MULTIPLE DEGRADATION PATHWAYS FOR MISFOLDED MUTANTS OF THE YEAST PLASMA MEMBRANE ATPASE, PMA1

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Running title: Multiple degradation pathways for Pma1 mutants

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Abstract

To understand protein sorting and quality control in the secretory pathway, we have analyzed intracellular trafficking of the yeast plasma membrane ATPase, Pma1. Pma1 is ideal for such studies because it is a very abundant polytopic membrane protein, and its localization and activity at the plasma membrane are essential for cell viability and growth. We have tested whether the cytoplasmic amino and carboxy terminal domains of Pma1 carry sorting information. As the sole copy of Pma1, mutants truncated at either N- or C-termini are targeted at least partially to the plasma membrane and have catalytic activity to sustain cell viability. The mutants are also delivered to degradative pathways. Strikingly, N- and C-terminal Pma1 mutants are differentially recognized for degradation at distinct cellular locales. C-terminal mutants are recognized for destruction by ER-associated degradation. By contrast, N-terminal mutants escape detection by ERAD entirely, and undergo endocytosis for vacuolar degradation after apparently normal cell surface targeting. Both N- and C-terminal mutants are conformationally abnormal, as revealed by increased sensitivity to tryptic cleavage, but are able to assemble to form oligomers. We propose that different quality control mechanisms may assess discrete domains of Pma1 rather than a global conformational state.

Introduction

There are multiple mechanisms for recognition and destruction of misfolded, and/or unassembled proteins in the secretory pathway (1). ER-associated degradation (ERAD) is one major pathway that uses conformational recognition by chaperones to select defective proteins for ubiquitination via ER-associated ubiquitin ligases; these proteins then undergo dislocation from the endoplasmic reticulum, and destruction by the 26S proteasome (2). In addition to ERAD, however, Golgi-based targeting of proteins for lysosomal/vacuolar destruction has been described. Moreover, there is accruing evidence for plasma membrane quality control in which selected proteins are identified for turnover from the cell surface via endocytosis and lysosomal/vacuolar delivery (3). The molecular mechanisms by which proteins of the secretory pathway are recognized by each of these degradative pathways is of considerable interest.

We have studied the yeast plasma membrane ATPase, Pma1, as a model protein to understand protein sorting and quality control in the secretory pathway. Pma1 is a H+−ATPase which pumps protons out of the cell to generate the membrane potential and regulate cytosolic pH (4). Therefore, proper delivery of Pma1 to the cell surface is essential for cell viability. Pma1 belongs to the P-type ATPase family of ion transporters which includes the Na+K+−ATPases of mammalian cells (5,6). The 8Å and 2.6 Å structures of Neurospora Pma1 and
mammalian sarcoplasmic reticulum Ca\(^{++}\) ATPase have been determined (6,7), and based on these data, a structural model for fungal Pma1 has been proposed (8). Like other P-type ATPases, Pma1 is embedded in the membrane by 10 transmembrane segments, and there are 3 major cytoplasmic domains which contain the nucleotide-binding and catalytic phosphorylation sites and have critical roles in catalytic activity. The amino and carboxy termini are cytoplasmic; in several family members, these domains represent regulatory domains (9). For instance, the fungal H\(^{+}\) pumps have C-terminal regulatory domains that modulate activity by kinase-mediated phosphorylation (10). Pma1 forms a hexamer (7), or two hexamers may come together to form a dodecamer (11), and the C-terminal domain has also been proposed to participate in the oligomeric structure of Pma1 (8).

Newly synthesized wild-type Pma1 undergoes efficient intracellular transport to the plasma membrane where it acquires remarkable longevity (12). ERAD destroys misfolded Pma1 mutants; however, some Pma1 mutants have been characterized that escape ER retention and ERAD (1). For example, Pma1-10 is misfolded, and yet properly targeted to the plasma membrane; however, its turnover from the plasma membrane is increased by comparison with wild-type Pma1 (13). Of relevance to the question of ER export versus retention is the identification of ER export signals in the cytoplasmic C-terminal domain of some polytopic membrane proteins (14). Diacidic and dihydrophobic motifs appear to promote entry into COPII coated vesicles, in some cases, acting combinatorially (14,15). Moreover, arginine-based sorting motifs have been identified in the cytosolic domains of multimeric membrane proteins that become masked upon proper protein assembly, leading to ER exit of the complexes (16). To ask whether the cytoplasmic N- and C-terminal domains of Pma1 carry sorting motifs, we constructed mutants truncated at either termini and analyzed their intracellular transport and stability at the cell surface.

In this paper, we report that mutants at either N and C-termini are conformationally abnormal, as revealed by increased sensitivity to tryptic cleavage. Nevertheless, these Pma1 mutants are differentially recognized for degradation at distinct cellular locales. N-terminal mutants escape ERAD entirely; after apparently normal targeting to the cell surface, they are unstable at the cell surface and undergo endocytosis for vacuolar degradation. By contrast, C-terminal mutants are recognized by ERAD, although some of the protein is able to escape to the plasma membrane and sustain cell viability. Although trafficking through the secretory pathway is clearly affected by C-terminal truncation, Pma1 oligomerization is not impaired. We propose that different quality control mechanisms may assess discrete domains of Pma1 rather than a global conformational state.

**Methods**

**Media and Strains**

Standard yeast media and genetic manipulations were as described (17). Yeast transformations were performed by the lithium acetate method (18). Strains are W303-derived (ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100; also referred to as F1105) unless otherwise noted. Strains with C-terminal and N-terminal truncations in PMA1 were generated by PCR-based gene modification (19).

SSX5 (Δ40C diploid) is a heterozygote with one wild-type PMA1 and one chromosomal copy of a truncation of the carboxy terminal 40 residues of PMA1 followed by a triple HA tag; to generate the strain, a W303 diploid was transformed with products made by PCR amplification of pFA6a-3HA-HIS3MX6 (19) using primers SS11 & SS13. All primer sequences are available upon request. SSX5-1a is a MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 pma1Δ40C-HA::HIS3 haploid generated by sporulation and tetrad dissection of SSX5. SSX9 (Δ30C diploid) is a heterozygote with wild-type PMA1 and a truncation of the carboxy terminal 30 residues of PMA1 followed by a triple HA tag; this strain was generated as
described for Δ40C except that primers SS11 & SS19 were used to generate PCR products for transformation. SSX9-1b is a MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 pma1Δ30C-HA::HIS3 haplotype generated by sporulation and tetrad dissection of SSX9. pma1Δ30C-HA::HIS3 fails to grow at 37°C; this phenotype is complemented by PMA1. SSY3 was similarly constructed except that primers SS11 and SS10 were used to generate PCR products for transformation of haploid W303 (F1105). Correct integration was confirmed by checking insertion of the HA tag by Western blot and/or PCR. SSY38 (MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 pma1Δ30C-HA::HIS3 end4Δ::clonNAT) was made by transformation of SSX9-1b with PCR products to knockout END4 marked by resistance to clonNAT; primers 294 and 295 were used to amplify pAG25. SSY39 (MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 pma1Δ30C-HA::HIS3 pep4Δ::LEU2) was made by transformation of SSX9-1b with a PEP4 disruption construct pSN273 cut with SacI and XhoI (20). SSY24 was constructed by first transforming MHY1703 (MATa his3Δ200 leu2-3,11 ura3-52 lys2-801 trp1-1 doa10Δ::HIS3 hrd1Δ::LEU2) (21) with pHT6 to swap the HIS3 marker for a TRP1 marker to generate SSY23 (22); SSY23 was then crossed with SSX5-1a, the diploid was sporulated and dissected to produce a MATa strain that is also pma1Δ40C::HIS3 hrd1Δ::LEU2 doa10Δ:: TRP1. SSY25 was made by first transforming MHY552 (MATa his3Δ200 leu2-3,11 ura3-52 lys2-801 trp1-1 ubc6Δ::HIS3 ubc7::LEU2) with pHT6 to replace the HIS3 marker with TRP1, generating SSY15; SSY15 was then crossed with SSX5-1a, the diploid was sporulated and dissected to produce a MATa strain that is also pma1Δ40C::HIS3 ubc6Δ::TRP1 ubc7Δ::LEU2. Similarly, SSY37 (MATa ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 pma1Δ30C-HA::HIS3 ubc6Δ::TRP1 ubc7Δ::LEU2) was made by crossing SSX9-2b (MATa) with SSY15. ACX134 is a W303 diploid transformed with PCR products to make a PMA1 knockout marked by resistance to clonNAT; primers 397 and 398 were used to amplify the template pAG25 bearing natMX4, conferring resistance (obtained from Charlie Boone, University of Toronto, Toronto, Canada) to the antibiotic nourseothricin (clonNAT, Werner BioAgents) (23). The knockout was confirmed by PCR. SSY29 is a MATa pma1Δ::clonNATþ [pXG29] haplotype generated upon transformation of ACX134 followed by sporulation and tetrad dissection.

KKY39 is a heterozygous diploid with wild-type PMA1 and another chromosomal copy of an HA-tagged pma1 mutant with an N-terminal truncation of 40 residues; the strain was generated by transformation of a W303 diploid with products made by PCR amplification of pFA6a-HIS3MX6-pGAL1-3HA (19) using primers 305 & 272. KKY44 is a HIS3⁺ haplotype Δ40N strain generated by sporulation and dissection of KKY39. KKY40 is a heterozygous diploid with GAL1-HA-Δ60N-pma1, generated as described for Δ40N except that primers 306 & 272 were used for PCR amplification. KKY37 is a heterozygous diploid with wild-type PMA1 and GAL1-HA-PMA1, constructed as described for Δ40N except that primers 306 & 272 were used for PCR amplification. KXX24-1B is a HIS3⁺ haplotype HA-PMA1 strain generated by sporulation and dissection of KKY37. KKY93 is pma1Δ::clonNAT⁺ [pKK107]; KKY94 is pma1Δ::clonNAT⁰ [pKK109]. Both strains were constructed by plasmid shuffle: SSY29 was transformed with pKK107, a LEU2-marked centromeric plasmid bearing HA-tagged wild-type PMA1 or pKK109, a LEU2-marked centromeric plasmid bearing the HA-tagged 4D2E/A mutant; loss of wild-type PMA1 (pXG29) was selected on 5-fluoro-orotic acid (24).

Other yeast strains used to examine the behavior of plasmid-borne pma1-4D2E/A are L3852 (MATa his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2) and isogenic ACY67 (MATa his3Δ200 lys2Δ201 leu2-3,112 ura3-52 ade2 pep4), and RH266-1D (MATa end3-1 leu2 his4 ura3 bar1-1) (25).

**Molecular Biology**

pXG29 is a URA3-marked centromeric plasmid constructed by placing a 5 kb HindII-HindIII fragment containing PMA1 promoter and coding sequence from pAC4 into pRS316 (26).
pKK107 and pKK109 are LEU2-marked centromeric plasmids bearing HA-tagged \( \text{PMA1} \) and HA-tagged \( \text{pma1-4D2E/A} \), respectively, under control of the native promoter. pKK107 is derived from a \( \text{URA3} \)-marked YIp bearing a 4.5 kb SacI-XhoI fragment containing \( \text{MET3-HA-PMA1} \) (pKK98). A 0.5 kb SacI-XmaI fragment was excised to replace the \( \text{MET3} \) promoter with an 0.8 kb fragment containing \( \text{PMA1} \) promoter sequence amplified with introduced sites using primers 395 and 396. pKK103, a \( \text{URA3} \)-marked YIp bearing \( \text{pma1-4D2E/A} \) under the control of \( \text{MET3} \), was constructed by using Quikchange site-directed mutagenesis (Stratagene, LaJolla, CA) was used to introduce D\( _{39-41} \)/A with primers 393 & 394 followed by D\( _{42} \)A\( _{48} \)A\( _{49} \) changes with primers 399 & 400. PCR mutagenesis was performed using as template pKK74, an excised 1.8 kb fragment bearing \( \text{MET3-HA-PMA1} \) up to the unique BamH1 site, derived from pCC2 (27). Mutations were confirmed by DNA sequencing. pKK109 was similar to that of pKK107 except that the \( \text{MET3} \) promoter was excised as a 0.5 kb SacI-XmaI fragment for replacement with \( \text{PMA1} \) promoter sequence.

For co-IP experiments, inducible constructs were used. pND542 is a LEU2-marked centromeric plasmid bearing HA-tagged wild-type Pma1 under the control of the \( \text{GAL1} \) promoter, described in (28). pWQ3 is a \( \text{URA3} \)-marked centromeric plasmid bearing myc-tagged wild-type Pma1 under \( \text{GAL1} \) control, pSS16 is a \( \text{URA3} \)-marked centromeric plasmid bearing \( \text{GAL-myc-pma1A40C} \); to construct this, pWQ3 was used as a PCR template to introduce a stop codon TAA after Thr\( _{878} \) using primers SS31 and SS32. pSS17 is a LEU2-marked centromeric plasmid bearing \( \text{GAL-HA-pma1A40C} \); this was constructed by using pND542 as template for PCR to introduce a stop codon after Thr\( _{878} \) using primers SS31 & SS32.

**Indirect immunofluorescence**

Indirect immunofluorescence was as described previously (29). Cells were fixed with 4% formaldehyde for 2h at room temperature. Cells were spheroplasted with zymolyase 100T (ICN) and permeabilized with methanol and acetone. Cells were stained with monoclonal anti-HA (Covance, Inc) or monoclonal anti-Pma1 (gift from John Aris, University of Florida), followed by fluorescent CY3, Texas Red and/or DTAF-conjugated secondary antibodies (Jackson Immunoreagents). Cells were visualized with an Olympus fluorescence microscope and images were collected with a Hamamatsu Orca CCD camera. Anti-Kar2 antibody was a gift from Mark Rose (Princeton University, Princeton, New Jersey).

**Protein induction, metabolic labeling, cell fractionation, trypsinolysis and Western blot**

For induction of \( \text{GAL1} \)-regulated constructs, cells were grown overnight in medium containing 2% raffinose. Mid-log cultures were then resuspended in medium containing 2% galactose for 2-4h. For \( \text{MET3} \)-regulated constructs, cells were grown overnight in medium with 600µM methionine to repress \( \text{MET3} \); exponentially growing cells were then washed with water and transferred to methionine-free to induce protein synthesis.

For metabolic labeling, cells were grown overnight without cysteine and methionine (usually minimal medium) to mid-log phase. Cells were resuspended in fresh medium at a density of 1 OD\( _{600} \)/ml and incubated at room temperature for 15 min before pulse-labeling with \( \text{Expre} \, ^{35} \text{S} \) (0.4 mCi/OD\( _{600} \) cells) (Perkin-Elmer). Cells were labeled for 10 min before chasing with an equal volume of synthetic complete medium supplemented with 20 mM methionine and cysteine. At various times of chase, aliquots were removed and added to 10 mM azide on ice. Lysate was prepared by vortexing with glass beads (30), and immunoprecipitation was normalized to acid-precipitable cpm. IPs with anti-HA or anti-Pma1 antibodies were analyzed by SDS-PAGE and fluorography.

Cell fractionation by Renografin density gradients were as described (31). RenoCal-76 was substituted for Renografin-76. Fourteen fractions were collected and diluted with buffer (50 mM Tris, pH 7.5, 1 mM EDTA), membranes were pelleted by centrifugation at 100,000 g for
1h, and analyzed by Western blotting. Markers for ER, Golgi and plasma membrane were localized using antibodies against Sec61 (from Randy Schekman, UC-Berkeley, Berkeley, CA), Gda1 (from Greg Payne, UCLA, Los Angeles, CA), and Gas1 (from Howard Riezman, University of Geneva, Geneva, Switzerland). Quantitation was performed using NIH Image on scanned Western blots.

Limited trypsinolysis was performed as described (32). Total membranes were generated by centrifugation of cell lysate at 100,000 g for 1h. Membranes were resuspended in buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 2 mM DTT), and incubated at 30°C at a 1:25 trypsin:protein ratio. At various times, 10% ice-cold trichloroacetic acid was added, protein was precipitated on ice for >20 min, precipitates were washed with cold acetone. Samples were analyzed by Western blot with anti-Pma1 and/or anti-HA antibodies. For Western blot of lysate, samples were normalized to protein content measured by Bradford assay. Immune complexes were visualized by an ECL detection system.

**Gel filtration chromatography**

Lysate (1.5 mg protein) was solubilized with 1% digitonin in 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA. Insoluble material was pelleted by centrifugation at 100,000 g for 1h. One ml of extract was fractionated over a gel filtration column (Sephacryl S300HR) with a range of 10^4 to 1.5 x 10^6 daltons using the FPLC system (Amersham Biosciences). A void volume of ~39 ml was estimated using blue dextran. Fractions (40 x 1 ml) were collected from 36 ml. Protein was precipitated with trichloroacetic acid, and every two fractions were pooled by analysis by Western blot. For determination of molecular weight, a high molecular weight calibration kit (Amersham Biosciences) was fractionated.

**Results**

**Pma1 truncation mutants**

Strains were constructed in which chromosomal PMA1 was truncated at the C-terminus. According to hydropathy predictions, removal of the last 40 amino acid residues, Δ40C, should result in loss of the entire C-terminal cytoplasmic domain; Δ30C, was also constructed to avoid possible perturbation of the preceding transmembrane segment. Heterozygous diploid strains were generated by PCR-mediated transformation to generate one chromosomal copy of a PMA1 truncation that were also HA-tagged at the C-terminus and HIS3-marked. Sporulation and tetrad dissection resulted in viable haploid Δ30C and Δ40C pma1 mutants. Haploid Δ30C and Δ40C strains were also readily generated by plasmid shuffle (not shown).

In parallel with truncations at the C-terminus, a series of truncations were also made at the N-terminus. These mutants made by PCR-mediated transformation of diploid cells are HA-tagged at the N-terminus, marked with HIS3, and under the control of the GAL1 promoter. Diploids were sporulated and dissected onto galactose-containing medium to determine the effect of N-terminal truncations on cell growth. Like the C-terminal truncations, strains without 40 residues at the N-terminus, Δ40N, are viable with no obvious growth defect (not shown); however, there is impaired trafficking (see below). Δ60N mutants are not able to support growth as the sole copy of Pma1 (not shown), in agreement with a previous report (33).

**Localization of Pma1 C-terminal truncation mutants**

We expected Pma1 C-terminal truncation mutants to localize at the plasma membrane because haploid cells with these mutations are viable. Indirect immunofluorescence localization of HA-tagged Δ40C was therefore surprising because significant perinuclear staining was seen with anti-HA antibody, overlapping with the ER marker Kar2 (Fig. 1A). By contrast, anti-Pma1 antibody revealed predominant plasma membrane staining (Fig. 1A) with faint perinuclear staining in most of these cells. Δ30 Pma1 localization was observed at the cell surface as well as in punctate spots; the cell surface and punctate staining pattern is similar with both anti-HA and anti-Pma1 antibodies (Fig. 1A, left panels). In a pep4
In which vacuolar proteolysis is prevented, Δ30C Pma1 staining was observed in vacuoles, seen as indentations by DIC, whereas vacuolar localization was not seen for Δ40C Pma1 (not shown). By contrast with the mutant strains, wild-type Pma1 HA-tagged at the N-terminus appeared exclusively localized at the plasma membrane (Fig. 2A); wild-type Pma1 HA-tagged at the C-terminus appeared predominantly cell surface-localized (Fig. 1A, top right panel).

To quantitate cell surface and intracellular Δ30C and Δ40C Pma1, cell lysate was fractionated on Renografin density gradients. As shown previously, Renografin density gradients effectively separate plasma membrane from intracellular membranes (31). The distribution of membrane markers analyzed by Western blot is shown in Fig. 1B, showing that the plasma membrane marker Gas1 (triangle) fractionates in denser gradient fractions 9-12 whereas intracellular membrane markers, both Golgi GDPase (open circle) and ER Sec61 (diamond), are in fractions 2-5 at the top of the gradient. Peak fractions containing HA-tagged wild-type Pma1 (square) are coincident with the Gas1 marker (Fig. 1B). Similarly, the preponderance of Pma1Δ30C is present in plasma membrane-enriched fractions but there is also a fraction coincident with intracellular membranes (Fig. 1B, right panel, square), consistent with the puncta seen by indirect immunofluorescence (Fig. 1A). Fractionation of Δ40C Pma1 and detection by anti-HA antibody results in a majority of Δ40C Pma1 (square) distributed in intracellular membrane fractions 2-5, coincident with the ER marker Sec61. Detection of Δ40C Pma1 with anti-Pma1 monoclonal antibody (closed circle) revealed the converse distribution with the majority in fractions 9-12 with a small shoulder trailing to the top of the gradient, in agreement with the indirect immunofluorescence results in Fig. 1A. These data are consistent with the idea that the HA epitope at the C-terminus of Δ40C Pma1 is removed after ER export, and the majority of Δ40C Pma1 is detected at the plasma membrane by the monoclonal antibody.

Localization of N-terminal truncation mutants

Localization of newly synthesized Δ40N was detected by indirect immunofluorescence by inducing synthesis of HA-tagged Δ40N Pma1 in a heterozygous diploid. Δ40N staining is at the cell surface as well as at the vacuole, seen as indentations by DIC microscopy, whereas newly synthesized wild-type Pma1 is exclusively at the plasma membrane (Fig. 2A). Δ60N staining appeared over vacuoles as well as intracellular dots surrounding vacuoles, and cell surface staining was undetectable (Fig. 2A). According to a proposed structural model (8), the N-terminal domain abuts the “A domain”, one of the major cytoplasmic domains proposed to have critical catalytic function, as well as membrane-spanning domains; it therefore seems plausible that removal of 60 residues leads to exposed hydrophobic regions and a nonfunctional molecule that cannot support viability. Because cell surface delivery was not detected and Δ60N Pma1 cannot sustain cell viability (not shown), this mutant was not studied further.

The residues between N-terminal 40 and 60 are predicted to form an alpha helix followed by a loop (8). Residues 41-52 DDIDALIEELQS, when modeled as an α-helical wheel, forms an amphipathic helix, with hydrophobic residues lying on one face of the helix and positively charged residues on the opposing face. This sequence is conserved in all fungal P-type ATPases, and has been proposed to play a role in cell surface delivery (33). Therefore, mutagenesis was performed to change four aspartate (D39-42) and two glutamate residues (E48-49) to alanine to test whether the charged face of the helix might mediate protein-protein interaction during protein sorting and intracellular transport; this mutant is called Pma1-4D2E/A. Fig. 2B shows that newly synthesized Pma1-4D2E/A is predominantly delivered to the cell surface, but localization at both the plasma membrane as well as in intracellular puncta was seen by indirect immunofluorescence (Fig. 2A). Pma1-4D2E accumulation in vacuoles was only obvious in pep4 cells, likely because degradation is efficient in PEP4+ cells (Fig. 2A). Pma1-
Both Pma1Δ30C and Pma1Δ40C truncation mutants are partially degraded by ER-associated degradation

Because of its accumulation at the ER, we tested whether HA-Pma1Δ40 is a substrate for ER-associated degradation (ERAD) by pulse-chase analysis. Cells were pulse-labeled with 35S-cysteine and methionine, and chased for various times. By contrast with wild-type Pma1 which has remarkable stability [(12) & Figs. 4A & B], Δ40C, detected by immunoprecipitation with anti-HA, is rapidly degraded (Fig. 3A). No stabilization was observed in pep4 or end4 cells, impaired in internalization from the plasma membrane (not shown), but newly synthesized HA-tagged Δ40C is stabilized in mutants of the ER-associated ubiquitin conjugating enzymes Ubc6 and Ubc7, and the ER-associated ubiquitin ligases Doa10 and Hrd1 (21) (Fig. 3A). These results suggest that degradation of HA-tagged Δ40C Pma1 requires ERAD machinery. Immunoprecipitation with anti-Pma1 antibody, which recognizes both tagged and untagged Δ40C, also shows loss of newly synthesized Pma1 (Fig. 3A, bottom panel).

Pulse-chase experiments show that newly synthesized Δ30C, like Δ40C, is also rapidly degraded (Fig. 3B). Accumulation of Δ30C Pma1 in pep4 cells by indirect immunofluorescence suggests vacuolar delivery and degradation (not shown). Localization in punctate structures seen by indirect immunofluorescence (Fig. 1A) could indicate Golgi or endosomal accumulation prior to vacuolar degradation. To test whether Δ30C Pma1 is transported for vacuolar degradation and whether it travels via the plasma membrane, pulse-chase experiments were performed in pep4Δ and end4Δ mutants. Surprisingly, degradation of newly synthesized Δ30C Pma1 over a 2h time course is not significantly affected by pep4 or end4 cells (Fig. 3B). However, stabilization of newly synthesized Δ30C Pma1 occurs in ubc6 ubc7 cells (Fig. 3B), even though punctate accumulation of Δ30C seen by indirect immunofluorescence is not in a typical perinuclear ER pattern (Fig. 1A). Thus, degradation of newly synthesized Δ30C occurs mostly via ERAD. It is also likely that some Δ30C undergoes vacuolar degradation on a time scale slower than that assayed during the 2h pulse-chase time course as vacuolar accumulation was seen in Δ30C pma1 pep4 cells by indirect immunofluorescence (not shown).

Trafficking pathways and quality control of N-terminal mutants

Although Δ40N Pma1 undergoes eventual vacuolar delivery as revealed by indirect immunofluorescence (Fig. 2A), pulse-chase experiment indicates that newly synthesized Δ40N Pma1 is stable for up to 2h chase (Fig. 4A). Taken together, these results are consistent with increased turnover of Δ40N Pma1 from the plasma membrane. Degradation of Pma1-4D2E/A is increased by comparison with wild-type Pma1 (Fig. 4B & C). Δ40N Pma1-4D2E/A degradation is inhibited in pep4 and end3-1 (Fig. 4C), indicating mutant Pma1 is delivered for vacuolar degradation by endocytosis after cell surface arrival. No effect on Pma1-4D2E/A degradation was detected when ERAD was prevented in doa10 hrd1 cells (Fig. 4C). Thus, it appears that Pma1-4D2E/A escapes detection by ER quality control.

Conformational condition of wild-type and mutant Pma1 and its effect on cell growth

The first step in ERAD is thought to involve conformational recognition of a misfolded protein (2). Because both Δ40C and Δ30C are at least partially ERAD substrates, the conformation of these mutants was assayed by limited tryptic digestion. Membrane fractions were incubated with trypsin for various times and tryptic fragments were visualized by Western blot. Previous work from Carolyn Slayman’s lab has shown that tryptic cleavage occurs initially at the N-terminus so it is possible to detect tryptic fragments with either anti-Pma1 or anti-HA antibody recognizing the C-terminal tag (35). Fig. 5A (top panel) shows Δ30C Pma1 has increased susceptibility to tryptic cleavage and a different digestion pattern from that of wild-type Pma1; blotting with anti-HA revealed a similar tryptic cleavage patterns as that seen with anti-Pma1 (not shown). These results
suggest a conformational difference between Δ30C and wild-type Pma1. Although the preponderance of Δ30C Pma1 is properly targeted to the plasma membrane (Fig. 1B), it appears that its conformational difference is detected by multiple quality control mechanisms as Δ30C undergoes ERAD (Fig. 3B) as well as vacuolar delivery.

No significant difference in trypsin sensitivity was readily detectable between Δ40C and wild-type Pma1 as assayed by Western blot with anti-Pma1 antibody (Fig. 5A, right lower panel). However, when the same blot was probed with anti-HA antibody, increased sensitivity of HA-tagged Δ40C Pma1 compared with wild-type Pma1 was visualized (Fig. 5A, lower left panel), suggesting that HA-Δ40C is conformationally distinct. Thus, it appears that the pool of Δ40C Pma1 that is recognized for ERAD is conformationally different from the pool that undergoes proper targeting to the plasma membrane. Interestingly, the trypsin assay reveals that accessibility of the Pma1 N-terminus is increased by truncation at the C-terminus.

Consistent with its trafficking defect, Pma1-4D2E/A has increased sensitivity to trypsin (Fig. 5B, right panel), even as it sustains viability as the sole copy of Pma1. Interestingly, Δ40N, is resistant to trypsin (Fig. 5B), and yet it is delivered to the vacuole (Fig. 2A).

Consistent with their severe conformational defects, cells with Δ30C Pma1 and Pma1-4D2E/A display temperature-sensitive growth (Fig. 6). Pma1-4D2E/A also has growth defects at 25°C and 30°C (Fig. 6).

**Pma1 truncation mutants are oligomers**

According to a proposed structural model, the C-terminal domain mediates interaction between monomers to make the Pma1 oligomer (8). Moreover, ER retention of Δ40C Pma1 is consistent with impaired oligomerization affecting ER export. To test the effect of Pma1 truncation on oligomerization, gel filtration chromatography was used after solubilization in digitonin. Previous work by Lee et al. using native gel analysis showed that Pma1 oligomers are stable after digitonin solubilization (11). Using a Sephacryl column with a fractionation range of $10^4$- $1.5 \times 10^6$, wild-type Pma1 was detected in the void fraction by Western blot (Fig. 7A); this is consistent with Pma1 being a large oligomeric complex $\geq 1$ MDa, as previously reported (11). Similarly, Δ30C and Δ40C were found predominantly in the void fraction (Fig. 7A), suggesting that the C-terminal domain is not the sole determinant of oligomerization. Moreover, we observed that faster turnover of Pma1-4D2E/A from the plasma membrane (Fig. 4B) is not related to its oligomerization state (Fig. 7A).

To confirm that the C-terminal domain is not essential for oligomer formation, co-immunoprecipitation was assayed using myc-tagged and HA-tagged Pma1 constructs. Fig. 7B, lane 2, shows Western blot detection of myc-tagged wild-type Pma1 in a non-denaturing immunoprecipitate of HA-tagged wild-type Pma1, in agreement with previous work (36). Fig. 7B, lane 3, shows Δ40C Pma1 (myc-tagged) co-immunoprecipitates with wild-type Pma1 (HA-tagged). Δ40C Pma1 has a faster electrophoretic mobility compared with full-length Pma1 (compare lane 2 with other lanes). Δ40C Pma1 (myc-tagged) associates with Δ40C Pma1 (HA-tagged) (Fig. 7B, lane 4). Control experiments show that anti-HA antibody cannot immunoprecipitate myc-tagged Pma1 or recognize myc-tagged Pma1 by Western blot (Fig. 7B, lane 1); nor can anti-myc recognize HA-tagged Pma1 by Western blot (lane 3, upper band). These data suggest that the C-terminal domain is not essential for Pma1 oligomerization.

**Discussion**

We examined the effect of truncations at the N and C-termini on Pma1 assembly and trafficking in the secretory pathway. We find that Pma1 with truncation of C-terminal 30 or 40 residues is delivered to the plasma membrane and can sustain cell viability as the sole provider of proton pumping activity. Our results are in agreement with an earlier study reporting that Pma1 is detectable at the plasma membrane after
46 residues are truncated from the C-terminus (33). Our findings differ from a recent study reporting that loss of 37 C-terminal residues results in failure to escape ERAD (37); one possible explanation is that Mason et al. analyzed the short-term fate of newly synthesized Pma1 mutant expressed in the presence of wild-type Pma1 whereas there are likely compensatory physiologic responses to promote cell surface delivery of the Δ30C and Δ40C mutants as the sole Pma1 in the cell. Although cell surface delivery occurs, removal of the last 30 residues affects Pma1 conformation (Fig. 5), and trafficking. Some Δ30C Pma1 molecules are delivered for ERAD (Fig. 3), and Δ30C displays additional impaired trafficking in the secretory pathway, as revealed by localization in puncta and in the vacuole (Fig. 1A). The puncta may represent a slow step in Δ30C trafficking through endosomes or Golgi. It is not clear why Δ30C is delivered to multiple different transport pathways; however, we have previously observed that misfolded Pma1 mutants can escape ERAD but are then detected by quality control steps in other parts of the secretory pathway (1). It seems reasonable to propose that different quality control sites independently collect information about a discrete domain rather than assessing a global conformational state. It is also possible that conformational changes occur during intracellular transport so that a mutant protein can assume certain requisite conformations but not others in distinct organelles. In this way, a mutant protein can escape entirely or partially one quality control checkpoint, but undergo detection at another.

Removal of 40 residues from the C-terminus resulted in two distinct populations of molecules: one population bearing the HA-tag is retained for ERAD whereas a second population is correctly targeted to the plasma membrane. Because no significant ER peak of Δ40C was seen by Western blot with anti-Pma1 antibody after cell fractionation (Fig. 1B), it appears that the ER-retained form might represent a very small fraction of total Δ40C Pma1. By contrast, the plasma membrane population is not well-recognized by Western
unpublished data), suggesting numerous interactions participate in oligomer formation.

By contrast with C-terminal truncation mutants, the N-terminal Pma1-4D2E/A mutant is not detected by ERAD. Indeed, Pma1-4D2E/A has no detectable targeting defect (Fig. 2) but has impaired stability at the plasma membrane (Fig. 4). Our results also suggest increased cell surface turnover of Δ40N. Our results are consistent with previous studies that concluded that the N-terminal domain has little direct effect on overall enzyme function (33,41). The diminished plasma membrane stability of Pma1-4D2E/A mutant is like that of Pma1-10 which has point mutations in the cytoplasmic loop between transmembrane domains 2 and 3, comprising the “A domain” thought to play an important role in catalysis (13,27). Because the cytoplasmic N-terminal domain is proposed to abut the A domain (5), it seems possible that perturbations in either domain could affect protein-protein interactions that promote plasma membrane stability. Pma1-10 has a slow ER export phenotype which was detected by indirect immunofluorescence after inducing expression of the tagged protein (27). Using the same method in this work (Fig. 2A), no effect was detected on ER export of N-terminal mutants. Thus, it seems unlikely that the N-terminal domain carries an ER export motif.

Further work is necessary to understand whether the C-terminal domain of Pma1 carries an ER export signal or how some Δ30C and Δ40C molecules escape ERAD. Cells with Δ30C Pma1 as the sole Pma1 cannot grow at 37°C; Pma1-4D2E also confers temperature-sensitivity (Fig. 6). One possible explanation for temperature-sensitive growth is that there is increased ERAD and/or turnover from the cell surface to the vacuole at 37°C, resulting in limited plasma membrane protein and activity at the cell surface. The temperature-sensitive phenotype provides the opportunity to use a genetic screen to gain further insight into these mutants.
Figures

Fig. 1: Intracellular and plasma membrane localization of C-terminal truncation mutants

(A) Indirect immunofluorescence localization. Cells were grown to mid-log phase at 25°C and then fixed with formaldehyde, spheroplasted, and permeabilized for staining. Staining with a single primary antibody was visualized with a CY3-conjugated secondary antibody; DTAF- and Texas Red-conjugated secondary antibodies were used for staining with two primary antibodies. HA-tagged Δ30C Pma1 (SSX9-1b) and Δ40C Pma1 (SSX5-1A) are localized intracellularly and at the plasma membrane. Cells were stained with both polyclonal anti-Kar2 and monoclonal anti-HA, or anti-Pma1 polyclonal antibody alone. Staining with anti-HA and anti-Kar2 is coincident in Δ40 Pma1 cells (right middle panels). (B) Cell fractionation by Renografin density gradients. Yeast strains were grown at 25°C. Cells were lysed and fractionated, as described in Methods. Membranes from each fraction were pelleted and analyzed by SDS-PAGE and Western blot. Anti-Gas1 (triangle), anti-GDPase (open circle), anti-Sec61 (diamond) were used to detect plasma membrane, Golgi, and ER membrane fractions. Steady-state distribution of HA-tagged wild-type and mutant Pma1 detected by anti-HA (square), Δ40C-Pma1 detected by anti-Pma1 monoclonal antibody (closed circle) and membrane markers in each fraction is plotted as a percentage of total across the gradient. HA-tagged wild-type Pma1 (SSY3) are predominantly in peak fractions 9 & 10 whereas Pma1 mutants, Δ30C’ and Δ40C display varying degrees of intracellular accumulation.

Fig. 2: Intracellular and plasma membrane localization of N-terminal Pma1 mutants

(A) Indirect immunofluorescence localization. Strains with HA-tagged wild-type Pma1 (KKY37), Δ40N (KKY39) and Δ60N (KKY40) under the control of GAL1 were shifted from raffinose-containing synthetic complete medium to galactose-containing medium to induce Pma1 synthesis. Wild-type (L3852) and pep4 (ACY67) cells with pMET-pma1-4D2E/A were washed with water and shifted to methionine-free medium to derepress mutant Pma1. After 2h induction cells were fixed and processed for staining with anti-HA antibody. (B) Cell fractionation by Renografin density gradients. Distribution of newly synthesized Pma1 (detected by anti-HA) and membrane markers is plotted as a percentage of total. Triangle, square, cross indicate Gas1, HA-Pma1, and alkaline phosphatase, respectively. Cells with HA-tagged wild-type Pma1 (pKK98) or HA-tagged mutant Pma1-4D2E/A (pKK103) under the control of MET3 were grown to mid-log at 30°C in minimal medium with 600µM methionine, and induced by resuspending in methionine-free medium for 2h. Cells were then lysed for fractionation. The majority of newly synthesized Pma1 is delivered to the plasma membrane.

Fig. 3: Decreased stability of C-terminal truncation mutants as revealed by pulse-chase analysis

Cells were grown in minimal medium overnight at 25°C before pulse-labeling with 35S-methionine and cysteine for 10 min at 25°C and chasing for various times. Immunoprecipitation from lysate was normalized to acid-precipitable cpm, and analyzed by SDS-PAGE and fluorography. (A) A fraction of newly synthesized Δ40 is degraded by ERAD. Anti-HA immunoprecipitates were from pulse-labeled Δ40 pma1 (SSX5-1a), Δ40 pma1 doa10 hrd1 (SSY24), and Δ40 pma1 ubc6 ubc7 (SSY25) cells. Bottom panel, immunoprecipitation from Δ40 pma1 cells with anti-Pma1 antibody. (B) A fraction of newly synthesized Δ30 Pma1 undergoes ERAD. Anti-HA immunoprecipitates were from Δ30 pma1 (SSX9-1b), Δ30 pep4 (SSY39), Δ30 end4 (SSY38), Δ30 ubc6 ubc7 (SSY37) cells.

Fig. 4: Trafficking pathway of N-terminal mutants: stabilization of Pma1-4D2E/A by preventing endocytosis and vacuolar proteolysis.
(A) Pulse-chase analysis. Cells with GAL-HA-PMA1 (KKY37) and GAL-HA-pma1-Δ40N (KKY39) were grown in synthetic complete medium without cysteine and methionine with 2% raffinose overnight before induction of Pma1 by resuspending cells in 2% galactose for 1h. Cells were then pulse-labeled at 25°C with Expre$^{35}$S$^{35}$S for 10 min and chased for various times. Immunoprecipitation from lysate was normalized to acid-precipitable cpm, and analyzed by SDS-PAGE and fluorography. (B) & (C) Degradation of Pma1-4D2E/A and its stabilization in end3-1 (RH266-1D) and pep4 (ACY67) but not doa10 hrd1 (MHY1703) cells. Cells bearing pMET3-HA-pma1-4D2E/A (pKK103) were grown to mid-log in the presence of methionine (off), induced to express mutant Pma1 by washing with water and resuspending in methionine-free medium for 2h (on), and chased by addition of 2 mM methionine for up to 4h.

Fig. 5: Conformational analysis of wild-type and mutant Pma1 by limited trypsinolysis

Strains were grown at 25°C in synthetic complete medium. Lysate was prepared, total membranes were pelleted by centrifugation at 100,000 g for 1h. Membranes were resuspended in buffer and incubated with trypsin (1:25 trypsin: protein) at 30°C for various times. Digestion was terminated by acid-precipitation, and products were analyzed by Western blot with anti-HA and/or polyclonal anti-Pma1 antibody. (A) Comparison of C-terminal Pma1 mutants (SSX5-1a, SSX9-1b) with wild-type Pma1 (SSY3). (B) Comparison of N-terminal mutants with wild-type Pma1. Δ40N (KKY44) cells were grown in synthetic complete medium with galactose because the mutant is under the control of the GAL1 promoter and the sole copy of Pma1 in the cell; the corresponding wild-type strain is KKY24-1B. The Pma1-4D2E/A mutant (KKY94) is under the control of the PMA1 promoter and it is the sole copy of Pma1 in the cell; the corresponding wild-type control is KKY93. Western blots are with anti-Pma1 antibody.

Fig. 6: Growth of wild-type and Pma1 mutants

Strains were grown on synthetic complete medium at 25, 30, and 37°C. Strains are wild-type (F1105), WT-HA (SSY3), HA-WT (KKY93), Δ30C (SSX9-1b), Δ40C (SSX5-1a), and Pma1-4D2E/A (KKY94).

Fig. 7: Pma1 mutants are not impaired in oligomerization.

(A) FPLC analysis. Cell lysate was prepared from strains grown to mid-log in synthetic complete medium at 25°C. Lysate was solubilized with 1% digitonin for 45 min at 25°C. After removal of nonsolubilized material by centrifugation at 100,000 g for 1h, samples were fractionated by fast protein liquid chromatography using a column with a sizing range of 10$^4$ to 1.5 x 10$^6$. Protein was acid-precipitated and analyzed by Western blot with anti-HA and/or anti-Pma1 antibody. Analyzed strains (from top to bottom) are: Δ30C (SSX9-1b), Δ40C (SSX5-1a), HA-WT (KKY93), 4D2E/A (KKY94). Migration of markers, blue dextran (void), aldolase (150 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), β-amylase (200 kDa) are indicated by arrows.

(B) Co-IP. Cells were grown at 25°C in synthetic complete medium with 2% raffinose. Cells were harvested and resuspended in synthetic complete medium with 2% galactose for 4h. Lysate was prepared from wild-type cells bearing (1) GAL-myc-Δ40pma1 only (pSS16), (2) both GAL-myc-PMA1 (pWQ3) and GAL-HA-PMA1 (pND542), (3) both pSS16 and pND542, and (4) both pSS16 and GAL-HA-Δ40pma1 (pSS17). IPs (normalized to lysate protein) were with monoclonal anti-HA antibody and protein G-agarose beads in the presence of 1% NP40, and analyzed by Western blot with polyclonal anti-myc antibody; lysates were examined by Western blot with both anti-myc and anti-HA antibodies.
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References

Fig. 3
Fig. 6

The figure shows petri dishes with bacterial growth at different temperatures: 25°C, 30°C, and 37°C. The dishes are labeled with different combinations of strains: WT-HA, WT, Δ30C, 4D2E, Δ40C, and HA-WT.