

## Altered Distribution of the Yeast Plasma Membrane H<sup>+</sup>-ATPase as a Feature of Vacuolar H<sup>+</sup>-ATPase Null Mutants\*

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**The effect of vacuolar H<sup>+</sup>-ATPase (V-ATPase) null mutations on the targeting of the plasma membrane H<sup>+</sup>-ATPase (Pma1p) through the secretory pathway was analyzed. Gas1p, which is another plasma membrane component, was used as a control for the experiments with Pma1p. Contrary to Gas1p, which is not affected by the deletion of the V-ATPase complex in the V-ATPase null mutants, the amount of Pma1p in the plasma membrane is markedly reduced, and there is a large accumulation of the protein in the endoplasmic reticulum. Kex2p and Gef1p, which are considered to reside in the post-Golgi vesicles, were suggested as required for the V-ATPase function; hence, their null mutant phenotype should have been similar to the V-ATPase null mutants. We show that, in addition to the known differences between those yeast phenotypes, deletions of *KEX2* or *GEF1* in yeast do not affect the distribution of Pma1p as the V-ATPase null mutant does. The possible location of the vital site of acidification by V-ATPase along the secretory pathway is discussed.**

Proton pumps play a major role in providing energy for several secondary uptake processes as well as maintaining the pH homeostasis required for the living cell and subcellular compartments (1). In the yeast *Saccharomyces cerevisiae*, there are two mechanistically distinct ATP-dependent proton pumps that play a pivotal role in these processes. One is Pma1p, which functions in the plasma membrane, and the second is the V-ATPase,<sup>1</sup> which functions in the vacuolar system (2, 3). Pma1p is an essential enzyme that provides the protonmotive force for the yeast plasma membrane and plays a major role in the pH homeostasis of the cell (4). The enzyme is capable of generating very high membrane potentials, and in the presence of permeant anions, pH gradients of more than 3 units are observed (5). To maintain its proper activity as well as to prevent its deleterious activity in different aspects of the secretory pathway, its transport to the cell surface must be strictly controlled. The amount of Pma1p in the plasma membrane is also under strict control, and usually the enzyme is present at relatively constant amounts of 25–50% of the mem-

brane proteins (4). Because of its importance for the viability of the cell, Pma1p was subjected to extensive studies aimed at understanding its mechanism of action as well as its potential as a drug target for antifungal agents (6). For these reasons, the gene *PMA1* was subjected to mutagenesis and numerous mutants that influenced its catalytic activity and/or targeting to the plasma membrane have been generated (7–10). Some of these mutants exhibited reduced amounts of Pma1p in the plasma membrane and resulted in growth sensitivity at low pH (11, 12).

While Pma1p is vital, and null mutations in this protein are lethal, null mutations even in one of the numerous V-ATPase subunits, while lethal in other eukaryotes, are conditionally lethal in yeast. Their vitality can be sustained when yeast mutants are grown at low pH (13–15). It was proposed that yeast V-ATPase null mutants could grow at low pH because they are able to energize their vacuolar system by fluid phase endocytosis, which provides an acidic medium for the lumen. Indeed, inactivation of the genes involved in endocytosis on the background of a V-ATPase null mutation caused lethality (16). Due to its location in the organelles of the secretory pathway, the V-ATPase plays a role in protein targeting (17). Inhibition of the V-ATPase with the drug bafilomycin A<sub>1</sub>, as well as V-ATPase null mutations, resulted in the accumulation and missorting of precursor forms of various vacuolar proteins (18–22). In addition, it was shown that, under those conditions, precursor forms of vacuolar hydrolases were accumulated within the Golgi complex and/or post-Golgi compartments (22) and that proteins having lumenally oriented targeting signals were directly dependent on compartment acidification for efficient vacuolar delivery (23).

Studies with different vacuolar proteins revealed involvement of at least two distinct targeting pathways to the vacuole (24). One goes through the endosomes, and the other delivers the proteins directly to the vacuole from the post-Golgi vesicles. Similarly, multiple delivery pathways of plasma membrane proteins may exist (25). Studies of Pma1p sorting revealed involvement of several proteins needed specifically for its transport to the plasma membrane (9, 26, 27). In this work, we report on such a differential effect of V-ATPase null mutations on the sorting of Pma1p to the plasma membrane. We show that while the targeting of Pma1p is highly affected by the V-ATPase null mutations, these mutations have very little effect on the targeting of Gas1p, Sec61p, or Sed5p into their respective compartments.

Until now, every V-ATPase mutant studied that abolished the activity of the enzyme shared more than one feature with the other V-ATPase mutants' phenotypes. Since medium pH growth requirements are common to a variety of different mutations, in order to characterize a new V-ATPase-defective mutant, we have to demonstrate several features that coexist with and are similar to a known subunit of the V-ATPase-deleted

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<sup>1</sup> The abbreviations used are: V-ATPase, vacuolar H<sup>+</sup>-ATPase; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; kb, kilobase pair(s); ER, endoplasmic reticulum; WT, wild type.

mutant. The sorting profile of Pma1p in the V-ATPase mutants can be added to this assortment of features.

Kex2p and Gef1p, two membrane proteins residing in the post-Golgi vesicles, were indicated as influencing V-ATPase activity. Their null mutants were created in two different yeast strains, and both had intact V-ATPase activity and regular quinacrine accumulation in the vacuole as well as wild-type profile of Pma1p distribution. The conclusions from these observations are that neither Kex2p nor Gef1p has a general effect upon the V-ATPase activity; nor do they affect the sorting path of Pma1p in the secretory pathway in the way that the V-ATPase null mutant does.

#### MATERIALS AND METHODS

**Strains, Media, and Reagents**—The “wild type” that was used is *S. cerevisiae* W303 (*MATa1*  $\alpha$  *trp1 ade2 his3 leu2 ura3*). The other strains used in this work are  $\Delta$ VMA8 (*MATa ade2 ura3 his3 leu2 trp1 VMA8::LEU2*);  $\Delta$ VMA3 (*MATa ade2 his3 trp1 leu2 VMA3::URA3*);  $\Delta$ VMA10 (*MATa ade2 his3 trp1 leu2 VMA10::URA3*);  $\Delta$ KEX2 [*W303*](*MATa ade2 ura3 his3 trp1 leu2 KEX2::URA3*);  $\Delta$ KEX2[RH448] (*MATa lys2 his4 ura3 leu2 bar1 KEX2::URA3*);  $\Delta$ KEX2[RSY257] (*MATa ura3 leu2 KEX2::URA3*);  $\Delta$ GEF1 (*MATa ade2 ura3 his3 leu2 trp1 GEF1::URA3*); RSY271 (*MATa his4 ura3 sec18-1*); RSY782 (*MATa his4 ura3 sec1-1*); RSY978 (*MATa his4 ura3 sec7-5*); HSMF136 (*MATa sec6-4*); and RSY20 (*MATa pep4 sec14-3*). *Sec* mutants were kindly provided by R. Schekman.

The cells were grown in a YPD medium containing 1% yeast extract, 2% bacto-peptone, and 2% dextrose. For metal ion limitation experiments, the cells were grown in a medium containing 0.25% yeast extract, 0.5% bacto-peptone, 2% dextrose, 50 mM MES, and 50 mM MOPS, and the pH was adjusted by NaOH (13, 28). Agar plates were prepared by the addition of 2% agar to the YPD buffer medium at the given pH. Yeast transformation was performed as described previously (29), 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.

**Gene Disruption**—The gene knockout of the new strains was performed as follows. All or part of the target gene was replaced by the selectable marker *URA3*, leaving flanking DNA sequences of about 0.3 kb. When polymerase chain reaction was used for the construct, the DNA fragments were cloned into the TA plasmid of pGEM-T Easy (Promega). Transformed colonies that grew on the selective medium were selected, checked by polymerase chain reaction for homologous recombination, and analyzed for their phenotype. The genes containing approximately 0.3-kb flanking sequences were cloned by polymerase chain reaction into pYES2. The various null mutants were analyzed for the disruption of each gene by polymerase chain reaction using one primer from the *URA3* gene and one primer flanking the interrupted gene.

The interruption of *KEX2* was performed as follows. The 2.150-kb *HindIII*–*HindIII* fragment within the *KEX2* open reading frame in plasmid p-GEM-T Easy was replaced by a 1.3-kb *HindIII* fragment containing the *URA3* gene. The resulting fragment was digested with *EcoRI* to release the *URA3*-disrupted allele from the vector, and the linear DNA fragment generated was used to transform three yeast strains (W303, RH448, and RSY257). Replacement of the chromosomal *GEF1* gene with an allele disrupted with the *URA3* gene was constructed as follows: A 1290-base pair *HpaI*–*NarI* fragment within the *GEF1* open reading frame in plasmid p-GEM-T Easy was replaced by the *URA3* gene. The *GEF1::URA3* construct, liberated from p-GEM-T Easy by digestion with *PvuII*, was transformed into the wild-type strain. Null V-ATPase mutants were generated as described previously (30).

**Yeast Transformation**—Yeast transformation was performed either by the method of Ito *et al.* (29) or by a bench-top method according to Elble (31). 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  $\mu$ l of salmon sperm (10 mg/ml) was added to the pellet as a DNA carrier. Then about 1  $\mu$ g of plasmid or 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% PEG 4000, and 0.1 M lithium acetate. The suspension was incubated overnight at room temperature and plated on the appropriate culture media (28).

**DNA Isolation from Yeast**—Yeast cells were grown in 5 ml of selective or YPD medium to stationary phase. The cells were harvested by

centrifugation for 2 min at 2500 rpm. The pellet was suspended in 100  $\mu$ l 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  $\mu$ l of STET was added, and the mixture was boiled for 3 min, cooled for 1 min on ice, and centrifuged for 10 min at 18,000  $\times g$ . 100  $\mu$ l was removed from the supernatant, and 50  $\mu$ l of 7.5 M ammonium acetate was added. The mixture was incubated for 1 h at  $-20^\circ\text{C}$  and centrifuged for 10 min at 18,000  $\times g$ . 100  $\mu$ l of supernatant was removed to a fresh tube, 200  $\mu$ l of cold ethanol was added, and the mixture was centrifuged for 30 min at 18,000  $\times g$ . The pellet, containing the DNA, was washed with 70% ethanol and dissolved in 20  $\mu$ l of 10 mM Tris and 1 mM EDTA (pH 8).

**Antibody and Western Analysis**—Antibody to Pma1p was raised in rabbits using the purified protein that was electroeluted from polyacrylamide gels as described previously (32–34). Western blots were performed also using anti-Sec61, anti-Sed5 (provided by R. Schekman, University of California, Berkeley, CA), anti-Gas1 (provided by H. Riezman, Biozentrum, University of Basel), and anti-CPY (purchased from Molecular Probes, Eugene, OR; catalog no. A-6428).

The antibody detection system (ECL) was from Amersham Pharmacia Biotech. Western blot analysis was performed according to the protocol of the ECL antibody detection system from Amersham Pharmacia Biotech. Samples were denatured by SDS sample buffer and electrophoresed on 10 or 12% polyacrylamide mini gels (Bio-Rad) as described previously (34). Following electrotransfer at 0.5 A 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 dried milk. Antibodies were incubated for 30 min at room temperature at a dilution of 1:1000 in a similar solution containing 2% dried milk. Following five washes in the same solution, peroxidase-conjugated second antibody or protein A was added to the filters. After incubation for 30 min 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 5–60 s.

**Membrane Preparations**—Yeast cells were grown in 500 ml of YPD medium (pH 5.5) to an OD of 1 at 600 nm. The suspension was centrifuged at 3000  $\times g$  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 a 30-min incubation at  $30^\circ\text{C}$ , the suspension was centrifuged in 15-ml Corex tubes at 3000  $\times g$  for 5 min. 1 ml of glass beads was added to the pellet as well as 1 ml of a 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 for centrifugation at 1000  $\times g$  for 5 min to give a pellet containing the cell debris and nuclei. The supernatant was centrifuged at 115,000  $\times g$  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  $-80^\circ\text{C}$ . Sucrose gradients were also used to estimate the relative density of various membrane fractions. The gradients were made as described by Lupashin *et al.* (35) except that gradients of 20–60% sucrose were used, and the centrifugation was for 14 h. Fractionation of yeast lysates by sucrose gradient centrifugation for Pma1p activity analysis was performed essentially as described by Sorin *et al.* (36), except that 1 ml of the yeast lysate was layered on top of a 10-step gradient containing 1 ml each of sucrose (w/w; 18–54% in 4% increments), and gradients were spun for 2 h at 150,000  $\times g$  in a Beckman SW40 rotor. Gradients were fractionated from bottom to top, manually, in 1-ml aliquots (fractions 1–10, respectively).

**Detection of Vacuole Acidification by Quinacrine Fluorescence**—Yeast cells were grown in 5 ml of YPD to an OD of 0.8 at 600 nm. The cells were cooled on ice for 5 min, and 1 ml of the cells were sedimented by centrifugation and resuspended in 100  $\mu$ l of YPD containing 100 mM Hepes (pH 7.6) and 200  $\mu$ M freshly prepared quinacrine. The suspension was incubated for 5–10 min at  $30^\circ\text{C}$  and cooled on ice for 5 min. The cells were sedimented by centrifugation and resuspended in 1 ml of 100 mM Hepes (pH 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  $\mu$ l of the cell suspension was mixed on the microscope slide with 4  $\mu$ l of 0.5% low melting agarose kept at about  $45^\circ\text{C}$  and covered with a glass cover. The 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.

**Metabolic Labeling and Immunoprecipitation**—Yeast metabolic la-

being was performed by the method of Chang and Slayman (37). Briefly, cells were grown to midlogarithmic phase in low sulfate minimal medium (100  $\mu$ M  $\text{Na}_2\text{SO}_4$ ) containing 2% glucose and harvested by centrifugation at  $1000 \times g$  for 5 min. The cells (usually 300  $A_{600}$  units) were resuspended at 1  $A_{600}$ /ml in minimal medium containing 25 mM  $\text{Na}_2\text{SO}_4$  and 2% glucose, preincubated at 30 °C for 30 min, and then labeled with 10–20  $\mu\text{Ci}/A_{600}$  unit [ $^{35}\text{S}$ ]methionine (ARC Inc., St. Louis MO) at 30 °C for 3 min. In pulse-chase experiments, radiolabeling was terminated by adding an equal volume of minimal medium containing 20 mM cysteine and 20 mM methionine, adjusted to pH 6. The cells were then centrifuged and resuspended in chase medium with 10 mM cysteine and 10 mM methionine, adjusted to pH 6. After chase, the cells were chilled on ice, centrifuged, resuspended at 200  $A_{600}$  units/ml in buffer (30 mM MOPS, pH 7), and lysed with glass beads in the presence of 1 mM phenylmethylsulfonyl fluoride, and cell membranes were prepared as described under "Membrane Preparations." For immunoprecipitation, 0.2-ml aliquots of membranes from sucrose gradients were diluted in NET buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 120 mM NaCl, 1% SDS, 1% Triton X-100). Tubes were incubated overnight at 4 °C with rabbit anti-Pma1 antibody and protein A-Sepharose (Amersham Pharmacia Biotech). Immunoprecipitates were washed three times with NET buffer, followed by one wash with NET buffer containing 0.5 M NaCl and one wash with  $\text{H}_2\text{O}$ . Immunoprecipitates were analyzed by electrophoresis on 10% polyacrylamide gels after resuspension in dissociation buffer. Fluorography was performed by impregnating the gels with Amplify (Amersham Pharmacia Biotech); the gels were then dried and exposed to film (Kodak X-Omat AR film) at  $-70$  °C.

**ATPase Assays**—Analysis of plasma membrane ATPase activity was performed by the method of McCusker *et al.* (12). ATP hydrolysis associated with isolated plasma membrane vesicles was assayed in 0.5 ml of a reaction medium consisting of 5 mM ATP (vanadate-free), 5 mM KSCN, 1 mM  $\text{NaN}_3$ , and 10 mM morpholine ethanesulfonic acid-KOH (pH 6.0) in the presence and absence of 100  $\mu$ M sodium vanadate. The reaction was initiated by the addition of 0.1 ml from gradient fractions, allowed to proceed for 10 min at 30 °C, and stopped by the addition of a combined stop-colorimetric development reagent consisting of 0.5 M  $\text{H}_2\text{SO}_4$ , 10% ammonium molybdate, and 5%  $\text{FeSO}_4$ . Color development was allowed to proceed for 15 min and was read at 660 nm.

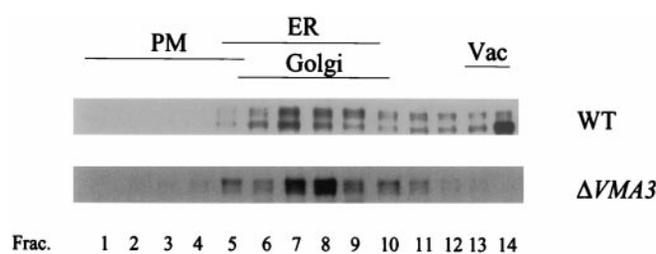
## RESULTS

**The Change in Distribution of Pma1p in the V-ATPase Null Mutants**—Sucrose gradient fractionation (35) was used to elucidate the role of the V-ATPase in compartment acidification. Previous works have established that sucrose density gradient efficiently resolves plasma membrane from intracellular (ER, Golgi, and vacuole) membranes (38). This technique of subcellular fractionation of the yeast membranes enables a demonstration of the altered sorting of proteins in the V-ATPase null mutants. Golgi membranes were well resolved from plasma membrane and were identified by Western blot analysis with the anti-Sed5p (Golgi marker). Plasma membrane was identified with anti-Pma1p or -Gas1p. The ER fractions were detected using the Sec61p antibody, and the vacuolar fractions were identified with CPYp antibody.

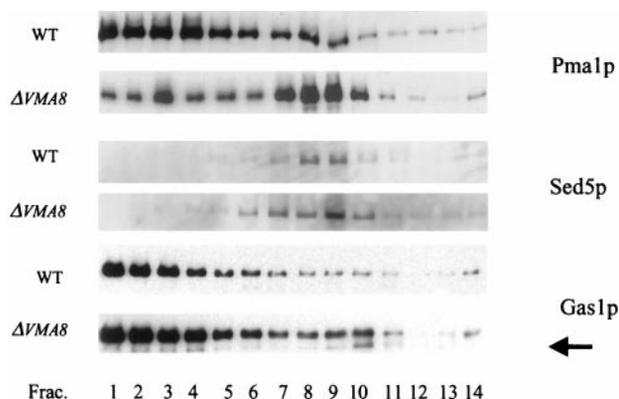
In agreement with previously published results (21, 22), in our strain as well, V-ATPase mutants accumulate precursor forms of the soluble vacuolar hydrolase CPY in the Golgi complex, and its processing to mature form is markedly inhibited (Fig. 1). To further characterize the V-ATPase mutants, we checked their ability to sort the plasma membrane protein Pma1p.

To analyze the trafficking and distribution of the Pma1p in null V-ATPase strains in comparison with the wild-type strain, we subjected their membrane preparations to sucrose gradient fractionation. Fraction samples both of wild type and  $\Delta\text{VMA8}$  mutant were assayed by Western blot analysis with Pma1p antibody. The data, summarized in Fig. 2, show drastically changed distribution patterns of Pma1p in the sucrose gradient of the V-ATPase null mutant in comparison with the wild-type strain.

In the V-ATPase null mutants, Pma1p appeared in much lighter fractions, and its amounts in more dense fractions (plas-



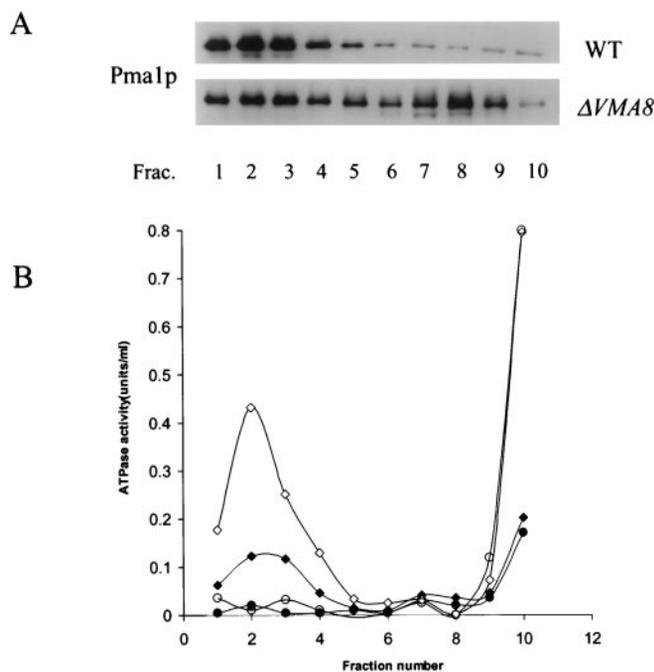
**FIG. 1. Carboxypeptidase Y is accumulated in the precursor form in V-ATPase mutant strains.** 500 ml of wild-type and  $\Delta\text{VMA3}$  cells grown in YPD medium at pH 5.5 to an OD of about 1 were lysed and subjected to sucrose gradients from 20 to 60% as described under "Materials and Methods." The gradients were centrifuged in an SW-40 rotor at  $150,000 \times g$  for 14 h. 15 fractions of 0.7 ml each were collected from the bottom of the tube, and the first 14 fractions were subjected to SDS-polyacrylamide gel electrophoresis. The first fraction from the left corresponds to the bottom of the tube. The location of CPY was determined by immunoblotting as described under "Materials and Methods." PM, plasma membrane; Vac., vacuoles.



**FIG. 2. Null mutation in V-ATPase subunit alters the distribution of Pma1p in the membranes.** Yeast cells (grown as described in the legend to Fig. 1) from the wild-type and  $\Delta\text{VMA8}$  strains were treated by zymolyase as described under "Materials and Methods." The cells were broken by glass beads and centrifuged ( $1500 \times g$ ), and 0.5 ml of supernatant was applied 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 an SW-40 rotor at  $150,000 \times g$  for 14 h. The first 14 fractions collected from the bottom of the tube were assayed by immunoblotting with antibodies against Pma1p, Gas1p (the 105-kDa ER form of Gas1p is marked with an arrowhead), and Sed5p.

ma membrane-containing fractions) were diminished. We examined several V-ATPase null mutants ( $\Delta\text{VMA8}$ ,  $\Delta\text{VMA3}$ , and  $\Delta\text{VMA10}$ ) and found the same effect in redistribution of Pma1p (data not shown). This phenomenon may be explained either in terms of general changes in the cell membrane compartments' protein distribution, altering the specificity of those membranes, or changes in distribution of specific proteins without a marked change in the characteristics of the various membrane compartments. As shown in Fig. 2, Golgi membranes were well resolved from plasma membrane and were present in fractions 7–9 in both strains as determined by the Golgi marker Sed5p. ER fractions, as detected using the Sec61p, overlapped with Golgi fractions in wild-type cells and in  $\Delta\text{VMA8}$  cells as well (not shown).

A different profile from the Pma1p was achieved on the Western blot when another plasma membrane marker, Gas1p, was used. As shown in Fig. 2, Gas1p from wild-type cells fractionated in one peak in the heavy fractions, coinciding with plasma membrane fractions. In the  $\Delta\text{VMA8}$  gradient, contrary to the diminished amounts of Pma1p, the amounts of Gas1p appear to be similar to the wild-type strain. In addition, decorating the gradient fractions with antibody to Gas1p revealed an additional band at 105 kDa in fractions 9–11 (see Fig. 2,

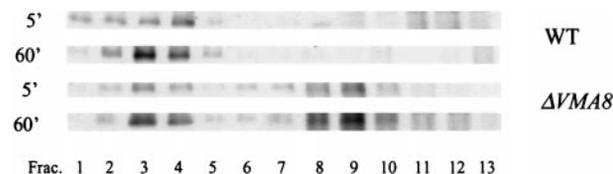


**FIG. 3. Pma1p activity in the V-ATPase null mutants is reduced, and Pma1p accumulated in lighter fractions is not catalytically active.** Yeast lysates from the wild-type and  $\Delta VMA8$  strains were fractionated and assayed as described under "Materials and Methods," except that membranes were fractionated on sucrose density gradients (18–54%), spun for 2 h at  $150,000 \times g$  in a Beckman SW40 rotor at 4 °C. *A*, distribution of Pma1p from the bottom (fraction 1) to the top (fraction 10) of the gradient. *B*, ATPase activity in gradient fractions was performed in the presence or absence of 100  $\mu M$  sodium vanadate. *Empty diamonds*, wild-type membranes; *empty circles*, wild-type membranes in the presence of vanadate; *closed diamonds*, membranes of  $\Delta VMA8$ ; *closed circles*, membranes of  $\Delta VMA8$  in the presence of vanadate. The membrane fractionation and determination of ATPase activity were performed as described under "Materials and Methods". 1 unit of ATPase activity is defined as 1  $\mu mol$  of P<sub>i</sub>/min.

arrow). This 105-kDa form was previously characterized as an ER intermediate that moves rapidly to the Golgi, where it shifts to the 125-kDa form as it acquires further glycosylation (39, 40). The observation that the 105 kDa form appeared in elevated amounts on the Western blot of  $\Delta VMA8$  may suggest that damage in compartment acidification in V-ATPase mutants slowed down the ER to Golgi export of proteins in a differential manner. It has a marked effect on Pma1p and a much milder effect on Gas1p. Hence, while in V-ATPase null mutants there is a distinct reduction of Pma1p in the plasma membrane and its accumulation in lighter fractions, the amounts of Gas1p on the cell surface are not altered, and its relative amounts in the various compartments are not drastically changed.

Acidification of the vacuolar system is therefore important for proper transport of plasma membrane proteins, as it is for the transport of the vacuolar membrane proteins and vacuolar hydrolases (21–23). However, the V-ATPase mutation does not alter the basic properties of the various compartments, and every marker we used, except Pma1p, appeared at the fraction parallel to the one in wild-type strain.

**Pma1p Present in Lighter Fractions in V-ATPase Mutants Is Not Active**—It was tempting to assume that Pma1p, which is specifically influenced by the null V-ATPase mutants, would fulfill the V-ATPase function in crucial locations along the secretory pathway. The ATPase activity was therefore measured in the sucrose gradient fractions of wild type and the null mutant. Vanadate, a known inhibitor of Pma1p, was used as well, to attribute the ATPase activity to Pma1p.



**FIG. 4. Kinetics of Pma1p transport.** Wild-type and  $\Delta VMA8$  cells were pulse-labeled for 3 min with [<sup>35</sup>S]methionine, centrifuged to remove the free radiolabeled material, and resuspended in chase medium containing 10 mM methionine and 10 mM cysteine at 30 °C. At two time points of chase (5 and 60 min), aliquots were removed, and total membranes were prepared and subjected to sucrose density fractionation. Pma1p was immunoprecipitated with its antibody from individual fractions of the gradients and analyzed by SDS-PAGE and fluorography, as described under "Materials and Methods."

The data, summarized in Fig. 3, show that in comparison with plasma membrane in wild type, the H<sup>+</sup>-ATPase activity in the  $\Delta VMA8$  strain was strongly reduced in the plasma membrane (denser fractions 1–4). These results also confirm the immunological data (Fig. 3A) of the decreased amount of the Pma1p in fractions 1–4 in  $\Delta VMA8$  strain in comparison with the wild-type strain. In fractions 7–9, which on Western blot show the second peak of Pma1p accumulation in the V-ATPase null mutant when assayed for ATPase activity in the absence of vanadate, an apparent activity of ATP hydrolysis (35% of maximal activity in fractions 2 and 3 in  $\Delta VMA8$  strain) was observed (Fig. 3B). However, this activity was not reduced upon the addition of the vanadate. Therefore, the Pma1 protein accumulated in these fractions is not enzymatically active. It is possible that the ATPase activity demonstrated in fractions 7–9 (also in wild-type strain) belongs to a different type of ATPase present in these fractions. These results are in agreement with previously published results (37), which demonstrated that maturation of the Pma1p occurs after multiple phosphorylation events during intracellular transport. Significantly, arrival of Pma1p at the plasma membrane coincides with phosphorylation of specific site(s), which appear to regulate the enzyme's ATPase activity. Probably, in V-ATPase null mutants, Pma1p is accumulated in the early steps of the secretory pathway, where the appropriate phosphorylation events that control the onset of enzymatic activity do not operate.

**ER Localization of Accumulated Pma1p in V-ATPase Mutants**—We first checked if the newly synthesized Pma1p in a wild-type strain passes the same site in which the enzyme is accumulated in the  $\Delta VMA8$  strain. A pulse-chase experiment was carried out in which the wild-type and  $\Delta VMA8$  cells were labeled with [<sup>35</sup>S]methionine and were then transferred to chase medium either for 5 min or for 1 h. A total membrane fraction was prepared for each sample and was fractionated on sucrose density gradients. Pma1p was immunoprecipitated from individual fractions and analyzed by SDS-PAGE. Already at the 5-min chase point, a large amount of the Pma1p was detected in denser fractions (2–4); it was further increased at the 1-h chase in both strains (Fig. 4). In both strains at the 5-min chase, we detected the second peak of the protein in fractions 8 and 9. However, in wild-type strain, the second peak of Pma1p in fraction 8 disappeared after a 1-h chase, probably reflecting transit of Pma1p from the ER-Golgi complex to the plasma membrane, whereas in the  $\Delta VMA8$  strain it intensified after a 1-h chase. Thus, the Pma1p accumulates in the V-ATPase mutants in a compartment along the secretory pathway, where the newly synthesized Pma1p in the wild-type strain does indeed appear to pass.

Normally, Pma1p quickly reaches the cell surface via the secretory pathway (37, 41), but in several additional mutants, an accumulation of Pma1p in ER was observed. During catalysis, the conserved aspartate residue in Pma1p (Asp<sup>378</sup>) under-

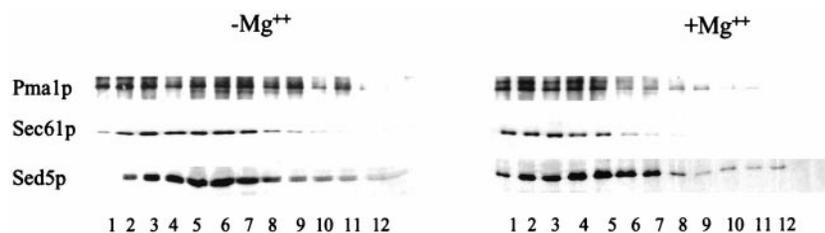


FIG. 5. Significant amounts of Pma1p are localized to the ER membranes in  $\Delta VMA8$  cells. Total membranes from  $\Delta VMA8$  cells were fractionated on a density gradient of 20–60% sucrose containing either 10 mM EDTA or 2 mM  $Mg^{2+}$ . Relative levels of Pma1p, Sed5p, and Sec61p in each fraction were quantitated by Western blotting.

goes phosphorylation. In the mutant D378A and other Asp substitutions, the targeting of newly synthesized Pma1 to the plasma membrane is impaired (42–44). It was suggested that most of the mutant protein was misfolded and retained in the ER, due to its quality control in yeast (25, 45). Another example of a reduced amount of Pma1p in plasma membrane and its retention in the ER is in the Lst1p mutant (27). Lst1p is an ER resident and interacts with COPII subunit Sec23p. Its deletion specifically inhibits the transport of Pma1p to the cell surface and causes its accumulation in the ER without affecting the distribution of Gas1p. It is interesting to check whether in our  $\Delta VMA8$  mutant the Pma1p specifically accumulates in the ER as well.

Since the separation of the ER from Golgi in our sucrose gradient standard procedure was inadequate for good resolution of these compartments, we employed a sucrose gradient fractionation on whole-cell extracts, in the presence of  $MgCl_2$ . These conditions were reported to produce a better separation between membranes of Golgi and ER; due to probable  $Mg^{2+}$ -dependent association of ribosomes with the ER membranes, they fractionate at a much higher density than Golgi membranes (38). Fig. 5 depicts such  $Mg^{2+}$ -containing gradients. It can be clearly seen that the Sec61p-containing fractions (ER) shift from the lighter to the denser fractions (co-localizing with the plasma membrane fractions) as does the Pma1p second peak from the  $\Delta VMA8$  strain, that now only shows one peak. The localization of Sed5p (Golgi marker) is unchanged as compared with fractionation without  $Mg^{2+}$ . With this procedure, a fine separation between the ER and Golgi compartments was obtained, and the accumulation of Pma1p is clearly associated with the ER. This accumulation of Pma1p in the V-ATPase null mutant is in the same compartment as the above mentioned mutants of *PMA1* or the  $\Delta LST1$  mutant, namely the ER.

Yet another way to confirm the Pma1p location on the gradient is to use temperature-sensitive *sec* mutants blocked at discrete steps of the secretory pathway. Such mutants, when shifted from the permissive (30 °C) to the restrictive (37 °C) temperature for 20 h, should demonstrate accumulation of the Pma1p in different compartments; in *sec18*, accumulation is known to occur in ER, in *sec7* and *sec14* it occurs in Golgi, and in *sec6* it occurs in the secretory vesicles. Only membranes of the *sec18* mutant, when applied to the gradient should imitate the picture of distribution of Pma1p in V-ATPase null mutants. As shown in Fig. 6, there is a Pma1p shift to lighter fractions (fractions 8 and 9) in the *sec18* mutant in restrictive conditions resembling the distribution of Pma1p in V-ATPase null mutants. The Western blot analysis obtained in other *sec* mutants with antibody against Pma1p did not resemble the same distribution (not shown). These data further indicate that the secretory pathway compartment in which the Pma1p accumulates in the V-ATPase null mutants is indeed the ER.

*pH-sensitive Phenotype of V-ATPase Null Mutants Has a Different Origin than the Same Phenotype in  $\Delta KEX2$  and  $\Delta GEF1$  Mutants*—The precise metabolic process that prevents the growth of V-ATPase null mutants at high pH is not known.

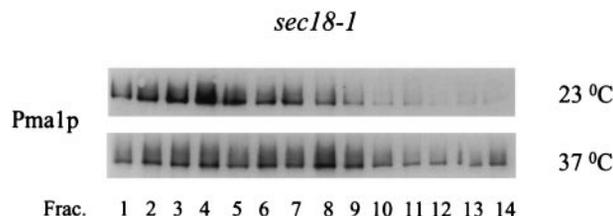
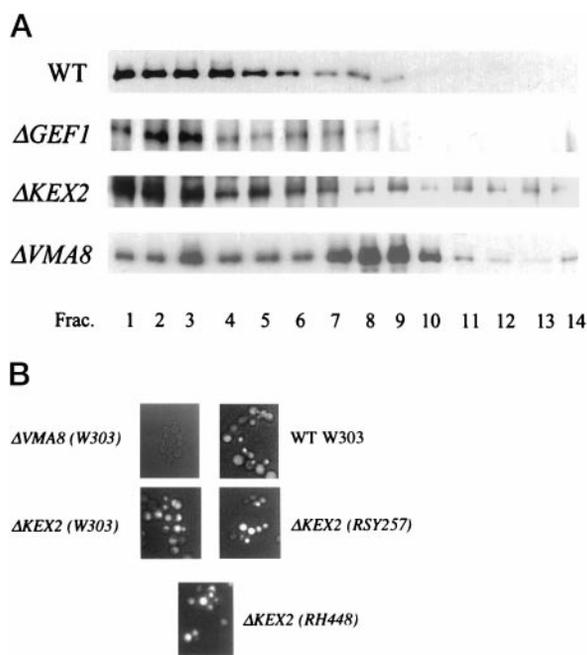


FIG. 6. Pma1p accumulated in ER in *sec18* ts-mutant under restrictive temperature. A *sec18* mutant was grown either continuously at 24 °C or shifted to 37 °C for 20 h. Total membranes prepared from both cell cultures were fractionated on a density gradient of 20–60% sucrose. Pma1p localization was evaluated for each fraction on Western blot as described under “Materials and Methods.”

Moreover, the location of the vital acidic compartment in the vacuolar system is not apparent. Indirect evidence indicates that the vacuole is not the vital acidic compartment (46). Recently, we characterized the *svf* (suppressor of V-ATPase function) mutants that suppress the V-ATPase null phenotype and are able to grow at pH 7.5 (34, 47). We proposed that one of the possibilities to explain the *svf* phenotype was replacement of V-ATPase, in the crucial cellular organelle, by another mechanism that generates a protonmotive force. The most likely localization for the acidification of the crucial organelle is one or more of the Golgi structures.

Two recent observations were reported to indicate that post-Golgi vesicles are responsible for yeast growth arrest on pH 7.5 medium (like the V-ATPase null mutants). Inactivation of the Kex2p and the Gef1p resulted in growth arrest at high pH and were suggested to be involved in the activity of V-ATPase (48, 49). Since both proteins are localized in the post-Golgi structures, the localization of the defects in their corresponding mutants was suggested at the post-Golgi site. It is possible that from that same site both of them can also cause the observed V-ATPase null mutant-linked Pma1p accumulation in the ER.

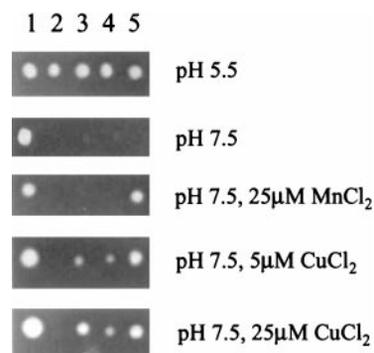
To compare the Pma1p distribution in the *KEX2* and *GEF1* null mutants with its distribution in V-ATPase null mutants, null mutants of *KEX2* in three different yeast strains as well as a null mutant of *GEF1* in W303 strain were prepared (see “Materials and Methods”). Total membranes of  $\Delta KEX2$  (W303) and  $\Delta GEF1$  null mutants as well as the V-ATPase null mutant were subjected to fractionation on sucrose density gradient, and the distribution of the Pma1p in these strains was assayed. Pma1p in all  $\Delta KEX2$  and  $\Delta GEF1$  strains is present in plasma membrane fractions (between fractions 1 and 5), resembling the distribution in wild-type cells. In both strains, no shift in Pma1p distribution was detected to less dense fractions as in V-ATPase null mutants (Fig. 7A). CPY as well as efficiently sorted to the vacuole in these strains as in the wild-type strain, and the 105-kDa ER form of the Gas1p was not observed (data not shown), in contrast to the V-ATPase mutants (21, 22). Previously, it was shown that the vacuole acidification, analyzed by quinacrine accumulation, was not impaired in  $\Delta GEF1$  but was abolished in the  $\Delta KEX2$  mutant (48, 49). Experiments with quinacrine staining in  $\Delta KEX2$  (W303),  $\Delta KEX2$  (RSY257),



**FIG. 7.  $\Delta KEX2$  and  $\Delta GEF1$  null mutants show normal Pma1p distribution and vacuolar acidification.** A, total membranes from wild-type,  $\Delta VMA8$ ,  $\Delta GEF1$ , and  $\Delta KEX2$  (W303) cells were fractionated on sucrose density gradients. The location of Pma1p was determined by immunoblotting as described under "Materials and Methods." B, vacuolar acidification was assessed by quinacrine accumulation in the vacuole as described under "Materials and Methods." Log phase yeast cells were incubated in 0.1 ml of YPD containing 100 mM Hepes (pH 7.6) and 200  $\mu$ M freshly prepared quinacrine for 10 min at 30 °C. Cells were washed twice with 1 ml of a solution containing 100 mM Hepes (pH 7.6), 2% glucose and resuspended in 0.1 ml of the same buffer. 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.

and  $\Delta KEX2$  (RH448) demonstrated that, like the wild-type cells,  $\Delta KEX2$  null mutants in the three yeast strains accumulate quinacrine and thus do not appear to be defective in vacuolar acidification (Fig. 7B). Together with the original finding (48) that the  $\Delta KEX2$  mutant exhibited the same V-ATPase assembly and activity as the wild-type strain, there is no clear evidence that Kex2p is indeed involved in regulation of V-ATPase activity.

However, it is possible that inactivation of  $KEX2$  and  $GEF1$  led to inappropriate proton uptake activity of V-ATPase specifically at the post-Golgi network, resulting in phenotypes having some, but not all, of the properties of V-ATPase null mutants. Alternatively, the pH-sensitive phenotype of  $\Delta KEX2$  and  $\Delta GEF1$  mutants could result from different sources that are not related to the vacuolar system acidification. One example of such a mutant is the triple null mutant in the three *SMF* genes ( $\Delta SMF1+2+3$ ), encoding for metal ion transporters, that is also unable to grow on a medium at pH 7 or above (50). The growth arrest of this mutant was alleviated by the inclusion of micromolar concentrations of  $Cu^{2+}$  or  $Mn^{2+}$  in the medium, suggesting that Smf-depleted yeast cells need a higher concentration of metals in their growth medium, especially at high pH. To clarify the origin of the pH sensitivity of the  $\Delta KEX2$  and  $\Delta GEF1$  mutants, we tried complementation of their media in high pH with 5 and 25  $\mu$ M  $Cu^{2+}$  or 25  $\mu$ M  $Mn^{2+}$ . Fig. 8 shows that, at basic pH where all of the mutants fail to grow, 5  $\mu$ M  $Cu^{2+}$  did not support the  $\Delta VMA8$  mutant growth but was sufficient to promote growth of all of the other mutants, including the  $\Delta KEX2$  and  $\Delta GEF1$  mutants. As a control, 25  $\mu$ M  $Mn^{2+}$  specifically promoted the growth of the triple null mutant  $\Delta SMF1+2+3$  and did not affect the others.



**FIG. 8. Growth complementation of  $\Delta KEX2$ ,  $\Delta GEF1$ , and the triple *SMF* disruptant mutant ( $\Delta SMF1+2+3$ ) at high pH by copper.** YPD plates buffered by 50 mM MOPS at pH 7.5 were prepared as described under "Materials and Methods." The indicated concentrations of filter-sterile  $CuCl_2$  or  $MnCl_2$  were added right before the pouring of the warm medium. Cultures of the various yeast strains were washed by sterile distilled water, and 2  $\mu$ l of the cell suspension was placed in the indicated positions. The plates were incubated for 3 days at 30 °C. The following strains were used: wild type (W303 $\alpha$ ) (lane 1);  $\Delta VMA8$  (lane 2);  $\Delta GEF1$  (lane 3);  $\Delta KEX2$  (W303) (lane 4);  $\Delta SMF1+2+3$  (lane 5).

These data may support the possibility that a wide spectrum of yeast mutants could result in growth arrest at pH 7.5. However, the probability cannot be excluded that V-ATPase and Gef1p, for example, cooperate functionally in the acidification of a certain segment in the vacuolar system (51).

#### DISCUSSION

Pma1p is an essential enzyme that provides the protonmotive force for the yeast plasma membrane and plays a major role in the pH homeostasis of the cell. The amount of Pma1p in the plasma membrane is also under strict control, and the enzyme is present usually in a relatively constant amount (4). The V-ATPase complements the function of Pma1p in organelles and membranes of the vacuolar system (52). In addition to its function as the main energizing enzyme in the vacuolar system, the V-ATPase was found to be involved in the sorting processes of proteins to the vacuolar compartment in yeast (53). It has long been known that acidification of a lysosomal compartment is required for proper sorting of soluble lysosomal proteases in mammalian cells, utilizing the mannose 6-phosphate receptor pathway (54, 55). The pre-Golgi and cis-Golgi membranes were also shown to contain a V-ATPase activity. There it regulates retrograde membrane traffic at the ER-Golgi pathway (56). A compartment acidification requirement was demonstrated in the targeting of GPP130 (Golgi phosphoprotein of 130 kDa) to the early Golgi (57). It would be expected that the other secretory sites would also be sensitive to the pH in their respective compartments.

In this study, we examined the influence of V-ATPase null mutations in yeast on the processes of targeting to the plasma membrane by following their effect on the Pma1p distribution. Numerous studies investigated the influence of deletion or overexpression of proteins, known to reside in the secretory pathway, on Pma1p sorting and targeting to the plasma membrane in yeast. Among them, Ast1p, Ast2p, Vps8p, and Vps36p were investigated and found to influence the Pma1p distribution (9, 58). By comparison with the Gas1p, another plasma membrane resident, several proteins with a differential effect on Pma1p were discovered. Among them is Lst1p, which was suggested to take part in the transport of Pma1p from ER to the Golgi compartment (27). Another was identified as Eps1p (ER-retained *PMA1* suppressor), which functions in ER quality control (45). The mutant of Eps1p suppresses the D378N phenotype by allowing both the mutant and wild-type Pma1p molecules to travel to the plasma membrane. Its null mutant

does not affect the sorting of native Pma1p at all, whereas it causes a marked accumulation of the 105-kDa ER form of Gas1p in the ER.

We found that V-ATPase null mutants have a specific effect on Pma1p as well. To determine its distribution on the sucrose gradient, we used Sec61p as a marker of the ER, Sed5p as a marker for the Golgi compartment, and Gas1p as the other plasma membrane marker. There was no change in the distribution of Sec61p and Sed5p in the mutant in comparison with the wild-type strain; nor was the amount of Gas1p in the plasma membrane reduced, although its 105-kDa intermediate was elevated in the ER in comparison with the wild-type strain. However, Pma1p amounts in the plasma membrane in the ATPase-depleted mutants were markedly reduced, and a large amount of the protein was accumulated in the ER in a nonactive form.

The fact that, in V-ATPase null mutants, the distribution of Gas1p differs from Pma1p suggests that lack of acidification of the various compartments differentially changes the protein composition of their membranes but not the characteristics of the membranes in general. The decline in Pma1p in plasma membrane may be a unique feature of this protein, whereas the slowdown of movement from ER to Golgi, as demonstrated by Gas1p in the mutant yeast, might be a general feature of the mutant.

The question remains as to the site of the crucial secretory pathway compartment, whose acidification by V-ATPase is vital. The fact that the site of Pma1p accumulation in the mutant is in the ER suggests that the passage from ER to Golgi in the V-ATPase mutants is impaired. This hypothesis is supported by several previous results. It was shown in mammalian cells that retrograde transport from the pre-Golgi intermediate compartment and the Golgi complex is affected by the V-ATPase-specific inhibitor Bafilomycin A1 (56). In yeast, the deletion of the *LST1* gene that encodes for a peripheral ER membrane protein results in a profile of Pma1p distribution on sucrose gradient similar to the one of V-ATPase null mutants (27). It was suggested that Lst1p takes part in efficient packaging of Pma1p into vesicles derived from the ER. Moreover, it was shown that the plasma membrane  $H^+$ -ATPase undergoes pH-dependent conformational changes (59). If we assume that the V-ATPase functions in *cis*-Golgi, then the impaired sorting of Pma1p in the V-ATPase null mutant could be explained by damage to the retrograde transport of factors such as Lst1p or conformational changes in Pma1p, imposed by the improper pH conditions.

The defect that V-ATPase null mutants are mostly checked for is their inability to grow on a buffered pH 7.5 medium. However, these mutants are also defective in growth on a low pH medium (13). The inability to grow on very acidic medium was demonstrated for a wide range of Pma1p mutants as well and was explained by a low activity of the mutant, stemming from either the mutation or competition of the mutant on the targeting to the membrane with the intact protein (12, 60). Since we find that in V-ATPase null mutants the amount of Pma1p in the plasma membrane is markedly reduced, it is tempting to suggest that the reduced growth rate at low pH is caused by reduced levels of Pma1p in the plasma membrane of those mutants.

In this study, we show that the altered distribution of Pma1p in V-ATPase null mutant can serve as another characteristic feature of it. Kex2p and Gef1p were both suggested to be involved in the proper function of the acidification by the V-ATPase (48, 49). In the case of the  $\Delta$ KEX2 null mutant, there has been a discrepancy in the original paper, where the ATPase and proton pumping activities in isolated vacuoles of this mu-

tant were undamaged, whereas the vacuoles failed to accumulate quinacrine. To exclude the possibility that in our strain (W303), uniquely, the deletion of Kex2p did not affect the quinacrine accumulation in the vacuoles, we disrupted this gene in two additional strains and in them observed as well that the  $\Delta$ KEX2 mutant was fully active in quinacrine accumulation into their vacuoles. In addition, when we checked the distribution of the Pma1p in the  $\Delta$ KEX2 and  $\Delta$ GEF1 mutants, it was similar to the wild type and not to the V-ATPase mutant. By metal supplementation in the growth medium, we showed that the addition of  $5 \mu\text{M}$   $\text{Cu}^{2+}$  rescued the  $\Delta$ KEX2 and  $\Delta$ GEF1 mutants and enabled their growth on pH 7.5 medium, whereas it did not have any effect upon the V-ATPase mutant. Therefore, a direct interaction between Kex2p or Gef1p and V-ATPase can be excluded. However, their activity may be involved in some cellular functions, such as anion transport or metal ion homeostasis, in specific organelles of the secretory pathway that also affect the growth at high pH levels in the medium.

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