Cytoplasmic Hsp70 Promotes Ubiquitination for Endoplasmic Reticulum-associated Degradation of a Misfolded Mutant of the Yeast Plasma Membrane ATPase, PMA1*

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Cells have a variety of strategies for dealing with misfolded proteins. Heat shock response involves transcriptional induction of chaperones to promote and/or correct folding, and also activation of the ubiquitin/proteasome system to degrade defective proteins. In the secretory pathway, it is primarily luminal misfolded or unassembled proteins that trigger the unfolded protein response which, like heat shock, induces chaperones and components of the endoplasmic reticulum (ER)-associated degradation (ERAD) pathway. To understand cellular response to a misfolded polytopic membrane protein of the secretory pathway, we studied Pma1-D378S, a model ERAD substrate. Expression of misfolded Pma1 induces heat shock response in the absence of increased temperature. Overexpression of *HSF1*, the transcription factor that mediates heat shock response, increases degradation of Pma1-D378S without temperature upshift. Nevertheless, efficient Pma1-D378S degradation occurs in an *hsf1* mutant that maintains basal transcription levels but cannot mediate transcriptional activation. Thus, heat shock protein induction enhances but is not necessary for ERAD. The Ssa group of cytoplasmic Hsp70 chaperones is required for ERAD of both Pma1-D378S and another transmembrane ERAD substrate, Ste6*. In the absence of Ssa chaperones, ubiquitination of both substrates is impaired, resulting in stabilization. We suggest a role for Hsp70 cytoplasmic chaperones in recognition by the endoplasmic reticulum-associated ubiquitination machinery.

ER quality control refers to the process by which newly synthesized proteins of the secretory pathway undergo folding and assembly with the assistance of cytoplasmic molecular chaperones and/or chaperones residing in the ER lumen. When proteins fail to fold or assemble properly, ER quality control is responsible for recognition and delivery of these proteins for ER-associated degradation (ERAD). The basic elements of the ERAD pathway are conserved in eukaryotes; after conformationally abnormal proteins are detected, they are tagged with ubiquitin via ER-associated enzymes; in yeast the major E2 enzymes of this pathway are Ubc6, Ubc7, and Cue1, and the E3 enzymes are Doa10 and Hrd1. The ubiquitin-recognizing cytoplasmic complex comprised of the AAA ATPase Cdc48, Npl4, and Ufd1 plays a role in extracting substrate from the ER membrane for degradation by the 26 S proteasome (1–3).

The unfolded protein response (UPR) pathway represents another mechanism for dealing with misfolded proteins. UPR is activated upon recognition of abnormal proteins in the ER lumen by the luminal Hsp70 protein Kar2/BiP. On the other hand, some (transmembrane) ERAD substrates that are not recognized by Kar2/BiP do not elicit UPR. Signaling of UPR via Ire1 leads to transcriptional activation of a subset of genes, including those encoding molecular chaperones and components of the ERAD pathway. UPR also induces proteins involved in alternative mechanisms to get rid of abnormal secretory pathway proteins (4).

Like UPR, heat shock response involves transcriptional activation of genes whose functions include chaperones that assist protein folding or catalyze refolding of denatured proteins and ubiquitin- and proteasome-dependent degradation. Induction of heat shock proteins occurs in response to heat stress (5). It is thought that heat stress generates denatured or misfolded proteins that trigger activation of the transcription factor Hsf1, the mediator of heat shock response.

Involvement of cytoplasmic heat shock proteins has been established previously in degradation of some misfolded transmembrane proteins (called ERAD-C substrates) with lesions in their cytoplasmic domains (6, 7). By contrast, cytoplasmic chaperones are not involved in ERAD of luminal (ERAD-L) or ERAD-M substrates with lesions in their membrane domains (8). Although efficient degradation of transmembrane substrates such as Ste6* and cystic fibrosis transmembrane regulator ∆F508 requires cytoplasmic heat shock proteins, it is not clear at which step(s) of the ERAD pathway these chaperones play their substrate-specific role. One possibility is that chaperone association with an aberrant protein may prevent or alleviate its aggregation prior to destruction. Alternatively, cytosolic chaperones may promote the function of components of the ERAD machinery; for instance, it has been proposed that Hsp70 association with the 19 S subunit may help disassemble tightly folded ubiquitinated substrates prior to degradation (9).

Mutants of Pma1 have been studied as a paradigm of a misfolded membrane protein that undergoes ERAD (10, 11). In wild-type yeast, Pma1, the plasma membrane [H⁺]ATPase, is...
an abundant protein that pumps protons out of the cell to generate the membrane potential. Because of its critical function, Pma1 localization and activity at the cell surface are essential for cell viability (12). Structural evidence indicates that fungal Pma1 is an oligomeric molecule with an asymmetric membrane topology; it has cytoplasmic amino and carboxyl termini, a large central cytoplasmic domain, and the extracytoplasmic domain consists of small stretches connecting 10 transmembrane segments (13). Mutation of the Asp-378 residue, which is the catalytic phosphorylation site lying in the large central cytoplasmic domain, or changes in other residues in the surrounding region (14), results in ER accumulation and degradation dependent on Ubc6, Ubc7, and Doa10 (15). Substitutions of Asp-378 have a dominant negative effect on cell growth because wild-type Pma1 is degraded as a result of its oligomerization with mutant protein (16).

We have investigated the role of cytoplasmic chaperones in ERAD of Pma1-D378S. Expression of Pma1-D378S causes activation of heat shock response but not stress response or UPR. Heat shock response facilitates ERAD of misfolded Pma1 because overexpression of HSF1 increases the rate of Pma1-D378S degradation. However, heat shock response is not required for efficient Pma1-D378S degradation as basal transcription levels of cytosolic chaperones are presumably sufficient. We find that ubiquitination of Pma1-D378S is impaired in a mutant defective in all four members of the Hsp70 Ssa family, resulting in stabilization of mutant Pma1. Similarly, another transmembrane ERAD substrate Ste6 is also stabilized with decreased ubiquitination in ssa cells. We suggest a role for Hsp70 cytoplasmic chaperones in recognition of ERAD-C substrates by the ER-associated ubiquitination machinery.

MATERIALS AND METHODS

Media and Strains—Standard yeast media and genetic manipulations were as described (17). Yeast transformations were performed by the lithium acetate method (18). The following strains used in this study are derived from W303 (MATα ura3-1 leu2-3,112 his3-11 trpl-1 ade2-1 can1-100; also referred to as F1105): Bas12 (MATα trpl-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2+ met2-Δ1 lys2-Δ2 ssa2::TRP1 ssa3::LYS2 ssa4::ADE2) and Bas15 (MATα trpl-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2+ met2-Δ1 lys2-Δ2 ssa2::TRP1 ssa3::LYS2 ssa4::ADE2 ssa1-45::URA3) (from E. Craig, University of Wisconsin, Madison). The hsp26 strain has a TRP1-marked centromeric plasmid bearing an hsp26-G170D mutant under the control of the constitutive GPD promoter; this construct covers chromosomal deletions of hsp26 and hsc82 (∆hsc82::LEU2 ∆hsp82::LEU2 (pTGPDT/T1-101)); the control strain has chromosomal deletions covered by a plasmid with wild-type HSP26, hsp26, and hsp104Δ::LEU2 cells were from S. Lindquist, Whitehead Institute for Biomedical Research, Cambridge, MA (19). msn2-Δ3::HIS3 msn4-1::TRP was from Francisco Estruch (Universitat de Valencia) (20). The ydj1 hji1 mutant (MATα leu2 ura3 his3 trpl1 ade2 can1-100 ydj1-2::HIS3 ydj1-151::LEU2 hji1::TRP1) was from J. Brodsky (University of Pittsburgh) (21). ire1Δ::LEU2 was from J. Warner (Albert Einstein College of Medicine) (22).

Some strains were from the EUROSCARF deletion strain collection background BY4741 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0); these are MATα npl4Δ and MATα doa10Δ. Other ERAD mutants used in this study include MHY1703, which is MATα his3Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 doa10::HIS3 hrd1::LEU2 from M. Hochstrasser (Yale University), and npl4-2 (MATα ura3-52 leu2Δ1 trp1Δ63 npl4-2) from M. Hochstrasser (Yale University). This strain was crossed with a MATα W303 strain; the diploid was sporulated and dissected to generate ACX137-4A (MATα ssa1-45Δ8 ssa2::LEU2 ssa3::TRP1 ura3Δ leu2-3,112 his3-11 trp1-1 LYS2+). HSI70T (MATα ade2 leu2 trpl1 trp1 his3 can1 hisf1::HIS3 YCP·TRP·HSF1 (pK157)) and YAY22 (YCP·TRP·hsf1·R206S·F256S (pAY22) of HS170T) are HSF1+ and hsf1 mutant strains, respectively, obtained from Hiroshi Sakurai (Kanazawa University).

Plasmids—pWQ3 and pWQ4 are URA3-marked centromeric plasmids bearing GAL-myc-PMA1 and GAL-myc-pma1-D378S, respectively, and pWQ1 and pWQ2 are LEU2-marked pGAL1-myc-PMA1 and pGAL1-myc-pma1-D378S (16). pWQ13 and pWQ12 are URA3-marked centromeric plasmids bearing MET25-HA-PMA1 and MET25-HA-pma1-D378S, as described previously (15). pSH13 is like pWQ12 except HIS3-marked. In previous reports from our laboratory, Pma1-D378S was erroneously described as Pma1-D378N; however, Ser and Asn substitutions at Asp-378 have the same phenotype (11). p414MET25-VMA12-PrA was from M. Hochstrasser (Yale University). pCM64-SSA3-lacZ is a URA3-marked 2-micron plasmid bearing the SSA3 HSE fused to lacZ (23) (from D. Thiele, Duke University). pSTRE-lacZ (TRP1) was obtained from D. Engelberg (Hebrew University, Israel). pJC104 is a URA3-marked 2-μm plasmid bearing a UPRE-lacZ reporter (24) obtained from P. Walter (University of California, San Francisco). YEp LEU2 HSF1 and pAKS92, a TRP1-marked 2-μm plasmid bearing constitutively active hsf-R206S, were provided by D. Winge (University of Utah) (25). pSM1694 is a URA3-marked 2-μm plasmid bearing the STE6 promoter and the open reading frame for Ste6-166 tagged with both HA epitope and green fluorescent protein, provided by S. Michaelis (The Johns Hopkins University) (26).

Protein Induction, Western Blot, Immunoprecipitation, and Indirect Immunofluorescence—Pma1 synthesis under the control of the GAL1 promoter was induced by transferring cells from growth in synthetic complete medium with 2% raffinose to medium with 2% galactose. After a 4-h induction period, 3% glucose was added to inhibit further synthesis. Pma1 synthesis under the control of the MET25 promoter was repressed by the presence of 600 μM methionine in minimal medium. For induction (derepression) of Pma1 expression, cells were washed in water and transferred to fresh minimal medium without methionine for 2 h. To shut off further synthesis, 2 mM methionine was added.

Cell lysis was prepared by vortexing with glass beads in the presence of protease inhibitor mixture, including 1 mM phenylmethylsulfonyl fluoride, as described previously (27).
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FIGURE 1. Degradation of mutant Pma1 is dependent on cytoplasmic Hsp70 and Hsp40. A, Pma1-D378S degradation is impaired in ssa1 cells. SSA1 (W303), ssa2A3A4Δ, and ssa1–2Δ, ssa2A3A4Δ (BAS15) cells bearing pGAL-myc-pma1-D378S (pWQ2) were grown at 25 °C to mid-log phase in synthetic complete medium with 2% raffinose. Cells were then shifted to medium with 2% galactose for 4 h to induce expression of mutant Pma1 (time 0). Both SSA1 and ssa1–2Δ cells were shifted to 37 °C, and 3% glucose was added for chase. Samples were normalized to lysate protein for SDS-PAGE and Western blot with anti-Myc antibody. Bands were quantitated by using NIH image on scanned blots and plotted as a percentage of the band seen at time 0. The blot is representative of at least three independent experiments. WT, wild type. B, Pma1-D378S degradation is not dependent on new protein synthesis. SSA1 (W303) and ssa1–2Δ, ssa2A3A4Δ (BAS15) bearing MET-HA-pma1-D378S (pSH13) were grown at 25 °C, induced to express mutant Pma1 for 1 h by resuspending in methionine-free medium, and then shifted to 37 °C for 15 min. Cells were labeled with Expre Norris 5 for 10 min and then chased for various times in the presence of 10 μg/ml cycloheximide. At various times of chase, cells were harvested; lysate was prepared, and mutant Pma1 was immunoprecipitated with anti-HA antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. C, degrom-mediated degradation is dependent on cytoplasmic Hsp70. SSA1 (W303) and ssa1–2Δ, ssa2A3A4Δ (BAS15) bearing a p414MT25-Deg1-Vma12-PrA plasmid were induced to express the fusion protein by resuspending in methionine-free medium for 2 h at 25 °C (on). Cells were then shifted to 37 °C for 20 min. Cells were then harvested at various times after addition of 2 μM methionine. Off indicates before induction. Fusion protein was detected by Western blot with rabbit antibody coupled to horseradish peroxidase. Detection of 3-phosphoglycerate kinase (Pgk) was used as a loading control. D, Pma1-D378S degradation is slowed in ydj1 hlj1 cells. Because the strain of ydj1 hlj1 cells used in this experiment is not GAL−, ERAD was analyzed using the pMET25-HA-pma1-D378S (pWQ12) construct. Cells were grown at 25 °C to mid-log phase in minimal medium with 600 μM methionine to repress expression of mutant Pma1 (off). Cells were then washed and resuspended in fresh medium without methionine for 2 h to de-repress mutant Pma1 expression (on); at the same time, cells were shifted to 37 °C. Methylionine was added, and HA-tagged mutant Pma1 was measured by Western blot at various times of chase. Bands were quantitated by scanning and plotted. Results are representative of two experiments. E, Pma1-D378S degradation is not affected in hsp104Δ and hsc82Δ hsp40Δ cells. hsc82Δ hsp40Δ and its isogenic wild-type strain bearing pWQ12 were analyzed as described in D except the cells were grown at 30 °C. Results are representative of at least three independent experiments.

Galactosidase assay was performed as described (28); activities were normalized to lysate protein measured by Bradford assay (29). For Western blot, samples were also normalized to lysate protein; after SDS-PAGE, proteins were transferred to nitrocel- lulose for blotting with antibody. Antibody binding was visualized by peroxidase-conjugated secondary antibody followed by a chemiluminescence detection system. Rabbit antibody to Ssa protein was from Elizabeth Craig (University of Wisconsin, Madison, WI). Monoclonal antibody to 3-phosphoglycerate kinase was from Molecular Probes (Eugene, OR). For blots with anti-ubiquitin, nitrocellulose filters were autoclaved (30) before incubating with monoclonal antibody (Zymed Laboratories Inc.).

For immunoprecipitation for analysis of ubiquitination, lysate protein was prepared in the presence of 5 mM N-ethylmaleimide to inhibit deubiquitination and solubilized in 1% SDS before diluting into RIPA buffer without SDS to a final SDS concentration of 0.1%. After removing nonsolubilized material and preclearing, samples were then incubated overnight with anti-HA monoclonal antibody (Covance, Inc.) and protein A-agarose beads pre-bound with rabbit anti-mouse antibody.

Co-immunoprecipitation was performed by pelleting lysate (200 μg of protein) for 10 min at 20,000 × g in a microcentrifuge. The membrane pellet was resuspended in bead buffer (300 mM sorbitol, 100 mM NaCl, 5 mM MgCl2, 10 mM Tris, pH 7.4), and 50 μl was solubilized in non-denaturing buffer (1% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, 10 mM Tris, pH 7.4). Immunoprecipitations were with monoclonal anti-HA antibody and either rabbit anti-mouse and protein A-agarose beads or protein G-agarose beads. Immunoprecipitates were washed by centrifugation for 5 min at 400 × g.

Indirect immunofluorescence was as described previously (28). Cells were fixed with 4% formaldehyde overnight 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.) and polyclonal anti-Kar2 (gift from M. Rose, Princeton University) followed by Texas Red and/or dichlorotriazinylamino fluorescein-conjugated anti-mouse IgG (Jackson Laboratories Inc.).
RESULTS

A Requirement for Cytoplasmic Chaperones in ERAD—Preceding, the Ssa family of cytoplasmic Hsp70 proteins was reported to play a role in ERAD of some misfolded transmembrane proteins (6). To test whether degradation of Pma1-D378S was also dependent on Ssa proteins, a strain was used bearing deletions in ssa2, ssa3, and ssa4, and a temperaturesensitive mutation in ssa1 (ssa1−45). Because Pma1-D378S has a dominant negative effect on cell growth (16), its expression was placed under the control of either GAL1 or MET25. Mutant Pma1 synthesis was induced (or derepressed), and the cells were then shifted to 37 °C at the same time mutant Pma1 synthesis was shut off, and the level of mutant Pma1 protein was analyzed by Western blot at various times of chase. Fig. 1 shows that mutant Pma1 is degraded efficiently in control SSA+ cells even at 37 °C when exacerbation of misfolding may occur. By contrast, inactivation of all the cytoplasmic Hsp70 chaperones upon shifting ssa1+/+ ssa2Δ ssa3Δ ssa4Δ cells to 37 °C resulted in impaired degradation of mutant Pma1. It appears that Ssa1 plays the major role in facilitating ERAD as degradation of mutant Pma1 proceeds at a roughly normal rate in SSA1 cells in the absence of Ssa2, Ssa3, and Ssa4 (Fig. 1A). Fig. 1B shows a pulse-chase experiment using metabolic radiolabeling and cycloheximide during chase (Fig. 1B); in SSA+ cells, Pma1-D378S is degraded efficiently in the presence of cycloheximide, indicating that new protein synthesis is not required for ERAD of mutant Pma1. This result also implies that stabilization of mutant Pma1 when all Ssa proteins are inactivated is not because of a requirement for Ssa proteins in the folding of newly synthesized components of the ERAD machinery.

Pma1-D378S is targeted for ERAD because it is misfolded, and indeed, it has been shown to have increased sensitivity to limited trypsinolysis (11). The Ssa dependence of Pma1-D378S degradation (Fig. 1, A and B) is similar to that of several other misfolded or damaged membrane proteins that also undergo Doa10-mediated ubiquitination and degradation by ER quality control (7). We next asked whether there is a similar Ssa requirement for a membrane protein whose degradation is dependent on Doa10 but signaled by a degron, a sequence that targets a protein for degradation in order to regulate its function (31). It has been shown that attachment of the Deg1 signal of the MATa2 transcriptional regulator to the membrane protein Vma12 leads to rapid degradation of the construct that is dependent solely on the degron signal (32). Fig. 1C shows that protein A-tagged Deg1-Vma12 is slowed in ssa1+/+, ssa2Δ, -3Δ, and -4Δ by comparison with SSA+ cells. Thus, a misfolded conformation as well as a degron signal, both leading to Doa10-mediated degradation, have a requirement for Ssa proteins.

The Hsp40 proteins Ydj1 and Hlj1 are ER-membrane associated and act as co-chaperones for the Ssa family of Hsp70 proteins (21). ERAD of Pma1-D378S was also examined in ydj1Δ hlj1Δ cells. As in ssa1+/+ cells, mutant Pma1 is stabilized in the absence of the Hsp40 proteins as shown by Western blot (Fig. 1D) and metabolic pulse-chase (not shown). These results suggest a role for one or both of these co-chaperones in degradation of mutant Pma1.

Cytoplasmic chaperones belonging to other families were also tested for a role in ERAD of Pma1-D378S. A role for the Hsp90 family of chaperones has been suggested previously in ERAD of some substrates (21, 33). The two members of the family in yeast are Hsp82 and Hsc82; no significant effect on Pma1-D378S degradation was detected in hsp82Δ hsc82Δ cells (Fig. 1E). Hsp104 belongs to the Hsp100 AAA ATPase family of chaperones that participates in disaggregating aggregated proteins (19, 34). We tested degradation of Pma1-D378S in hsp104Δ cells; no significant effect on the degradation of Pma1-D378S was apparent (Fig. 1E, left panel).
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Mutant Pma1 Induces Heat Shock but Not General Stress Response—Chaperones of the heat shock family undergo transcriptional activation when cells are exposed to high temperature. Induction of this gene family occurs via Hsf1 binding to a conserved promoter element called a heat shock element (HSE) (35, 36). Because members of this family participate in ERAD (39), the general stress response to various environmental conditions (including heat shock, osmotic shock, and oxidative stress). The general stress response is mediated by the transcription factors Msn2 and Msn4 which bind to a conserved STRE promoter element of many genes. Some genes are only responsive via HSEs (e.g. SSA1) or STREs, whereas others are responsive to both (e.g. HSP26) (37). Induction of the STRE regulon in the presence of Pma1-D378S was tested by using an STRE-lacZ reporter (38). Pma1 protein levels are shown by Western blot (Fig. 2B). General stress response is not activated by Pma1-D378S, although STRE reporter activity is increased by temperature shift (Fig. 2B). Accordingly, in the absence of general stress response in msn2A msn4Δ cells, ERAD of Pma1-D378S is unaffected (Fig. 2B, bottom panel).

The Unfolded Protein Response Is Not Required for ERAD of Pma1-D378S—Although Kar2/BiP, the ER luminal Hsp70, is required for UPR and for ERAD of luminal misfolded substrates, it does not play an important role in degradation of transmembrane ERAD substrates which have misfolded cytoplasmic domains (6, 7). Fig. 3A shows that mutant Pma1 induces a low unfolded protein response, similar to that induced by wild-type Pma1, as measured by a UPRE-lacZ reporter. Consistent with this observation, Fig. 3B shows degradation of Pma1-D378S is not significantly changed in the absence of the UPR signaling protein Ire1. Even so, indirect immunofluorescence localization shows considerable overlap in staining of HA-tagged Pma1-D378S and Kar2 (Fig. 3C). Pma1-D378S and Kar2 were observed co-localized in a ring around the nucleus, which is a typical ER staining pattern; both mutant Pma1 and Kar2 were also seen accumulated in bright puncta adjacent to ER rings (Fig. 3C). Kar2 is only present in puncta in the presence of mutant but not wild-type Pma1 (Fig. 3C). Other misfolded membrane proteins have also been localized to such puncta, named ER-associated compartments (ERACs) (39). ERACs appear to represent proliferated ER membranes, and a number of ER resident proteins have been observed to localize to these structures (39). Thus, it appears that co-localization of Kar2 and Pma1-D378S may not reflect a
functional interaction. In \textit{ssa} cells, Pma1-D378S degradation is impaired but localization to ERACs remains unaffected (Fig. 3D). Targeting of wild-type Pma1 to the plasma membrane is unaffected in \textit{ssa} cells (Fig. 3D), suggesting that Ssa family chaperones are not essential for biogenesis of wild-type Pma1 but are necessary for ERAD of mutant Pma1 (Fig. 1A).

\textit{HSF1 Facilitates but Is Not Necessary for ERAD}—Because misfolded Pma1 induces heat shock response (Fig. 2A), it was of interest to determine whether heat shock response is required for Pma1-D378S degradation. A combination of two mutations R206S/F256S has been described previously to generate an \textit{hsf1} mutant that is viable and able to maintain basal gene transcription levels at 30 °C, but fails to sustain growth or generate transcriptional activation in response to shift to high temperature (35). Impaired activation of an HSE-lacZ reporter by shift to 37 °C in these mutant \textit{hsf1} cells was confirmed (Fig. 4B); similarly, response to misfolded Pma1 in the absence of temperature shift was defective (not shown). Although these \textit{hsf1} cells are unable to mount a heat shock response, degradation of misfolded Pma1 is not different from that in \textit{HSF1} cells (Fig. 4A). These results suggest that although heat shock response is elicited by misfolded Pma1, it is not necessary for efficient ERAD.

To assess whether heat shock response facilitates ERAD, Pma1-D378S degradation was examined upon Hsf1 overexpression. Previous work has shown that transcriptional activation of heat shock genes can occur in the absence of temperature shift upon overexpression of wild-type \textit{HSF1} on a 2-micron plasmid (25). Moreover, the single R206S Hsf1 mutant is constitutively active in the absence of heat shock (25). In Fig. 5, Pma1-D378S degradation was compared in cells shifted to 37 °C in the presence and absence of high copy wild-type \textit{HSF1}; additionally, the effect of the Hsf1-R206S mutant was tested at 25 °C. In both cases, there is increased Hsf1 activity, and the rate of Pma1-D378S degradation is increased (Fig. 5), suggesting ERAD is enhanced by heat shock response.

\textbf{Cytoplasmic Ssa Family Chaperones Interact with Mutant Pma1 and Facilitate Its Ubiquitination}—Although cytoplasmic Hsp70 and Hsp40 chaperones participate in ERAD of several transmembrane substrates (Fig. 1) (6, 7), it is unclear at which step in the pathway these chaperones act. One model proposes that because Hsp70 chaperones are involved in promoting folding of newly synthesized proteins, they may also have the dual function of detecting misfolding, as demonstrated for the ER luminal Hsp70, BiP/Kar2 (40). To test the possibility that the Ssa proteins may participate in substrate recognition, we assayed interaction with mutant Pma1. In Fig. 6, \textit{left panel}, HA-tagged mutant Pma1 was immunoprecipitated under native (nondenaturing) conditions, and associated Ssa1 was revealed by Western blotting the immunoprecipitate with anti-Ssa1 antibody. (The anti-Ssa1 antibody also detects Ssa3 and probably other Ssa proteins.)3 The control experiment, in which HA-tagged Pma1-D378S was first denatured with 1%

3 E. Craig, personal communication.
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**FIGURE 6. Interaction of Ssa protein with mutant Pma1.** Wild-type (W303) or doa10 hrd1 (MHY1703) cells bearing vector (v), pMET25-HA-pma1-D378S (pWQ12), or pMET25-HA-PMA1 (pWQ13) were grown to mid-log in minimal medium with 600 μL methionine at 30 °C. Cells were induced to express mutant Pma1 after washing with water and resuspending in minimal medium without methionine for 2 h. Left panel, mutant Pma1 was immunoprecipitated (IP) with anti-HA antibody under native (nondenaturing) or denaturing conditions (solvilization in 1% SDS followed by dilution to 0.1% in RIPA buffer without SDS) and analyzed by SDS-PAGE and Western blot with anti-HA and anti-Ssa antibodies. Arrowhead indicates HA-tagged Pma1-D378S protein; lower band in left bottom panel is IgG. Right panels, Ssa proteins interact with mutant Pma1 but not wild-type Pma1; interaction between Ssa proteins and Pma1-D378S is independent of Doa10 and Hrd1.

SDS before immunoprecipitation, shows loss of interaction with Ssa proteins (Fig. 6, left panel). By contrast with mutant Pma1, interaction of Ssa with wild-type Pma1 is not detectably above background (Fig. 6, top right panel). Strikingly, co-immunoprecipitation of Ssa with Pma1-D378S is not affected in *doa10Δ hrd1Δ* cells (bottom right panel), consistent with the idea that this interaction occurs before Doa10-mediated ubiquitination.

If cytoplasmic Hsp70 and Hsp40 proteins participate in substrate recognition, it seems possible that impaired function of these proteins would affect subsequent substrate ubiquitination. We therefore tested ubiquitination of ERAD substrates in *ssa* and *hlj1 ydj1* mutants. In the experiment in Fig. 7A, epitope-tagged Pma1-D378S protein was expressed at the permissive temperature and shifted to 37 °C for chase. Mutant Pma1 was immunoprecipitated under denaturing conditions for analysis by Western blot with anti-ubiquitin antibody. A high molecular weight smear extending above the position of Pma1-D378S (Fig. 7A, arrow) was observed in SSA+ cells, indicating polyubiquitination. Ubiquitinated forms of Pma1-D378S are detectable only by Western blot with anti-ubiquitin as these forms are insufficiently abundant for detection with antibody against tagged mutant Pma1. In *ssa*− cells, the level of polyubiquitination of Pma1-D378S was reduced by comparison with that seen in SSA+ cells (Fig. 7A, right panel). The amount of myc-Pma1-D378S recovered by immunoprecipitation is comparable in SSA+ and *ssa*− cells (Fig. 7A, bottom right panel). Similarly, in *hlj1 ydj1* cells, ubiquitination of (HA-tagged) Pma1-D378S is decreased by comparison with wild-type cells (Fig. 7A, left panel). These results are consistent with cytoplasmic chaperones participating in an early step in the ERAD pathway. As a control for incubation of *ssa*− cells at 37 °C, wild-type and *npl4Δ* cells were also shifted to 37 °C, resulting in increased ubiquitination of Ste6* (Fig. 7C, left panel). As a negative control, Ste6* ubiquitination was examined in *doa10Δ* cells; ubiquitination signal was virtually undetectable from cells grown at 30 °C, consistent with the reported role of Doa10 as a ubiquitin ligase for Ste6* (Fig. 7C, both panels). Strikingly, when *doa10Δ* cells were shifted to 37 °C, a low level of Ste6* ubiquitination was apparent (Fig. 7C), suggesting a possible alternative ubiquitination pathway available at 37 °C.

**DISCUSSION**

A number of different cytoplasmic chaperones were screened for involvement in ERAD of Pma1-D378S. Although no effect was noted in *hsp104* and *hps90* mutants, we report a requirement for Ssa family chaperones, especially Ssa1, and their co-chaperones Ydj1 and Hlj1 in ERAD of misfolded mutant Pma1 (Fig. 1). Although a role for the Hsp70 chaperones was reported previously in ERAD of some other transmembrane substrates, it has not been clear in what way(s) these proteins facilitate degradation of misfolded membrane proteins such as Ste6* and cystic fibrosis transmembrane regulator ΔF508 (7). Among several possibilities that have been suggested, the cytosolic Hsp70 and Hsp40 chaperones may prevent aggregation of transmembrane ERAD substrates before or during dislocation, analogous with participation by luminal Hsp70, Kar2/BiP, in maintaining the solubility of luminal proteins for retrotranslocation (42). Cytosolic Hsp70 chaperones may also...
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mutant Pma1 and Ste6*, is dependent on Ubc6, Ubc7, and Doa10. The observation that ERAD of Pma1-D378S is not dependent on continuing protein synthesis (Fig. 1B) rules out the possibility that the Ssa requirement is in folding of newly synthesized components of the ERAD machinery.

Ssa proteins are dispensable for ERAD of substrates such as the luminal protein CPY* (8) and the membrane protein Sec61-2 (43), so-called ERAD-L and ERAD-M substrates with lesions in their luminal and membrane domains, respectively (44). A subset of ERAD components involved in disposal of these substrates is shared by those recognizing mutant Pma1 and Ste6* (ERAD-C substrates). These components of the ERAD machinery include Ubc6 and Ubc7, the Cdc48-Ufd2-Npl4 complex, and the 26 S proteasome (6), and because they are competent to dispose of ERAD-L and ERAD-M substrates in ssa− cells, we can infer these components do not require Ssa proteins for activity. Therefore, stabilization and impaired ubiquitination of Pma1-D378S and Ste6* are not the indirect results of an Ssa requirement for these ERAD components.

A proposed role for cytosolic Hsp70 and their Hsp40 co-chaperones in facilitating substrate ubiquitination does not preclude their participation at multiple steps in the ERAD pathway. However, it is not unreasonable that these cytosolic chaperones may facilitate interaction between substrate and Doa10 or promote formation of the E2-E3 complex, or somehow stimulate Doa10 activity. In mammalian cells, the ubiquitin ligase CHIP (chaperone-interacting protein) has a tetratricopeptide repeat domain that recognizes Hsp70-bound substrates; CHIP serves as a physical link between molecular chaperones and the ubiquitin/proteasome system (45). In yeast, there is no identified CHIP homolog, but it is interesting to speculate that there is a direct interaction of a chaperone-substrate complex with Doa10. Currently, it is not known how Doa10 recognizes its transmembrane ERAD clients; by contrast, the Hrd1 ubiquitin ligase appears to recognize its luminal substrates via interaction with Yos9, Kar2, and Hrd3, components of the ERAD machinery that bind misfolded proteins (44, 46).

Misfolded Pma1-D378S does not induce a significant unfolded protein response but does cause induction of heat shock response in the absence of temperature upshift (Figs. 2

A major finding of this study is that the Ssa proteins are necessary to promote ubiquitination of misfolded membrane proteins Pma1-D378S and Ste6* (Fig. 7), suggesting the cytosolic Hsp70 proteins act at an early step in the ERAD pathway. A simple model to explain these results is that these chaperones participate in substrate recognition, as suggested for luminal chaperones such as Kar2/Bip and protein-disulfide isomerase. Co-immunoprecipitation experiments show that Ssa binds to mutant Pma1 but not wild-type Pma1 (Fig. 6), and wild-type Pma1 delivery to the plasma membrane is not affected in ssa− cells (Fig. 3D). These results support the idea that the Ssa proteins play a major role in disposal of mutant Pma1 (versus biogenesis of wild-type Pma1). In this regard it is interesting that the membrane-bound fusion protein Deg1-Vma12 also requires Ssa proteins for degradation (Fig. 1C), as its destruction is degron-signalized, but its ubiquitination, like that of

B

FIGURE 7. The role of cytoplasmic chaperones in ubiquitination of transmembrane ERAD substrates. A, Pma1-D378S ubiquitination (Ub) is decreased in ssa− ydj1− cells. Right, wild-type (F1105) and ssa− cells (BAS15) bearing pGAL1-myc-pma1-D378S (pWQ2) were shifted to galactose-containing medium for 4 h to induce mutant Pma1. Cells were shifted to 37 °C and chased for 2 h. Cells were then lysed, and Pma1-D378S was immunoprecipitated from cell lysate with anti-HA antibody and analyzed by Western blot with anti-ubiquitin; Filter was stripped and reprobed with anti-HA. Left, ydj1− cells bearing pMET25-HA-pma1-D378S (pWQ12) were derepressed by transfer to methionine-free medium. Cells were chased at 37 °C for 1 h. Mutant Pma1 was immunoprecipitated from cell lysate with anti-HA antibody and analyzed by Western blot with anti-ubiquitin; Filter was stripped and reprobed with anti-HA (bottom panel). Arrows indicate the position of nonubiquitinated Pma1. B, Ste6 degradation is impaired in ssa− cells. MATα ssa− cells (ACX137–4A) were transformed with a plasmid bearing HA-Ste6* (pSM1694). Cells were shifted to 37 °C for 40 min and then at various times after addition of cycloheximide (10 μg/ml) to inhibit further protein synthesis, aliquots were lysed and analyzed for Ste6* by Western blot with anti-HA antibody. C, Ste6 ubiquitination is impaired in ssa− cells. MATα wild-type (BY4741), npl4Δ, doa10Δ, and MATα ssa− were grown at 30 °C or shifted to 37 °C for 40 min. HA-Ste6* was immunoprecipitated from cell lysates. Because Ste6* levels are less in wild-type than in doa10Δ and npl4Δ cells, immunoprecipitations were from 2× protein concentrations. For ssa− cells, immunoprecipitations were from 1× and 2× protein concentrations. Immunoprecipitates were blotted with anti-ubiquitin followed by re-probing with anti-HA. Hatch marks indicate molecular weight markers, and arrow indicates the position of nonubiquitinated Ste6*.
and 3A). It is unlikely that heat shock response is an indirect result of loss of functional Pma1 at the cell surface; because of the extreme longevity of wild-type Pma1 at the plasma membrane (47), no change in growth rate is even detectable until ≥6 h after expression of Pma1-D378S (15). Induction of heat shock response without temperature change has also been observed upon treatment of cells with a toxic analog of proline, azetidine-2-carboxylic acid (AZC) (48). Neither AZC treatment nor expression of mutant Pma1-D378S activates the general stress response (Fig. 2B) (48)). Both reports support the idea that heat shock response is mediated by misfolded protein. AZC treatment causes global protein misfolding, whereas in the case of Pma1-D378S, activation of Hsf1 is induced by a single misfolded protein. We suggest that Hsf1 activation is signaled by the balance between misfolded protein and the availability of specific cytoplasmic chaperones.

Degradation of Pma1-D378S is not affected in an hsf1 mutant unable to mediate translational activation (Fig. 4), suggesting that heat shock response is not required for ERAD of mutant Pma1. By contrast, overexpression of HSF1 to mimic heat shock response increases the rate of Pma1-D378S degradation (Fig. 5). Thus, heat shock response enhances the ERAD pathway, i.e. a basal transcriptional level of heat shock genes is sufficient for ERAD but the pathway is enhanced by heat shock protein induction. For example, Ssa1 is induced by heat shock and necessary for ERAD of Pma1-D378S (Fig. 1); however, the significant level of constitutive Ssa1 expression (49) is apparently sufficient for its role in ERAD.

We propose that heat shock response may enhance ERAD by activating additional pathway(s). Like UPR triggered by misfolded ER luminal proteins, induction of heat shock proteins in response to transmembrane ERAD substrates could reflect an attempt to repair misfolding or mitigate aggregation. Moreover, heat shock response could recruit additional components to participate in the ERAD pathway. We observed that Ste6* undergoes increased ubiquitination at 37 °C in wild-type and npl4 cells (Fig. 7C). Remarkably, Ste6* ubiquitination is also increased at 37 °C in the absence of Doa10 (Fig. 7C). Although it is likely that Ste6* misfolding is increased at 37 °C, mild heat shock may also enlist one or more E3s to assist Doa10 in Ste6* ubiquitination. Because the ubiquitin ligase Hrd1 has previously been reported to make a minor contribution toward Ste6* degradation (26), work is underway to test whether Hrd1 or other E3s play a larger role under heat shock conditions.

A relationship between heat shock and protein degradation has been noted previously by Riezman and co-workers (50); impaired heat shock induction in leb1-100 cells (defective in serine palmitoyltransferase activity and ceramide biosynthesis) results in temperature-sensitive growth, which is suppressed by overexpression of ubiquitin. Thus, increased ubiquitin-mediated degradation can replace the essential requirement for heat shock response, perhaps by disposal of toxic protein aggregates. Conversely, our results suggest that induction of heat shock genes can augment ERAD. Further work is necessary to dissect the multitude of cellular events that comprise quality control in the face of protein misfolding.

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