Targeting of the Yeast Plasma Membrane [H+]ATPase: A Novel Gene AST1 Prevents Mislocalization of Mutant ATPase to the Vacuole

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Abstract. We have characterized a class of mutations in PMA1, (encoding plasma membrane ATPase) that is ideal for the analysis of membrane targeting in Saccharomyces cerevisiae. This class of pma1 mutants undergoes growth arrest at the restrictive temperature because newly synthesized ATPase fails to be targeted to the cell surface. Instead, mutant ATPase is delivered to the vacuole, where it is degraded. Delivery to the vacuole occurs without previous arrival at the plasma membrane because degradation of mutant ATPase is not prevented when internalization from the cell surface is blocked. Disruption of PEP4, encoding vacuolar proteinase A, blocks ATPase degradation, but fails to restore growth because the ATPase is still improperly targeted.

The plasma membrane mediates interaction between the cell and the extracellular environment. To maintain the unique identity and function of this organelle, it is essential that newly synthesized plasma membrane proteins are accurately sorted and targeted. Defects in intracellular transport and cell surface expression of plasma membrane proteins, including ion transporters and channels, are well-known causes of human disease (Amara et al., 1992; Ashcroft and Roper, 1993).

Proteins destined for the cell surface enter the secretory pathway at the ER. Folding and assembly of newly synthesized proteins at the ER is promoted by molecular chaperones that interact selectively with nascent polypeptides (Gething and Sambrook, 1992; Bergeron et al., 1994; Shinde et al., 1993). Chaperones comprise one component of a quality control mechanism functioning at the ER (Klausner, 1989). This quality control system prevents the export of misfolded and improperly assembled proteins, and it degrades retained proteins (Klausner and Sitia, 1990). Since assembly of at least one integral plasma membrane protein occurs after exit from the ER, quality control may occur at multiple steps of the secretory pathway (Musil and Good- enough, 1993). Thus, the requirements for transit through the secretory pathway may be unique for individual proteins. For example, amino acid permeases in yeast require a novel gene, SHR3, for transport from the endoplasmic reticulum (Ljungdahl et al., 1992). Analogously, a specific receptor is required exclusively for targeting of carboxypeptidase Y to the vacuole (Marcusson et al., 1994).

One of these pma1 mutants was used to select multicopy suppressors that would permit growth at the non-permissive temperature. A novel gene, AST1, identified by this selection, suppresses several pma1 alleles defective for targeting. The basis for suppression is that multicopy AST1 causes rerouting of mutant ATPase from the vacuole to the cell surface. pma1 mutants deleted for AST1 have a synthetic growth defect at the permissive temperature, providing genetic evidence for interaction between AST1 and PMA1. Ast1 is a cytoplasmic protein that associates with membranes, and is localized to multiple compartments, including the plasma membrane. The identification of AST1 homologues suggests that Ast1 belongs to a novel family of proteins that participates in membrane traffic.
The plasma membrane \[\text{H}^+\]-ATPase of Saccharomyces cerevisiae, encoded by \textit{PMA1} (Serrano et al., 1986), presents an excellent model for targeting studies because it is a major component of the cell surface, making up 10–20% of the total plasma membrane protein. Pmal is predicted to have a polytopic membrane topology with 10 transmembrane segments, a large cytoplasmic domain containing ATP-binding and catalytic phosphorylation sites, and \textasciitilde 4\% of the protein is extracytoplasmic (Serrano et al., 1986). Newly synthesized Pmal is delivered to the plasma membrane via the secretory pathway, and its intracellular transport is accompanied by kinase-mediated phosphorylation on multiple Ser and Thr residues (Chang and Sluyman, 1991). At the cell surface, the ATPase is quite stable with a half-life of \textasciitilde 1 h (Benito et al., 1991).

The plasma membrane \[\text{H}^+\]-ATPase belongs to the superfAMILY of P-type ion transporters, with which it shares structural and functional similarity (Gaber, 1992). By pumping protons out of the cell, the plasma membrane ATPase regulates cytoplasmic pH and creates the electrochemical proton gradient that drives nutrient uptake (Serrano et al., 1986; Gaber, 1992). ATPase activity is modulated by a variety of environmental factors, including glucose metabolism (Eraso and Portillo, 1994; Chang and Sluyman, 1991) and acidification of the medium (Eraso and Gancedo, 1987). The \textit{PMA1} gene is regulated transcriptionally (Garcia-Arranz et al., 1994; Kuo and Grayhack, 1994) and, because of its critical physiological role, its function is essential for viability.

Other members of the P-type ATPase family, e.g., the mammalian Na+,K+ and H+,K+-ATPases, have a \(\beta\) subunit whose assembly with the catalytic subunit is required for plasma membrane delivery and stability of the complex (Renaud et al., 1991; Jaunnin et al., 1993; Eakle et al., 1994). The \(\beta\) subunit is a glycosylated transmembrane polypeptide with highly conserved structure; however, a subunit of this type has not been found for the yeast plasma membrane \[\text{H}^+\]-ATPase.

In this study, we have characterized temperature-sensitive \textit{pma1} mutants that undergo growth arrest when ATPase protein and activity become limiting. We find that newly synthesized ATPase in these cells is defective for delivery to the plasma membrane and is, instead, degraded in the vacuole. We used the \textit{pma1} \(\alpha\) mutants as a basis for selection of genes involved in quality control and plasma membrane targeting. We identified \textit{AST1}, a multicopy suppressor of temperature-sensitive growth of \textit{pma1} cells, which reroutes mutant ATPase from the vacuole to the plasma membrane.

Materials and Methods

Strains and Media

Standard yeast media and genetic manipulations were as described in Sherman et al. (1986). Mutations in \textit{PMA1} were made in vitro by hydroxylamine mutagenesis and transformed into yeast containing a deletion of \textit{PMA1} on the chromosome and a functional \textit{PMA1} copy on a plasmid. Temperature-sensitive yeast were isolated by plasmid shuffle (Boeke et al., 1987). \textit{pma1} and \textit{pma1}-8 were cloned into pRS306 (Sikorski and Hieter, 1989) as 5-kb HindIII fragments to generate \textit{pCp7} and \textit{pCp8}. The plasmids were linearized with BstEII, and \textit{pma1} (ACY5) and \textit{pma1}-8 (ACY6) were integrated into the genome of strain L3852 (Antebi and Fink, 1992), replacing \textit{PMA1} by pop-in, pop-out gene replacement. \textit{AST1}-disrupted strains were constructed by transformation of diploid strains ACX7 (\textit{PMA1/pma1}-7) with \textit{pAC19} and ACX24 with \textit{pAC69}. Strains ACX7 and ACX24 were from crosses between ACY7 and L3854, and L3852 and L4364, respectively. Temperature-sensitive \textit{pma1 sec} double mutants were derived from crosses between ACY7 and L5077 (Mat a sec7-1 leu2-3,112 lys2-Delta00 ural3-52) and L5435 (Mat a sec6-4 his3 Delta00 ade2-3,112 ural3-52) (Antebi and Fink, 1992). The \textit{pma1}-7 end3-1 strain came from a cross between ACY7 and RH266-1D (Mat a end3-1 leu2 his4 ura3 bar1-l) (Raths et al., 1993). A list of the strains and plasmids used in this work are shown in Table I. Yeast transformations were performed by the lithium acetate method (Gietz et al., 1992).

Metabolic Labeling, Immunoprecipitation, Western Blot and ATPase Assay

Cultures were grown overnight in synthetic complete medium without methionine and uracil. Mid-log cells were harvested and resuspended in fresh medium. Temperature-sensitive mutants were shifted to 37°C for 5 min (except as noted) before pulse labeling for 2 min with Expre35S (New England Nuclear, Boston, MA) at 2 mCi/25 OD600 cells. An equal volume of complete medium plus 20 mM cysteine and methionine was added to start the chase. At various times of chase, aliquots were placed on ice in the presence of 10 mM Na azide and 2 mg/ml cycloheximide. Cell lysis and immunoprecipitation in RIPA buffer were as previously described (Chang and Sluyman, 1991). Immunoprecipitations were normalized to acid-precipitable cpm, and analyzed by SDS-PAGE and autoradiography or phosphorimaging with a Fujix Bas2000 image analyzer. Rabbit polyclonal anti-ATPase antibody was from Carolynn Sluyman (Yale University, New Haven, CT). A mouse monoclonal anti-ATPase antibody 9E10 was from Harvard University Cell Culture Facility (Boston, MA). For Western blot analysis and ATPase assays, samples were normalized to lysate protein as measured by Bradford (1976). For quantitative immunoblots, \textasciitilde 250 kDa protein A (Amersham Corp., Arlington Heights, IL) was used to detect the primary antibody; otherwise, immune complexes were visualized with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and chemiluminescence detection reagents (ECL Western blotting detection system; Amersham). For ATPase assays, total membranes were prepared by centrifugation of lysate at 100,000 g for 1 h. Membranes were resuspended in buffer containing 250 mM sucrose, 20 mM Hepes, pH 7.5, and vanadate-sensitive ATPase activity was assayed in the presence and absence of 100 \muM vanadate, as described (Chang and Sluyman, 1991).

Indirect Immunofluorescence

Immunofluorescent staining of cells was done essentially as described (Rose et al., 1990). For ATPase staining, mid-log cultures were shifted to 37°C for 1 h, before fixation with 4.4% formaldehyde in 0.1 M K phosphate, pH 6.5, for 2 h at room temperature. Cells were permeabilized with methanol and acetone before staining with affinity-purified rabbit anti-ATPase antibody or mouse anti-ATPase antibodies, followed by a Texas red- or Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories).

Plasmid Construction and Molecular Biology Techniques

High copy suppressors of \textit{pma1} cells were isolated by transforming ACY7 with a yeast 2\# library (Connelly, C., and P. Hieter, personal communication). Plasmids were rescued from yeast transformants (Hoffman and Winston, 1987). A 2-kb Xhol-Sacl fragment containing \textit{AST1} was subcloned from pAC21 (the original isolate from the 2\# library) into centromeric and high-copy-number plasmids. Disruption of PE/\textit{ATPS} was accomplished by transformation with the 4.7-kb EcoRi-XhoI fragment of pASI73 (Sachs, A., unpublished data). \textit{AST1} deletion plasmids were constructed by using PCR to amplify sequences from pAC21. pAC69 was cloned by converting a 1.9-kb KpnI-BamHI PCR...
Table 1. Yeast Strains and Plasmids Used in This Study

| Yeast Strain | Genotype | Source |
|--------------|----------|--------|
| L3852*       | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | Antebi and Fink (1992) |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACX24* This work |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACY7* * |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACY8* * |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACY12* * |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACX21* 6D * |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACX18* 4D * |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACX19* 4A * |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACX19* 4C * |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACX7* 8A * |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACX7* 8B * |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACX7* 8D * |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACX30* 2B* |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACY21* |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACY22* |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACY49* |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACY63* |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACY65* |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACY69* |
| L3854        | Mat a his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 | ACY19* |

**Table LEGEND:**
- *: All strains marked with asterisks are isogenic with L3852.
- **pAC21**: AST1 URA3 2 µ
- **pAC22**: AST1 URA3 CEN
- **pAC49**: AST1 URA3 2 µ
- **pAC63**: AST2 URA3 2 µ
- **pAC56**: AST1::myc URA3 CEN
- **pAC64**: AST1::myc LEU2 CEN
- **pAC65**: AST1::myc URA3 2 µ
- **pAC69**: ast1:URA3 pBluescript
- **pAC19**: ast1:URA3 pBluescript
- **pAC62**: ast1:URA3 pBluescript

**Results**

Three Temperature-sensitive pmal Mutants Are Defective for ATPase Stability

We characterized three temperature-sensitive pmal mutants that are defective in plasma membrane ATPase stability. Temperature-sensitive mutations were identified by plasmid shuffle after in vitro mutagenesis of PMAI (Boeke et al., 1987). Two of the three mutations (pmal-7 and pmal-8) were then integrated into the genome, replacing wild-type PMAI, by pop-in, pop-out gene replacement. As shown in Fig. 1A, pmal-7 cells grew at the permissive temperature (25°C or 30°C), but became growth-arrested at 37°C. This growth phenotype is reversible since the cells resumed growth when the plate was shifted back to the permissive temperature. Sequencing of the pmal mutation revealed two nucleotide changes in pmal-7 resulting in changes at Pro434→Ala, lying near the conserved catalytic phosphorylation domain, and Gly789→Ser, predicted to lie in a cytoplasmic loop between transmembrane segments 8 and 9. Two additional alleles, pmal-8 and pmal-9, caused similar temperature-sensitive growth arrest; pmal-8 has a single change at Gly783→Ala, and pmal-9 has two missense mutations at Pro198→Ser and Thr837→Ile. Fig. 1B shows that the time course of growth arrest of pmal-7 cells in liquid culture was slow, with the first detectable decrease in the growth rate occurring at >6 h after shifting the cells to 37°C.

Western blot analysis of the steady state level of ATPase protein revealed that pmal-7 cells at the permissive temperature have ~30% of the ATPase protein of isogenic wild-type cells (Fig. 2A). After a shift to 37°C, there was a dramatic loss of ATPase protein in pmal-7 cells, with ~20% of that seen at 25°C remaining after 6 h (Fig. 2A, middle, a vs c). Since newly synthesized ATPase is degraded in the mutant (see below), the ~20% remaining appears to represent dilution of preexisting surface ATPase, since the cells undergo about two doublings in 6 h. A similar loss of ATPase protein was observed when pmal-8 and pmal-9 cells were shifted to the restrictive temperature.

After 6 h at 37°C, vanadate-sensitive ATPase activity of pmal-7 cells also decreased, although to a lesser extent than
of proteins involved in quality control and plasma membrane delivery. \textit{pma1-7} cells were transformed with a multicopy yeast library (Connelly, C., and P. Hieter, unpublished data) and plated at 37°C. Plasmids were rescued from transformants that grew at 37°C. Several genes were isolated by this selection process, including \textit{PMAI}. One of these genes, \textit{AST1} (ATPase stabilizing), was chosen for further analysis because it suppressed the growth arrest phenotype of all three \textit{pma1} alleles, as well as two \textit{pma1} alleles previously characterized by Cid and Serrano (1988) [Ala547→Val and Gly254→Ser, but not Thr212→Ile or the double-mutant Asp91→Tyr, Glu92→Lys]. \textit{pma1-7} cells could grow at 37°C when transformed with \textit{AST1} on a multicopy 2μ plasmid or even on a low copy centromeric plasmid (Fig. 1 A).

Loss of mutant ATPase protein and activity was suppressed by multicopy \textit{AST1}. Quantitation of Western blots showed that the steady-state level of ATPase at 25°C was increased in \textit{pmal-7[AST1 2μ]} cells compared with \textit{pmal-7} cells (Fig. 2, left a vs middle a). Furthermore, the presence of multicopy \textit{AST1} decreased the loss of mutant ATPase protein after shifting to 37°C for 6 h (Fig. 2, left, b vs c). ATPase activity in membranes from \textit{pmal[AST1 2μ]} cells was also increased, reflecting increased ATPase stability; multicopy \textit{AST1} did not directly affect ATPase specific activity (Fig. 2 B). By contrast with \textit{pmal-7} cells, shifting \textit{PMAI} cells to 37°C had no effect on the steady-state ATPase level or ATPase activity (Fig. 2, right).

\textbf{Newly Synthesized ATPase in \textit{pmal-7} Cells Is Degraded in the Vacuole}

Loss of mutant ATPase could result either from degradation of preexisting ATPase from the cell surface or from degradation of newly synthesized ATPase. The slow time course of growth arrest (Fig. 1 B) and loss of steady-state ATPase activity (Fig. 2) supported the latter hypothesis. Indeed, pulse-chase experiments (Fig. 3 A) show that newly synthesized ATPase in the \textit{pmal-7} mutant was degraded with a half-time of ~20 min.
Figure 4. Newly synthesized mutant ATPase is degraded before delivery to the plasma membrane. Pulse-chase analysis of temperature-sensitive mutants at 37°C. (Top to bottom) pma1-7 (ACY7); pma1-7 sec6-4 (ACX19-4D); PMAI end3-1 (ACX 19-4C); pma1-7 end3-1 (ACX19-4A); and pma1-7 see18-1 (ACX21-6D). pma1-7 cells were shifted to 37°C for 5 min before labeling, while end3-1 and sec6-4 cells were shifted for 10 and 30 min, respectively.

Mutant ATPase Is Not Delivered to the Plasma Membrane, but It Goes Directly to the Vacuole

The pathway by which mutant ATPase is delivered to the vacuole for degradation was studied using a series of temperature-sensitive strains that are blocked in intracellular transport at discrete steps of the secretory pathway (Novick et al., 1981). Pulse-chase experiments showed stabilization of mutant ATPase by inhibition of export from the ER in sec18-1 cells at 37°C (Fig. 4). By contrast, mutant ATPase is not stabilized by sec6-4, a mutation that results in inhibition of secretory vesicle fusion with the plasma membrane. Furthermore, when internalization from the cell surface was inhibited by end3-1 (Fig. 4) or end4-1 (not shown), no stabilization of newly synthesized mutant ATPase was seen. Since end4-1 results in a temperature-sensitive block in endocytosis, whereas end3-1 cells are defective in internalization at all temperatures (Raths et al., 1993), these data indicate that mutant ATPase is degraded before arrival at the plasma membrane.

Multicopy AST1 Stabilizes Mutant ATPase during Intracellular Transport

The idea that delivery to the vacuole occurs before arrival at the plasma membrane was further supported by the kinetics of degradation of newly synthesized ATPase. In Fig. 5 A, pma1-7 cells were pulse labeled with [35S]Cys and Met at 25°C, and were then shifted to 37°C at various times of chase. ATPase was immunoprecipitated, analyzed by SDS-PAGE, and quantitated by phosphorimaging. Even at the permissive temperature, a fraction of newly synthesized ATPase was degraded. The rate of degradation was increased when the cells were shifted from 25 to 37°C at 5 min of chase.
ATPase degradation occurs at a specific interval during the chase. Thus, by ~30 min of chase, intracellular transport of the absence and presence of AM/ (Fig. 6). At 37°C, phosphorylation of mutant ATPase appeared defective since the electrophoretic mobility of mutant ATPase was seen during the chase in both 25°C and 37°C. (a) Chase at 25°C; (c) samples shifted to 37°C after 5 min of chase; (Δ) samples shifted to 37°C after 30 min of chase.

However, after 30 min of chase at 25°C, ATPase degradation was no longer increased by shifting to 37°C, i.e., increased ATPase degradation occurs at a specific interval during the chase. Thus, by ~30 min of chase, intracellular transport of mutant ATPase has progressed beyond the (Golgi) compartment from which delivery to the vacuole can occur.

In pmal-7 cells overexpressing ASTI, a 30-min chase at 25°C also allowed escape from increased ATPase degradation at 37°C. However, multicopy ASTI decreased mutant ATPase degradation at all chase times (Fig. 5 B).

Fig. 6 shows an SDS polyacrylamide gel comparing the stability and electrophoretic mobility of newly synthesized ATPase in pmal-7 cells in the absence and presence of multicopy ASTI. Previous work has shown that wild-type ATPase undergoes a decrease in electrophoretic mobility during intracellular transport; the mobility change is caused by kinase-mediated phosphorylation, not glycosylation (Chang and Slayman, 1991). At 25°C, a shift in the electrophoretic mobility of mutant ATPase was seen during the chase in both the absence and presence of ASTI (Fig. 6). At 37°C, phosphorylation of mutant ATPase appeared defective since the electrophoretic mobility was unchanged (Fig. 6, arrowhead). Nevertheless, multicopy ASTI caused stabilization of mutant ATPase at 37°C.

**Figure 6.** High copy ASTI stabilizes newly synthesized mutant ATPase. Pulse-chase analysis of pmal-7 cells with and without multicopy ASTI (pAC21) at 25°C and 37°C. At 5 min and 1 h of chase, cells were lysed, and ATPase was immunoprecipitated, separated by SDS-PAGE, and analyzed by phosphorimaging. At 37°C, ATPase is stabilized by high copy ASTI, but it does not undergo a mobility shift (arrowhead).

Molecular Characterization and Deletion of ASTI

ASTI was cloned and sequenced. Physical mapping revealed that ASTI lies on the left arm of chromosome II between CDC27 and ILS1. The ASTI open reading frame of 1,289 nucleotides predicts a 429–amino acid polypeptide (Fig. 8). The predicted polypeptide does not have an amino-terminal signal sequence nor any apparent transmembrane domains. Database searches revealed that ASTI is 26% identical to an uncharacterized yeast open reading frame RFI095 on chromosome XIII (Behrens et al., 1991), and 70% identical to a second orf on chromosome V (Dietrich, F., and D. Botstein, unpublished data). The second orf has functional similarity to ASTI since it was able, when expressed in high copy (pAC63), to suppress growth arrest of pmal-7 (Fig. 1 A); it was named AST2. An alignment of the three genes is shown in Fig. 8. These data suggest that ASTI belongs to a family of proteins with overlapping function.

Diploid strain ACX24 was transformed with the Asp718RK sacII fragment of pAC69 to delete precisely the ASTI open reading frame and replace it with a URA3 marker. Sporulation and tetrad dissection resulted in four viable spores, 2:2 Ura+:Ura− and growth of PMAI cells was not affected. A strain carrying deletion of both ASTI and AST2 was viable and displayed no growth defect. However, a synthetic growth defect was observed in astl Δpmal-7 cells at 30°C (Fig. 9 A), and the rate of ATPase degradation was increased in these cells (Fig. 9 B). Since one of the physiologic roles of Pmal is to buffer cytosolic pH, the effect of buffering the medium was tested. Fig. 9 A shows that astl Δpmal cells grew better in medium buffered to pH 5 when environmental stress and the demand for Pmal was reduced (buffering the medium cannot, however, rescue pmal-7 cells from growth arrest at 37°C). The synthetic growth defect represents genetic evidence for interaction between PMAI and ASTI.

**PMAL**

### pmal-7

**pmal-7<AST1 2μ>**

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**Figure 5.** Degradation of newly synthesized mutant ATPase occurs at a discrete step during intracellular transport. pmal-7 (ACY7) cells were pulse-labeled for 2 min with [35S]Cys and Met, and chased at 25°C, as described in Materials and Methods. At 5 and 30 min of chase, aliquots were shifted to 37°C, and incubation continued. ATPase was immunoprecipitated from cell lysates, analyzed by SDS-PAGE, and quantitated by phosphorimaging. At 37°C, ATPase is stabilized by high copy ASTI, but it does not undergo a mobility shift (arrowhead).

**Chase time (min)**

| Chase time (min) | 0 | 20 | 40 | 60 | 80 | 100 | 120 |
|------------------|---|----|----|----|----|-----|-----|
| pmal-7           | □ | □  | □  | □  | □  | □   | □   |
| pmal-7<AST1 2μ>  | □ | □  | □  | □  | □  | □   | □   |

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**Figure 6.** High copy ASTI stabilizes newly synthesized mutant ATPase. Pulse-chase analysis of pmal-7 cells with and without multicopy ASTI (pAC21) at 25°C and 37°C. At 5 min and 1 h of chase, cells were lysed, and ATPase was immunoprecipitated, separated by SDS-PAGE, and analyzed by phosphorimaging. At 37°C, ATPase is stabilized.
Ast1 Protein Is Membrane-associated and Is Localized to Multiple Membrane Compartments

To characterize Ast1 further, a c-myc epitope was engineered at the amino terminus. Constructs with either a single copy or three tandem copies of the 11-amino acid epitope behaved as wild-type Ast1 in the ability to suppress growth arrest of pmal1Δ. By Western blot (Fig. 10 A), myc-tagged Ast1 protein was visualized as a single band of \( M_r \sim 49 \text{kD} \). Upon fractionation of cell lysate, essentially all Ast1 protein was associated with a 100,000-g membrane pellet. Consistent with the behavior of a peripheral membrane protein, Ast1 was progressively extracted into a soluble fraction with 0.5 M NaCl and 0.1 M Na carbonate, pH 11.5 (Steck and Yu, 1973). Ast1 was also insoluble in 1% Triton X-100, suggesting that it may be a part of a protein or lipid complex.

Indirect immunofluorescence was used to localize Ast1.

**Figure 7.** ATPase localization in PMA1, pmal-7 pep4Δ, and pmal-7[AST1 CEN] cells. Nomarski imaging and indirect immunofluorescent localization of ATPase with polyclonal anti-ATPase antibody followed by Texas red-conjugated goat anti-rabbit IgG. Exponentially growing pmal-7 (ACY7) cells bearing pAC22 (AST1 URA3 CEN), pmal-7 pep4Δ (ACY12) cells, and PMA1 (L3852) bearing vector only, were harvested and resuspended in fresh synthetic complete medium without uracil. The cells were shifted to 37°C for 1 h before fixation. There is striking intracellular staining in pmal-7 pep4Δ cells, which overlies the vacuolar membrane (middle panel). The vacuole is seen by Nomarski optics as cell indentations. The majority of pmal-7[AST1 CEN] cells display a surface staining pattern, although some intracellular staining is also present.
Figure 8. Alignment of AST1, AST2, and a third homologous gene. Sequences were aligned with the Megalign program (DNastar, Madison, WI) using the Clustal method (Higgins and Sharp, 1989). Identical amino acid residues are boxed and hyphens indicate gaps introduced to maximize alignment. AST1 sequence is available from EMBL under accession number X81843. AST2 sequence is unpublished data from F. Dietrich and D. Botstein (Stanford University, Stanford, CA). The peptide sequence for RF1095 is found in the SWISS-PROT protein sequence database under accession number P28625.

Discussion

We have characterized a novel class of mutants that is defective for delivery of newly synthesized [H+]ATPase to the plasma membrane. Based on in vitro assay of ATP hydrolysis, the mutants do not appear to have substantially defective catalytic activity. Furthermore, the cells can grow if a fraction of newly synthesized ATPase is delivered to the cell surface at the permissive temperature (Fig. 6), or in the presence of multicopy AST1 or several other AST genes. This behavior is similar to that observed for the temperature-sensitive ΔF508 mutant of the cystic fibrosis transmembrane conductance regulator, which is defective in intracellular transport but functional once delivered to the plasma membrane (Welsh and Smith, 1993). The slow time course of growth arrest of pmalΔ cells at 37°C is consistent with continuing function and a slow turnover rate of preexisting ATPase at the plasma membrane. A comparably slow time course of growth arrest is also observed when ATPase expression is shut off in cells where the PMA1 is under the control of a galactose-dependent promoter (Cid et al., 1987).

Loss of mutant ATPase is caused by rerouting of the newly synthesized enzyme to the vacuole, based on indirect immunofluorescence localization of mutant ATPase (Fig. 7). This interpretation is further supported by pulse-chase experiments, which demonstrate ATPase stabilization in a pep4A mutant (Fig. 3). However, PEP4 disruption causes ATPase stabilization without suppressing growth arrest.
consistent with the idea that ATPase delivery to the plasma membrane is required for growth. Since the end3 mutation, which blocks internalization from the cell surface, does not circumvent ATPase degradation (Fig. 4), it appears that delivery of mutant ATPase to the vacuole occurs without previous arrival at the plasma membrane. This conclusion is supported by the kinetics of ATPase degradation (Fig. 5), where newly synthesized ATPase was degraded during a specific interval of intracellular transport, and it was protected when transported beyond that step.

The behavior of the ATPase in temperature-sensitive pMal mutants bears directly on the mechanism of membrane protein transport in yeast. One model for the delivery of mutant ATPase to the vacuole is that it occurs by default. Recently, it has been proposed that delivery to the vacuole is the default pathway for membrane protein traffic in yeast (Stack and Emr, 1993; Nothwehr and Stevens, 1994), implying that cell surface membrane proteins in yeast have specific targeting signals. According to this model, mutant ATPase would be delivered to the vacuole because the plasma membrane targeting signal has been lost.

An alternative model is that defective proteins are spe-
cifically identified and targeted to the vacuole by a dedicated quality control mechanism. A mechanism for degradation of misfolded or unassembled proteins within the yeast ER has been reported (McCranken and Kruse, 1993), and some pma1 mutants do accumulate ATPase at the ER (Harris et al., 1994). Although a mechanism for clearing unwanted cell surface proteins by endocytosis and subsequent degradation in the vacuole has been well characterized (Trowbridge et al., 1993; Davis et al., 1993), none has been uncovered for clearing defective plasma membrane proteins from the biosynthetic pathway by “direct” delivery to the vacuole. Nevertheless, the mutant ATPase described in this study is not the only example of a plasma membrane protein that is defective for cell surface delivery and is routed instead to the vacuole for degradation. In mammalian cells, a pentameric T cell receptor complex lacking 2 subunits is not efficiently transported to the plasma membrane, but is delivered instead to lysosomes (Minami et al., 1987). This observation is especially intriguing since most partial complexes and individual subunits of the T cell receptor are degraded at the ER (Klausner and Sitia, 1990). In addition, certain mutations in Ste3, the yeast a mating factor receptor, cause PEP4-dependent degradation without transport to the cell surface (Horecka, J., and G. Sprague, personal communication). At the present time, we cannot distinguish between default and quality control models for vacuolar delivery of mutant ATPase.

Overexpression of AST1 stabilizes mutant ATPase protein without directly affecting ATPase activity (Fig. 2). The mutant protein is stabilized because there is increased ATPase delivery to the plasma membrane (Fig. 7), and thus growth arrest is suppressed.

Based on our understanding of ATPase traffic in the pma1 mutant, we reason that Astl must affect the ATPase at a step in intracellular transport before delivery to the vacuole, e.g., at the ER, Golgi, or endosome. Kinetic analysis indicates that mutant ATPase is stabilized by Astl at 30 min of chase (Fig. 5). Interestingly, newly synthesized ATPase moves into a Triton-insoluble fraction during intracellular transport (Chang, A., unpublished data), perhaps associating with Astl, which is also Triton-insoluble. Astl behaves like a peripheral membrane protein and is localized to a cytoplasmic compartment(s), as well as to the plasma membrane (Fig. 10). These findings are consistent with a role for Astl in membrane traffic, in which it interacts with the cytoplasmic surface of one or more organelles of the secretory pathway, perhaps sculpting between cytoplasmic compartments and the plasma membrane.

Although Astl does not have sequence similarity with known chaperones, a simple model to explain how multicopy Astl allows cell surface delivery of mutant Pma1 is that Astl acts as a novel targeting/chaperone molecule. The synthetic growth defect seen in pma1-7 astlΔ cells (Fig. 9) provides genetic evidence for interaction between Astl and Pma1. In addition, we have been able, under specific conditions, to coimmunoprecipitate Astl with the ATPase, supporting the idea that the two proteins may directly interact (Chang, A., unpublished data). Thus, Astl could facilitate delivery to the cell surface by interacting specifically with the ATPase.

Alternatively, Astl could play a general role in plasma membrane targeting as a component of the targeting machinery. Similar to a model that has been proposed for protein sorting in epithelial cells (Simons and Wandinger-Ness, 1990), Astl might participate in cell surface targeting by interacting with several plasma membrane proteins and lipids at the cytosolic side to form a subdomain that could bud to form a surface-directed vesicle. By either a specific or general model for Astl function, defective targeting of mutant ATPase would result from diminished interaction with Astl.

Deletion of AST1 and AST2 did not significantly alter cell viability. The viability of the double-deletion strain is consistent with the hypothesis that there are additional members in the AST gene family. Future genetic and biochemical analyses should reveal the molecular mechanism for AST1 function in membrane traffic.

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