Yeast mutants lacking the intracellular V-ATPase proton pump (vma mutants) have reduced levels of the Pma1p proton pump at the plasma membrane and increased levels in organelles including the vacuolar lumen. We examined the mechanism and physiological consequences of Pma1p mislocalization. Pma1p is ubiquitinated in vma mutants, and ubiquitination depends on the ubiquitin ligase Rsp5p and the arrestin-related adaptor protein Rim8p. vma mutant strains containing rsp5 or rim8 mutations maintain Pma1p at the plasma membrane, suggesting that ubiquitination is required for Pma1p internalization. Acute inhibition of V-ATPase activity with concanamycin A triggers Pma1p ubiquitination and internalization. In an endocytosis-deficient mutant (end4Δ) Pma1p is ubiquitinated but retained at the plasma membrane during concanamycin A treatment. Consistent with specificity in signaling loss of V-ATPase activity to Pma1p, another plasma membrane transporter, Mup1p, is not internalized in a vma mutant, and loss of the Mup1p adaptor Art1p does not prevent Pma1p internalization in a vma mutant. Very poor growth of vma2 rsp5-1 and vma2 rim8Δ double mutants suggests that Pma1p internalization benefits the vma mutants. We hypothesize that loss of V-ATPase-mediated organellar acidification signals ubiquitination, internalization, and degradation of a portion of Pma1p as a means of balancing overall pH homeostasis.

Cellular pH homeostasis involves a complex array of mechanisms operating in the cytosol and organelles to support localized pH-dependent functions and adapt to changing metabolic conditions (1). Vacuolar H⁺-ATPases (V-ATPases)² acidify several organelles, including vacuoles/lysosomes, endosomes, and Golgi apparatus of virtually every eukaryotic cell (2). These intracellular pumps must act in concert with mechanisms for proton export. In plants and fungi, a P-type proton pump, Pma1p, is responsible for direct export of metabolic protons (3), whereas in mammalian cells the P-type Na,K-ATPase drives activity of Na⁺/H⁺-exchangers at the plasma membrane to help achieve proton export (1). Although plasma membrane and organellar pumps have often been treated as independent, their activities are intrinsically interdependent as both draw protons from the cytosol and require cytosolic ATP for function. In yeast, PMA1 is an essential gene, suggesting an indispensable role in pH homeostasis (4), but mutations eliminating V-ATPase activity (vma mutants) are conditionally lethal (5). In this context it was somewhat surprising to find that vma mutants have more alkaline vacuoles than wild-type cells as expected but also a more acidic cytosol, which is slow to recover from acid stress (6, 7). This poor control of cytosolic pH likely derives not only from compromised organellar acidification but also from loss of proton export through Pma1p. In the vma mutants Pma1p levels are lower in the plasma membrane, and internal Pma1p is detected in multiple organelles, including the vacuole lumen. Internal Pma1p appears to be degraded by vacuolar proteases (6).

Neither the source nor the cellular consequences of Pma1p mislocalization have been fully explored. Huang and Chang (8) reported that newly synthesized Pma1p-GFP as well as certain other plasma membrane proteins such as arginine transporter Can1 were mislocalized under conditions of reduced V-ATPase activity and attributed this mislocalization to protein missorting in the altered pH at the Golgi apparatus. This may well be true given the importance of tight pH control in biosynthetic traffic to vacuoles and lysosomes (9), but targeting and/or retention of some Pma1p to the plasma membrane is essential for viability. In addition, it remains unclear whether mislocalization of Pma1p is solely a sorting error arising from poor Golgi pH control (8) or also a compensatory mechanism that somehow helps to offset loss of pH control in the vma mutants. A number of plasma membrane transporters are down-regulated by ubiquitination and endocytosis in response to ligand excess (10, 11). Certain of these transporters are ubiquitinated by yeast Rsp5p, a homologue of mammalian Nedd4, which can

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1 To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, SUNY Upstate Medical University, 750 East Adams St., Syracuse, NY 13210. Tel.: 315-464-8742; E-mail: kanepm@upstate.edu.

2 The abbreviations used are: V-ATPase, vacuolar H⁺-ATPase; YEPD, yeast extract-peptone-dextrose; SC, synthetic complete medium; Art protein, arrestin-related transport adaptor protein; BCECF, 2′,7′-bis(2-carboxyethyl)-5′-(and-6′)-carboxyfluorescein; Ub, ubiquitin; end, endocytosis.
be recruited to the transporter by 1 of 10 arrestin-related trafficking adaptors (Art) proteins (11, 12). The Art proteins bind via PY motifs to one or more of the three WW domains of Rsp5p (11, 12). Furthermore, it is clear that the Art proteins are downstream of signal transduction cascades capable of sensing specific ligand excess and altering Art protein modification or localization to direct Rsp5p-dependent ubiquitination toward their target transporter (13–16). Ubiquitination then targets the transporter for endocytosis and insertion into intraluminal vesicles of the multivesicular body for eventual vacuolar degradation (12, 16, 17). Transporter targets for several Art proteins have now been elucidated (11, 12). Rim8, also known as Art9, belongs to the Art protein family and contains PY sites capable of binding to specific ligands and triggering endocytosis of plasma membrane receptors. Rim8 is a critical component of a pathway that senses V-ATPase activity and triggers ubiquitination and endocytosis of plasma membrane Pma1p when V-ATPase activity is lost.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized by Eurofins MWG Operon. TaKaRa LaTaq polymerase was purchased from Fisher, and Pfu I DNA polymerase was from Stratagene. Concanamycin A was purchased from Axxora. All other biochemical reagents were purchased from Sigma.

Yeast Strains, Transformation, and Growth Conditions—Yeast strains used in this study are listed in Table 1. A plasmid (pHA-Ub) expressing an epitope-tagged 3HA ubiquitin was constructed by standard genetic techniques and grown in YEPD (1% yeast extract, 2% peptone, 2% dextrose) buffered to pH 5.0 or 7.5 with 50 mM potassium succinate, 50 mM potassium phosphate, or fully supplemented minimal (SC) media (0.67% yeast nitrogen base, 2% dextrose including all supplements except as indicated) (21). The BY4741 rim8Δ::kanMX, vma2Δ::kanMX, and art1Δ::kanMX mutant strains were obtained from Open Biosystems (Huntsville, AL). The temperature-sensitive rss5-1 strain, LHY23 (22), was provided by Scott Emr. The VMA2 gene was deleted in the rim8Δ, the art1Δ, and the rss5-1 mutant strains by lithium acetate transformation (23) with PCR-amplified DNA using genomic DNA from a wild-type strain, LHY23 (22), as a template and primers VMA2-2 (840) and VMA2-4 (Table 2). Transformants were selected on SC plates lacking uracil. The RH299–1C end4Δ::LEU1 strain was provided by Howard Riezman, the plasmid pCHL642, a pRS416 plasmid expressing Mup1-GFP, was provided by Scott Emr (24), and the pTS422-GCK plasmid was provided by David Amberg.

A plasmid (pHA-UB) expressing an epitope-tagged 3HA-ubiquitin under the control of an actin promoter was constructed for this study. A fusion PCR protocol was used in which the 3HA tag was first amplified by PCR from the pFA6a-3HA-KanMX6 plasmid (25) using the oligos HindIII-HA and HA-Ub (Table 2). Transformants were selected on SC plates lacking uracil. The RH299–1C end4Δ::LEU1 strain was provided by Howard Riezman, the plasmid pCHL642, a pRS416 plasmid expressing Mup1-GFP, was provided by Scott Emr (24), and the pTS422-GCK plasmid was provided by David Amberg.

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TABLE 1

Yeast strains used in this study

| Strain          | Genotype                                | Source                  |
|-----------------|-----------------------------------------|-------------------------|
| BY4741          | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0       | Open Biosystems         |
| BY4741 vma2Δ    | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vma2Δ::kanMX | Open Biosystems         |
| BY4741 rim8Δ    | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rim8Δ::kanMX | Open Biosystems         |
| BY4741 rim8Δ vma2Δ | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rim8Δ::kanMX | This study              |
| LHY23 rss5Δ–1  | MATa rss5Δ–1 ura3Δ leu2Δ0 met1Δ0 bar1Δ1 GAL | Scott Emr               |
| RH299–1C end4Δ  | MATa ura3 his4Δ leu2Δ0 bar1Δ1 end4Δ::LEU1 | This study              |
| BY4741 art1Δ    | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art1Δ::kanMX | Open Biosystems         |
| BY4741 art1Δ vma2Δ | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art1Δ::kanMX | This study              |
| BY4741 pHA-UB   | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pHA-UB | This study              |
| BY4741 vma2Δ pHA-UB | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vma2Δ::kanMX | This study              |
| By4742          | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vma2Δ::kanMX | This study              |
| BY4742 pMup1-GFP | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pMup1-GFP | This study              |
| BY4742 vma2Δ pMup1-GFP | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vma2Δ::kanMX | This study              |
| SF938–5A pHA-UB | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vma2Δ::kanMX | This study              |
| RH299–1C end4Δ pHA-UB | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vma2Δ::kanMX | This study              |
10 \(A_{600}\) units of cells was harvested, washed once in YEP, then resuspended in 1 ml of YEP and incubated for 10 min at 30 °C. At time 0, glucose was restored to 2% final concentration, and then concanamycin A (2 \(\mu\)M final concentration) was added, and the culture was returned to 30 °C. At various times equal volumes of culture and the methylene blue solution were mixed and visualized immediately by microscopy. 200–300 cells were counted for each condition to determine the percentage of cells that were stained blue.

**Immunoprecipitations**—For the \(rsps-1\) and \(rsps-1\ vma2\Delta\) strains, cells were grown in YEPD buffered to pH 5.0 with 50 mM sodium phosphate, 50 mM sodium succinate media to mid-log phase (0.6 \(A_{600}\) /ml) at the permissive temperature of 30 °C and then divided into separate cultures. Half of the cells were maintained at 30 °C and half were resuspended in media prewarmed to 37 °C, the non-permissive temperature for the \(rsps-1\) mutant for 2 h. All other strains were grown to mid-log phase at 30 °C in YEPD pH 5.0 media and harvested by centrifugation. Pellets were frozen at –80 °C. Total membranes were prepared as described in Chang and Slayman (27) with some modifications. Frozen cell pellets (100 \(A_{600}\) units) were suspended at 60 \(A_{600}\) /ml in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA) with added protease inhibitors (1 mM PMSF, 1 \(\mu\)g/ml leupeptin, 5 \(\mu\)g/ml aprotinin, 2 \(\mu\)g/ml pepstatin) and 25 mM N-ethylmaleimide to preserve ubiquitination. Cells were lysed by agitation with glass beads, and the cell lysate was centrifuged at 400 \(\times\)g for 5 min to remove unbroken cells. Total membrane fractions were prepared by centrifugation of the supernatant at 100,000 \(\times\)g for 1 h in a Beckman TL-A100 ultracentrifuge, and pellets were resuspended in immunoprecipitation buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1% Triton-X 100, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitors and 25 mM N-ethylmaleimide. Insoluble material was removed by centrifugation in an Eppendorf microcentrifuge for 5 min. For Pma1p immunoprecipitations, the supernatant was incubated overnight at 4 °C with a monoclonal antibody 40B7 against Pma1p (Abcam ab4645) followed by 60 \(\mu\)l of a 50% (v/v) suspension of Protein A Sepharose CL-4B. Immunoprecipitates were washed 3 times with lysis buffer and solubilized at 55 °C in SDS-PAGE cracking buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% SDS, 5% \(\beta\)-mercaptoethanol) for 10 min. Immunoprecipitated proteins were analyzed by electrophoresis and Western blotting using either a polyclonal antibody against Pma1p (a generous gift from Amy Chang) or monoclonal antibody P4D1 against ubiquitin (Santa Cruz Biotechnology). Alkaline phosphatase-conjugated secondary antibodies used for detection by colorimetric assay were purchased from Promega. The ratio of the ubiquitin and Pma1p signals was determined using NIH ImageJ (28) to quantitate the signals in each blot and determine the ratio of ubiquitin and Pma1p signals and then normalized to the highest ratio on each set of blots. The relative level of Pma1p was also determined by quantification with NIH ImageJ and normalization to the indicated sample on each blot. Results for multiple experiments are reported in the text as the means ± S.E. for \(n\) independent experiments.

For testing of Pma1p ubiquitination in concanamycin A-treated cells, a wild-type strain containing the pHA-UB plasmid was grown to mid-log phase (0.6 \(A_{600}\) /ml) at 30 °C in SC lacking uracil. Cell volumes corresponding to 50 \(A_{600}\) units were pelleted in separate tubes for each time point to be tested. Each sample was washed in growth media lacking glucose and finally resuspended in 1 ml of SC lacking uracil and glucose. After 10 min glucose was added to a 2% final concentration followed immediately by concanamycin A to 2 \(\mu\)M final. The 0-min time point was pelleted immediately, and the pellet was frozen at –80 °C. The remaining samples were incubated with shaking at 30 °C for the indicated times and pelleted and frozen at –80 °C. Equal numbers of cells, based on \(A_{600}\) reading, were pelleted for each sample. Frozen cell pellets were resuspended in ice-cold lysis buffer as described above except that 1.0 mM sodium orthovanadate and 10 mM sodium fluoride were included. Cells were lysed by agitation with glass beads followed by the addition to a final concentration of 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS and incubated on ice for 30 min to solubilize membranes. After removal of insoluble material by centrifugation, the supernatant was incubated overnight at 4 °C with monoclonal antibody 16B12 against the HA epitope (Covance HA.11) followed by Protein A-Sepharose as described above. Immunoprecipitates were washed and analyzed as described above, except that monoclonal antibody 16B12 was used to detect ubiquitinated proteins.

**Fluorescence Microscopy**—For visualization of Pma1p, cells were fixed and permeabilized for immunofluorescence microscopy as described (29). Fixed cells were incubated with mouse monoclonal antibody 40B7 (Abcam ab4645) against Pma1p followed by Alexa-Fluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen) (6). Cells were visualized under fluorescence optics on a Zeiss Imager Z1 fluorescence microscope. All fluorescent images were taken under identical exposure. For \(rsps-1\ vma2\Delta\) double mutant cells, Pma1p was localized by immunofluorescence in cells that were grown at 30 °C and either maintained at that temperature or shifted to media prewarmed to 37 °C for 2 h before the fixation and permeabilization step. For experiments demonstrating acute loss of V-ATPase activity, cells were grown to early log phase at 30 °C and deprived of glucose for 15 min followed by restoration of glucose and addition of 2 \(\mu\)M concanamycin A. Incubation with concanamycin A continued at 30 °C for the indicated times before fixation and permeabilization. Plasma membrane flu-
rescence was quantitated using NIH ImageJ Version 1.43r. Polygons were drawn surrounding the cell just outside the plasma membrane (whole cell) and just inside the plasma membrane (cell interior), and area and mean fluorescence signals for these polygons and background polygons of the same size were quantitated. Plasma membrane fluorescence was obtained by subtracting background signals (area × mean) from both the whole cell and cell interior signals, then subtracting the background-corrected cell interior signal from the corrected whole cell signal. Plasma membrane staining was quantitated for 8–10 cells at each time point.

Strains carrying the pMup1-GFP construct were grown in SC media lacking uracil and methionine at 30 °C to 1.0 A₆₀₀/ml and GFP fluorescence was observed using GFP optics as described above. Methionine was added at a concentration of 20 μg/ml to induce internalization of Mup1-GFP (12).

Measurement of Vacuolar pH—Vacuolar pH was measured using the BCECF method as described (30). Cells were grown in YEPD, pH 5, washed, and resuspended in medium without glucose before initiating the fluorescence measurements in a Horiba FluoroMax-4 spectrometer. Fluorescence was measured before and 5 min after the addition of glucose to a final concentration of 2%. Calibration of fluorescence ratios to pH was performed as described (30).

RESULTS

Loss of Pma1p from the Plasma Membrane in a vma Mutant Requires an E3 Ubiquitin Ligase and an Adaptor—vma2Δ cells have reduced levels of plasma membrane Pma1p and accumulate Pma1p in intracellular organelles and the vacuole, particularly in cells with reduced vacuolar protease activity (6). To test whether Pma1p is ubiquitinated in a vma2Δ mutant, we immunoprecipitated Pma1p from wild-type and vma2Δ cells and probed the immunoprecipitates for both Pma1p and ubiquitin by immunoblotting. As shown in Fig. 1A, less total Pma1p is immunoprecipitated from a vma2Δ mutant strain (6, 7) as expected. Over three independent experiments, vma2Δ cells displayed 41.0 ± 17.5% as much Pma1 signal as wild-type cells. The ratio of the ubiquitin and Pma1 signals (Ub/Pma1) in Fig. 1A demonstrates that there is much more ubiquitination of Pma1p in the vma2Δ mutant than in wild-type cells; we determined that the Ub/Pma1 ratio in the wild-type cells was 2.3 ± 1.0% of the vma2Δ ratio (n = 3). In addition, we calculated the approximate change in molecular mass between the Pma1p and ubiquitinated Pma1p bands. The average difference over three different experiments was 6.5 kDa, suggesting that Pma1p may be monoubiquitinated in the vma2Δ mutant.

The E3 ubiquitin ligase Rsp5p is responsible for ligand- or stress-responsive ubiquitination of a number of plasma membrane transporters (10). We tested whether the Pma1p ubiquitination observed was dependent on Rsp5p. RSP5 is an essential gene, but the temperature-sensitive rps5-1 mutant exhibits a loss of function at 37 °C (22). Pma1p was immunoprecipitated from the rps5-1 mutant and the rps5-1 vma2Δ double mutant after incubation at the permissive temperature of 30 °C or after 2 h at the non-permissive temperature of 37 °C. As indicated by the Ub/Pma1p ratio in Fig. 1B, there is little ubiquitination of Pma1p in the rps5-1 single mutant at either temperature (<3% of the rps5-1vma2Δ ratio at 30 °C in three independent experiments). However, in the rps5-1vma2Δ double mutant, Pma1p is ubiquitinated at the permissive temperature of 30 °C but is much less ubiquitinated after a 2-h incubation at the non-permissive temperature of 37 °C (Ub/Pma1 ratio of 15.7 ± 3.6% of the ratio at 30 °C (n = 3)). Therefore, ubiquitination of Pma1p in the vma2Δ mutant is dependent on the Rsp5p ubiquitin ligase.

Rsp5p ubiquitinates multiple cellular substrates (31). Although it binds directly to some substrates, other targets are recognized indirectly via Art proteins or other adaptors (10). One Art protein that has not been associated with a transporter is Rim8p. Rim8p has been implicated in cellular adaptation to ambient pH and shown to bind to Rsp5p (18, 32, 33). We hypothesized that the response to alkalization of organelles as a result of loss of V-ATPase activity might share factors such as...
Rim8p with the response to external alkalization. We, therefore, tested whether loss of Rim8p affects Pma1p ubiquitination in a vma2Δ mutant by constructing a rim8Δ vma2Δ double mutant and testing for Pma1p ubiquitination. Fig. 1A shows much less Pma1p ubiquitination in the rim8Δ vma2Δ double mutant than in the vma2Δ mutant (the Ub/Pma1p ratio for the double mutant was 12.4 ± 7.3% that of the vma2Δ single mutant (n = 3)). These results indicate that efficient Pma1p ubiquitination also requires Rim8p function.

If Pma1p ubiquitination targets it for degradation, we would predict that failure to ubiquitinate Pma1p might stabilize the enzyme in the presence of the vma2Δ mutation and lead to higher overall amounts. In fact, there was more total Pma1p immunoprecipitated from the rim8Δ vma2Δ mutant (68.0 ± 2.6% of wild-type, n = 3) than from the vma2Δ mutant (41% of wild type). There was no significant difference in Pma1p levels between rim8Δ and wild-type cells. The amount of Pma1 immunoprecipitated from the rsp5Δvma2Δ mutant rose from 41 ± 17% that from the rsp5-1 strain at 30 °C to 59 ± 15% after the 37 °C incubation, suggesting that Pma1p is also stabilized by loss of Rsp5p function. There was no significant difference between Pma1p levels in the rsp5-1 mutant at 30 and 37 °C.

If Pma1p ubiquitination signals its mislocalization to the vacuole and other intracellular compartments, loss of ubiquitination would be expected to result in retention of Pma1p at the plasma membrane. In Fig. 2A, the cellular distribution of Pma1p was monitored by immunofluorescence microscopy in wild-type and vma2Δ mutant cells; as shown previously, there are lower levels of Pma1p at the plasma membrane in the vma2Δ mutant and much higher levels of internal Pma1p. In Fig. 2C Pma1p is present in the vacuole and other internal compartments in the rsp5-1 vma2Δ mutant at the permissive temperature (30 °C) but is primarily at the plasma membrane after this mutant is shifted to 37 °C for 2 h. Pma1p also remains primarily at the plasma membrane in the rim8Δ vma2Δ double mutant (Fig. 2B), and its localization is unaffected in the rim8Δ single mutant. Taken together, these data indicate that under conditions where Pma1p ubiquitination is compromised (i.e. rsp5-1 vma2Δ mutant at 37 °C and rim8Δ vma2Δ mutant), loss of Pma1p from the plasma membrane is also reduced.

Pma1p Internalization in the vma Mutants May Be Compensatory—The vma mutants have significant growth defects, with optimal growth at extracellular pH 5 and little or no growth at pH 7.5 (5). It is still not known whether reduced levels of Pma1p at the plasma membrane are an underlying cause of the growth defects in vma mutants or a compensatory factor that allows better growth of the mutants. We asked whether cell growth was altered in the rim8Δ vma2Δ and rsp5-1 vma2Δ mutants, in which Pma1p remains at the cell surface in the absence of V-ATPase activity. As shown in Fig. 3A, neither the rim8Δ nor the rsp5-1 mutation alone compromised cell growth at pH 5 or 7.5, in contrast to the growth defect in the vma2Δ mutant at pH 7.5. The rim8Δ vma2Δ mutant grew very poorly even at pH 5, however, as did the rsp5-1 vma2Δ mutant at the non-permissive temperature. These results indicate a negative synthetic genetic interaction between vma2Δ and both rim8Δ and rsp5-1 and are consistent with ubiquitination and internalization of Pma1p having a beneficial effect on growth of vmaΔ mutants. However, we cannot exclude the possibility that other, indirect effects of the rim8Δ and rsp5-1 mutations are responsible for the synthetic growth defect. One surprising result in Fig. 3 is the suppression of the rsp5-1 temperature sensitivity at pH 5. The source of this suppression is not known.

It is possible that Pma1p down-regulation benefits vmaΔ mutants by promoting vacuolar acidification. Previous experiments revealed no evidence of Pma1p activity in vacuoles isolated from vmaΔ mutants (6), but reduced proton export as a result of Pma1p internalization might reduce pH in both the cytosol and vacuole. To address this possibility, we compared vacuolar pH responses between vma2Δ and rim8Δ vma2Δ mutants. Wild-type vacuoles are acidified in response to glucose addition, partially as a result of glucose-activated assembly of the V-ATPase. In the vma2Δ mutant, the vacuole is consistently more alkaline than in wild-type cells and becomes more alkaline upon glucose addition to glucose-deprived cells, presumably as a result of glucose activation of Pma1p unopposed by proton pumping into organelles (6). We briefly deprived (<20 min) wild-type, rim8Δ, vma2Δ, and rim8Δ vma2Δ cells of glucose, then measured vacuolar pH with the ratiometric pH sensor BCECF before and after glucose readdition. Fig. 3B
V-ATPase Loss Triggers Pma1p Ubiquitination and Endocytosis

**Acute Loss of V-ATPase Activity Results in Endocytosis of Pma1p from the Membrane—**
Pma1p is constitutively mislocalized in the vma2Δ mutant. We tested whether an acute loss of V-ATPase activity would also result in Pma1p mislocalization. To test whether acute inhibition of the V-ATPase results in Pma1p mislocalization, wild-type cells were treated with 2 μM concanamycin A in the presence of glucose for various time points, and Pma1p localization was assessed by immunofluorescence microscopy (Fig. 5A). Internal Pma1p is visible within 30 min of initiating concanamycin treatment, and the appearance of internal Pma1p was accompanied by a reduction in Pma1p at the cell surface. After 120 min, the concanamycin A-treated cells resembled the vma2Δ mutant. To quantitate loss of plasma membrane Pma1p, we measured the plasma membrane Pma1p signal in several cells as described under “Experimental Procedures.” As shown by the quantitation in Fig. 5B, reduced plasma membrane staining was observed even at the 30-min time point, and after 120 min ~50% of the signal remained at the plasma membrane. Loss of Pma1p from the cell surface during concanamycin treatment was accompanied by the appearance of Pma1p in internal structures, and the distribution of these structures appeared to change with time, with 1–2 larger internal structures predominant at the early times, and ≥3 small stained structures predominating after 60 min of concanamycin treatment (Fig. 5C). It should be noted that we previously reported little endocytosis of Pma1p in concanamycin-treated cells (6), but these experiments were done at a lower concanamycin A concentration, which also caused incomplete alkalization of vacuolar pH.

To determine whether Pma1p is removed from the plasma membrane by endocytosis after concanamycin treatment, we treated end4Δ mutant cells, which have a severe endocytosis defect (34), with concanamycin A. Synthetic lethality between the endocytosis (end) and vma mutations is well established.
and has even been used as a means of isolating new end mutants (35). Therefore, we first tested the tolerance of the end4Δ mutant to concanamycin A treatment. Methylene blue exclusion provides a measure of cell integrity, and as shown in Fig. 6A, wild-type cells exhibit very few methylene blue positive cells even after 90 min of concanamycin A treatment. In contrast, the end4Δ mutant showed a >2-fold increase in methylene blue-positive cells after only 30 min of concanamycin A treatment, and the proportion of stained cells continued to rise. In addition, the proportion of misshapen cells and cell “ghosts” that appear to have lost internal content had substantially increased by 60 min. Based on these results, we examined localization and ubiquitination of Pma1p in the end4Δ during the first 30 min of concanamycin A treatment. As shown in Fig. 6B, Pma1p remains predominantly at the plasma membrane in the end4Δ mutant treated for 30 min with concanamycin A. We also scored the pattern of Pma1p staining before and after the 30 min of concanamycin A treatment as in Fig. 6C. As shown in Fig. 6C, 95 ± 0.8% (mean ± range of two independent experiments) of end4Δ cells had no internal Pma1p before concanamycin A treatment, and this decreased to 83 ± 0.6% after 30 min of concanamycin A treatment. In contrast, the proportion of wild-type cells with no internal Pma1 decreased from 95% to ~40% after 30 min with concanamycin A (Fig. 6C). In addition, the major population of cells that had 1–2 large spots of internal Pma1 at 30 min with concanamycin A was missing in the end4Δ cells; instead, the small proportion of cells with internal Pma1 had only 1–2 small spots. These results indicate that internalization of Pma1p requires an intact endocytic pathway.

If ubiquitination of Pma1p precedes its endocytosis, we would expect that acute loss of V-ATPase activity would still stimulate Pma1p ubiquitination in the end4Δ mutant. We tested this hypothesis by transforming end4Δ cells with the pHA-Ub plasmid as in Fig. 4. As shown in Fig. 6D, a 30-min treatment with concanamycin A did stimulate ubiquitination of Pma1p in the end4Δ mutant even though little internalization occurred. This indicates that Pma1p can be ubiquitinated at the plasma membrane in response to loss of V-ATPase activity. The electrophoretic mobility of the ubiquitinated Pma1p in end4Δ cells is the same as in wild-type cells, again suggesting mono-ubiquitination.

Specificity of the Pma1p Internalization Pathway—Several of the Art proteins respond to distinct cellular signals and target specific transporters for ubiquitination and internalization (10), whereas others have a broader specificity and appear to impact many transporters as part of plasma membrane quality control (36). We tested whether loss of V-ATPase activity would result in mislocalization of another transporter, Mup1, which has been shown to respond to methionine levels via the

for the indicated times with 2 μM concanamycin A. Pma1p localization was visualized by immunofluorescence microscopy as described in Fig. 2. Nomarski images (right panels) and indirect immunofluorescence using a monoclonal antibody against Pma1p followed by an Alexa Fluor 488-conjugated secondary antibody (left panels) are shown for each field. B, plasma membrane fluorescence was quantitated (see “Experimental Procedures”) and normalized to the 0 time point. C, changes in the distribution of Pma1p between the plasma membrane (PM) and internal structures with time in concanamycin A (the average of two independent experiments is shown; error bars correspond to the range. At least 95 cells were counted for each time point).
adaptor Art1 (12). As shown in Fig. 7A, Mup1-GFP remains at the plasma membrane in vma2Δ/H9004 cells deprived of methionine, indicating that loss of V-ATPase activity does not result in a global decrease in protein localization to the plasma membrane. Mup1-GFP could be mobilized to the vacuole in the vma2Δ/H9004 strain upon methionine addition, suggesting that the targeted Mup1 endocytosis pathway is active in the vma2Δ/H9004 mutant. Cycloheximide treatment of wild-type cells results in Rsp5p-dependent ubiquitination and internalization of a number of plasma membrane amino acid transporters including Mup1 (12, 14). However, after cycloheximide treatment, Pma1p remains at the plasma membrane (Fig. 7B); this again suggests that Pma1p responds specifically to a distinct signal related to loss of V-ATPase activity. We also tested whether loss of an Art1 would affect Pma1p trafficking. Art1 is responsible for internalization of Mup1 and Can1 in response to cycloheximide (14) and is also implicated in endocytic down-regulation of a number of plasma membrane transporters in response to severe heat stress (36). As shown in Fig. 7, C and D, Pma1p was internalized in the art1Δ vma2Δ mutant, and growth of the art1Δ vma2Δ mutant was indistinguishable from that of the vma2Δ mutant, suggesting that loss of V-ATPase activity is not transmitting a general plasma membrane quality control signal via Art1. Taken together, these results indicate that Pma1p internalization requires a distinct signal that arises from loss of V-ATPase activity and is mediated through Rim8p and Rsp5p.

**DISCUSSION**

Our previous results suggest an unexpected interdependence between plasma membrane Pma1p and organellar V-ATPases. Experiments described here demonstrate that 1) Pma1p is ubiquitinated in an Rsp5p- and Rim8-dependent manner when V-ATPase activity is lost, 2) mutations that compromise ubiquitination also result in retention of Pma1p at the plasma membrane, and 3) both chronic and acute inhibition of V-ATPase activity trigger ubiquitination and endocytosis of Pma1p. In wild-type cells, Pma1p is a very long-lived and abundant plasma membrane protein (37). Although ubiquitination and internalization of mutant forms of Pma1p have been documented previously (38–41), this may be the first evidence of stimulus-dependent ubiquitination and endocytosis of wild-type Pma1p.

This work suggests that down-regulation of Pma1p in response to loss of V-ATPase activity may share common elements with signal-specific endocytic down-regulation of other plasma membrane transporters such as Mup1, Lyp1, Can1, and Dip5 (11, 12, 17) in response to intracellular excess of their ligands. Signals for ligand excess originate in different pathways and are funneled through specific Art proteins that then facilitate Rsp5p-dependent ubiquitination of the transporter (14–16). There is some redundancy among Art proteins (12), and this may account for the residual ubiquitination and incomplete stabilization of Pma1p in the rim8Δvma2Δ mutant. Sig-
V-ATPase Loss Triggers Pma1p Ubiquitination and Endocytosis

FIGURE 7. Loss of V-ATPase activity results in a signal that is specific for Pma1p internalization mediated by the Art protein Rim8. A, wild-type and vma2Δ mutant strains expressing Mup1-GFP from a plasmid (pMup1-GFP) were grown to log phase in selective media lacking uracil and methionine (or 20 μg/ml methionine added where indicated). Subcellular localization was visualized by fluorescence microscopy (right panels). Nomarski images of the same frames are shown on the left. B, wild-type cells were grown to log phase and treated for 3 h with 100 μg/ml cycloheximide. Pma1p localization was visualized as in Fig. 2. C, Pma1p was localized by immunofluorescence microscopy as described in Fig. 2 in both the art1Δ single and the art1Δ vma2Δ double mutant strains. D, log phase cells from art1Δ single and art1Δ vma2Δ double mutant strains were serial-diluted and spotted on plates that were buffered to pH 5.0 and grown at 30 °C as described in Fig. 3.

FIGURE 8. Working model for the Pma1p internalization pathway.

significantly, loss of V-ATPase activity neither triggers endocytosis of the well characterized Art1 substrate Mup1 nor requires Art1 for signaling Pma1p endocytosis, suggesting some signaling specificity. This specificity and the rapid response to acute loss of V-ATPase activity upon concanamycin treatment argue against Pma1p ubiquitination and endocytosis occurring as part of a general plasma membrane quality control mechanism.

The signaling pathways that communicate loss of V-ATPase activity to Rim8p remain unclear. Rim8p is conserved in fungi and involved in adaptation to alkaline extracellular pH (18, 32, 42, 43). Previously identified Rim8p binding partners include both plasma membrane “sensors” of extracellular alkalinity, Dfg16 and Rim21, and proteins of the ESCRT (endosomal sorting complexes required for transport) complex at the late endosome/multivesicular body, Vps23 and Vps28 (18). In addition, Rim8p has PY domains that bind to the WW domains of Rsp5p (12, 18, 19). In our experiments, Pma1p internalization occurs in the acidic extracellular environment optimal for growth of the vma mutants, an environment where Dfg16 and Rim21 should not stimulate the Rim alkaline response pathway. However, endosomal proteins play a key role in the alkaline pH response (44), and loss of V-ATPase activity is likely to impact endosomal acidification and function. Failure to internalize Pma1p in vph1Δ and rav1Δ mutants, which compromise vacuolar acidification to the same extent as vma deletions, suggests that the signal for Pma1p internalization does not originate at the vacuole (7, 45). Pumps containing the Golgi/endosomal V-ATPase subunit isoform Stv1 may maintain acidification of earlier organelles in these mutants, suppressing a signal for Pma1p relocation. Only upon a full loss of V-ATPase activity, in the vph1Δ stv1Δ double mutant, is Pma1p internalized, suggesting that Rim8p signaling is initiated when acidification of one or more of these “pre-vacuolar” organelles is compromised.

A working model for the Pma1p internalization pathway is shown in Fig. 8. Rim8p has been reported to be predominantly cytosolic (46), but it also interacts with endosomal proteins (18, 19) and has been detected at the plasma membrane when over-expressed (18). We depict Rim8p as cycling from the cytosol to the endosome, sensing pH changes at the endosome and then recruiting Rsp5p to the plasma membrane where it ubiquitiates Pma1p, triggering its endocytosis and eventual degradation. A number of aspects of this model remain to be proven. As described above, the nature of the signal for loss of V-ATPase activity is a critical question. In addition, we were not able to detect an interaction between Rim8p and Pma1p by immunoprecipitation from vma2Δ cells, but interactions between Art proteins and their transporter targets have proven to be very transient (12, 47).

It is also possible that Pma1p endocytosis is not the exclusive pathway for its mislocalization in vma mutants. Huang and Chang (8) induced expression of GFP-tagged Pma1p and saw that newly synthesized GFP-Pma1p was diverted to the vacuole from the secretory pathway but saw no evidence of ubiquitination or internalization of Pma1p from the cell surface. Here we have examined the total rather than the newly synthesized population of Pma1p. Reduced Pma1p levels at the cell surface were observed within 30 min of V-ATPase inhibition with concanamycin A and required an intact endocytic pathway, suggesting that endocytic down-regulation is a major mechanism of reducing Pma1p levels at the plasma membrane during the initial period after V-ATPase inhibition. Although most of the reduction in plasma membrane Pma1p levels occurs within 30 min of concanamycin addition (Fig. 5), the intracellular distribution of
Pma1p continues to change. Diversion of newly synthesized Pma1p from the secretory pathway through the pathway described by Huang and Chang (8) may account for an increasing portion of the total internal Pma1p as time goes on, after the Pma1p initially internalized is degraded in the vacuole. However, in the rsp5-1vma2Δ mutant, Pma1p shifts from being predominantly internal at the permissive temperature to being primarily at the plasma membrane after 2 h at the rsp5-1 non-permissive temperature. This suggests that newly synthesized Pma1p is being sent to the plasma membrane and retained there when Rsp5p is inactive, although it is also possible that Rsp5p activity is required for both internalization of Pma1p and diversion from the secretory pathway (40). Further experiments will be necessary to resolve these issues.

One ongoing question has been whether mislocalization of Pma1p is a side effect of loss of V-ATPase activity, resulting from perturbed trafficking pathways, or a compensatory response to loss of activity. The synthetic growth phenotypes between the vma2Δ mutation and rim8Δ, rsp5-1, and end4 mutations (Fig. 3A; Ref. 35) indicate that cell growth is compromised under conditions where Pma1p cannot be internalized, supporting a compensatory mechanism. The molecular basis of synthetic growth phenotypes can be complex, particularly for pleiotropic mutations such as rsp5-1. However, the rim8Δ mutation causes a mild phenotype on its own, but a strong synthetic phenotype in combination with vma2Δ, and end4Δ is not only synthetically lethal with vma mutants but also very sensitive to acute V-ATPase inhibition with concanamycin A (Fig. 6A). All of these results suggest that down-regulation of Pma1p activity at the plasma membrane is beneficial to cells with compromised V-ATPase activity.

The cellular advantages of Pma1p down-regulation are not clear. There is no evidence that mislocalized Pma1p contributes to vacuolar acidification (Ref. 6 and Fig. 3B), and in fact it appears to be trafficked to the vacuolar lumen and degraded rather than retained at the organelle membrane. Cytosolic acidification arising from loss of Pma1p activity might indirectly support some acidification of organelles in the absence of an active V-ATPase, but there is no obvious difference in vacuolar pH responses in the vma2Δ mutant, where Pma1p is internalized, and the rim8Δ vma2Δ, where Pma1p is at the plasma membrane (Fig. 3B). In the future, the acidometric pH sensors targeted specifically to endosomes may help to clarify this issue. Alternatively, Pma1p endocytosis may have a more indirect compensatory effect. For example, loss of V-ATPase activity appears to induce multiple stresses (48–50), and Pma1p is a major consumer of cellular ATP. Down-regulation of Pma1p might help to preserve energy stores in times of stress.

Although V-ATPases are highly conserved and present in virtually all eukaryotes, Pma1p is not present in mammalian cells. Instead, the functions of Pma1p in pH control are assumed by Na⁺/H⁺ exchangers (NHE proteins) at the plasma membrane in most cells, powered by gradients established through the Na⁺/K⁺-ATPase (1). Interestingly, ubiquitination and internalization of Nhe1, dependent on an arrestin family adaptor and the Rsp5p homolog Nedd4-1 (51), has been demonstrated. No stimulus for Nedd4-1 ubiquitination was described, but NHE1-mediated transport was significantly up-regulated when Nedd4-1 or β-arrestin 1 were absent, suggesting that ubiquitination and endocytosis act as a regulatory mechanism that limits NHE1 activity. Ubiquitin-dependent endocytosis could be a conserved mechanism for controlling and coordinating activity of major pH-regulating systems in eukaryotes.

The results presented here suggest an “inside-out” signaling system that senses organelle pH and down-regulates Pma1p at the plasma membrane. This system likely requires tight control mechanisms. Although other transporters targeted by ART proteins become dispensable in the presence of ligand excess and thus can be removed almost completely, PMA1 is an essential gene, and complete removal from the plasma membrane would be lethal. This suggests that any signaling mechanism triggering Pma1p down-regulation must be balanced by mechanisms to ensure that sufficient Pma1p is left at the plasma membrane to support essential functions. These opposing mechanisms may be previously unrecognized parts of the extensive, delicate balance that supports cellular pH homeostasis.

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