Phosphorylation Region of the Yeast Plasma-membrane H\textsuperscript{+}-ATPase

ROLE IN PROTEIN FOLDING AND BIOGENESIS

(Received for publication, April 10, 1998, and in revised form, June 11, 1998)

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Mutations at the phosphorylation site (Asp-378) of the yeast plasma-membrane H\textsuperscript{+}-ATPase have been shown previously to cause misfolding of the ATPase, preventing normal movement along the secretory pathway; Asp-378 mutations also block the biogenesis of co-expressed wild-type ATPase and lead to a dominant lethal phenotype. To ask whether these defects are specific for Asp-378 or whether the phosphorylation region as a whole is involved, alanine-scanning mutagenesis has been carried out to examine the role of 11 conserved residues flanking Asp-378. In the sec6–4 expression system (Nakamoto, R. K., Rao, R., and Slayman, C. W. (1991) \textit{J. Biol. Chem.} 266, 7940–7949), the mutant ATPases displayed varying abilities to reach the secretory vesicles that deliver plasma-membrane proteins to the cell surface. Indirect immunofluorescence of intact cells also gave evidence for a spectrum of behavior, ranging from mutant ATPases completely arrested (D378A, K379A, T380A, and T384A) to partially arrested in the endoplasmic reticulum in those that reached the plasma membrane in normal amounts (C376A, S377A, and G381A). Although the extent of ER retention varied among the mutants, the endoplasmic reticulum appeared to be the only secretory compartment in which the mutant ATPases accumulated. All of the mutant proteins that localized either partially or fully to the ER were also malfolded based on their abnormal sensitivity to trypsin. Among them, the severely affected mutants had a dominant lethal phenotype, and even the intermediate mutants caused a visible slowing of growth when co-expressed with wild-type ATPase. The effects on growth could be traced to the trapping of the wild-type enzyme with the mutant enzyme in the ER, as visualized by double label immunofluorescence. Taken together, the results indicate that the residues surrounding Asp-378 are critically important for ATPase maturation and transport to the cell surface.

In eukaryotic cells, the first step in the biogenesis of plasma membrane proteins takes place in the endoplasmic reticulum,

\textsuperscript{a} This work was supported by National Institutes of Health Research Grant GM15761 (to C. W. S), National Institutes of Health Postdoctoral Fellowship GM18623 (to N. D. D.), and a fellowship from Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (to C. F. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisiment" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\textsuperscript{1} The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; HA, influenza hemagglutinin; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate.

\textsuperscript{2} Published in \textit{J. Biol. Chem.} 273, 21744 (1998). This paper is available on line at http://www.jbc.org
Slayman, manuscript in preparation.

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sensitive

bub 3–52; LEU3–52; LEU2–3, 112; his4–6; GAL1::PM1::URA3; sec6–4; GAL2 is isogenic with NY605, and

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brane. When transformed with the appropriate plasmid (see below), SY4 allows newly synthesized ATPase to be arrested in the secretory vesicles for study (19).

Plasmids Containing Epitope-tagged PMA1—For ease in detecting the ATPase during biogenesis studies, two different epitope-tagged versions of PMA1 were constructed, containing either a 10-amino acid c-Myc epitope plus linking sequences (MTASEQKLISEEDLNDTS....) or a 9-amino acid HA epitope (MTYPYDVPDYADTS....) or a N terminus of the ATPase.7 As shown in Table I, the Myc-tagged and HA-tagged PMA1 genes were placed under control of the GAL1 promoter in plasmids YCPGAL1rPMA1-myc (centromeric; URA3) and YCPGAL1rPMA1-HA (centromeric; LEU2). The lithium acetate method (21) was used to transform the plasmids into NY605 cells to study the delivery of the ATPase to the plasma membrane. The tagged genes were also placed under heat-shock control in plasmid YCP2HSE-

PMA1-myc (centromeric; LEU2). These plasmids were transformed into SY4 cells to study the early stages of ATPase biogenesis. Site-directed Mutagenesis—The polymerase chain reaction was used to introduce mutations into a subcloned Sty1-BamHI fragment of PMA1, as described previously (22). To Myc-tag the mutant alleles and place them under heat-shock or GAL1 control, DNA fragments carrying the mutations were subcloned into the centromeric expression plasmids described above. The presence of the mutations in the final constructs was confirmed by automated DNA sequencing of the Sty1-BamHI region.

Immunoblotting of Myc-tagged pma1 Mutants in Yeast Total Mem-

branes—Quantitative immunoblotting was carried out to measure the steady-state expression levels of Myc-tagged wild-type and mutant ATPases in total membranes. SY4 cells transformed with either the wild-type or a mutant version of YCP2HSE-PMA1-myc were grown on minimal medium containing 2% galactose at 23 °C, transferred to me-

dium containing 2% glucose for 3 h, and then shifted to 39 °C for 30 min (19). After zymolase treatment and Dounce homogenization of the cells, total membranes were isolated by differential centrifugation (18).

An aliquot of membranes containing 10 µg of protein was precipitated with trichloroacetic acid, solubilized in loading buffer, subjected to SDS-PAGE, transblotted to polycvinydene difluoride membrane, and immunoblotted with rabbit anti-Myc polyclonal antibody (Medical and Biological Laboratories, Nagoya, Japan), followed by incubation with 125I-protein A and fluorography.

Immunofluorescence—Subcellular structures containing Myc-tagged ATPase were visualized by indirect immunofluorescence using a modi-

ification of the method of Redding et al. (23). For these experiments, NY605 cells were transformed with YCPGAL1rPMA1-myc alone (mutant or wild-type version; URA plasmid) or YCPGAL1rPMA1-HA (wild-type PMA1 gene; LEU plasmid) in combination with YCPGAL1rPMA1-myc (mutant; pma1 gene; URA plasmid). The transformed strains were grown at 30 °C in synthetic complete medium (24) lacking uracil (or uracil and leucine) and containing 4% raffinose, which was used as a carbon source to prevent glucose repression in the next step. At 1 OD/ml, the cells were shifted to medium with 4% galactose for 4 h to induce expression of the plasmid-encoded ATPase gene(s). Cells were fixed, treated with Zymolyase-101, and permeabilized by Triton X-100 as described previously (22).

Fixed, permeabilized spheroplasts were stained for immunofluores-

cence essentially as described by Redding et al. (23), but with the addition of a blocking step in modified WT buffer, in which the dry milk and bovine serum albumin were replaced by 5% normal goat serum (Sigma). Four different primary antibodies were used: HA polyclonal (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan), diluted 1:100; Myc monoclonal 9E10.2 from ascites fluid (provided by H. Doh-

lan), diluted 1:100; Sec7 large domain polyclonal antibody (provided by R. Schekman; Ref. 25), diluted 1:150; and Kar2 polyclonal antibody (provided by M. Rose; Ref. 26), diluted 1:5000. Goat anti-rabbit FITC and goat anti-mouse Texas Red IgG (Jackson Immunoresearch, West Grove, Pa) served as fluorescent secondary antibodies and were diluted 1:100. 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 cross-reactivity of the c-Myc and HA antibodies, uninduced cells were simultaneously fixed and stained with each set of antibodies. Cells were then stained with 4,6-diamidino-2-phenylindole (Sigma), and mounted in Citifluor (Ted Pella, Redding, Pa).

For fluorescence microscopy, cells were examined with a Nikon Microphot-FXA (Melville, NY) and photographed using Tmax 400 black-

and-white film (Eastman Kodak Co.). For confocal microscopy, cells were observed on a Bio-Rad MRC-600 scanning confocal microscope using dual channel filters for simultaneous visualization of Texas Red and FITC fluorochromes. For all images, the slit width was set to provide an optical slice of <1 µm. The absence of nonspecific antibody binding was verified by examination of uninduced controls using the same settings, and the absence of bleed-through, by confirming that the signal disappeared when viewed with single-wavelength filter blocks. Images were collected with Comos software (Bio-Rad) and modified by contrast stretching, application of pseudocolor, and merging, using Adobe Photoshop 4.0 (Adobe Systems Inc., San Jose, CA).

Isolation of Secretory Vesicles, ATPase Assay, and Limited Trypsi-

nolysis—Secretory vesicles were isolated from SY4 cells transformed with the wild-type or a mutant version YCP2HSE-PMA1-myc using a two-step discontinuous sucrose gradient (27). Assays of ATPase hydro-

ysis, ATP-dependent proton transport, and protein were carried out as described previously (17, 28), as were limited trypsinolysis experiments (18, 29).

Genetic Tests of Dominance or Recessiveness—To examine the genetic behavior of the various mutations, NY605 cells transformed with wild-
type or mutant YCPGAL1rPMA1-myc plasmid were grown on synthetic medium lacking uracil and containing 2% galactose. Cells were diluted in water and tested for growth on solid medium, lacking uracil and con-
taining either 2% glucose (wild-type gene expressed) or 2% galactose (both mutant and wild-type genes expressed). The plates were incubated at 30 °C for 42 h and photographed.

### Table I

| Plasmid | PMA1 gene | Promoter | Epitope tag | Selectable marker | Origin |
|---------|-----------|----------|-------------|-------------------|--------|
| YCPGAL1rPMA1-myc | GAL1 | c-Myc | URA3 | pRN93, derived from Ycpplac33 (46) |
| YCPGAL1rPMA1-HA | GAL1 | HA | LEU2 | pRN94, derived from Ycpplac111 (46) |
| YCP2HSErPMA1-myc | HSE | c-Myc | URA3 | pRN90, derived from Ycpplac111 (46) |
| YCP2HSErPMA1-HA | HSE | HA | LEU2 | pRN90, derived from Ycpplac111 (46) |

### Yeast Strains—NY605 (MATa; ura3–52; leu2–3, 112; GAL2) was kindly provided by P. Novick. SY4 (MATa; ura3–52; leu2–3, 112; his4–619; GAL1::PM1::URA3; sec6–4; GAL2) is isogenic with NY605, and was described previously by Nakamoto and co-workers (19). In this strain, the chromosomal copy of the PMA1 gene has been placed under control of the GAL1 promoter (20). SY4 also contains the temperature-sensitive sec6–4 allele that blocks the last step of the secretory pathway: namely, the fusion of secretory vesicles with the plasma mem-

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1. C. F. Tourinho dos Santos, N. D. DeWitt, K. E. Allen, and C. W. Slayman, manuscript in preparation.
**RESULTS**

**Definition of the Region to Be Studied**—As described above, a central goal of this work was to explore structure-function relationships in the phosphorylation region of PMA1, surrounding the site (Asp-378) at which the β-aspartyl phosphate reaction intermediate is formed. To choose residues for study, sequences were aligned from 15 P-type ATPases differing in cation specificity: 1) fungal, algal, and plant H\(^+\)-ATPases; 2) bacterial Mg\(^{2+}\)-ATPases; fungal and plant Ca\(^{2+}\)-ATPases; 3) mammalian Ca\(^{2+}\)-, Na\(^{+}\)-, K\(^{+}\)-, and gastric H\(^+\)-K\(^{+}\)-ATPases; 4) heavy metal transporting P-ATPases; 5) the E. coli K\(^{+}\)-ATPase. Residues that are conserved in all known P-ATPases are boxed. From top to bottom of the figure, GCG accession numbers for the sequences are X03534, F54210, P20649, U07843, M25488, M96324, M12898, X63575, P20648, P05023, L10909, L13292, U03464, L06133, and P03960. B, the Robson-Garnier secondary structure prediction for PMA1 was determined using the GCG PeptideStructure program. Residues likely to contribute to α-helices are indicated by H, β-turns by T, and those of indeterminate nature by dots.

**TABLE II**

| Mutant | Expression\(^{a}\) | ATPase activity\(^{b}\) | Uncorrected | Corrected | % |
|--------|------------------|---------------------|-------------|-----------|---|
|        |                   | units/mg            | units/mg    |           |   |
| Wild type | 100 | 3.85 | 3.85 | 100 |
| Vector | 2 | 0.05 | d | d |
| I374A | 55 | 0.37 | 0.67 | 16 |
| L375A | 31 | 0.39 | 1.30 | 29 |
| C376A | 104 | 3.02 | 2.90 | 94 |
| S377A | 103 | 0.62 | 0.62 | 15 |
| D378A | 5 | 0.08 | d | d |
| K379A | 29 | 0.25 | d | d |
| T380A | 20 | 0.06 | d | d |
| G381A | 81 | 0.28 | 0.34 | 9 |
| T382A | 14 | 0.07 | d | d |
| L383A | 12 | 0.20 | d | d |
| T384A | 9 | 0.10 | d | d |

\(^{a}\) Expression levels are calculated as a percentage of the yield of wild-type protein per milligram of secretory vesicles, as determined by quantitative immunoblotting (see “Experimental Procedures”). Values are the mean of two to five experiments, with an average standard error of 13%.

\(^{b}\) Measured at 30 °C in the presence of 50 mM MES/Tris, pH 5.7, 5 mM Na\(_{2}\)ATP, 10 mM MgCl\(_{2}\), 5 mM KN\(_{3}\), 5 mM phosphoenolpyruvate, and 50 µM pyruvate kinase, as described under “Experimental Procedures.” Values are the mean of two to three experiments with an average standard error of 13%. One unit is defined as 1 µmol of Pi/min.

\(^{c}\) Corrected values were not calculated for mutant preparations in which expression levels were less than 30% of wild type.
apparent defect in either the synthesis or the short term stability of the mutant ATPases; rather, as reported earlier for other substitutions at Asp-378 (15–17, 22), the failure of many of the Ala mutants to reach the secretory vesicles reflects a problem in their trafficking to the cell surface.

ATP hydrolysis was also severely affected in most of the mutants (Table II). Among those examined, only C376A displayed near-normal activity (94%), while much lower values (9–29%) were seen in I374A, L375A, T382A, and T384A. The remaining mutants had activities that were not significantly above background.

Subcellular Location of the Mutant ATPases—To examine the trafficking defects in greater detail, a different expression strategy was used, coupled with indirect immunofluorescence to visualize the location of the mutant ATPases. In this case, each Myc-tagged mutant allele was placed under GAL1 control on a centromeric plasmid and transformed into yeast strain NY605, which contains a wild-type copy of PMA1 on the chromosome. Expression of the mutant protein was induced by growth on galactose medium, and the cells were examined by immunofluorescence microscopy using c-Myc antibody.

Once again, the mutants displayed a range of behavior (Fig. 3). In C376A, S377A, and G381A, there was a smooth, ringlike labeling of the plasma membrane by c-Myc antibody, and little if any epitope-tagged ATPase was seen intracellularly; this was also the case for the wild-type control. By contrast, in I374A, L375A, T382A, and L383A, the Myc-tagged ATPase was distributed to varying degrees between the plasma membrane and intracellular structures. Finally, there were mutants (D378A, K379A, T384A) in which the Myc-tagged ATPase appeared only intracellularly; in these cells, no smooth labeling of the plasma membrane was observed.

It seemed likely that the intracellular structures were derived, at least in part, from the endoplasmic reticulum. The yeast ER typically surrounds the nucleus, with extensions to the cell periphery (30). During the expression of abnormal proteins, however, it can proliferate into enlarged structures (31) that are labeled by antibody against the luminal ER protein, Kar2 (32–34). Indeed, Harris et al. (16) and Portillo (17) have recently reported such proliferation in cells expressing certain mutant forms of PMA1 ATPase, including D378N and D378E. Thus, an important goal of the present study was to determine whether any or all of the current group of mutant ATPases accumulate in these ER-derived structures, and at the same time, whether there is also accumulation in other intracellular compartments such as the Golgi.

To address these questions, double-labeling immunofluorescence and confocal microscopy were carried out on cells expressing mutant PMA1 ATPases of the second, “intermediate” type and the third, severely affected type. In addition to c-Myc antibody, which labeled the epitope-tagged mutant ATPases, Kar2 antibody served as a marker for the ER (26) and Sec7 antibody, for the Golgi (25). In the case of I374A (an intermediate mutant), some of the ATPase reached the plasma membrane (Fig. 4A). However, much of it co-localized with Kar2 in the perinuclear region and in brightly staining cytoplasmic structures, reminiscent of those described by Harris et al. (16); the regions of co-localization appear as yellow and orange areas on the merged image of panel A/B. In the more than 20 cells that were closely examined, I374A was not detected in the Sec7-containing compartment, even when large amounts of I374A were seen in both the plasma membrane and ER (Fig. 4, C, D, and C/D). Thus, there did not appear to be a uniform slowing of transit through the entire secretory pathway; rather, I374A ATPase that managed to leave the ER could move all the way to the cell surface. Similar observations were made for each of the other intermediate mutants including L375A, T382A, and L383A. In severely affected mutants such as D378A, there was no evidence of epitope-tagged ATPase at the plasma membrane or in the Golgi (Fig. 4G/H); rather, staining was limited to the Kar2-containing cytoplasmic structures (Fig. 4E/F).

Protein Folding as Studied by Limited Trypsinolysis—In previous work, we have shown that D378N ATPase is integrated into the lipid bilayer but is poorly folded, with greatly increased sensitivity to trypsin (18); it is presumably the folding defect that causes the ATPase to be retained in the ER (15–17). As described above, the present study now provides evidence for a spectrum of trafficking problems in mutants throughout the phosphorylation region of the ATPase. Some, like D378A, display virtually complete ER retention; in other cases, from 10% to 100% of the protein can travel to the secretory vesicles and then to the cell surface. It was thus interesting to determine whether the severity of the trafficking problem might correlate
with the extent of the protein folding defect, as judged by limited trypsinolysis.

For this set of experiments, total $^{35}$S-labeled membranes containing each of the mutant ATPases were digested with trypsin, and the resulting cleavage products were isolated by immunoprecipitation with ATPase antibody and analyzed by SDS-PAGE and autoradiography. Indeed, the mutants varied in their sensitivity to limited trypsinolysis, as shown in Fig. 5. Some (C376A and S377A) were virtually indistinguishable from the wild-type control; after 5 min of digestion at a trypsin:protein ratio of 1:20, there were roughly equal amounts of full-length 100-kDa ATPase and a 97-kDa fragment. Others (I374A and G381A) displayed intermediate behavior; after 5 min of digestion, both 100- and 97-kDa bands were still visible, but in significantly reduced amounts. By contrast, most (L375A, D378A, K379A, T380A, T382A, L383A, and T384A) were extremely sensitive to trypsinolysis; after as little as 1 min of digestion, the 100- and 97-kDa species had been almost completely degraded and were barely perceptible by autoradiography. Thus, there was a rough correlation between poor protein folding and intracellular retention of the mutant ATPase; the apparent exceptions will be discussed below.

Effect on Co-expression of Wild-type ATPase—As described previously, mutations at the phosphorylation site (Asp-378) of PMA1 have a dominant negative phenotype; they are lethal when co-expressed with the wild-type allele (15–17). It was therefore of interest to survey the genetic behavior of mutations across the phosphorylation region. For this purpose, the cells used for the immunofluorescence experiments of Figs. 3 and 4 were plated on galactose or glucose medium to induce or repress expression of the mutant allele. As shown in Fig. 6, the various mutations exerted a range of effects on cell growth. At one extreme, D378A, K379A, T380A, and T384A displayed a dominant negative phenotype, with D378A inhibiting growth on galactose most severely. I374A, L375A, G381A, T382A, and L383A had an intermediate effect, slowing but not completely preventing growth on galactose, and C376A and S377A were recessive, growing on galactose as well as the wild-type and empty vector controls. Thus, with one exception (G381A, which will be discussed below), mutant ATPases with trafficking defects were either partially or completely dominant in the growth test.

To determine whether, as expected, the co-expressed mutant and wild-type ATPases were trapped in the same intracellular compartment, mutant and wild-type alleles were epitope-tagged (with c-Myc and HA, respectively), placed behind the GAL1 promoter on separate plasmids, and transformed to-

![Fig. 4](image-url) **Retention of biogenesis-defective mutant ATPases in intracellular structures derived from the ER.** NY605 cells were transformed with centromeric plasmids encoding GAL1''-I374A-myc (panels A–D), and GAL1''-D378A-myc (panels E–H) and grown to early log phase in medium containing 4% raffinose. Expression of the Myc-tagged ATPase was induced by shifting to medium containing 4% galactose. The cells were then fixed with formaldehyde and processed for double-label immunofluorescence as described under “Experimental Procedures,” using antiserum to c-Myc (9E10.2), combined with antiserum to either Kar2 (panels A, B, E, and F) or Sec 7 (panels C, D, G, and H), and detected by FITC- and Texas Red-conjugated secondary antibodies. Staining of both fluorochromes was visualized simultaneously by confocal microscopy using dual channel filters, and the images were merged (A/B, C/D, E/F, G/H) using Adobe Photoshop. Bar = 5 μm.

![Fig. 5](image-url) **Time course of trypsinolysis of wild-type and mutant ATPases.** Secretory vesicles (10 μg of protein) containing [35S]methionine-labeled wild-type (WT), S377A, D378A, K379A, G381A, or L383A ATPase were incubated at a trypsin:protein ratio of 1:20 for 0, 1, and 5 min. Samples were immunoprecipitated with anti-ATPase antibody and analyzed by SDS-PAGE and fluorography.
GAL1 42 h at 30 °C, and photographed. shown in Fig. 7 (mutant with an intermediate block in biogenesis (L383A) is together into NY605 cells. An example of this experiment using a medium containing 2% galactose (procedures). Drops of each diluted strain were placed onto synthetic NY605 cells, containing a constitutively expressed chromo-

somal copy of the PMA1 gene, were transformed with either Empty plasmid

ATPases.

YCplac33 (3 A. Ambesi, unpublished data.)

FIG.6 . Growth of strains co-expressing mutant and wild-type ATPases. NY605 cells, containing a constitutively expressed chromosomal copy of the PMA1 gene, were transformed with either GAL1-YCplac33 (Empty plasmid), GAL1^pr-PMA1-myc gene (PMA1), or GAL1^pr-pma1-myc (mutant listed) as described under “Experimental Procedures.” Drops of each diluted strain were placed onto synthetic medium containing 2% galactose (Gal) or 2% glucose (Glu), grown for 42 h at 30 °C, and photographed.

Together into NY605 cells. An example of this experiment using a mutant with an intermediate block in biogenesis (L383A) is shown in Fig. 7 (bottom panel). When cells containing L383A and wild-type plasmids were shifted to galactose medium and examined by immunofluorescence, there was co-localization of newly synthesized mutant and wild-type ATPases in perinuclear and peripheral intracellular structures, as revealed in the yellow and yellow-orange regions of the merged images. Combining this observation with the results of Fig. 3, we conclude that the two forms of the ATPase were arrested together in the ER-derived membranes. In contrast, when recessive mutants such as C376A were co-expressed with the wild type, both ATPases reached the cell surface (Fig. 7, top panel).

DISCUSSION

As described in the Introduction, the starting point for this study was the discovery that mutations at position Asp-378 cause misfolding of the yeast PMA1 ATPase, leading to retention in the ER and dominant lethality (15–17). One goal of the present work was to learn whether these features are specific to Asp-378 or whether they are shared by other residues throughout the phosphorylation region. It is now clear that the latter is the case; 8 of 11 alanine substitutions in the stretch between Ile-374 and Thr-384 caused noticeable defects in folding and biogenesis, resulting in some degree of dominant lethality. By contrast, similar problems have been seen in only 6 out of 48 site-directed mutants in the adjacent stalk3 and M4

regions of PMA1 (28). Based on these findings, the local sequences surrounding Asp-378 must play a special role in ATPase maturation and transport to the cell surface.

When looked at in detail, mutants in the phosphorylation region can be arrayed along a continuum based on their behavior during biogenesis (Table III). Some of the mutant proteins (C376A, S377A, G381A) travel normally along the secretory pathway as evidenced by either of two independent assays: immunoblotting of ATPase that has reached the secretory vesicles (in the sec6–4 expression system; Ref. 19) or indirect immunofluorescence of ATPase in the plasma membrane of intact cells (after epitope-tagging and expression in strain NY605). Not surprisingly, these mutant ATPases appear to be either normally folded (C376A, S377A) or reasonably well folded (G381A) when tested by limited trypsinolysis. It is worth pointing out that two of the three mutants, S377A and G381A, are virtually inactive; rates of ATP hydrolysis, assayed in isolated secretory vesicles, were only 7–15% of the wild-type control (before correction for expression), implying that delivery of the ATPase to the secretory vesicles does not require PMA1-mediated proton transport into the compartments of the secretory pathway.

At the other end of the continuum lie two mutants (D378A and T384A) with very severe blocks in biogenesis. Both were virtually absent from isolated secretory vesicles, and both were seen only in ER-derived structures upon indirect immunofluorescence of intact cells. Based on their extreme sensitivity to trypsinolysis, these two mutant ATPases appear to be poorly folded; it therefore seems likely that they are retained intracellularly by a quality control mechanism at the ER-to-Golgi step of the secretory pathway.

The remaining six mutants were intermediate in their behavior, exhibiting a range of abilities (between 55% and 12%) to reach the secretory vesicles in the sec6–4 system. Four of them (I374A, L375A, T382A, L383A) also showed intermediate sub-

3 A. Ambesi, unpublished data.
cellular localization based on indirect immunofluorescence microscopy; some ATPase clearly reached the cell surface, but much of it remained in ER-derived intracellular structures. In the other two mutants (K379A and T380A), the epitope-tagged ATPase appeared to be largely, if not completely, arrested in the ER. Structurally, one member of this group (I374A) was intermediate in its sensitivity to trypsin and the rest were highly sensitive, again pointing to abnormalities in protein folding.

It is intriguing that ATPase biogenesis is so vulnerable to substitutions in the phosphorylation region. Previous studies have shown that the spacing of residues in this part of the protein is critically important, since the insertion of a single glycine at successive points between Cys-376 and Thr-384 leads to biosynthetic arrest of the ATPase (22). When the mutational data are superimposed upon secondary structural predictions for the phosphorylation region, there is a clear correspondence between the cluster of biogenesis-defective mutations and a predicted \( \beta \)-turn in the vicinity of Asp-378. According to the same algorithm (29), substitution by alanine at each position should introduce a more helical nature to the \( \beta \)-turns and could therefore perturb the structure of the cytoplasmic mid-region of the ATPase, preventing insertion of the C-terminal transmembrane domains into the lipid bilayer. Indeed, it has been shown that \( \beta \)-turns play a major role in defining the three-dimensional organization of many proteins (35) and that mutations in \( \beta \)-turns may have a special propensity to disrupt the formation of folding intermediates (36, 37).

Based on the current view of protein folding, it seems plausible that the ER-retained ATPases exist in a dynamic equilibrium between intermediate and mature folded states, as proposed for \( \Delta F_{508} \) CFTR (38), with the more severe mutations causing kinetic traps that favor non-productive or off-pathway folding intermediates. According to this model, the population of mutant protein that attains normal conformation can exit the ER, while the malfolded forms are retained. It may be significant that the trypsin-sensitive mutant ATPases, particularly D378A, are generally more prone to aggregation on SDS-PAGE than well folded ATPases such as C376A, S377A, and G381A (data not shown). At the moment, it is unclear whether the mutants capable of reaching the plasma membrane are malfolded forms that escape the ER quality control machinery, or whether some proportion of the mutant proteins eventually manage to fold correctly; our observation of a trypsin-resistant subpopulation of I374A that reaches secretory vesicles lends support to the latter possibility. Severe biogenesis mutants such as D378A are probably incapable of achieving correct conformation and for this reason undergo complete biosynthetic arrest.

Finally, it is interesting to speculate on the mechanism by which dominant lethal ATPase mutants cause co-expressed wild-type ATPase to be retained in the ER (Refs. 16 and 17; this study). One obvious possibility is that the mutant protein forms aggregates (42), leading to a general disruption of ER function. At first glance, this idea fits with the striking proliferation of ER membranes that takes place during overexpression of the closely related PMA2 ATPase (32), \( H^+ \)-ATPases from higher plants (39), and the integral ER proteins hydroxymethylglutaryl-coenzyme A reductase (31) and cytochrome \( b_5 \) (40). As reviewed by Kerchove et al. (41), the morphology of the proliferated ER can vary, depending on the expressed protein, but in all cases is thought to occur as the cell’s response to increased levels of these proteins in the ER.

However, although expression of the dominant lethal PMA1 mutants clearly disrupts the morphology of the ER membranes, there does not appear to be a complete block in ER function that would explain the retention of the wild-type ATPase. This point was shown in a recent study in which secretory vesicles, isolated from cells producing the D378N ATPase, contained a normal profile of Coomassie-stained proteins (38), and the integral ER proteins hydroxymethylglutaryl-coenzyme A reductase (31) and cytochrome \( b_5 \) (40). As reviewed by Kerchove et al. (41), the morphology of the proliferated ER can vary, depending on the expressed protein, but in all cases is thought to occur as the cell’s response to increased levels of these proteins in the ER.

| Mutant    | Expression in secretory vesicles | Localization in cells | Conformation | Catalytic activity | Genetic phenotype |
|-----------|----------------------------------|-----------------------|--------------|-------------------|-------------------|
| C376A     | 104                              | PM                    | Well folded  | Normal            | Recessive         |
| S377A     | 103                              | PM                    | Well folded  | Low               | Recessive         |
| G381A     | 81                               | PM                    | Intermediate| Low               | Intermediate      |
| I374A     | 55                               | ER/PM                 | Intermediate| Low               | Intermediate      |
| L375A     | 31                               | ER/PM                 | Malfolded    | /                 | Dominant lethal   |
| K379A     | 29                               | ER                    | Malfolded    | /                 | Dominant lethal   |
| T380A     | 20                               | ER                    | Malfolded    | /                 | Dominant lethal   |
| T382A     | 14                               | ER/PM                 | Malfolded    | /                 | Intermediate      |
| L383A     | 12                               | ER/PM                 | Malfolded    | /                 | Intermediate      |
| T384A     | 9                                | ER                    | Malfolded    | /                 | Intermediate      |
| D378A     | 5                                | ER                    | Malfolded    | /                 | Dominant lethal   |

a As shown by quantitative immunoblotting.

b As shown by indirect immunofluorescence. ER, endoplasmic reticulum; PM, plasma membrane.

c As shown by limited trypsinolysis.

d As determined by ATP hydrolysis measurements in secretory vesicles.

e As determined by a test of dominant lethality, in which the mutant ATPase was co-expressed with the wild-type ATPase.

f Activity could not be determined because the mutant ATPase was retained in the ER.
the ATPase. It also calls attention to the quality control mechanisms that govern the exit of the ATPase from the ER, and suggests that further analysis of those mechanisms will be fruitful. The recent finding of several new genes affecting ATPase biogenesis and stability (43–45) will serve as a useful point of departure for such analysis.

Acknowledgments—We thank Drs. Henrik Dohlman for providing the c-Myc monoclonal antibody and for advice on yeast immunofluorescence, Robert Nakamoto for supplying the pRN93 and pRN94 expression vectors, Philippe Mâle for assistance with confocal microscopy and photomicrographs, Michael Caplan and Vincent Marchesi for the use of their microscopes, and Anita Panek for support throughout this study. We are also grateful to Drs. Mark Rose and Randy Schekman for generously providing the Kar2 and Sec7 antibodies, respectively.

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