Degradation of unassembled Vph1p reveals novel aspects of the yeast ER quality control system

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The endoplasmic reticulum quality control (ERQC) system retains and degrades soluble and membrane proteins that misfold or fail to assemble. Vph1p is the 100 kDa membrane subunit of the yeast Saccharomyces cerevisiae V-ATPase, which together with other subunits, assembles into the V-ATPase in the ER, requiring the ER resident protein Vma22p. In vma22Δ cells, Vph1p remains an integral membrane protein with wild-type topology in the ER membrane before undergoing a rapid and concerted degradation requiring neither vacuolar proteases nor transport to the Golgi. Failure to assemble targets Vph1p for degradation in a process involving ubiquitylation, the proteasome and cytosolic but not ER lumenal chaperones. Vph1p appears to possess the traits of a ‘classical’ ERQC substrate, yet novel characteristics are involved in its degradation: (i) UBC genes other than UBC6 and UBC7 are involved and (ii) components of the ERQC system identified to date (Der1p, Hrd1p/Der3p and Hrd3p) are not required. These data suggest that other ERQC components must exist to effect the degradation of Vph1p, perhaps comprising an alternative pathway.

Keywords: degradation/ER quality control/proteasome/ubiquitylation

Introduction

Proteins destined for the secretory pathway are translocated across the endoplasmic reticulum (ER) membrane where they fold and may be modified by disulfide bond or carbohydrate addition, proteolytic processing, oligomerization and/or assembly into multi-subunit complexes. A quality control system associated with the ER (ERQC) monitors these processes and selectively retains and degrades those proteins that have failed to fold, oligomerize or assemble correctly (Bonifacino and Weissman, 1998). ERQC substrates include: CFTR-AF508, α1-antitrypsin inhibitor, unassembled T-cell receptor subunits, apoB under conditions of limited lipid availability and CPY*, a mutant form of the yeast vacuolar carboxypeptidase Y (CPY), which is retained in the ER (Bonifacino and Weissman, 1998). The ERQC degradative machinery also acts in a regulatory role to moderate the half-life of correctly folded ER resident proteins in response to cellular signals. The cholesterol biosynthesis enzyme HMG-CoA reductase (HMG-R) is turned over rapidly in response to elevated levels of mevalonate and its metabolites (Hampton et al., 1996).

ERQC substrate degradation occurs independently of the lysosome (or yeast vacuole) and proteolysis proceeds in the absence of traffic from the ER to the Golgi (Finger et al., 1993). Inhibition of ERQC degradation was accomplished with drugs that inactivate the cytosolic proteasome or with mutations that reduce proteosomal activity (Ward et al., 1995; Hiller et al., 1996). Substrates of the 26S proteasome require ubiquitylation, as do a number of ERQC substrates (Bonifacino and Weissman, 1998). In yeast, two ubiquitin-conjugating enzymes, Ubc6p and Ubc7p, are localized to the cytosolic face of the ER membrane, and mutations in UBC6 or UBC7 have been found to reduce the degradation rate of ERQC substrates (Biederer et al., 1996; Hiller et al., 1996; Hampton and Bhakta, 1997; Galan et al., 1998; Loayza et al., 1998). The ERQC system utilizes cytosolic proteasome–ubiquitylation components for degradation and requires ERQC substrates to be exported from the ER to the cytosol. Results of co-immunoprecipitation and genetic data demonstrated that components of the translocon were also involved in the retro-translocation of ERQC substrates (Wiertz, 1996b; Pilon et al., 1997; Plemper et al., 1997).

Several approaches in yeast have identified components of the ERQC machinery. In the ER lumen, the molecular chaperone hsp70 protein Kar2p (BiP homolog), but not cytosolic molecular chaperones, participates in the degradation of the soluble substrates CPY* (Plemper et al., 1997), pro-α-factor and a mutant form of α1-antitrypsin inhibitor (Brodsky et al., 1999). Additionally, three genes encoding membrane-associated ER proteins have been identified: Der1p, required for the turnover of CPY*; Hrd3p, for the degradation of HMG-R; and Hrd1p (also named Der3p), as necessary for the degradation of both CPY* and HMG-R (Hampton et al., 1996; Knop et al., 1996; Bordallo et al., 1998).

Vph1p is a 100 kDa polytopic membrane subunit of the yeast vacuolar H+-ATPase (V-ATPase), which resembles in the ER with other subunits into the vacuolar membrane where the final 13 subunit V-ATPase complex functions to acidify the organelle (Graham and Stevens, 1999). The absence of any one of three ER assembly factors (Vma22p, Vma21p or Vma12p) results in the rapid and specific degradation of the now unassembled Vph1p. Degradation of unassembled Vph1p was found to occur independently of vacuolar proteases and did not require transport from the ER to the Golgi (Hill and Stevens, 1994, 1995; Jackson and Stevens, 1997).

Here we demonstrate that Vma22p is required for Vph1p assembly into the V-ATPase and characterize the degradation of unassembled Vph1p. Our findings that unassembled Vph1p is an integral membrane protein in
the ER prior to its degradation and that its turnover involves an energy-dependent step(s), ubiquitylation and
the 26S proteasome, classify Vph1p as an ERQC substrate.
We have assessed the role of chaperones on both sides of the
ER membrane in degrading Vph1p and found that cytosolic but not ER luminal chaperones are required.
The novel attributes of Vph1p turnover include not only
the employment of a different subfamily of Ubc enzymes
but also that degradation occurs independently of the
previously identified DER/HRD components of the yeast
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Results

Vph1p is localized to the ER membrane in vma22Δ cells
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Although Vma22p is an integral membrane protein in
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assembly role of Vma22p, a protease shaving assay was
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membrane and assumed the same membrane topology as that of wild-type Vph1p. However, as our anti-Vph1p antibodies recognize the N-terminal half of Vph1p, we cannot completely eliminate the possibility of differences in the C-terminal insertion pattern. The defect seen in vma22Δ cells is therefore consistent with a failure of Vph1p to assemble with other subunits of the V-ATPase complex. The degradation of a fully translocated but unassembled protein in the ER membrane establishes Vph1p as an ERQC substrate.

Degradation of unassembled Vph1p is an energy-requiring process

ERQC degradation is an energy-requiring process as ATP is thought to be necessary for retro-translocation, ubiquitylation and proteasome-mediated degradation (McCracken and Brodsky, 1996). To assess whether Vph1p turnover was energy requiring, a radiolabeling kinetic analysis was performed in which energy poison sodium azide was absent or present during the chase period. The half-life of unassembled Vph1p was extended to >800 min in the presence of azide as compared with 25 min in KHY125 cells without azide (Figure 4). The degradation of another ERQC substrate, CPY*, was also greatly stabilized in the presence of azide, extending the half-life of the protein from ~26 min to >800 min (Figure 4).

Degradation of unassembled Vph1p involves ubiquitylation and the 26S proteasome

Another hallmark of ERQC-mediated degradation is ubiquitylation involving Ubc enzymes. VMA22 was disrupted in a number of ubcΔ strains to determine whether the absence of a particular Ubc protein retarded the turnover of Vph1p (Table 1). Of the ubc mutations tested, the ubc1 ubc4 (Figure 5A) and ubc2 ubc4 double mutants slowed the degradation of Vph1p ~2-fold. Surprisingly, Vph1p was not stabilized in a ubc6Δ ubc7Δ double mutant.
Degradation of unassembled Vph1p

Table I. Effect of ubc mutations on Vph1p turnover in vma22Δ cells

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<tr>
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Degradation of unassembled Vph1p

The involvement of the 26S proteasome in the degradation of unassembled Vph1p was investigated utilizing mutants with defects in proteasome activity. CIMS encodes a regulatory subunit of the 19S cap of the 26S proteasome, and a cin5-1 mutation previously has been shown to result in the reduced degradation rate of proteasome substrates (Ghislain et al., 1993). VMA22 was deleted in a strain carrying a cin5-1 mutation, and a kinetic analysis was performed to determine the half-life of Vph1p compared with that of a vma22Δ CIM5 parent strain. Figure 5B shows that the cin5-1 mutation significantly reduced the degradation rate of unassembled Vph1p, extending the half-life of the protein from 25 min to 90 min.

DOA4 encodes a proteasome-associated isopeptidase thought to promote proteolysis by cleaving ubiquitin from ubiquitylated substrates during proteasome action (Papa et al., 1999). Introduction of a vma22Δ mutation into a doa4Δ strain was also found to reduce the turnover kinetics of Vph1p (Figure 5C), increasing the half-life of Vph1p ~3-fold and further implicating ubiquitylation and the 26S proteasome in Vph1p degradation.

Degradation of Vph1p is a concerted process

Vph1p consists of an N-terminal half exposed in the cytosol (K. Hill and A.A. Cooper, unpublished data) and a C-terminal half containing six or seven potential membrane-spanning domains. We investigated whether the proteasome degrades only those domains of Vph1p exposed to the cytosol or, alternatively, if Vph1p is extracted from the ER membrane to be fully accessible to the proteasome. To detect any proteolytic fragments that may represent degradative intermediates, we used a functional allele of VPH1 that contained an insertion of three hemagglutinin (HA) epitopes at the C-terminus of Vph1p (Vph1p-HA). This allowed us to monitor the cytosolic N-terminal domain of Vph1p with anti-Vph1p antibodies and the C-terminus with antibodies directed against HA. vma22Δ vph1Δ cells expressing Vph1p-HA were radiolabeled and aliquots harvested at specified time points. The resulting lysates were divided in two and immunoprecipitated with antibodies directed against either Vph1p or HA. Samples were resolved by SDS–PAGE on both 7% (data not shown) and 15% gels to detect both small and large proteolytic fragments. Other than full-length Vph1p, no significant proteolytic species were detected during the course of the degradation using either antibody (Figure 6). Furthermore, the half-life of Vph1p-HA was very similar when calculated from data derived from either antibody. These data suggest that the proteolytic degradation of Vph1p occurs in a concerted process to degrade the entire Vph1p molecule and that Vph1p must be extracted from the ER membrane to be accessible to the proteasome.

Unassembled Vph1p is not extracted into the cytosol prior to degradation

Extraction of Vph1p from the ER membrane for degradation might proceed in one of two ways: complete extraction
from the membrane into the cytosol and subsequent degradation by the proteasome or, as each portion of Vph1p is extracted from the membrane, it is degraded immediately by the proteasome. We undertook to determine whether Vph1p was extracted as a full-length molecule from the ER membrane prior to its degradation. Such a species of Vph1p would be expected to accumulate in a supernatant fraction from a strain with reduced proteasome activity. Alkaline carbonate extraction experiments were performed on wild-type, vma22Δ and vma22Δ cim5-1 radiolabeled cell extracts. Vph1p was immunoprecipitated from total, supernatant and pellet fractions after carbonate treatment, the samples resolved by SDS–PAGE and quantified. The fractionation of Vph1p in vma22Δ and vma22Δ cim5-1 cells shown in Figure 2A was very similar to that of wild-type Vph1p, and we could observe no obvious indications of an accumulation of an extracted soluble form of Vph1p. However, this does not completely dispel a two-step membrane extraction/degradation model. The highly hydrophobic character of the C-terminal half of Vph1p might cause a membrane-extracted, pre-degraded form of Vph1p to aggregate in the cytosol with sedimentation properties similar to those of the microsomes used for the carbonate extraction experiments. To distinguish an aggregated extracted form of Vph1p from a membrane-spanning form, we employed the membrane floatation approach of Xiong et al. (1999). Microsomes were first confirmed to contain all of the Vph1p remaining in vma22Δ cells (Figure 7A) and then placed beneath a two-step sucrose gradient prior to centrifugation, during which membranes and associated proteins would be expected to float up through the gradient while aggregates remain pelleted (Xiong et al., 1999). The gradient profiles of wild-type Vph1p and Vph1p from both vma22Δ and vma22Δ cim5-1 cells were very similar and located in the center of the gradient (Figure 7B), indicating that unassembled Vph1p in proteasome-inhibited cells is membrane-associated instead of an aggregate of extracted protein. These data support a model in which Vph1p is extracted and degraded in a concerted process.

**Cytoplasmic but not ER molecular chaperones are required for Vph1p degradation**

Molecular chaperones are thought to distinguish native from non-native proteins by binding to hydrophobic stretches of residues that are only exposed in non-native structure. Terminally unassembled or unfolded proteins would continue to display these residues and result in prolonged or repeated interactions with molecular chaperones that may then facilitate their degradation (Hayes and Dice, 1996), potentially by directing a terminally misfolded protein for entry into the ERQC degradative pathway.

Vph1p has domains exposed on both sides of the ER membrane and so it is possible that both cytosolic and ER luminal chaperones may contribute to the degradation of unassembled Vph1p. The yeast KAR2 gene encodes the major hsp70 molecular chaperone of the ER lumen, which facilitates both translocation into the ER (Vogel et al., 1990) and the subsequent folding of nascent proteins in the ER lumen (Simons et al., 1995). Kar2p also contributes to the degradation of the soluble ERQC substrates CPY*, pro-α-factor and α1-antitrypsin (Plempner et al., 1997; Brodsky et al., 1999). To determine if Kar2p is involved in Vph1p degradation, we employed the temperature-sensitive kar2-1 allele. At the non-permissive temperature, the mutant Kar2-1p is diminished in its protein-binding capacity but not in its ability to aid in the translocation of proteins into the ER (Brodsky et al., 1999). KHY190 (kar2-1 vma22Δ prc1-1) and KHY181 (KAR2 vma22Δ prc1-1) were radiolabeled at the permissive
Degradation of unassembled Vph1p

Fig. 8. Cytosolic but not lumenal molecular chaperones are involved in the turnover of unassembled Vph1p. KHY190 (vma22Δ Kar2-1 pcr1-1) and KHY181 (vma22Δ KAR2 pcr1-1) cells were radiolabeled at 24°C and chased at 37°C. Aliquots were taken at specific time points, the cells lysed and Vph1p immunoprecipitated from solubilized extracts (A). Vph1p-depleted extracts were then used to immunoprecipitate CPY* (B). ACY76 (vma22Δ ssa1-ts ssa2 ssa3 ssa4) cells carrying either pRS313 or pAC427 (SSA1) were radiolabeled at 24°C and chased at either 24°C (C) or 37°C (D). Vph1p was then immunoprecipitated as described.

Misfolded/unassembled integral membrane proteins in the ER also expose domains to the cytosol where they may be identified as ERQC substrates by cytosolic molecular chaperones. In yeast, the SSA sub-family of hsp70 cytosolic molecular chaperones, encoded by SSA1–SSA4, are required for the folding of certain nascent cytosolic proteins (Kim et al., 1998) in addition to assisting the translocation of some proteins into the ER (Becker et al., 1996). To determine if the SSA family of chaperones contribute to the turnover of Vph1p, the strain ACY76 (vma22Δ ssa1-ts ssa2Δ ssa3Δ ssa4Δ) was constructed which lacks Ssa2p, Ssa3p and Ssa4p and contains a temperature-sensitive mutation in the Ssa1p peptide-binding domain (Becker et al., 1996). ACY76 was transformed with either a vector plasmid (pRS313) or a plasmid containing the wild-type SSA1 gene (pAC427) and radiolabeled at 24°C. As it is not known whether Ssa1p function is required for Vph1p import into the ER membrane, the chase period proceeded for 8 min at 24°C to ensure the complete translocation of the radiolabeled Vph1p before either heated medium was added to raise the temperature immediately to the non-permissive temperature of 37°C or the chase was continued at 24°C. Aliquots were taken at specified time points, the cells lysed and solubilized Vph1p immunoprecipitated and found that the turnover of CPY* was slowed in kar2-1 cells as compared with that in KAR2 cells (Figure 8B).

Vph1p degradation occurs independently of the previously identified ERQC components Der1p, Hrd1p/Der3p and Hrd3p

Genetic screens in yeast employing CPY* and Hmg2p as ERQC substrates have identified three genes DER1, HRD1/DER3 and HRD3 required for their degradation. The potential involvement of these ER membrane proteins in the turnover of Vph1p was examined by disrupting the
This text discusses the radiolabeling experiment and double mutations in the Vph1p turnover. It also mentions the role of Vph1p in the ERQC-mediated degradation of Vph1p.

**Discussion**

Vma22p is required for assembling Vph1p into the V-ATPase

Vma22p has been identified as an ER resident protein required for the formation of the yeast V-ATPase. In the absence of Vma22p, the 100 kDa subunit of the V-ATPase, Vph1p, is degraded rapidly and specifically, requiring neither vacuolar proteases nor transport to the Golgi (Hill and Stevens, 1995). In this work, the authors characterize the fate of unassembled Vph1p in vma22Δ cells.

Fractionation experiments found the steady-state level of Vph1p in vma22Δ cells to be localized to the ER membrane and suggest two possible models for Vma22p function: one in which Vma22p is an assembly protein, required in the ER to assist Vph1p assembly into the V-ATPase, and another where Vma22p functions to aid in the insertion of Vph1p into the ER membrane. If Vma22p functions in the insertion of Vph1p into the ER membrane, we may expect that its absence would result in a Vph1p molecule that was only peripherally associated with the membrane. However, data from carbonate extraction and membrane floatation experiments (Figures 2 and 7) indicate that the Vph1p remaining in vma22Δ cells behaved as an integral membrane protein similar to wild-type Vph1p. Furthermore, the similar profile of cleavage products seen in protease shaving assays suggests that Vph1p attained the same topology in both vma22Δ and wild-type cells. It is therefore most likely that Vma22p acts to assist in the assembly of Vph1p into the V-ATPase after the protein has translocated successfully into the ER membrane. This role is very similar to that proposed for Vma12p (Jackson and Stevens, 1997), while Vma21p may play a different role in the V-ATPase assembly (Graham et al., 1998).

Vph1p degradation involves ubiquitylation and the 26S proteasome

Inhibition of Vph1p turnover by mutations in the 26S proteasome and UBC genes indicates that ubiquitylation contributes to the ERQC-mediated degradation of Vph1p. Other ERQC substrates have been found to be ubiquitylated during the course of their degradation, and it is likely that Vph1p is also ubiquitylated. Although approaches using tagged forms of ubiquitin so far have been unsuccessful in demonstrating that unassembled Vph1p is ubiquitylated, the authors have identified in vma22Δ cells a Vph1p-related species that migrates ~7 kDa larger (Figure 9A) and then CPY* (Figure 9C) from the same cell extracts, allowing a direct comparison of the data from our kinetic analysis. CPY* was found to be stabilized in radiolabeled der1Δ, hrd3Δ and hrd1Δ cells as expected, but again these mutations had no effect on the degradation of Vph1p.
92% identity with Ubc5p, and together they contribute to the selective degradation of many short-lived and abnormal proteins. Together, Ubc1p, Ubc4p and Ubc5p are considered to constitute a UBC subfamily providing an essential function as judged by the lethality of a \( ubc1\Delta \) \( ubc4\Delta \) \( ubc5\Delta \) mutation (Seufert et al., 1990). Our evidence suggests that Ubc1p, Ubc4p and Ubc5p would contribute the majority of the Vph1p-ubiquitylation activity and predicts that a \( ubc4\Delta \) \( ubc5\Delta \) double mutant would severely inhibit the degradation of unassembled Vph1p. To investigate this, we have repeatedly attempted to construct a \( vma22\Delta \) \( ubc4\Delta \) \( ubc5\Delta \) strain by both gene disruption and genetic crossing, but so far have been unsuccessful, suggesting that the disruption of \( VMA22 \) in a \( ubc4\Delta \) \( ubc5\Delta \) strain is lethal.

In contrast to the yeast ERQC substrates characterized to date, the ER-associated ubiquitin-conjugating enzymes Ubc6p and Ubc7p are not involved in the degradation of Vph1p. The subcellular localization of Ubc6p and Ubc7p at the ER membrane made them attractive candidates for the ubiquitylation of ERQC substrates, but this does not disqualify other Ubc proteins from participating in the process. Although novel, it is perhaps not surprising that Ubc proteins other than Ubc6p and Ubc7p contribute to the turnover of ERQC substrates, as it is unlikely that two Ubc proteins can recognize all possible ERQC substrates. Even within the pair of Ubc6p and Ubc7p enzymes, there is a difference in their contributions to substrate ubiquitylation as shown by the varying effects of their absence on ERQC substrate degradation. The deletion of \( UBC6 \) has no effect on HMG-R degradation (Hampton and Bhakta, 1997) yet significantly reduces the turnover of \( CPY^* \) (Hiller et al., 1996) and Sec61-2p (Biederer et al., 1996).

The molecular basis for being classified as an ERQC substrate is at present unknown, and it is possible that the process that identifies unassembled proteins such as Vph1p as ERQC substrates may differ from that used to identify misfolded ERQC substrates such as \( CPY^* \), and this process may therefore involve different Ubc proteins. Furthermore, the above discussion centers on the UBC/E2 class of enzymes dictating substrate selection for ubiquitylation and it may be that E3 or ubiquitin–protein ligases alone, or in conjunction with E2s, determine substrate specificity.

The impairment of Vph1p degradation in \( cim5 \) or \( doa4 \) cells demonstrates that the 26S proteasome participates in the degradation of unassembled Vph1p. The lack of proteolytic intermediates observed during Vph1p turnover and the absence of full-length Vph1p extracted into a soluble fraction prior to degradation suggest that Vph1p is simultaneously extracted from the membrane and degraded by the proteasome in a concerted manner, as has been suggested for other ERQC substrates (Mayer et al., 1998; Plemper et al., 1998; Xiong et al., 1999). Such an event would presumably involve the recruitment of the 26S proteasome to the ER membrane. A significant portion of 26S proteasomes have been localized to the ER and nuclear envelope network in \( Saccharomyces cerevisiae \) (Enenkel et al., 1998), and such positioning of the proteasomes, especially if bound in the vicinity of the cytosolic face of the translocon, would simplify delivery of an ERQC substrate for degradation. In addition, the six ATPase subunits within the 19S cap of the proteasome might contribute to the driving force required to retro-translocate a ubiquitylated substrate (Mayer et al., 1998; Plemper et al., 1998; Brodsky et al., 1999). However, the attractive possibility that the proteasome solely provides the required extraction force presents an apparent conflict with the findings that certain ERQC substrates, both soluble and membrane-spanning, are released as soluble proteins into the cytosol when proteasome activity is inhibited (McCracken and Brodsky, 1996; Wiertz et al., 1996a,b).

### Molecular chaperone involvement in Vph1p degradation

Kar2p, the yeast BiP homolog, is required for the degradation of soluble ERQC substrates including \( CPY^* \) (Plemper et al., 1997; this study), pro-\( \alpha \)-factor and the PiZ variant of \( \alpha \)-antitrypsin (Brodsky et al., 1999). BiP is also implicated in the degradation of soluble proteins associated with the mammalian ER in that a strong correlation has been found between the extent of BiP interaction with unassembled immunoglobulin light chains and the rate of light chain degradation (Skowronek et al., 1998). Vph1p is the first ERQC substrate whose turnover has been examined for the role of both ER lumenal molecular chaperones such as BiP and cytosolic molecular chaperones including Ssa1p. We have found that peptidyl-binding activity of Kar2p is not required for the degradation of unassembled Vph1p. The turnover of another ERQC substrate, the membrane-spanning Pdr5p\(^* \) (a mutant yeast ABC transporter), is unaffected in a different \( kar2 \) mutant strain (Plemper et al., 1998). This BiP-independent degradation of membrane-spanning ERQC substrates is also suggested in mammalian cells where truncated fragments of the influenza type I membrane protein HA do not associate with BiP during degradation (Zhang et al., 1997).

We have found that the SSA family of cytosolic hsp70s is required for the degradation of Vph1p. Similarly in mammalian cells, cytosolic hsp70 maintains a prolonged interaction with the misfolded membrane-spanning protein CFTR-\( \Delta F508 \) prior to its degradation (Yang et al., 1993). Furthermore, apolipoprotein B interacts with cytosolic hsp70 when lipid availability is reduced in the ER, and undergoes rapid degradation that is enhanced upon overexpression of hsp70 (Fisher et al., 1997). However, the SSA family of hsp70s are not needed for the turnover of yeast soluble ERQC substrates including pro-\( \alpha \)-factor and the PiZ variant of \( \alpha \)-antitrypsin (Brodsky et al., 1999). Soluble ERQC substrates appear to require luminal but not cytosolic chaperones for their degradation and, conversely, membrane-spanning substrates require cytosolic but not luminal chaperones to effect their turnover. Different components of the ERQC system may participate in the degradation of soluble and membrane-spanning substrates. Soluble ERQC substrates are probably released from the translocon into the ER lumen (Plemper et al., 1999) where BiP/Kar2p, having identified and retained the protein as a misfolded protein/ERQC substrate, may also act to return it to the translocon for retro-translocation into the cytosol. Kar2p’s ability to interact with the translocon via Sec63p may contribute to this step (Brodsky et al., 1999). Der1p might also participate in this step, as to date it has been found to be required only for the
Membrane-spanning ERQC substrates may either enter the ER membrane via a lateral gating of the translocon (Singer, 1990) or remain associated with the translocon. Re-entry of the membrane-spanning substrate into the translocon might occur by a reversal of the lateral gating mechanism. In either situation, membrane-spanning substrates could remain associated or re-associate with the translocon in a BiP-independent manner.

The functions of Ssa1p in the ERQC-mediated degradation of Vph1p might include: (i) acting as a motor or ratchet to extract Vph1p from the membrane into the cytosol in much the same manner as Kar2p is thought to provide directionality to the import of proteins into the ER (Brodsky et al., 1999); (ii) to maintain the unassembled Vph1p in a non-aggregated state accessible to the proteasome; or (iii) Ssa1p may act to partially unfold the cytosolic domain of Vph1p either to expose a ubiquitylation site for UBC enzymes in a manner similar to translocon might occur by a reversal of the lateral gating mechanism.

### Table II. Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNY28</td>
<td>MATa leu2-3,112 ura3-52 his4-519 ade6&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>KHY38</td>
<td>vma22Δ-1::URA3 in SNY28&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>RH448</td>
<td>MATa his4 leu2 ura3 lys2 bar&lt;sup&gt;i&lt;/sup&gt;</td>
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<td>KHY125</td>
<td>vph1Δ::KAN::LEU2 in KHY102</td>
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<tr>
<td>KHY127</td>
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<td>hrd3Δ::KAN in KHY125</td>
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<td>KHY140</td>
<td>hrd1Δ::LEU2 in KHY102</td>
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<td>KHY85</td>
<td>KAR2 vma22Δ-4::LEU2</td>
</tr>
<tr>
<td>KHY86</td>
<td>kar2-1 vma22Δ-4::LEU2</td>
</tr>
<tr>
<td>KHY181</td>
<td>pcr1-1 in KHY85</td>
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<tr>
<td>KHY190</td>
<td>pcr1-1 in KHY86</td>
</tr>
<tr>
<td>a1-45DU</td>
<td>MATa ssal-ts ssa2-1 ssa1-1 ssa4-2 his3-11,3-15 leu2-3,112 ura3-52 trp1-Δ1 lys2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACY76</td>
<td>MATa vma22Δ-2::URA3 ssa1-ts ssa2-1 ssa1-1 ssa4-2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Hill and Stevens (1994); <sup>b</sup>Hill and Stevens (1995); <sup>c</sup>Drs. L. Hicke and H. Riezman; <sup>d</sup>Dr. M. Hochstrasser; <sup>e</sup>Dr. R. Haguenaucr-Tsapis; <sup>f</sup>Dr. E. Craig; <sup>g</sup>Dr. M. Rose. Strains KHY183 and KHY184 are spores from a cross between RH3145 and KHY13. KHY85 and KHY86 are spores from a cross between KHY50 and MY767.
ER membrane, which would eliminate the substrate’s opportunity to aggregate in the cytosol.

Any role proposed for the participation of molecular chaperones in ERQC must account for a difference in the way that chaperones, both cytosolic and ER luminal, facilitate the folding of temporarily misfolded nascent proteins that ultimately will attain the correct mature structure in contrast to effecting the degradation of terminally misfolded proteins (Gottesman et al., 1997). It may be that misfolded proteins that never attain the correct mature structure, due to a mutation or the lack of subunit assembly, might either remain bound to, or continually be rebound by, a molecular chaperone. If the molecular chaperone remained bound to an ‘unfoldable’ protein, the increased time of association might result in entry into the ERQC pathway.

**Vph1p turnover occurs independently of DER/HRD gene products**

Three ER membrane proteins (Der1p, Hrd1p/Der3p and Hrd3p) were characterized previously as components of the yeast ERQC system. Der1p has so far been found to contribute only to the degradation of soluble ERQC substrates (Knop et al., 1996), whereas Hrd1p/Der3p plays a significant role in the turnover of HMG-R (Hampton et al., 1996), CPY* and Sec61-2p (Bordallo et al., 1998). Hrd3p is required for the degradation of HMG-R (Hampton et al., 1996), and our work has extended its involvement to the turnover of CPY*. Given the participation of Hrd1p/Der3p and Hrd3p in the degradation of both soluble and membrane-spanning ERQC substrates, it was surprising that the absence of either of these gene products had no effect on Vph1p turnover. Vph1p appears to possess the traits of a ‘classical’ ERQC substrate in that it is unassembled protein in the ER that is degraded rapidly in a process involving ubiquitylation and the proteasome. However, the ability to degrade Vph1p independently of the known components of the ERQC system identified to date (Ubc6p, Ubc7p, Der1p, Hrd1p/Der3p and Hrd3p) suggests that either (i) the ERQC system can bypass the normal requirements for these proteins to degrade Vph1p, perhaps because Vph1p is unassembled rather than misfolded, or (ii) that other ERQC components, perhaps comprising an alternative parallel pathway, exist to effect the degradation of Vph1p. The lack of complete stabilization of Vph1p in proteasome mutants supports the possibility of an alternative degradative system contributing to Vph1p turnover. An alternative pathway might employ the proteolytic activity recently identified in mammalian cells treated with proteasome inhibitors (Glas et al., 1998). Vacular proteases can be eliminated as candidates for this activity as the disruption of PEP4 in a cim5-1 strain did not stabilize Vph1p further (data not shown). Components of such a pathway should be identified by the genetic screen presently underway to isolate mutants that stabilize Vph1p. This plate-based assay utilizes a Vph1p-based reporter substrate which, as expected, is not stabilized by disruptions of DER1, HRD1/DER3 or HRD3 (K. Hill and A.A. Cooper, unpublished data).

### Table III. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKH2212</td>
<td>Hill and Stevens (1995)</td>
</tr>
<tr>
<td>pKH2213</td>
<td>Hill and Stevens (1995)</td>
</tr>
<tr>
<td>pMM322</td>
<td>Manolson et al. (1992)</td>
</tr>
<tr>
<td>pphpL:A--LEU2</td>
<td>Manolson et al. (1992)</td>
</tr>
<tr>
<td>YCp50-SSA1</td>
<td>A gift from Dr E.Craig</td>
</tr>
<tr>
<td>pDf65C</td>
<td>From Dr D.Jackson</td>
</tr>
<tr>
<td>pAC334</td>
<td>A 1.1 kb URA3 fragment from pJJ252 inserted into SryI-digested (blunted) pKH2212. pAC334 was cut by Spel–XhoI prior to transformation</td>
</tr>
<tr>
<td>pAC335</td>
<td>pphpL:A--LEU2 was digested with NheI–EcoRV, and a Smal–SpeI fragment from pF6-KanMX6 inserted. pAC335 was digested with BamHI–Apal prior to transformation</td>
</tr>
<tr>
<td>pAC336</td>
<td>pKH2212 was digested with SryI, blunt ended and treated with alkaline phosphatase. A Smal–EcoRV kan^2-containing DNA fragment from pF6-MX4 was then inserted, pAC336 was digested with Spel–XhoI prior to transformation</td>
</tr>
<tr>
<td>pAC338</td>
<td>DER1 was amplified by PCR from genomic DNA prepared from SEY6211 cells to yield an 842 bp fragment which was digested with BamHI–ClaI and inserted into BamHI–ClaI-digested pBluescript (Stratagene)</td>
</tr>
<tr>
<td>pAC341</td>
<td>pAC338 was digested with BsaI, and a Smal–EcoRI fragment from pF6-KanMX4 inserted. pAC341 was digested with BamHI–SalI prior to transformation</td>
</tr>
<tr>
<td>pAC356</td>
<td>The prc1-1 allele was amplified from genomic DNA prepared from SF648-5Da cells using PCR. The resulting 3 kb fragment was cloned into the SalI site of pCR-Script</td>
</tr>
<tr>
<td>pAC357</td>
<td>pAC356 was digested with HindIII, and a 1.1 kb HindIII DNA fragment containing URA3 from pJJ242 inserted. pAC357 was digested with BglII prior to transformation</td>
</tr>
<tr>
<td>pAC359</td>
<td>pKH2212 was digested with HindIII, and an XhoI linker (Stratagene) inserted</td>
</tr>
<tr>
<td>pAC361</td>
<td>pAC359 was digested with XhoI–SpeI and inserted into the corresponding sites in pRS306, pAC361 was digested with PstI prior to transformation</td>
</tr>
<tr>
<td>pAC367</td>
<td>pAC370 was digested with BglII–NsiI to release an 825 bp fragment which was then replaced with a BamHI–PstI LEU2 fragment and inserted into the corresponding sites in pRS306, pAC367 was digested with Drai prior to transformation</td>
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<tr>
<td>pAC368</td>
<td>A 2.5 kb fragment containing HRD3 was amplified by PCR from genomic DNA isolated from SEY6211 cells and inserted into the SalI site of pCR-Script</td>
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<td>pAC370</td>
<td>HRD1 was amplified by PCR as described above to yield a 1.8 kb fragment which was inserted into the SalI site of pCR-Script (Stratagene)</td>
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<td>pAC372</td>
<td>pAC368 was digested with BglII–HpaI, and a BamHI–EcoRV fragment from pF6-KanMX4 inserted. pAC372 was digested with BamHI–Nhel prior to transformation</td>
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<tr>
<td>pAC427</td>
<td>YCp50-SSA1 was digested with BamHI–HpaI and the SSA1-containing fragment inserted into EcoRV–BamHI-digested pRS313 to create pAC427</td>
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</table>
Materials and methods

Materials
Restriction enzymes were purchased from New England Biolabs. Chemicals were purchased from either Fisher or Sigma.

Strains, plasmids, media and microbiological techniques

Media were prepared as described by Hill and Stevens (1994). Yeast strains used in this study are listed in Table II and the plasmids used are described in Table III. In order to introduce the prc1-1 allele, pAC357 was transformed into KHY92 cells and Ura+ protoplasts were selected on 5-fluoro-orotic acid (5-FOA). Ura- colonies were screened for replacement of the wild-type {PRC1} gene by Western blot to detect CPY*. DER1 was disrupted in KHY125 cells by transformation with pAC341 and selection on YEPD pH 5.0 medium containing 200 μg/ml G418 (Sigma). G418-resistant candidates were screened by Western blotting to detect stabilized CPY*. The structure of the der1Δ disruption in KHY127 was confirmed by PCR analysis on genomic DNA utilizing oligonucleotides specific for the DER1 locus. HRD3 was disrupted in KHY125 cells using pAC372 and selection on G418-containing YEPD pH 5.0. The structure of the disruption in KHY138 was confirmed by PCR analysis as described. HB101 was deleted from KHY102 cells using pAC367 and selecting Leu+ protoplasts. Prototrophs were screened for stabilized CPY* by Western blotting, and the structure of the disruption in KHY140 confirmed by PCR analysis. KHY125, KHY127, KHY138 and KHY140 cells were transformed with pMM32 before radiolabeling/immunoprecipitation to detect Vph1p and CPY*. The vph1Δ::LEU2 allele contained in ACY73 and ACY77 was introduced as described (Manolishon et al., 1992). Leu+ protoplasts were examined by Western blotting to confirm the absence of Vph1p. The vma22Δ::URA3 allele contained in KHY38, KHY51, KHY67 and KHY180 was created by PCR amplification as described (Hill and Stevens, 1995). The vma22Δ::URA3 allele contained in KHY58, KHY70, KHY104, KHY154, KHY172, KHY173, KHY175 and KHY177 cells was introduced by transformation with pAC334, and Ura- transformants were tested for pH sensitivity. ACY73 and ACY77 contain vma22Δ::TRP1 introduced via PCR amplification as described (Hill and Stevens, 1995) except that pRS314 was used as a template. KHY13 contains vma22Δ::LEU2 introduced by transformation with pKH2213 (Hill and Stevens, 1995). The vma22Δ::STOP allele contained in KHY92 and KHY94 was introduced by transformation with pAC361. The vma22Δ::TRP1 allele was then excised via selection on 5-FOA, and pH-sensitive Trp- colonies selected. The vma22Δ::KAN allele contained in KHY77 and KHY78 was created using pAC336 and selection on YEPD pH 5.0 + G418. Resistant colonies were screened for pH sensitivity. The vph1Δ::Leu2Δ::KAN allele contained in ACY74, KHY102 and KHY125 cells was created by transformation with pAC335 and selection on YEPD pH 5.0 + G418. G418-resistant colonies were then tested for a Leu+ phenotype and the absence of Vph1p was confirmed by Western blot.

Protein preparation, antibodies and Western blotting

Whole-cell protein extracts were prepared as described (Hill and Stevens, 1994). Secondary antibodies were purchased from Bio-Rad. Immunoblots were detected using chemiluminescent or chemiluminescent detection (Amersham). Polyclonal antibodies were generated by HTI Bio-Products Inc. Monoclonal antibodies were purchased from Molecular Probes. Protease shaving experiments were performed as outlined by Jackson and Stevens (1997).

Radiolabeling and immunoprecipitation

Pulse-chase immunoprecipitations of Vph1p were performed essentially as described (Hill and Stevens, 1994). Samples were removed at set times during the chase period, processed for immunoprecipitation and resolved by SDS–PAGE. Gels were fixed, dried and exposed either to a phosphor cassette prior to quantitation using a phosphorimager (Molecular Dynamics) or to X-ray film. Immunoprecipitation of CPY was carried out as described previously (Hill and Stevens, 1994). Sodium carbonate fractionation was performed as described previously (Hill and Stevens, 1994).

Vesicle pelleting and flotation

Microsomal pellets were prepared as described above except that microsomes were collected at 100 000 g for 30 min. Microsomal flotation experiments were performed as described by Xiong et al. (1999).

Membrane fractionation

Membrane fractionation was performed as described by Roberg et al. (1997) except the gradients were centrifuged at 50 000 r.p.m. in an SW55.1 rotor for 14 h before samples were collected from the bottom of the tube.

Acknowledgements

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References

Degradation of unassembled Vph1p


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