 Da, respectively, and contain a predicted large globular domain in their N termini and a hydrophobic domain with predicted 2 to 3 transmembrane segments in their C-terminal part. A further search with *VTC2* or *VTC3* yielded an additional homologous gene *VTC4* of 75,483 Da, which is missing the hydrophobic domain and exhibited homology only to the N terminus hydrophilic part of *VTC2* and *VTC3*. Fig. 4 shows the multiple alignment of the predicted amino acid sequences of the four members of the *VTC* gene family. Because Vtc4p lacks the hydrophobic segment, it was interesting to see whether it is present in the cell as a soluble protein. Vtc4p sediments together with the yeast membranes and is not present in the cytoplasm as a soluble protein (not shown). Fig. 5 shows that Vtc4p could not be detected in the Δ*VTC1* strain or in the double disruptant mutant Δ*VTC2* + Δ*VTC3*. However, disruption of each of the latter genes had no effect on the presence of Vtc4p. These results suggest that Vtc1p and Vtc4p operate together as a complex, which resem-

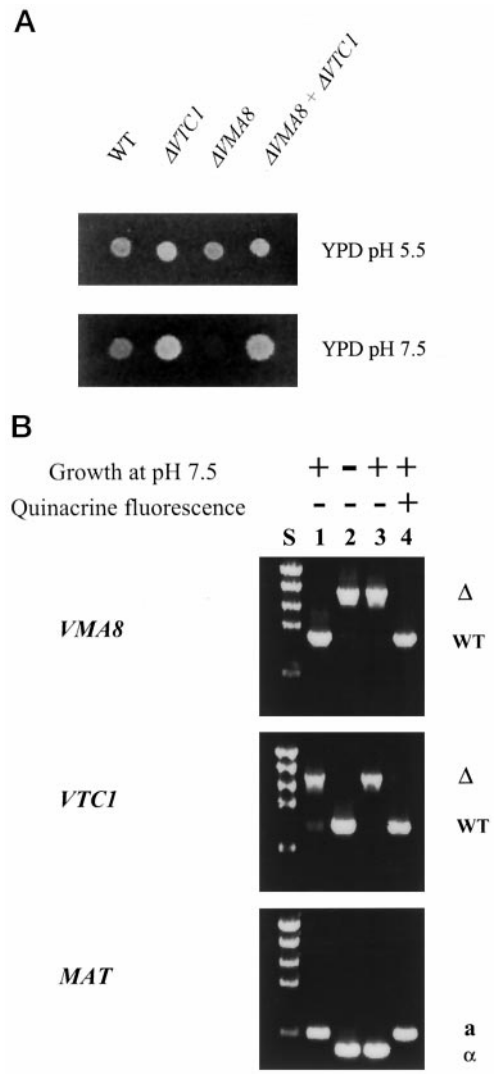

FIG. 3. **Disruption of *VTC1* in a V-ATPase null mutant results in growth at pH 7.5.** *A*, a diploid strain containing a single allele of Δ*VMA8* and Δ*VTC1* was obtained by mating the Δ*VMA8* (MATa) with the Δ*VTC1* (MATα). The diploid strain was sporulated and single spores were grown on YPD buffered at pH 5.5. The various strains were analyzed by PCR for the presence of native or interrupted genes. The various strains indicated in the figure were analyzed for growth at pH 5.5 or 7.5 as described under "Material and Methods." *B*, PCR analysis of the four spores obtained from a single ascus. The results show that spore 1 is a Δ*VTC1* strain; spore 2 is a Δ*VMA8* strain; spore 3 is a Δ*VMA8* and Δ*VTC1* double disruptant strain; and spore 4 is a wild-type strain. The mating type of each spore was analyzed. Quinacrine accumulation into the vacuoles of the various strains was measured as described under "Material and Methods."

bles the structure of Vtc2p and Vtc3p. However, indirect evidence suggests that for some cellular activities Vtc1p functions by itself without the assistance of Vtc4p (not shown).

A further search in the GenBank™ revealed a large family of proteins with limited but significant homology at the N terminus domain to the Vtc proteins. In *S. cerevisiae,* the search identified the proteins Pho81p, Pho87p, and Syg1p and the open reading frames YNR013C, YJL198W, and YDR089. Pho81p and Pho87p are involved in phosphate metabolism. Pho81p is thought to sense the low phosphate signal, perhaps at the transporter complex, followed by transportation into the nucleus to derepress the phosphate pathway (41, 42). Pho87p has a large globular N terminus of about 450 amino acids and up to twelve transmembrane domains in the C-terminal half of the molecule. It may be involved in inorganic phosphate uptake together with other potential phosphate permeases such as Pho86p and the proteins encoded by the open reading frames YNR013C and YJL198W (43). Their extended globular N termini suggest additional functions for their potential transport activity.

A search in protein data bases revealed potential homologous proteins in *Caenorhabditis elegans*, *D. melanogaster,* and a few expressed sequence tags in mouse and human libraries. The gene Y39A1A.22 (accession number AL031633) from *C. elegans* was denoted as an open reading frame in GenBank™. The gene is expressed and identified as cDNA expressed sequence tag yk304f12.5. Similar to the yeast genes, the predicted protein contains a globular N terminus and hydrophobic C terminus with predicted seven transmembrane domains. Only the globular N-terminal part exhibits sequence similarity with the Vtc proteins. The *Drosophila* gene is present on 163A7 cosmid with GenBank™ accession number AL031129. The predicted open reading frame shows high similarity to the *C. elegans* protein in its general structure, predicted transmembrane helices, and amino acid sequences. Even though the activity of these proteins is not known, in addition to their specific function, they are likely to have a similar mode of action. In line with this assumption, a common new amino acid motif of L*K*XXXX*E*X*Y*XX*L*XX*L*XX*F*XX*L*N*X*TG*F*X*K*I*X*KK*X*DK was identified in all of them. Fig. 6 depicts a multiple alignment of some of the above mentioned proteins at the region of the common motif.

VTC1 Is Involved in the Proper Distribution and/or Assembly of Membrane Proteins—The disruption of VTC genes resulted in different V-ATPase phenotypes as judged by quinacrine accumulation and ATP-dependent proton uptake into isolated vacuoles (Figs. 2 and 3*B*). Therefore the relative amounts of various V-ATPase subunits were analyzed by Western blots. Fig. 7 shows that the disruption of *VTC1* resulted in marked reduction of the amounts of subunits C, D, and E in the isolated vacuoles. This reduction is in line with the reduced activity of the isolated vacuoles. The disruption of other VTC genes did not alter the apparent amounts of these subunits. However, we observed reduced ATP-dependent proton uptake activity of isolated vacuoles from Δ*VTC4* (not shown). The proton uptake activity of Δ*VTC2* and Δ*VTC3* was at least at the wild-type level.

Because Δ*VTC1* suppresses the V-ATPase null phenotype, it is possible that the suppression may result from changes in the distribution of different transporters in the yeast membranes. As shown in Fig. 7, the amount of Pma1p was also reduced in membrane preparation of Δ*VTC1* in comparison to wild-type and other ΔVTC membranes. Sucrose gradients were used to identify changes in distribution of Pma1p in isolated membranes of the various mutants. As shown in Fig. 8*A*, the distribution of Pma1p in the sucrose gradient fractions of the membrane preparation was not altered in Δ*VTC1* in comparison with the wild-type strain. Quantitation of the band intensities indicated that the amounts of Pma1p in Δ*VTC1* are 2–3-fold less than in membrane preparations of the wild-type strain. Transformation of Δ*VTC1* with a plasmid bearing *VTC1* gene fully restored the amounts of Pma1p (not shown).

Null mutations in V-ATPase subunits result in missorting of vacuolar proteins (2). We therefore examined the distribution of Pma1p in the various V-ATPase null mutants and the *svf* suppressor mutations. As shown in Fig. 8*B*, the distribution of Pma1p in sucrose gradients of membrane preparations was drastically changed in the V-ATPase null mutants *versus* the wild-type strain. In all the V-ATPase null mutants examined (Δ*VMA3* and Δ*VMA8*), Pma1p appeared in much lighter fractions. We examined the distribution of Pma1p in sucrose gra-

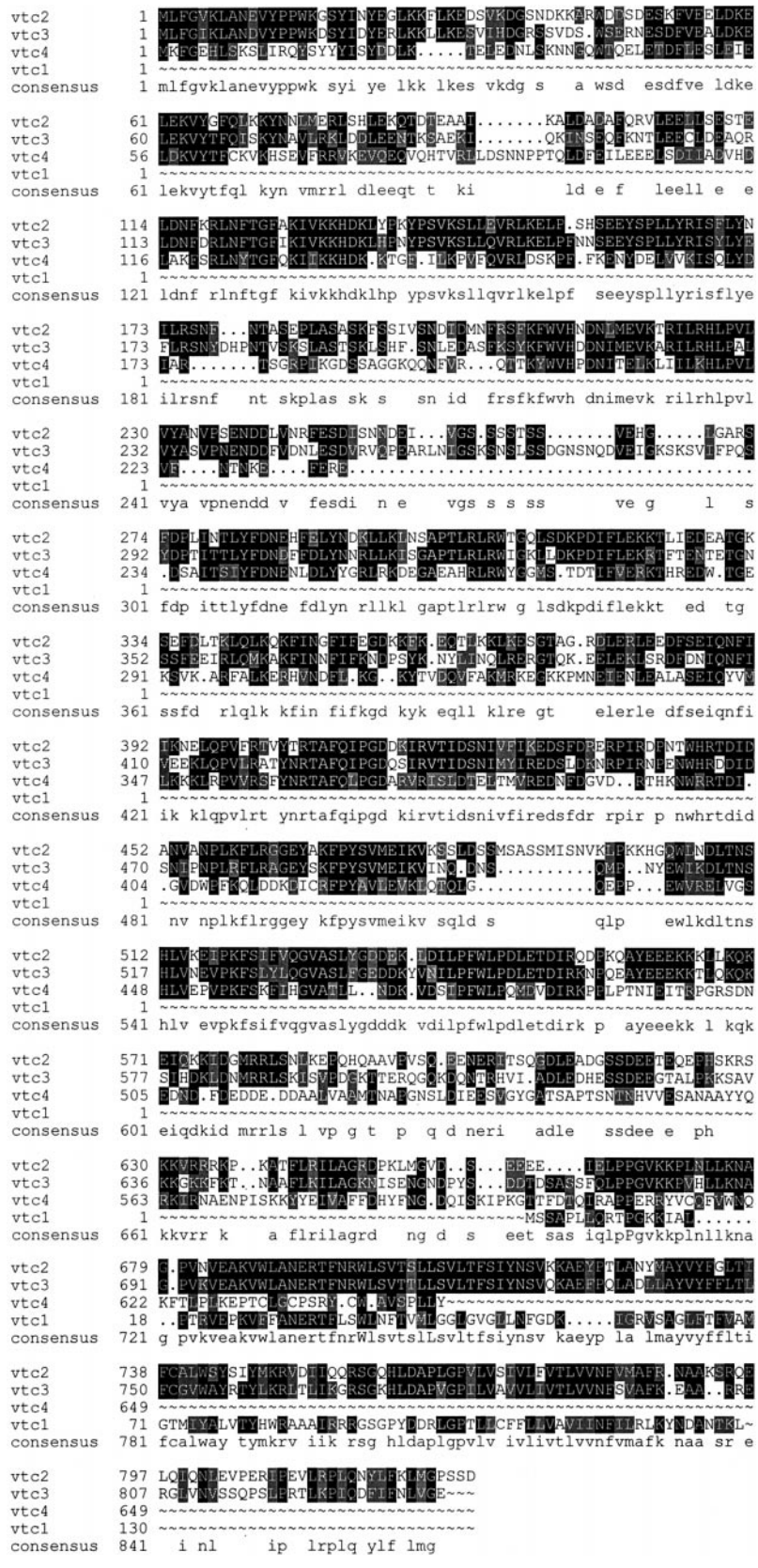

FIG. 4. **Multiple alignment of the amino acid sequences of the *VTC* family members.** The amino acid sequences of Vtc1p through Vtc4p were obtained from GenBank™ as the following reading frames: Vtc1p, YER072W; Vtc2p, YFL004W; Vtc3p, YPL019C; and Vtc4p, YJL012C. The multiple alignment was done using the program pileup. Boxshade program was used for visualizing the results (GCG software package).

dients of membrane preparations isolated from four different *svf* mutants in comparison with their corresponding V-ATPase null mutants (Fig. 8*B*). In each one, the peak fractions shifted slightly to heavier fractions in comparison to V-ATPase null mutant (see fractions 6 and 7 in the various gradients). In addition the *svf* mutants exhibited relatively higher amounts of Pma1p in more dense fractions (between fractions 1 and 5). These fractions represent the distribution of plasma membranes in wild-type cells. The distribution of Pma1p in the sucrose gradients of membranes isolated from the double mutant Δ*VMA8* + Δ*VTC1* shows reduced amounts of Pma1p with a distinct second peak in fraction 4. These phenomena may be

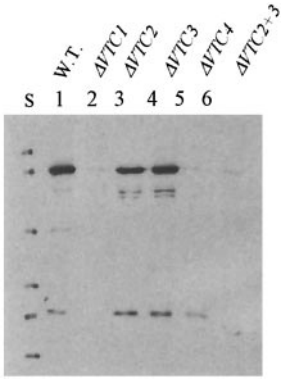

FIG. 5. **Detection of Vtc4p by its antibody in different *VTC* disruptant strains.** The antibody against Vtc4p was generated in rabbits by injecting a fusion protein containing polypeptide with a relatively unique amino acid sequence for Vtc4p (see "Materials and Methods"). The yeast strains were grown in YPD medium at pH 5.5 to an OD of about 1. The cells were harvested by centrifugation, washed by water, and spheroplasts were prepared as described under "Materials and Methods." The cells were broken by vortexing with glass beads, and the total membranes were collected by differential centrifugation between 10,000 × *g* for 10 min and 150,000 × *g* for 30 min. About 20 μg of protein were loaded in each lane, and the blotting and antibody decoration was performed as described under "Materials and Methods."

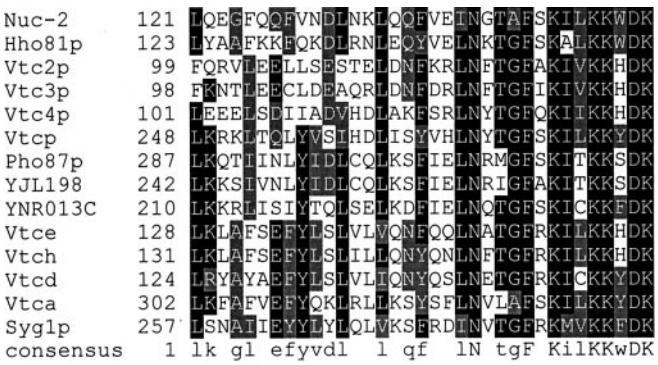

FIG. 6. **Multiple alignment of the amino acid sequences at the common motif region of the *VTC* family members from various phila.** The amino acid sequences of Vtc1p through Vtc4p were obtained from GenBank™ as follows: the yeast proteins Vtc2p, Vtc3p, and Vtc4p are as described in Fig. 4; Pho81p, YGR233c; Pho87p, YCR037C; Syg1p, YIL047C and two open reading frames YJL198W and YNR013C; Vtce is the *C. elegans* Y39A1A.22 gene product; Vtca is the *Arabidopsis thaliana* T18E12.7 gene product; Vtch is a human gene product encoding a murine leukemia virus receptor (53); Vtcp is a *Schizosaccharomyces pombe* SPBC3B8.04c gene product; Nuc-2 is a *N. crassa* Nuc-2 gene product that controls phosphorus acquisition.

explained in terms of differential distribution of Pma1p in different organelles and cellular membranes and/or marked changes in the lipid composition of the plasma and other cellular membranes in V-ATPase null mutants. The physiological consequences of this major redistribution of membrane proteins and/or the changes in physical properties of the membrane can explain some of the various phenotypes reported in this work.

DISCUSSION

The lack of V-ATPase activity in *S. cerevisiae* resulted in several conditional lethal phenotypes, including growth arrest at neutral pH, sensitivity to high and low metal-ion concentrations, as well as altered glycosylation pattern and missorting of vacuolar proteins (8, 11). Growth at low pH may correct some but not all of these defects, presumably by replacing acidification *via* the activity of V-ATPase by a fluid-phase endocytosis that brings the acidic external fluid into crucial positions in the secretory system of the cell (5, 12). In this study, we demonstrated that inactivation of some proteins may suppress the lack of growth of V-ATPase null mutants at neutral pH. One of those proteins is Vtc1p, a member of *VTC* family that was identified in the yeast genome project as open reading frames without known function. How can inactivation of a cellular protein suppress the phenotype of V-ATPase null mutations? One of the possibilities is a replacement of V-ATPase in the crucial cellular organelles, by another mechanism that generates protonmotive force. We propose that the lack of Vtc1p results in such rearrangement. As shown in Fig. 7, null mutation in *VTC1* changes the amounts of the plasma membrane H^+-ATPase (Pma1p) in comparison with wild-type membranes. Null mutation in V-ATPase subunits drastically changed the distribution of Pma1p in sucrose gradient fractions of the isolated membranes. In these mutants Pma1p appeared in much lighter membranes than in the wild-type strain. Disruption of *VTC1* in V-ATPase null mutants resulted in growth at pH 7.5 and a slight alteration of Pma1p in sucrose gradients in somewhat heavier membranes. This observation suggests that Vtc1p functions in the sorting of Pma1p to the plasma membrane, and in its absence, the distribution of this protein was altered. The missorting may lead to the presence of Pma1p in organelles that are being acidified in the wild-type strains by V-ATPase. This proposal is supported by a recent observation that the frequency of *svf* mutants increased 10-fold in Δ*VMA3* in background of secretory mutants either *sec1–1* or *sec18–1* but not *sec14–3* or *sec7–5*.[2] Although we do not know the identity of the organelle in which acidification is crucial for life, we do know that this is not the yeast vacuole. Several yeast mutants that fail to acidify their vacuoles are viable at neutral pH (13, 44, 45). Moreover, the elimination of Vtc1p in the background of V-ATPase null mutations did not result in the acidification of the yeast vacuole (see "Results"). The most likely candidate for the crucial acidifying organelle is one or more of the post-Golgi structures.

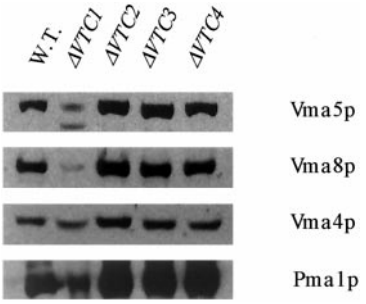

FIG. 7. **Null mutation in VTC1 causes reduced amounts of V-ATPase subunits in isolated vacuoles and reduced amounts of Pma1p in isolated membranes.** Yeast vacuoles and total membranes were isolated from the various yeast strains as described under "Material and Methods." The preparations were dissociated by SDS, and samples containing 10 μg of protein were applied in each lane. Following electrotransfer, the nitrocellulose membranes were decorated with the indicated antibodies. The polyclonal antibody against the purified Pma1p was raised in rabbits and was used at a dilution of 1:10,000. The polyclonal antibodies against V-ATPase subunits were raised in Guinea pigs and used at a dilution of 1:1,000.

Inactivation of the *VTC1* gene in wild-type W303 yeast strain resulted in a reduced vacuole acidification. The reduced acidification could be detected *in vivo* by a marked reduction in quinacrine accumulation and *in vitro* by reduced ATP-dependent proton uptake into isolated vacuoles. Western blot analysis with V-ATPase subunit-specific antibodies indicated that the reduced acidification resulted from reduced amounts of V-ATPase on the vacuolar membranes (Fig. 7). Therefore,

[2] N. Perzov and N. Nelson, unpublished observations.

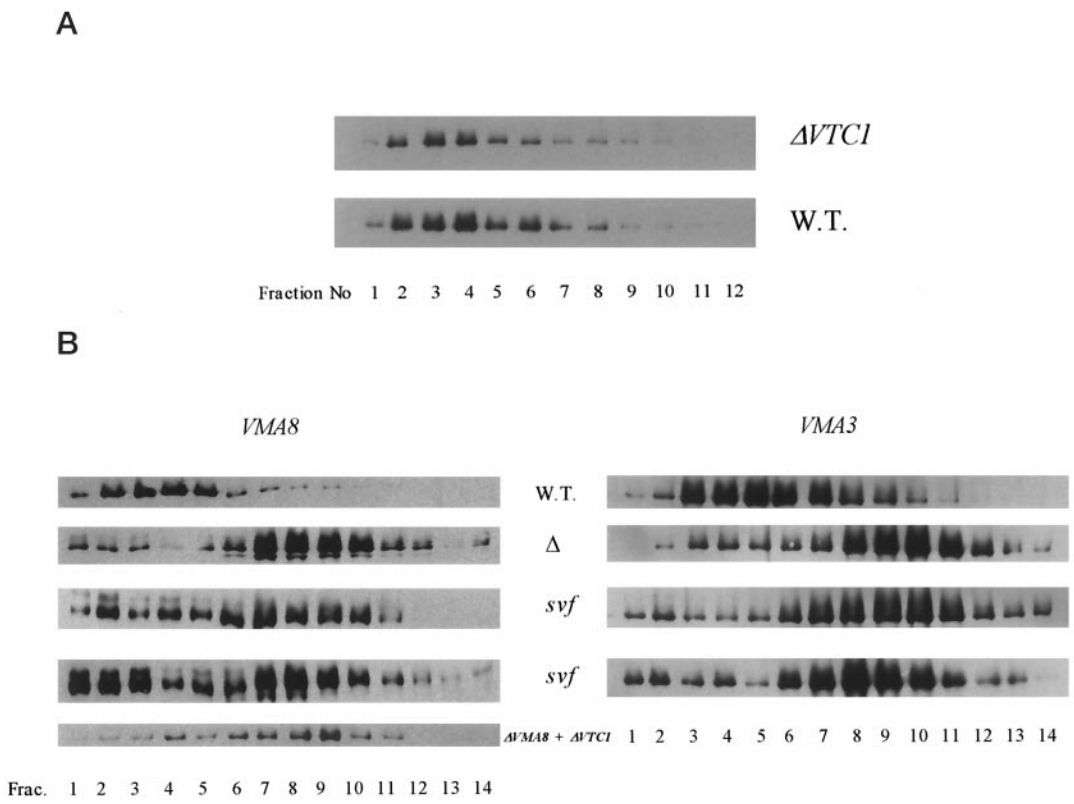

FIG. 8. **Null mutations in V-ATPase subunits and *VTC1* alter the quantity and distribution of Pma1p in the membranes.** Yeast cells were treated by zymolyase as described under "Materials and Methods." The cells were broken by glass beads and 0.5 ml of supernatant of 1,500 × *g* was placed on top of sucrose gradients from 20 to 60% in a buffer containing 20 mM MOPS (pH 7.2) and 1 mM EDTA. The gradients were centrifuged in a SW-40 rotor at 150,000 × *g* for 14 h. Sixteen fractions of 0.68 ml were collected from the bottom of the tube, the first 14 subjected to SDS-polyacrylamide gel electrophoresis, and the location of Pma1p was determined by immunoblotting as described under "Materials and Methods." The polyclonal antibody against the purified Pma1p was raised in rabbits and was used at a dilution of 1:10,000. The yeast strains analyzed in this experiment are wild-type strain W303 (*WT*), the V-ATPase null mutants of VMA8 and VMA3 that were treated with EMS to give *svf* mutants capable of growing at pH 7.5 as well as Δ*VMA8* + Δ*VTC1* double mutant. *Panel A,* membranes isolated from wild-type and Δ*VTC1* strains containing equal amounts of protein were applied on sucrose gradients and analyzed for the distribution and amounts of Pma1p. *Panel B*, wild-type, Δ*VMA8*, Δ*VMA3*, and two *svf* mutants generated from them by EMS treatment, as well as Δ*VMA8* + Δ*VTC1* strains were analyzed as described in *panel A*.

Vtc1p may be involved in the distribution or modulation of V-ATPase activity in different cellular organelles. The Δ*VTC4* mutant also exhibited reduced V-ATPase activity in isolated vacuoles but showed similar amounts of V-ATPase subunits to wild-type. This yeast strain showed normal quinacrine accumulation. The fact that Vtc4p could not be detected in Δ*VTC1* indicated that Vtc1p and Vtc4p function as a complex. Although the activity of Vtc4p is dependent on Vtc1p, the latter may function independently. The structure of this complex should be similar to Vtc2p or Vtc3p.

Until very recently the family of *VTC* seemed to be unique for yeast and a few other related fungi. A recent search in the GenBank™ raised the possibility that similar proteins may function in other organisms including mammals. The general structure of large globular proteins at the N terminus, followed by a membrane anchor of one or a few transmembrane domains at the C terminus, is quite abundant. The globular part may be situated in the cytoplasm or in the lumen of different organelles. Regardless of the position of the globular domain, several of these proteins fulfill sensory and/or regulatory functions (41, 42, 46). Very recently a human cDNA encoding cell-surface receptor for leukemia viruses was cloned and sequenced (47, 48). The protein contains a globular N terminus of about 200 amino acids and multiple hydrophobic potential membrane-spanning segments. The hydrophilic N terminus exhibited weak homology to the yeast proteins Syg1p and Pho81p. This part of the protein contains the VTC motif that was identified in this work (Fig. 6). The physiological function of the ectopic leukemia virus receptor is not known. It may function in the transport of substances across the membrane and/or serve as a membrane chaperon for the correct assembly of other membrane proteins. We propose that the identified amino acid sequence motif may suggest a similar function for all the VTC family members. Further studies may shed light on the possible function of this motif.

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