Within the large family of P-type cation-transporting ATPases, members differ in the number of C-terminal trans-membrane helices, ranging from two in Ca\(^{2+}\)-ATPases to six in H\(^{+}\), Na\(^{+}\), K\(^{+}\), Mg\(^{2+}\), and Ca\(^{2+}\)-ATPases. In this study, yeast Pma1 H\(^{+}\)-ATPase has served as a model to examine the role of the C-terminal membrane domain in ATPase stability and targeting to the plasma membrane. Successive truncations were constructed from the middle of the major cytoplasmic loop to the middle of the extended cytoplasmic tail, adding back the C-terminal membrane-spanning helices one at a time. When the resulting constructs were expressed transiently in yeast, there was a steady increase in half-life from 70 min in Pma1Δ452 to 348 min in Pma1Δ901, but even the longest construct was considerably less stable than wild-type ATPase (t\(_{1/2}\) = 11 h). Confocal immunofluorescence microscopy showed that 11 of 12 constructs were arrested in the endoplasmic reticulum and degraded in the proteasome. The only truncated ATPase that escaped the ER, Pma1Δ901, traveled slowly to the plasma membrane, where it hydrolyzed ATP and supported growth. Limited trypsinolysis showed Pma1Δ901 to be misfolded, however, resulting in premature delivery to the vacuole for degradation. As model substrates, this series of truncations affirms the importance of the entire C-terminal domain to yeast H\(^{+}\)-ATPase biogenesis and defines a sequence element of 20 amino acids in the carboxyl tail that is critical to ER escape and trafficking to the plasma membrane.

The single most abundant protein in the yeast plasma membrane is a 100-kDa H\(^{+}\)-ATPase that accounts for at least 10% of total plasma membrane protein (1). Encoded by the PMA1 gene (2) and belonging to the widely distributed family of P-type cation-transporting ATPases (3–5), the yeast H\(^{+}\)-ATPase acts physiologically to pump protons out of the cell, creating a trans-membrane potential that drives the uptake of amino acids, sugars, and other nutrients (1). Because of its abundance, the H\(^{+}\)-ATPase has attracted considerable attention over the past two decades, both for mechanistic studies of ATP-coupled proton transport and, more recently, as a model system to explore the biogenesis of polytopic membrane proteins.

A particularly fruitful approach to biogenesis studies has come from the use of site-directed mutagenesis to probe structure-function relationships in key regions of the H\(^{+}\)-ATPase polypeptide (1). Of the mutations that have been made to date, a significant subset cause partial or complete blocks in ATPase biogenesis, giving direct evidence for the critical nature of proper protein folding. Some of the mutant forms are severely defective and become arrested in the endoplasmic reticulum, from where they are shipped to the proteasome for degradation (e.g. see Refs. 6–8). Others, with more subtle abnormalities in folding, are transported further along the secretory pathway to reach the Golgi (pma1–7, Ref. 9) or even the plasma membrane (pma1–10, Ref. 10 and pma1G381A, Ref. 11), before being recognized as abnormal and committed to the endosomal/vacuolar pathway for degradation. In addition to their use in mapping amino acid residues within the Pma1 polypeptide that are important for folding and trafficking, both kinds of mutant have been used to identify novel components of the yeast secretory pathway, such as Eps1p, a protein-disulfide isomerase implicated in ER quality control (7), and Ast1p, a protein involved in transport from the Golgi to the plasma membrane (9).

The work to be described in this paper has taken a systematic look at features of the yeast H\(^{+}\)-ATPase structure and topology that are required for successful delivery to the plasma membrane. We have focused in particular on the C-terminal portion of the molecule, given the variability of this region during the evolution of P-type ATPases (12). On the one hand, members of the P\(_{1}\) subfamily, including mammalian Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\)-ATPases as well as the fungal and plant H\(^{+}\)-ATPases, have six C-terminal transmembrane helices, designated M5 through M10 (3). At the other extreme, heavy metal ATPases of the P\(_{1}\) family have only two C-terminal helices, whereas the P\(_{2}\)-type Kdp ATPase of Escherichia coli is intermediate between the first two groups, with four such helices.

To dissect the role of the C-terminal portion of yeast Pma1 ATPase in biogenesis and trafficking, we have constructed a set of mutants with truncations after each of the last six transmembrane segments, from M5 through M10. Whereas the results reveal a clear correlation between protein length and intracellular stability, all but one of the mutant ATPases fails to exit the endoplasmic reticulum. Only Pma1Δ901, truncated midway along the cytoplasmic C-terminal tail, is successfully targeted to the plasma membrane, where it is capable of splitting ATP and supporting growth. Thus, all six C-terminal membrane helices and at least a portion of the C terminus are essential for proper folding and trafficking of the H\(^{+}\)-ATPase through the secretory pathway.
C-terminal Truncations of Pma1 H\(^+\)-ATPase

**TABLE 1**

| Yeast strain | Relevant genotype | Reference |
|--------------|-------------------|-----------|
| BMY40        | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| BMY401       | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| BMY402       | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| BMY403       | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| BMY404       | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| BMY405       | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| BMY406       | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| BMY407       | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| BMY408       | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| BMY409       | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| BMY410       | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| BMY411       | MATa trp1–289 his32320 ura3–52 pma1::YIplac22-2HES\(^+\)-HA-PMA1 | This study |
| W303-1B pep4Δ | MATa trp1 ade2-1 his3 ura3-1 can1–100 pep4Δ | Thomas and Rothstein (14) |

**EXPERIMENTAL PROCEDURES**

Yeast Strains and Growth Conditions—Saccharomyces cerevisiae strains used in this study are listed in Table 1. For transient expression experiments, the mutant pma1 allele to be studied was introduced on either a centromeric or integrating plasmid into yeast strains with the appropriate mutant background (BMY40, W303-1B pep4Δ, W34a 11/22a) and in which the chromosomal copy of PMA1 had been placed under control of the GAL1 promoter (see Table 1). Transformants were selected on 2% galactose in synthetic medium lacking uracil and the relevant auxotrophic amino acid(s) (Bio 101 Inc., Carlsbad, CA). For the transient expression of hemagglutinin HA\(^2\)-tagged pma1 alleles, chromosomal GAL1P-PMA1 expression was first turned off by incubating the cells for 3 h in medium containing 2% glucose instead of galactose. Expression of the HA-tagged Pma1 mutant was then induced by incubating the cells at 39 °C for the appropriate time (15).

For the stable expression of chromosomally integrated pma1 alleles, yeast strain NY13 was used to make strain BMY58, a uracil auxotroph in which PMA2 is disrupted with the kanamycin resistance cassette (pma2::KanMX4).

Standard yeast media and genetic manipulations were as described by Sherman et al. (16). Yeast transformations were performed as described (17) using the Alkali-Cation Yeast Transformation kit (Bio 101).

Plasmids—Truncated pma1Δ alleles were made in plasmid YCplac22-2HES\(^+\)-HA-PMA1-Mul, a derivative of YCplac22-2HES\(^+\)-HA-PMA1 (11) containing an MluI site at the TAA stop codon of the PMA1 gene. Another MluI site was built into mutagenic oligonucleotides used to introduce a second TAA stop codon at the desired location within the PMA1 coding sequence in the plasmid template. Transforming DNA sequences were then removed by digestion with MluI, and the construct encoding a truncated ATPase product, with 3'-flanking sequence intact, was generated by circularization of the purified vector DNA fragment. The same strategy was used to create 2HES\(^+\)-HA-pma1 constructs in the integrative vector pRS303–2HSE (11) for targeting to the chromosomal HIS3 locus. Plasmid-encoded wild-type and mutant alleles of PMA1 used in this study were tagged with a single copy of the 9-amino acid HA epitope (YPYDVPDYA), inserted in frame between codons 2 and 3 of the PMA1 coding sequence (6). The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used for in vitro mutagenesis, and all mutant constructs were verified by DNA sequencing.

For the chromosomal integration of mutant pma1 alleles by homologous recombination at the PMA1 locus, plasmid pGW201 (18) was modified by adding two unique restriction sites: an NcoI site at the PMA1 ATG start codon and an MluI

\(^2\) The abbreviations used are: HA, hemagglutinin; ER, endoplasmic reticulum; TNP-ATP, 2', 3'-O-(2,4,6-trinitrophenyl)-adenosine-5'-triphosphate; FITC, fluorescein isothiocyanate; GM, glucose-metabolizing; CS, carbon-starved.
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 site at the PMA1 TAA stop codon. These two new restriction sites were used to introduce an HA-tagged copy of the PMA1 gene, producing plasmid pBM432. The pmaiΔ901 allele was constructed in pBM432 by replacement of the 1.1-kb BamHI-MluI fragment with the corresponding fragment from YCPlac22–2HSE-HA-pmaiΔ901. A 6-kb HindIII fragment from this construct was used to transform yeast strain BMY58 (Table 1). Transformants selected on complete synthetic medium lacking uracil were screened by immunoblotting for expression of truncated ATPase Pma1Δ901.

Immunofluorescence—Intracellular structures containing HA-tagged ATPase were visualized by confocal microscopy as described previously (6). Cells were fixed, treated with Zymol- yase T-100, permeabilized with 0.1% Triton X-100, and stained for immunofluorescence (11). Primary antibodies included rabbit anti-HA polyclonal (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan), diluted 1:100; anti-HA.11 monoclonal 16B12 (Babco, Richmond, CA), diluted 1:150; anti-vacu- lolar H\textsuperscript{+}-ATPase 60-kDa subunit monoclonal 13D11-B2 from raw ascites fluid (Molecular Probes, Inc., Eugene, OR), diluted 1:150; and rabbit anti-Kar2p polyclonal antibody (provided by M. Rose), diluted 1:2500. Goat anti-rabbit and goat anti-mouse IgGs conjugated, respectively, to FITC and Texas Red (Jackson Immunoresearch, West Grove, PA), served as fluorescent sec- ondary antibodies and were used at 1:100 dilutions. For double labeling experiments, both primary antibodies were present during the initial incubation, and both secondary antibodies were present during the subsequent incubation. To control for spurious antibody cross-reactivity, non-induced cells were simultaneously fixed and stained with each set of antibodies. Samples were then mounted in Citifluor (Ted Pella, Redding, PA).

Cells were observed under a Zeiss L510 scanning confocal microscope using dual channel filters for simultaneous visual- ization of FITC and Texas Red fluorochromes. All images were taken with a 63 × 1.4 numerical aperture Plan-Apochromat III differential interference contrast objective (Zeiss). In time course experiments, microscope settings were unchanged throughout the experiment in order to obtain a semi-quantita- tive signal. Cross-talk between FITC and Texas Red was avoided through the use of the Zeiss L510 digital signal proces- sor. The absence of bleed-through was confirmed by checking that the signal disappeared when viewed with single-wave- length filter blocks. Images were collected with LSM5 software (Zeiss) and modified by contrast stretching and merging, using Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA).

Total Protein Extracts and Immunoblotting—Total protein extracts were prepared as described by Volland et al. (19). Log phase cells (A\textsubscript{600 nm} = 1.5) were suspended in 0.5 ml of water and then lysed by adding 50 μl of 1.85 M NaOH, 5% (v/v) β-mer- captoethanol. Proteins were precipitated by adding 50 μl of 50% (m/v) trichloroacetic acid. The resulting pellets were resus- pended in 15 μl of 1 M Trizma Base and 30 μl of 3 × Laemmli SDS-PAGE loading buffer (20), resolved on 8% polyacrylamide gels (SDS-PAGE), and transferred to a polyvinylidene difluo- ride membrane (Immobilon, Millipore Corp., Bedford, MA). HA-tagged ATPases were quantified by immunoblotting with rabbit anti-HA polyclonal antibody diluted 1:4000 (Medical and Biological Laboratories, Nagoya, Japan) and Kar2p by immunoblotting with rabbit anti-Kar2p polyclonal antibody diluted 1:20,000 (provided by M. Rose), followed by incubation with 125I-protein A, autoradiography, and phosphorimaging analysis (Storm 840; Amersham Biosciences). Alternatively, blots were developed using horseradish peroxidase-conjugated secondary antibodies and the ECL chemiluminescent detection system (Amersham Biosciences). For quantitative immuno- blotting of the HA-tagged ATPase in sucrose gradient fractions (see below), proteins were trichloroacetic acid-precipitated from 100-μl samples. Dolichol phosphate mannosyl synthase (Dpmin1p) was detected on immunoblots using monoclonal antibody 5C5 (Molecular Probes, Inc., Eugene, OR).

Subcellular Fractionation—Sixty-ml cultures were grown to an A\textsubscript{600 nm} of ~1.0, shifted to glucose for 3 h, and then induced at 39 °C for 15 min as detailed above (see “Yeast Strains and Growth Conditions”). Samples equivalent to 25 A\textsubscript{600 nm} units were taken immediately before the temperature shift (t = 0) and at specified time points after induction. Sodium azide was added immediately to a final concentration of 10 mM, and cells were then centrifuged at 2000 × g for 5 min prior to resuspension in STE10 buffer (10% sucrose in 10 mM Tris, pH 7.5, 10 mM EDTA) supplemented with a protease inhib- itor mixture (leupeptin, pepstatin, aprotinin, and chymosta- tin, at 2 μg/ml each, plus phenylmethylsulfonyl fluoride to 1 mM final concentration). Cells were broken by vortexing with 0.45-mm glass beads (4 × 1-min pulses). Lysates were recovered, and the remaining beads were washed with 400 μl of STE10 plus protease inhibitors. Washes were combined with primary lysates, and these suspensions were centrifuged at 3000 × g for 5 min. Supernatants were applied to 5-ml 20–60% linear sucrose gradients, ultracentrifuged in an SW55Ti rotor for 16 h at 100,000 × g, and the gradients fractionated into 300-μl volumes.

Limited Trypsinolysis of Gradient-purified Plasma Mem- branes—Plasma membranes were prepared as described (21). Trypsinolysis was performed according to Nakamoto et al. (15) using trypsin/protien ratios as stated.

ATP Hydrolysis and Protein Determination—Activity of the plasma membrane H\textsuperscript{+}-ATPase was assayed at 30 °C using a standard (21) or microscale ATPase assay (22).

Protein concentrations were measured as described (23) or as modified by Ambesi et al. (24), using bovine serum albumin as a standard.

RESULTS

Design and Expression of Truncated ATPases—Fig. 1 illustrates the topology of yeast Pma1 H\textsuperscript{+}-ATPase, which is embed- ded in the lipid bilayer by four transmembrane helices (M1– M4) toward the amino terminus of the protein and six (M5– M10) toward the C terminus (25). The starting point for this study was a truncation at Glu\textsubscript{901}, midway along the C-terminal tail. This modest deletion of 18 amino acids was studied previ- ously and was shown to allow both measurable ATPase activity and growth (26). To judge the effects of longer C-terminal dele- tions on ATPase biogenesis and stability, stepwise terminations were introduced at eight additional points upstream of Glu\textsubscript{901} (Fig. 1). These were as follows: Ala\textsuperscript{865} (immediately following
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M10); Tyr\(^{773}\) (in the middle of M10); Asp\(^{851}\), Ile\(^{852}\), Ile\(^{785}\), Pro\(^{753}\), and Asp\(^{718}\) (in the small loops following M9, M8, M7, M6, and M5, respectively); and His\(^{866}\) (at the C-terminal end of the large cytoplasmic loop). The final and most amino-terminal truncation was at residue Asp\(^{452}\), near the middle of the large cytoplasmic loop and within the N (nucleotide-binding) domain of the ATPase (25). This mutation occurred spontaneously and was included in the series after preliminary studies suggested that it behaved distinctively when expressed in yeast.

Mutant pma1\(\Delta\) constructs tagged with a single HA epitope at the amino terminus were introduced into the yeast centromeric expression plasmid YCplac22–2HSE-HA-PMA\(1\)-MluI, placing them under control of two heat-activated promoter elements (2HSE). They were then transformed into yeast strain BMY40, in which the chromosomal copy of the PMA1 gene is under control of the GAL1 promoter (11). For expression studies, cells were grown in galactose medium at 23 °C (wild-type allele ON; mutant allele OFF) and then shifted to glucose medium at 23 °C for 3 h to turn off expression of the chromosomal PMA1 gene and empty the secretory pathway of wild-type ATPase. Cells were then incubated for 15 min at 39 °C to induce a short pulse of synthesis of mutant ATPase before being returned to 23 °C, with the HA tag providing a convenient way to track the synthesis, stability, and intracellular location of the mutant protein (11).

Stability of Truncated ATPase as a Function of Protein Length—The first step was to examine the stability of the truncated ATPases. For this purpose, total protein extracts were made from cells taken immediately before \((t = 0)\) and at various times after expression had been induced at 39 °C. Quantitative immunoblotting with anti-HA antibody revealed a striking relationship between the length of the mutant ATPase and its half-life (Fig. 2 and Table 2). The shortest polypeptide (Pma1\(1\)-H9004\(^{452}\)) was by far the least stable, with a \(t_{1/2}\) of only \(70\) min. Adding back the remainder of the large cytoplasmic loop (in Pma1\(1\)-H9004\(^{686}\)) increased the \(t_{1/2}\) to 116 min, and the successive addition of transmembrane helices M5–M10 led to further stepwise increases from 152 to 288 min. Pma1\(1\)-H9004\(^{901}\), which retained part of the C-terminal tail, had the longest half-life (\(\sim 348\) min), although still only about one-half that of wild-type Pma1.
ATPase (−11 h) (27). Thus, virtually the entire 100-kDa polypeptide chain is required for maximal stability, with critical contributions coming from all six transmembrane helices downstream of the major cytoplasmic domain and even from the C-terminal tail.

**Trafficking of Truncated ATPases**—To learn more about the processing of the truncated ATPases, their intracellular trafficking was monitored by confocal immunofluorescence microscopy (Fig. 3). In keeping with the results of earlier studies (11), HA-tagged wild-type ATPase could be detected as early as 15 min after induction of expression, and it was then exported efficiently through the secretory pathway to reach the plasma membrane by 60 min post-induction. The shortest truncation product, Pma1Δ452, was trapped in a predominantly perinuclear location and degraded rapidly, becoming undetectable after 60 min. By contrast, the next shortest truncation product (Pma1Δ686) gave a punctate staining pattern that persisted throughout the 180-min time course. Similar patterns were seen for Pma1Δ718 through Pma1Δ881, which also failed to reach the cell surface but remained visible for at least 180 min; confocal images for three of these mutants are included in Fig. 3. Of the mutants that were studied, only Pma1Δ901 was transported to the plasma membrane, with a trafficking behavior very similar to that of the wild-type ATPase.

The punctate staining patterns seen in Fig. 3 resemble ER-derived tubulo-vesicular structures that have been reported previously for misfolded full-length Pma1p mutants (7, 11, 28) as well as for trafficking-defective mutants of other plasma membrane proteins (29, 30). It therefore seemed likely that mutants Pma1Δ452 through Pma1Δ881 were arrested in the ER. Indeed, as illustrated in Fig. 4, these HA-tagged proteins co-localized strongly with the molecular chaperone Kar2p, a classical marker of the ER lumen (31). By comparison, wild-type and Δ901 ATPases could be seen at the cell surface, clearly distinct from the Kar2p marker.

Collectively, the results from confocal microscopy made it possible to divide the truncated ATPases into three distinct groups based on their trafficking patterns. The single mutant in Group I (Pma1Δ452) was unique in its perinuclear ER distribution with peripheral extensions. Eight mutants in Group II (Pma1Δ686 through Pma1Δ881) were sequestered in discrete, punctate, ER-associated bodies; and

### TABLE 2
Stability of truncated H^+-ATPase mutants

| Mutant H^+-ATPase | Number of membrane helices | t\(_{1/2}\)a |
|------------------|---------------------------|---------|
| Wild type        | 10                        | 11 h    |
| Δ452             | 4                         | 70 min  |
| Δ686             | 4                         | 116 min |
| Δ718             | 5                         | 152 min |
| Δ753             | 6                         | 162 min |
| Δ785             | 7                         | 186 min |
| Δ820             | 8                         | 217 min |
| Δ851             | 9                         | 244 min |
| Δ881             | 10                        | 288 min |
| Δ901             | 10                        | 348 min |

*a Calculated from an average of a least three separate time courses analyzed by quantitative immunoblotting.

![FIGURE 3. Expression and trafficking of wild-type (WT) and Pma1Δ ATPases.](image-url)
Pma1Δ901, the only example of Group III, was able to exit the ER and travel to the plasma membrane.

**Truncated ATPases in Groups I and II Are Degraded in the Proteasome**—Immunoblotting and immunofluorescence results indicated that the aggregates formed by the ER-arrested ATPases (Groups I and II) were gradually cleared from the cell, most likely by ER-associated degradation in the proteasome. To test this idea directly, the truncated ATPases were expressed in a pre1-1 pre2-2 yeast strain deficient in chymotrypsin-like activity of the proteasome (WG4a-11/22) (Table 1) (32). In the experiment of Fig. 5, total protein samples were taken both before induction at 39 °C and at intervals after induction, and assayed for HA-tagged ATPase by quantitative immunoblotting. Results are compared with control measurements carried out in the proteasomally active parent strain (WG4a) (Table 1).

In several cases (wild type, Pma1Δ452, and Pma1Δ686), ATPase levels in the pre1-1 pre2-2 mutant background were elevated even before induction (Fig. 5). This was probably due to some leakiness of the 2HSE promoter (15), resulting in accumulation of newly synthesized ATPase in the proteasome-defective strain. More important was the finding that all nine truncated ATPases in Groups I and II (Pma1Δ452 through Pma1Δ881) were stabilized when expressed in the pre1-1 pre2-2 mutant background, consistent with the idea that these mutant polypeptides undergo preferential degradation in the proteasome. This effect was most pronounced for early truncations, such as Pma1Δ452, Pma1Δ686, and Pma1Δ753, which were almost completely degraded by the 180-min time point without pre1-1 pre2-2 protection (Fig. 5); however, some stabilization was also evident for later truncations, such as Pma1Δ820 and Pma1Δ881.

Two strategies were tested without success in an attempt to override the ER quality control system and enable shipment of truncated ATPases to the plasma membrane. First, the eps1/H9004 mutation was introduced into strain BMY40 to see whether elimination of Eps1p, a member of the protein-disulfide isomerase family, might promote export of ER-arrested Pma1Δp mutants, as has been shown for a severely misfolded full-length Pma1 ATPase mutant (D378N) (7). Our experiments confirmed the published observations for HA-tagged Pma1-D378N but showed no effect of eps1/H9004 on the stability or trafficking of the truncated ATPases. The second, more general strategy, involved the addition of glycerol at concentrations as high as 10% to the galactose growth medium and/or the glucose medium used during induction of mutant ATPase expression. Other researchers have reported that glycerol stabilizes and even promotes the export of certain endogenous integral membrane proteins (33). However, no change was observed in either the ER arrest or instability of the truncated Pma1 ATPases (not shown).

Overall, the results up to this point show that truncations from the middle of the large cytoplasmic loop (Pma1Δ452) to the end of M10 (Pma1Δ881) create a mutant protein that is...
recognized as abnormal by the quality control machinery in the ER and degraded, with no discernible ability to be exported further along the secretory pathway.

\textit{Pma1Δ901 ATPase Is Partially Functional}—By contrast with the behavior of truncations \textit{Pma1Δ452} through \textit{Pma1Δ881}, \textit{Pma1Δ901} (the longest polypeptide of the present series) was able to reach the plasma membrane (Fig. 3), making it of interest to study under steady-state growth conditions. For this purpose, the mutant allele was inserted in place of the chromosomal copy of the \textit{PMA1} gene in a strain (BMY58) carrying a disruption of the closely related \textit{PMA2} gene (\textit{pma2::KanMX4}), creating strain BMY62 (Table 1). \textit{PMA2} is not expressed under standard laboratory conditions (34), but it has been shown to undergo gene conversion with certain mutant \textit{pma1} alleles under selective growth conditions (28) and could thus have interfered with studies of \textit{Pma1Δ901} expression.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{\textbf{Group I and Group II ATPases are degraded by the proteasome.} HA-tagged wild-type (WT) and mutant ATPases were expressed transiently in either the WG4a parental background \(\text{(open squares)}\) or the WG4a-11/22a \textit{pre1-1 pre2-2} strain \(\text{(solid squares)}\) using constructs based on plasmid YCplac22-2HSE-HA-Pma1-MluI. Mid-logarithmic phase cells growing at 23 °C in medium containing 2% galactose were shifted to medium containing 2% glucose for 90 min before the amino acid analog, canavanine, was added to a final concentration of 20 μg/ml to further stress the proteasome. After another 90-min incubation at 23 °C, HA-tagged ATPase expression was induced by shifting cultures to 39 °C for 15 min. Total protein extracts made from cell samples obtained immediately before \((t = 0 \text{ min})\) and at intervals after induction were analyzed as described in the legend to Fig. 2.}
\end{figure}
C-terminal Truncations of Pma1 H+-ATPase

**TABLE 3**

| ATPase     | Expression<sup>a</sup> | ATPase activity<sup>b</sup> | Activation<sup>c</sup> |
|------------|------------------------|-----------------------------|------------------------|
|            | CS         | GM         | CS   | GM   | CS   | Activation<sup>c</sup> |
| WT-HA      | 100        | 100        | 2.02 (0.09)<sup>d</sup> | 8.09 (0.12) | 4.01 |                      |
| Δ901-HA    | 65.0 (9.6) | 67.3 (1.2) | 3.81 (0.79) | 3.83 (1.15) | 1.01 |                      |

<sup>a</sup> Determined by quantitative immunoblotting with polyclonal rabbit anti-Pma1p; expressed as a percentage of wild-type HA (WT-HA).

<sup>b</sup> Vanadate-sensitive ATPase activity (μmol of P_i/min/mg of protein) measured at pH 6.25; values for Pma1Δ901-HA adjusted to account for expression levels.

<sup>c</sup> Activation =-fold increase in ATPase activity in GM cells compared with that of CS cells.

<sup>d</sup> Values in parentheses represent S.D. calculated from results for at least three different plasma membrane preparations. CS cells.

In the presence of glucose (glucose-metabolizing conditions), the integrated pma1Δ901 allele supported growth and was expressed in the plasma membrane at 67% of the wild-type level (Table 3). After correction for expression, the Δ901 polypeptide could be seen to hydrolyze ATP at almost one-half the rate measured for the wild-type control (3.83 versus 8.09 μmol/min/mg). Carbon starvation revealed contrasting responses between wild-type and Pma1Δ901 ATPases, consistent with results reported from studies in a different yeast background (26). Under carbon-starved (CS) conditions, wild-type ATPase displayed a rapid, 4-fold down-regulation of activity from 8.09 to 2.02 μmol/min/mg (Table 3). By contrast, Pma1Δ901 was constitutively active, with equilibrate ATPase activities under GM and CS conditions (3.83 and 3.81 μmol/mg/min). The difference between the mutant and wild-type strains provides added support for the widely accepted view that the C-terminal tail of Pma1 H+-ATPase acts as an autoinhibitory domain to down-regulate activity during carbon starvation (35).

*Pma1Δ901 ATPase Is Folded Poorly, and Its Passage through the Secretory Pathway Is Slowed*—To ask whether structural abnormalities could be detected that would account for the abnormal behavior of Pma1Δ901 ATPase, isolated plasma membranes from BMY58 (wild-type Pma1) and BMY62 (Pma1Δ901) were analyzed by limited trypsinolysis, which provides a sensitive way to examine the folded structure of yeast H+-ATPase (e.g. see Ref. 36). Pma1Δ901 proved to be significantly more sensitive to trypsin than its wild-type counterpart, with the mutant protein being almost completely degraded in 15 min at a trypsin/protein ratio of 1:5 (Fig. 6). Apart from the absence of a small amount of an ~71-kDa digestion product, the pattern and size of trypsin degradation products for Pma1Δ901 were almost identical to those of wild-type Pma1p, suggesting that the increased susceptibility of the mutant form to trypsin was due to the exposure of cleavage sites normally masked by the C terminus.

Not surprisingly, further investigation revealed that this structural abnormality was capable of slowing the rate at which Pma1Δ901 ATPase travels to the plasma membrane. For these experiments, lysates from cells expressing either wild-type or Pma1Δ901 ATPase were prepared 15, 60, or 120 min after induction at 39 °C and analyzed by ultracentrifugation on linear 20–60% sucrose gradients. Fractionation profiles were then analyzed by immunoblotting for the presence of HA-tagged wild-type or Pma1Δ901 ATPase (Fig. 7). At the earliest time point (15 min after induction), a substantial difference could be seen between the distributions of the two ATPases. Unlike the wild-type protein, which was spread uniformly throughout the gradient, most of the 15-min Pma1Δ901 ATPase was confined to the top half, where it co-fractionated with the ER membrane marker Dpm1p (dolichol phosphate mannose synthase). At 60 min, both ATPases were found predominantly in fractions 9–13, although a smaller peak of Pma1Δ901 was still associated with ER-containing fractions. Only at the 120-min time point did the peak of HA-tagged Pma1Δ901 co-locate with that of wild-type ATPase (Fig. 7). In all of the gradients, vanadate-sensitive ATPase activity was detected only in fractions 10–15 (not shown), confirming that these more dense fractions contained the active, plasma membrane-bound enzyme preformed prior to induction of tagged ATPase. Any HA-tagged ATPase synthesized during the 15-min induction period would be expected to make a negligible contribution to total measured ATPase activity.

*Pma1Δ901 Is Degraded in the Vacuole*—Although Pma1Δ901 displayed the ability to reach the plasma membrane in the present study (Fig. 3), it was much less stable than the wild-type control (Fig. 2). Thus, there was good reason to think that the two ATPases might ultimately be shipped at different rates to the endosomal/vacuolar system for degradation.

To test this idea, wild-type Pma1p, Pma1Δ901, and several other truncated ATPases were introduced via centromeric plasmids into strain W303-1B and its derivative W303-1B pep4Δ, which lacks vacuolar aspartyl protease activity. Protein profiles for transiently expressed wild-type, Pma1Δ686, and Pma1Δ901 ATPases are illustrated in Fig. 8A. Pma1Δ686, a mutant arrested in the ER, was not protected by the pep4Δ mutation. On the other hand, Pma1Δ901 ATPase was protected completely for at least 5 h after induction, implicating the vacuole as the primary site of degradation for this ATPase. At the 3-h time point in the pep4Δ background, immunofluorescence analysis showed both wild-type and Pma1Δ901 H+-ATPases at the
plasma membrane (Fig. 8B). Undigested Pma1Δ901 could also be seen in intracellular aggregates localized within vacuolar membranes, which were visualized with antibody against the 60-kDa subunit of yeast vacuolar H^+-ATPase.

**DISCUSSION**

In this study, a series of truncations have been used to examine the role of the C-terminal membrane domain in the stability of yeast Pma1 H^+-ATPase and in the delivery of the ATPase to the plasma membrane. Unlike previous work on P-type ATPases, where a typical approach has been to express fusion constructs either in vitro (37) or in heterologous cell backgrounds (38, 39), the transient expression system used in this study has enabled us to track the behavior of the Pma1 H^+-ATPase as a non-fusion protein in its native yeast background, thereby avoiding any complications that might have been caused by adding an unrelated protein sequence or by relying upon the membrane-trafficking machinery of an unrelated cell type.

**Contribution of the C-terminal Membrane Domain to Pma1 H^+-ATPase Stability**—The results of the present study show that stepwise truncations from the C terminus toward the middle of the ATPase lead to significant defects in stability and membrane trafficking. The most drastic change was seen in the shortest product, Pma1/H9004_452, which contained only half of the cytoplasmically exposed N (nucleotide-binding) domain. This polypeptide became arrested in perinuclear ER-derived structures and was shipped to the proteasome for degradation (t_1/2 = 70 min). Adding back the rest of the N domain caused the next mutant in the series, Pma1/H9004_686, to be sequestered in a different set of ER-derived structures, from which it underwent slower proteasomal degradation (t_1/2 = 116 min). Presumably, the cytoplasmic domain of Pma1Δ686 folded more tightly than that of Pma1Δ452, masking degradation signals that may be exposed.
C-terminal Truncations of Pma1 H⁺-ATPase

A

![Graphs showing relative expression over time](image)

B

![Images showing proteolysis](image)

in the shorter construct and slowing delivery to the proteasomal machinery. This idea is supported by the results of an earlier study in which the large cytoplasmic domain of yeast Pma1 H⁺-ATPase, when expressed by itself as a soluble protein in E. coli, folded well enough to bind TNP-ATP with high affinity (40); similar findings have been reported for the large cytoplasmic domain of mammalian sarcoplasmic reticulum Ca²⁺-ATPase (41, 42) and Na⁺,K⁺-ATPase (43). The slower degradation of Pma1Δ686 compared with Pma1Δ452 is also consistent with recent findings by Taxis et al. (44) on a series of modular protein substrates. In these constructs, the presence of a well folded cytoplasmic domain increased the time required for clearance of a membrane-anchored protein, due to the need for additional chaperones and accessory proteins to unfold the substrate prior to proteasomal degradation.

Further improvement in ATPase stability occurred when transmembrane helices M5–M10 were added back in constructs Pma1Δ718 through Pma1Δ881. Despite the fact that all of these mutant polypeptides were arrested in punctate ER-derived structures and ultimately degraded by the proteasome, they displayed steadily increasing half-lives from 152 to 288 min. Thus, it appears that individual helices from M5 through M6 contribute in a cumulative fashion to the stability of the Pma1 polypeptide. By contrast, a previous study by Béguin et al. (45) pointed to a more complex relationship between C-terminal helices and protein stability in the case of Na⁺,K⁺-ATPase. When truncated constructs of the Xenopus laevis α₁ subunit were expressed in a Xenopus oocyte system and assayed by immunoprecipitation, a polypeptide containing only M1–M4 and ~85% of the large cytoplasmic domain was stable, but the addition of M5 and M6 caused it to disappear completely during a 48-h chase, with no sign of residual proteolytic fragments. The authors traced this effect to a pair of Pro residues in the short extracellular loop between M5 and M6 (Pro⁷₉⁹-Leu-Pro), which appeared to act as a degradation signal under the conditions of their experiments. (It may be significant that there are no prolines in the M5-M6 loop of Pma1 H⁺-ATPase.) Further along in the same Na⁺,K⁺-ATPase/α₁ sequence, Béguin et al. (45) found evidence of a stabilizing effect when constructs containing M7 and M8 were co-expressed with the β₁ subunit, reflecting the association of β₁ with the extracytoplasmic

FIGURE 8. Pma1Δ901 ATPase is degraded in the vacuole. A, samples were taken from cultures grown and induced as described in the legend to Fig. 2. HA-tagged wild-type (WT) and mutant ATPases were expressed transiently in either the W303-1B parental background (open symbols) or strain W303-1B pep⁴ lacking aspartyl protease (protease A) activity (solid symbols). Total protein extracts were made at the time points indicated, separated by SDS-PAGE, immunoblotted using polyclonal anti-HA antibodies, and detected by ¹²⁵I-protein A and autoradiography. Each point represents the average of at least three determinations. Error bars have been omitted for clarity, but S.E. values were typically less than 20%. Top panel, wild type; middle panel, Pma1Δ686; bottom panel, Pma1Δ901. B, from time course experiments in A, cell volumes equivalent to an A₆₀₀nm of ~5.0 units were processed for double-label immunofluorescence as described under “Experimental Procedures.” In this experiment, monoclonal anti-HA antibody and an FITC-conjugated secondary antibody were used to detect HA-tagged plasma membrane H⁺-ATPase (left-hand panels), whereas a rabbit antibody against the 60-kDa vacuolar H⁺-ATPase subunit and a Texas Red-conjugated secondary antibody decorated vacuolar membranes (middle panels). Merged images are shown in the right-hand panels. Images show wild-type (top), Pma1Δ686 (middle), and Pma1Δ901 (bottom) ATPases at 180 min after induction of expression in strain W303-1B pep⁴. Bar, 5 μm.
M7-M8 loop. Worth noting in this respect is the fact that yeast Pma1p has no β subunit and appears to undergo membrane insertion and trafficking without the need for a “helper” protein of this kind. Indeed, yeast Pma1p may be a better model for the vast majority of P-type ATPases that lack auxiliary subunits as well as for other single subunit membrane proteins. Overall, our data showing a gradual increase in stability from Pma1Δ718 to Pma1Δ881 lend support to the idea advanced by Vashist and Ng (46) that the number of transmembrane segments in an integral membrane protein may influence the time required to extract misfolded forms of the protein from the lipid bilayer prior to proteasomal degradation.

A Plasma Membrane Targeting Sequence in the C-terminal Tail of Pma1 H\(^+\)-ATPase—Of the truncation mutants analyzed in the present study, only Pma1Δ901 successfully exited the ER and reached the cell surface, where it split ATP at nearly one-half of the wild-type rate in GM cells and was able to support growth. This result effectively defines a 20-amino acid sequence between Ala\(^{881}\) and Glu\(^{901}\), stretching from the end of growth. This result effectively defines a 20-amino acid half of the wild-type rate in GM cells and was able to support and reached the cell surface, where it split ATP at nearly one-half of the wild-type rate in GM cells and was able to support growth. This result effectively defines a 20-amino acid sequence between Ala\(^{881}\) and Glu\(^{901}\), stretching from the end of growth. This result effectively defines a 20-amino acid sequence between Ala\(^{881}\) and Glu\(^{901}\), stretching from the end of growth.

Among other P\(_2\)-type ATPases, most membrane-targeting studies have concentrated on the closely related Na\(^+\),K\(^+\)- and H\(^+\),K\(^+\)-ATPases. Here the picture is complicated, both by the presence of a β subunit and by the use of polarized cells to identify signals for basolateral versus apical targeting. Na\(^+\),K\(^+\)-ATPase is typically located in the basolateral membrane of epithelial cells, whereas H\(^+\),K\(^+\)-ATPase is mobilized to the apical surface of gastric parietal cells, where it pumps acid into the stomach lumen. To map the determinants of sorting, Dunbar et al. (47) constructed a set of chimeras between Na\(^+\),K\(^+\)- and H\(^+\),K\(^+\)-ATPase α subunits and tracked their behavior upon transfection into LLC-PK\(_1\) renal epithelial cells. The results were unexpectedly complex; substituting M4 of the H\(^+\),K\(^+\)-ATPase α subunit for the equivalent Na\(^+\),K\(^+\)-ATPase sequence was sufficient to redirect the chimera to the apical surface, but the same effect could be produced by incorporating the sequences flanking both ends of M4. Moreover, an earlier study (48) showed that the β subunit of H\(^+\),K\(^+\)-ATPase was delivered upon transfection to the basolateral surface of Madin-Darby canine kidney (MDCK) cells but to the apical surface of LLC-PK\(_1\) cells, indicating that identical targeting sequences can be “read” differently in two different cell types. By contrast with the intricacies of the results for Na\(^+\),K\(^+\)- and H\(^+\),K\(^+\)-ATPases, Pma1 H\(^+\)-ATPase offers a relatively straightforward way to identify targeting sequences in a P\(_2\)-type ATPase, uncomplicated by the presence of a β subunit or the need to distinguish between two alternative membrane destinations.

Looking beyond the P\(_2\)-type ATPase family, one finds many examples of membrane proteins in which the C-terminal region dictates delivery to the plasma membrane. For example, C-terminal tyrosine-containing motifs (e.g. NPXY) and dileucine motifs have long been known to direct proteins to the basolateral surface of polarized cells (reviewed in Ref. 49), and a C-terminal dileucine has recently been shown to play this role for Menkes protein, a copper-transporting P\(_1\)-type ATPase, during expression in MDCK cells (50). Less is known about motifs that underlie the apical targeting of proteins, such as the cystic fibrosis transmembrane conductance regulator, sodium/phosphate cotransporter (NpP, II), and GABA transporter (GAT-3). Once again, however, there is strong evidence for localization of the signal to the C terminus (51–55), and recent work has narrowed the responsible region to 39 residues in the case of rhodopsin (56) and 30 residues in the case of megalin (57) during expression in MDCK cells. Every indication is that these targeting events are achieved by interaction of the responsible sequence with PDZ domain-containing proteins (reviewed in Ref. 58).

Interestingly, the 20-amino acid sequence between Ala\(^{881}\) and Glu\(^{901}\) of the yeast Pma1 H\(^+\)-ATPase contains none of the C-terminal motifs previously associated with membrane protein targeting. Instead, it seems plausible that the highly charged nature of this sequence, which includes six positive and three negative residues, promotes interactions with other proteins that direct transport to the plasma membrane. This will be a fertile area for future study, with the long range aim of using the well recognized experimental advantages of yeast to map the molecular mechanism of targeting in detail.

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