

Two Distinctly Localized P-Type ATPases Collaborate to Maintain Organelle Homeostasis Required for Glycoprotein Processing and Quality Control

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Membrane transporter proteins are essential for the maintenance of cellular ion homeostasis. In the secretory pathway, the P-type ATPase family of transporters is found in every compartment and the plasma membrane. Here, we report the identification of *COD1/SPF1* (control of HMG-CoA reductase degradation/*SPF1*) through genetic strategies intended to uncover genes involved in protein maturation and endoplasmic reticulum (ER)-associated degradation (ERAD), a quality control pathway that rids misfolded proteins. Cod1p is a putative ER P-type ATPase whose expression is regulated by the unfolded protein response, a stress-inducible pathway used to monitor and maintain ER homeostasis. *COD1* mutants activate the unfolded protein response and are defective in a variety of functions apart from ERAD, which further support a homeostatic role. *COD1* mutants display phenotypes similar to strains lacking Pmr1p, a Ca²⁺/Mn²⁺ pump that resides in the medial-Golgi. Because of its localization, the previously reported role of *PMR1* in ERAD was somewhat enigmatic. A clue to their respective roles came from observations that the two genes are not generally required for ERAD. We show that the specificity is rooted in a requirement for both genes in protein-linked oligosaccharide trimming, a requisite ER modification in the degradation of some misfolded glycoproteins. Furthermore, Cod1p, like Pmr1p, is also needed for the outer chain modification of carbohydrates in the Golgi apparatus despite its ER localization. In strains deleted of both genes, these activities are nearly abolished. The presence of either protein alone, however, can support partial function for both compartments. Taken together, our results reveal an interdependent relationship between two P-type ATPases to maintain homeostasis of the organelles where they reside.

INTRODUCTION

During biosynthesis, nascent secretory proteins first pass the membranes of the endoplasmic reticulum (ER) through a proteinaceous pore called the translocon (Johnson and van Waes, 1999). In the lumen, ER chaperones and folding catalysts assist their folding and assembly. Because these factors are found only in the ER, the folding state of proteins is monitored to assure that only properly folded proteins traffic to their sites of function. This mechanism, termed “ER quality control,” also functions to select irreversibly damaged proteins for degradation. In this mode, misfolded proteins are exported back to the cytosol, presumably through the translocon, where they are ubiquitinated and degraded by the 26S proteasome (for review, see Brodsky and McCracken, 1999; Ellgaard and Helenius, 2001). Other than the

terminal step, termed ER-associated degradation (ERAD), the mechanisms governing ER quality control remain poorly understood.

Two pioneering studies used genetic methodologies to unravel the mechanisms underlying the degradation of proteins in the ER. One used the direct approach of screening for mutants defective in the degradation of two model misfolded soluble proteins: mutant carboxypeptidase Y (CPY*; Wolf and Fink, 1975) and mutant proteinase A (PrA*; Finger *et al.*, 1993; Knop *et al.*, 1996). The mutant strains, termed *der* (degradation in the endoplasmic reticulum), uncovered several genes of the ubiquitin/proteasomal degradation pathway that provided compelling evidence that misfolded proteins in the ER lumen are exported to the cytosol for degradation. One gene, *DER5*, did not fall into this category. Instead, *DER5* was allelic to *PMR1*. *PMR1* encodes a Ca²⁺/Mn²⁺ ion pump of the P-type ATPase family (Durr *et al.*, 1998; Strayle *et al.*, 1999). It was a surprising discovery because Pmr1p is a Golgi-localized enzyme without a

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Table 1. Strains used in this study

Strain	Genotype	Source
W303	<i>Mata, leu2-3,112, his3-11, trp1-1, ura3-1, can1-100, ade2-1</i>	P. Walter, UCSF
DNY507	<i>Mata, per9-1, ura3-1, can1-100, ade2-1, ade3, leu2-3-112, his3-11::HIS3-UPRE LacZ</i> [pDN336]	Ng <i>et al.</i> , 2000
JC408	<i>Mata, hac1::URA3, leu2-3-112::LEU-UPRE LacZ</i> , W303 background	Cox <i>et al.</i> , 1996
SMY8	<i>Mata, per9::HIS3</i> , W303 background	This study
SMY33	<i>Mata, pmr1::LEU2</i> , W303 background	This study
SMY36	<i>Mata, cod1::HIS3, pmr1::LEU2</i> , W303 background	This study
SMY15	<i>Mata</i> , W303, [pSM2]	This study
SMY16	<i>Mata</i> , W303, [pSM6]	This study
SMY24	<i>Mata, cod1::HIS3</i> , [pSM2], W303 background	This study
SMY44	<i>Mata</i> , W303, [pDN431], W303 background	This study
SMY46	<i>Mata, cod1::HIS3</i> , [pDN431], W303 background	This study
SMY42	<i>Mata, pmr1::LEU2</i> , [pDN431], W303 background	This study
SMY337	<i>Mata, cod1::HIS3, pmr1::LEU2</i> , [pDN431], W303 background	This study
SMY331	<i>Mata, cod1::HIS3, pRS315</i> , [pDN431], W303 background	This study
SMY332	<i>Mata, cod1::HIS3, pDN431</i> , [pSM2], W303 background	This study
SMY334	<i>Mata, cod1::HIS3</i> , [pSM1083], W303 background	This study
SMY336	<i>Mata, pmr1::LEU2</i> , [pSM1083], W303 background	This study
SMY339	<i>Mata, cod1::HIS3, pmr1::LEU2</i> , [pSM1083], W303 background	This study
SMY371	<i>Mata</i> , W303, [pSM1083]	This study
SS330	<i>Mata, ade2-101, his3Δ200, tyr1, ura3-52</i>	Vijayraghavan <i>et al.</i> , 1989
YG746	<i>Mata, ade2-101, his3Δ200, tyr1, ura3-52, mns1::KanMX4</i>	Jakob <i>et al.</i> , 1998

known equivalent in the ER of *Saccharomyces cerevisiae* (Antebi and Fink, 1992; Sorin *et al.*, 1997). The requirement of *PMR1* for ERAD is likely due to its role in maintaining normal Ca^{2+} levels in the ER (Durr *et al.*, 1998). *Pmr1p* is also needed for Golgi-specific functions dependent on divalent cations such as protein outer-chain glycosylation (Rudolph *et al.*, 1989; Durr *et al.*, 1998).

The second study sought to understand the regulation of hydroxymethylglutaryl-CoA reductase. In *S. cerevisiae*, two isoenzymes, Hydroxymethylglutaryl-CoA reductase (*Hmg1p* and *Hmg2p*), contribute to the HMG-CoA reductase activity resident in the ER membrane and play a key role in the biosynthesis of sterols and isoprenoids (for review, see Hampton, 1998). Whereas the *Hmg1* protein is responsible for basal constitutive activity, the *Hmg2p* activity is subject to regulatory processes in part by degradation in response to feedback signals from the mevalonate pathway. Combining genetic and biochemical approaches, Hampton and coworkers (1994) demonstrated that *Hmg2p* degradation uses core components of the ERAD pathway (Hampton and Rine, 1994; Hampton *et al.*, 1996; Hampton, 1998). For example, the *HRD1/DER3* gene, encoding an E3 ubiquitin ligase, is required for degrading misfolded proteins and *Hmg2p* (Bays *et al.*, 2001). Thus, ERAD is used for ER quality control and as a means to regulate the activity of *Hmg2p*. To investigate the signaling mechanism, mutants were isolated that allowed the constitutive degradation of *Hmg2p* under normally stabilizing conditions of reduced feedback signals (Cronin *et al.*, 2000). These were designated *cod* (control of HMG-CoA reductase degradation) and fell into a single complementation group, *cod1*. Cloning of *COD1* revealed its identity as *SPF1* (Cronin *et al.*, 2000). *SPF1* was previously identified to confer salt mediated killer toxin (SMKT) sensitivity, but the mechanism of action is unknown. Interestingly, *COD1/SPF1* encodes a putative P-type

ATPase of a class distinct from *PMR1* (Suzuki and Shimma, 1999). Nevertheless, phenotypic analysis of *COD1/SPF1* mutants suggested a possible role in Ca^{2+} homeostasis (Cronin *et al.*, 2000, 2002). Together, the two studies established roles for P-type ATPases in ERAD, but in seemingly different ways.

ERAD activity is regulated in part by the UPR (unfolded protein response) (Casagrande *et al.*, 2000; Friedlander *et al.*, 2000; Ng *et al.*, 2000; Travers *et al.*, 2000). The UPR is a signal transduction pathway between the ER and nucleus used by the cell to monitor and respond to the changing needs of the early secretory pathway (for review, see Patil and Walter, 2001; Spear and Ng, 2001). During ER disequilibrium, an array of target genes is transcriptionally activated to restore homeostasis (Travers *et al.*, 2000). To better understand the physiological role of the UPR, we previously performed a genetic screen to identify functions physiologically linked to the pathway (Ng *et al.*, 2000). Mutants obtained from this study, designated *per* (protein processing in the ER), lead to the constitutive activation of the UPR that, in turn, is required for their viability. A variety of functions involved in secretory protein biogenesis and ER quality control were revealed by this approach. One mutant, *per9-1*, is defective in the degradation of CPY*. Here, we report the identity of the *PER9* gene as identical to *COD1/SPF1*. We show that *Cod1p* is an ER-localized protein that functions together with *Pmr1p* to maintain glycoprotein processing activities in protein biosynthesis and ER quality control. Null mutants of either gene alone partially disrupt specific functions of both the ER and Golgi, irrespective of their primary sites of residence. Of cation-dependent functions, a *cod1 pmr1* double mutation nearly abolishes activity, suggesting that each protein can partially compensate for the loss of the other. Taken together, our data support a model of two P-type ATPases, one in the ER and another

in the Golgi, working together to maintain homeostasis in the two organelles.

MATERIALS AND METHODS

Plasmids Used in This Study

pSM2 is an expression vector with a hemagglutinin (HA) epitope-tagged version of *COD1*. To construct pSM2, the *COD1* gene was first subcloned into *SpeI* and *NotI* (blunt by T4 DNAP) of pRS315 (Sikorski and Heiter, 1989) as a 4580-base pair *SspI/SpeI* fragment to generate the complementing clone pSM1. A C-terminal HA-epitope tag was introduced in 3 steps: Purification of a *SacI/HpaI* fragment from pSM1 containing the *COD1* promoter and amino-proximal coding sequences; amplification of the 3' 1 kb of *COD1* coding sequences by high-fidelity polymerase chain reaction (PCR) using the primers S1 (5'-GCACACTTATCCACCTGGTCC-3') S2 (5'-CATAAAAGCCATGGCTTTAGAGGCAATCTT-3') followed by digestion with *NcoI*; and purification of the vector pDN413 bearing a single HA-epitope tag followed the *ACT1* terminator in pRS315 cleaved with *SacI* and *NcoI*. pSM2 was created by ligation of these three fragments.

pSM6 is the same as pSM2 except that HA-epitope tagged *COD1* was subcloned into pRS425 vector. The construction of pDN431 (HA-epitope tagged CPY*) was described previously (Ng *et al.*, 2000). pSM1346 was a gift from S. Michaelis (Johns Hopkins University, Baltimore, MD; Loayza *et al.*, 1998).

pSM5 contained the *cod1::HIS3* knockout construct. Upstream sequences (500 base pairs) of the *COD1* open reading frame were amplified by PCR using T7 and S3 primers (S3: 5'-GGGTTACCGATTCTATGT TTC-3') and pSM1 as template. Downstream sequences (447 base pairs) were amplified using T3 and S4 primers (S4: 5'-GGGTAAATCTTTTATGTAAGTAC-3'). The fragments were digested with *SacI* and *SpeI*, respectively, and were inserted into pBS SKII(+). Ligation of the two fragments created an internal *SmaI* site. The *HIS3* gene from pRS303 (Sikorski and Heiter, 1989) was inserted into the *SmaI* site to generate the plasmid pSM5. To generate a *COD1* null strain, pSM5 was digested with *SacI* and *SpeI*, and the fragments were transformed into W303 diploid cells. Tetrad dissection yielded four viable spores per tetrad with histidine prototrophs segregating 2:2 on replica plates (our unpublished data). Integration of *HIS3* into the *COD1* locus was confirmed by PCR analysis of genomic DNA.

Cloning of the PER9 Gene

DNY507 cells were transformed with pDN388 (*LEU2*, *IRE1*, and *ADE3*) to replace pDN366 (*URA3*, *IRE1*, and *ADE3*) by plasmid shuffle (Ng *et al.*, 2000). The resulting strain was transformed with a centromere-based genomic library (Lagosky *et al.*, 1987). Approximately 10,000 transformants were grown on synthetic complete (SC) media containing low adenine concentration (6 $\mu\text{g}/\text{ml}$) and lacking uracil, and were screened for the reversal of the red, nonsectoring phenotype. Five sectoring colonies were picked and streaked onto nonselective media to drop the reporter plasmid pDN388. The plasmid clones were recovered from each strain and restriction analysis was performed to estimate the length of each insert. The flanking regions of the shortest clone, p82-R2, were sequenced to reveal a 10,913-base pair fragment from chromosome V. The insert contained four intact open reading frames (*COD1/SPF1*, *ECM1*, *BUD16*, and *YEL028W*). Digestion of p82-R2 with *ClaI* followed by religation generates a plasmid (p82-R2 Δ Cla) containing *COD1/SPF1* as the only intact open reading frame. p82-R2 Δ Cla was found to complement all mutant phenotypes of *per9-1* and the *cod1* null strain.

Cell Labeling and Immunoprecipitation

Typically, 2 A_{600} OD units of log phase cells were pelleted and resuspended in 1.0 ml of SC media lacking methionine and cysteine.

After 30 min of incubation at the appropriate temperature, cells were labeled with 480 μCi of Tran³⁵S-label (ICN Biomedicals, Irvine, CA). A chase was initiated by adding cold methionine/cysteine to a final concentration of 2 mM. The chase was initiated 30 s before the end of the pulse to exhaust intracellular pools of unincorporated label. Labeling/chase was terminated by the addition of trichloroacetic acid to 10%. Preparation of cell lysates, immunoprecipitation procedures, gel electrophoresis, and quantification of immunoprecipitated proteins were performed as described previously (Ng *et al.*, 2000).

Indirect Immunofluorescence

Cells were grown in appropriate SC media to an OD_{600} of 0.5–0.9 and were treated with 2.5 $\mu\text{g}/\text{ml}$ tunicamycin for 60 min to induce the UPR. Formaldehyde (EM grade; Polysciences, Inc., Warrington, PA) was then added directly to the media to 3.7% at 30°C for 1 h. After fixation, cells were collected by centrifugation and were washed with 5 ml 0.1 M potassium phosphate buffer (pH 7.5). Cell walls were disrupted by incubation in 1.0 mg/ml zymolyase 20T (ICN Biomedicals, Aurora, OH) in 0.1 M potassium phosphate, pH 7.5, and 0.1% 2-mercaptoethanol for 30 min at 30°C. Spheroplasts were washed once with phosphate-buffered saline (PBS) and were resuspended. Thirty microliters of cell suspension was applied to each well of a poly-L-lysine-coated slide for 1 min and were washed three times with PBS. Slides were immersed in methanol for 5 min followed by immersion in acetone for 30 s at –20°C and were allowed to air dry. Subsequent steps were performed at room temperature. Thirty microliters of PBS block (3% bovine serum albumin in PBS) was added to each well and was incubated for 30 min. Primary antibodies α -HA or α -Kar2p applied were used at 1:1000 or 1:5000 dilutions in PBS block, respectively, for 1 h. Wells were washed three to five times with PBS block. Thirty microliters of secondary antibodies (Alexa Fluor 488 goat α -mouse or α -rabbit and Alexa Fluor 546 goat α -mouse or α -rabbit; Molecular Probes, Sunnyvale, CA) was added to wells and incubated for 45 min in the dark. Wells were washed five to seven times with PBS block and two times with PBS. Each well was sealed with 5 μl of mounting medium (PBS, 90% glycerol, and 0.025 $\mu\text{g}/\text{ml}$ 4,6-diamidino-2-phenylindole) and a glass coverslip. Samples were viewed on a Zeiss Axioplan epifluorescence microscope (Carl Zeiss, Thornwood, NY). Images were collected using a Spot 2 cooled charged-coupled device camera (Diagnostic Instruments, Sterling Heights, MI) and were archived using Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA).

Labeling, Extraction, and Analysis of N-linked Oligosaccharides

Cells were grown in yeast extract/peptone/dextrose (YPD) at 30°C to midlog phase. Cells (4×10^8) were harvested, washed once with low glucose medium (YP with 0.1% glucose [YP0.1D]), and resuspended in 200 μl of YP0.1D. Oligosaccharide labeling was started by the addition of 50 μl of YP0.1D containing 95 μCi of 2-[³H]D-mannose (21 Ci/mmol; Moravsek Biochemicals, Brea, CA). After incubation for 12 min at 30°C, lipid-linked oligosaccharides were extracted as described previously (Zufferey *et al.*, 1995). The pellet containing glycoprotein was dried, resuspended in 200 μl of 0.75% SDS and 2% 2-mercaptoethanol, and denatured for 10 min at 100°C. N-glycans were released by enzymatic hydrolysis for 14 h at 37°C in 300 μl of 0.5% SDS, 1% NP40, 50 mM sodium phosphate, pH 7.5, 1.33% 2-mercaptoethanol, and 2 U of N-glycosidase F (Roche Molecular Biochemicals, Indianapolis, IN). Cell debris was removed by ethanol precipitation. The N-linked oligosaccharides were analyzed by high-performance liquid chromatography as previously described (Zufferey *et al.*, 1995). A mixture of radiolabeled oligosaccharides of known structure served as standard.

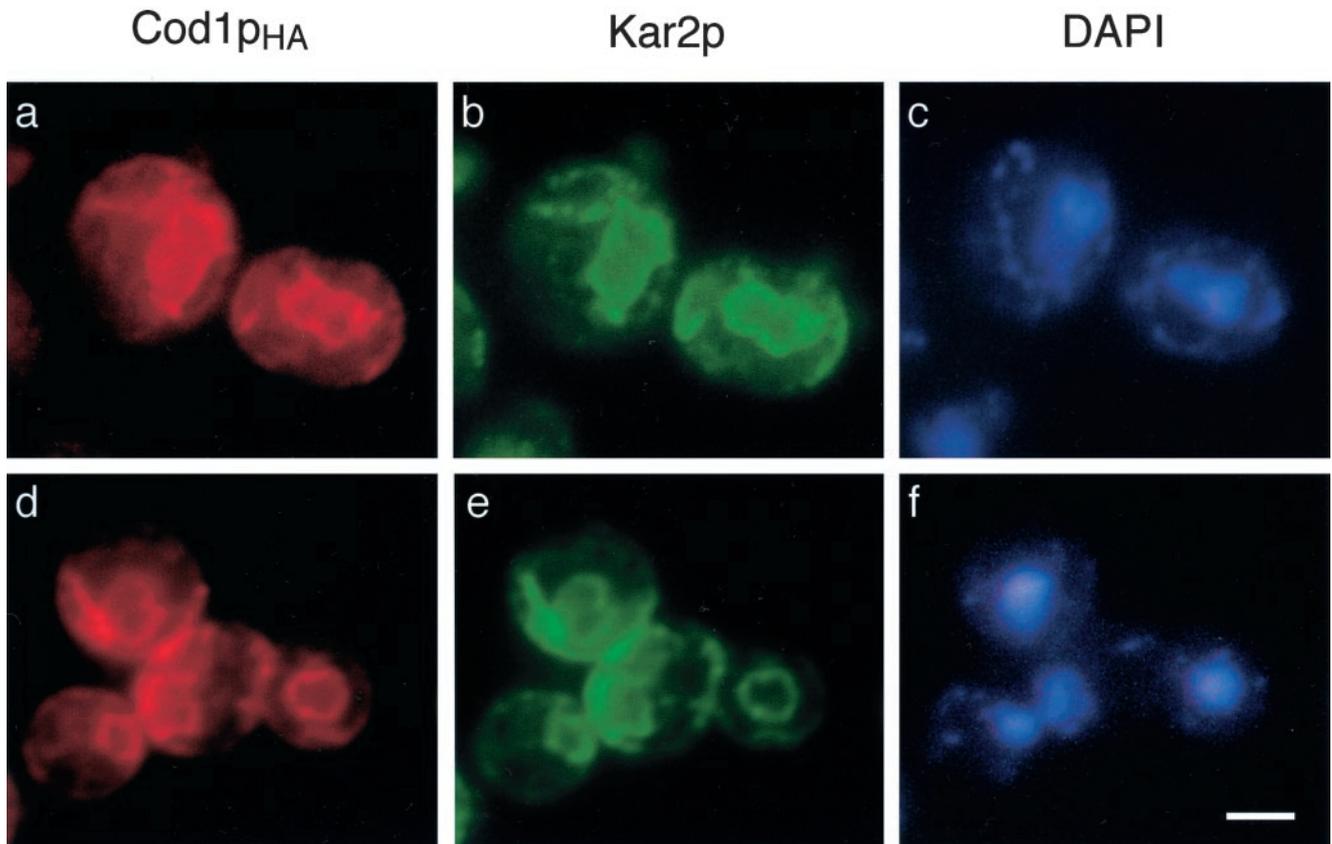


Figure 1. Intracellular localization of Cod1p_{HA}. Fixed and permeabilized wild-type cells expressing Cod1p_{HA} (SMY15) were decorated with anti-Kar2p polyclonal and anti-HA monoclonal (HA.11) antibodies. Antibody/antigen complexes were detected by applying Alexa Fluor 546 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit secondary antibodies. Cod1p_{HA} was visualized in the green channel (a and d), and the ER marker BiP was detected in the red channel (b and e). Cells were incubated in the absence (a-c) or presence (d-e) of 1 $\mu\text{g}/\text{ml}$ tunicamycin for 2 h. Because exposure times were optimized for image quality required for localization, the relative fluorescence intensities as shown do not reflect relative protein levels. Cells were stained with 4,6-diamidino-2-phenylindole to visualize nuclei (c and f). Bar, 2 μm .

RESULTS

The PER9 Gene Encodes a Putative P-type ATPase Localized in the ER

We previously reported that the unfolded protein response regulates multiple functions of the ER to maintain homeostasis (Ng *et al.*, 2000). In that study, a genetic strategy uncovered mutants (designated *per*) defective for a variety of ER functions, including ERAD. To better understand ER quality control mechanisms, we focused our efforts to clone the complementing genes of ERAD-defective *per* mutants. Among these, *PER8* and *PER16* were identified as the ERAD-related genes *SON1/RPN4* and *UBC7*, respectively (Biederer *et al.*, 1997; Mannhaupt *et al.*, 1999). One mutant, *per9-1*, was not complemented by known ERAD genes in our collection, suggesting that the *PER9* gene might be novel. Using a colony color sectoring assay, several complementing clones were isolated from a centromere-based genomic library (see "Materials and Methods"). By sequencing both ends of the shortest clone, we determined that *PER9* was contained within a 10,913-base pair fragment from chromosome V. Of four intact open reading frames found within the

insert, deletion mapping showed that *PER9* is identical to the *COD1/SPF1* gene (hereafter referred as *COD1* for simplicity; Suzuki and Shimma, 1999; Cronin *et al.*, 2000). Together, these data suggest that *COD1* is required for efficient degradation of misfolded proteins in addition to its previously defined roles in killer toxin sensitivity and regulation of HMGR stability.

To facilitate analysis of the Cod1 protein, an HA-epitope tagged version of the gene controlled by the native promoter was constructed (Cod1p_{HA}, see "Materials and Methods" for details). The tagged version is functional as it complements every mutant phenotype examined (our unpublished data). We next sought to determine the site of Cod1p function because Pmr1p, another P-type ATPase needed for ERAD, is a resident of the Golgi apparatus. Indirect immunofluorescence was performed to localize Cod1p_{HA}. As shown in Figure 1, Cod1p_{HA} staining is prominent in regions underlying the plasma membrane and within the nuclear envelope. These features are distinct from the Golgi apparatus, which appears punctate in budding yeast (Rosanese *et al.*, 1999). Instead, the pattern is characteristic of the ER. This assertion was confirmed by perfect coincident

staining with BiP, a well-established ER marker (Figure 1, compare a and b). In addition, induction of Cod1p expression by tunicamycin does not alter its localization under cellular stress (Figure 1, d and e). These data show that Cod1p is primarily localized in the ER and are in agreement with a recent report by Hampton and colleagues (Cronin *et al.*, 2002)

The COD1 Gene Is Regulated by the UPR Signaling Pathway

Among P-type ATPases with established functions, most couple ATP hydrolysis to the transport of ions across membranes (Catty *et al.*, 1997). These proteins are present in every compartment of the secretory pathway and function primarily to maintain transmembrane ion gradients. The *per9-1* mutant was isolated on the basis of a synthetic lethal interaction with *IRE1*, a key component of UPR signaling pathway. In *per9-1* cells, the UPR is constitutively activated, indicating that the loss of *COD1* activity causes ER disequilibrium (Ng *et al.*, 2000). This was not surprising given the established cellular roles of P-type ATPases. Because of its physiological link to the UPR, we wondered whether the pathway directly regulates *COD1*. For this, we measured the synthesis of *COD1* message and protein after treatment with the glycosylation inhibitor tunicamycin, a potent inducer of the UPR. As shown in Figure 2A, *COD1* mRNA is elevated 1.8-fold after 60 min of treatment. This is in agreement with data obtained by whole genome expression analysis (Travers *et al.*, 2000). The induction is UPR specific because no change is observed under identical conditions in the UPR-deficient strain $\Delta hac1$ (Figure 2A, lanes 3 and 4). To confirm that an increase in message level results in an increase in Cod1 protein synthesis, the translation rate was measured. As shown in Figure 2B, Cod1p_{HA} synthesis is elevated 3.3- and 3.8-fold after 60 and 120 min of tunicamycin treatment, respectively. Under stress, although Cod1p levels are elevated, it remains localized in the ER (Figure 1, d and e). These data show that *COD1* is part of the UPR regulatory program for maintaining ER homeostasis. By contrast, *PMR1* transcript levels do not change in response to ER stress, and a *pmr1* null mutant does not exhibit synthetic interactions with *IRE1* (Durr *et al.*, 1998 and our unpublished data).

The Requirement of COD1 and PMR1 in ERAD Is Substrate Specific

Because Cod1p and Pmr1p are localized to distinct compartments, we wished to understand their respective roles in ERAD. We constructed strains deleted of the *COD1* and *PMR1* genes to compare the effects when either or both proteins are absent. We measured the rates of CPY* degradation by metabolic pulse-chase analysis. As shown in Figure 3A, the delay of CPY* degradation was similar in the *cod1* and *pmr1* single mutants. By contrast, CPY* turnover was most compromised in the double mutant. Because the effects of the mutations are additive, the data suggest that the two proteins function independently in their respective compartments. The trivial explanation that the increased severity is a consequence of a general loss of ER function could be ruled out because translocation and core glycosyl-

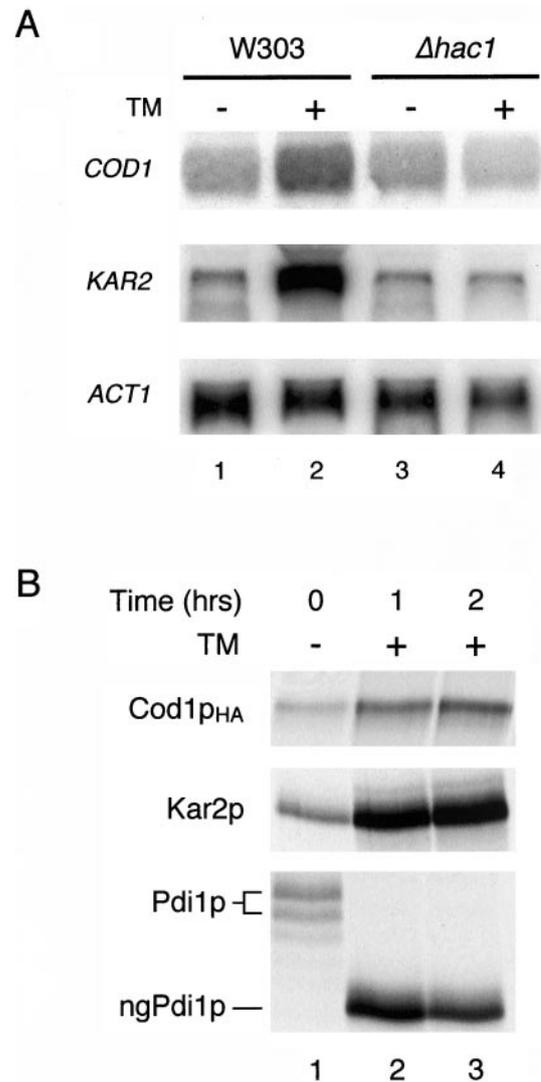


Figure 2. *COD1* expression is regulated by the unfolded protein response. (A) Wild-type (W303a) and UPR-deficient ($\Delta hac1$: JC408) cells were mock treated or incubated with 2.5 $\mu\text{g}/\text{ml}$ tunicamycin (TM) for 60 min. RNA was extracted, separated by agarose gel electrophoresis, and transferred to a nylon filter. The blot was probed sequentially using [^{32}P]labeled probes against *COD1*, *KAR2* (positive control), and *ACT1* (loading control) and was visualized by autoradiography. (B) Wild-type cells expressing Cod1p_{HA} from its native promoter (SMY15) were treated with tunicamycin (TM) for 0, 1, and 2 h. Equal cell numbers from each time point were pulse-labeled with [^{35}S]met/cys and detergent lysates were prepared. Cod1p_{HA}, Kar2p, and protein disulfide isomerase (Pdi1p) were immunoprecipitated, separated by SDS-PAGE, and visualized by autoradiography. Kar2p and Pdi1p expression is regulated by the unfolded protein response and serve as positive controls. The glycosylated (B, "Pdi1p \square ") and nonglycosylated (B, "ngPdi1p — ") forms of Pdi1p are indicated. Whole detergent lysates of each time point were analyzed by SDS-PAGE to determine that overall incorporation of label was equal (data not shown). Quantification of Northern blots (A) and labeled proteins (B) was performed by phosphorimager analysis.

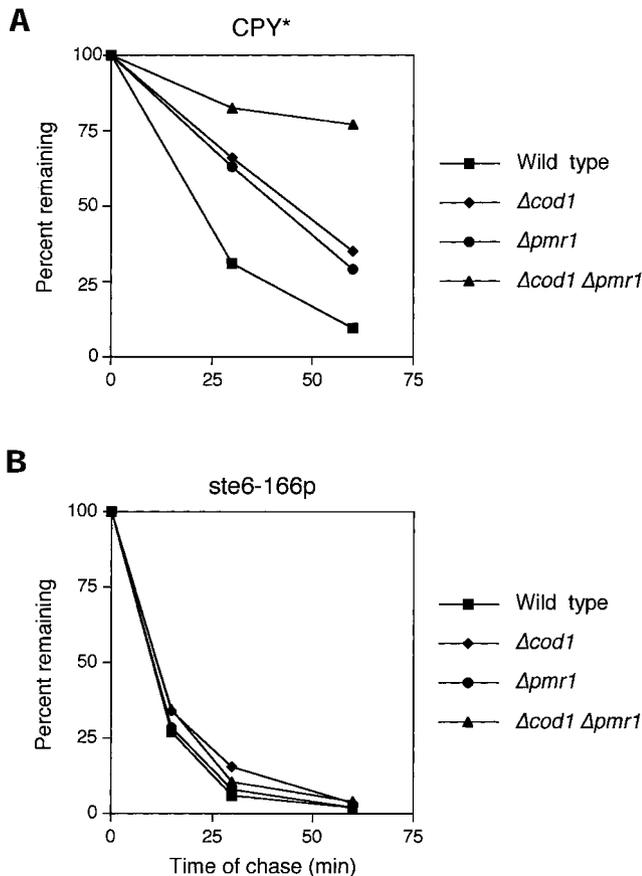


Figure 3. The requirement of Cod1p and Pmr1p for ERAD is substrate specific. HA epitope-tagged versions of Cod1p and Ste6-166p expressed in wild-type (SMY41 and SMY341), $\Delta cod1$ (SMY46 and SMY334), $\Delta pmr1$ (SMY42 and SMY336), and $\Delta cod1 \Delta pmr1$ (SMY337 and SMY339) strains were metabolically pulse-labeled with [35 S]met/cys and were chased with excess cold amino acids for 0, 30, and 60 min. Cod1p_{HA} and Ste6-166p were immunoprecipitated from detergent lysates normalized by trichloroacetic acid-precipitable counts and were separated by SDS-PAGE. Proteins were quantified by phosphorimager analysis and were plotted.

ation of proteins remained normal in the double mutant (Figure 5).

The requirement of *COD1* and *PMR1* in degrading CPY* was somewhat puzzling because HMGR is turned over rapidly in strains deleted of these genes (Cronin *et al.*, 2000). As both proteins are substrates of ERAD, these observations could reflect different requirements for misfolded versus folded proteins. Although reasonable, we explored an alternative possibility. We recently reported that at least two quality control mechanisms target substrates for ERAD (Vashist *et al.*, 2001). Some substrates like CPY* are transported to and recycled from the Golgi, whereas other mutant proteins like Ste6-166p are retained statically in the ER. Thus, we wondered whether the requirement for *COD1* and *PMR1* in degrading misfolded proteins is substrate and/or pathway specific. To address this question, we also measured the turnover of Ste6-166p in the various mutant strains. Ste6-166p is a misfolded version of Ste6p, a multi-

spanning integral membrane protein normally localized at the plasma membrane (Loayza *et al.*, 1998). As shown in Figure 3B, Ste6-166p is degraded normally in strains deleted of either gene. Furthermore, a *cod1 pmr1* double mutant also degraded Ste6-166p efficiently despite being growth impaired. These data show that *COD1* and *PMR1* are required only for a subset of substrates, and that core functions of the ERAD machinery are functional in the absence of these genes.

Loss of *COD1* and *PMR1* Alters the Trimming of N-linked Oligosaccharides

We next explored how the loss of *COD1* and *PMR1* disrupts CPY* degradation and not Ste6-166p. We noticed that the proteins differ in their glycosylation states (CPY* is N-glycosylated at four sites, whereas Ste6-166p is non-glycosylated [our unpublished data]). This might be important because some ERAD substrates, including CPY*, must contain properly processed carbohydrates for efficient degradation (Knop *et al.*, 1996; Jakob *et al.*, 1998). Along these lines, we tested whether the loss of *COD1* or *PMR1* might impair glycosylation and/or processing. To analyze the extent of glycosylation, we examined the synthesis of endogenous glycoproteins in the mutant strains. First, we observed that the addition of core carbohydrates immediately after a pulse-label is normal when compared with wild type (Figure 5, A and B, "Endo H"). We conclude that the core glycosylation of proteins is unimpaired in the mutant strains.

Next, we analyzed the processing of N-linked oligosaccharides. A key enzyme in the oligosaccharide processing is ER mannosidase I. Mannosidase I was shown to contain a coordinated Ca^{2+} that is essential for its activity (Vallee *et al.*, 2000). As Pmr1p and possibly Cod1p participate in maintaining calcium homeostasis, we reasoned that the mutants might display defects in N-glycan processing. We in vivo labeled oligosaccharides in various yeast cells and analyzed the N-glycan composition of the cells at steady-state level. Wild-type cells showed mainly the trimmed $Man_8GlcNAc_2$ N-linked oligosaccharide, as previously reported (Jakob *et al.*, 1998). The $\Delta cod1$ and $\Delta pmr1$ single mutants contained $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ oligosaccharides at approximately equal quantities (Figure 4). The $\Delta cod1 \Delta pmr1$ double mutant, however, accumulated most prominently the $Man_9GlcNAc_2$ oligosaccharide, the N-glycan that does not support degradation when attached to CPY* (Figure 4; Knop *et al.*, 1996; Jakob *et al.*, 1998). From these data, we conclude that oligosaccharide processing by the Ca^{2+} -dependent mannosidase I is most significantly impaired in the $\Delta cod1 \Delta pmr1$ double mutant; oligosaccharide trimming in the single mutants is also affected, but to a lesser degree. The extent of the trimming defects correlates well with their respective CPY* degradation rates (Figure 3). As ERAD is not generally disrupted in these mutants, these data provide a biochemical basis for why CPY* degradation is impaired, whereas Hmg2p (Cronin *et al.*, 2000) and Ste6-166p (Figure 3B) can be degraded efficiently.

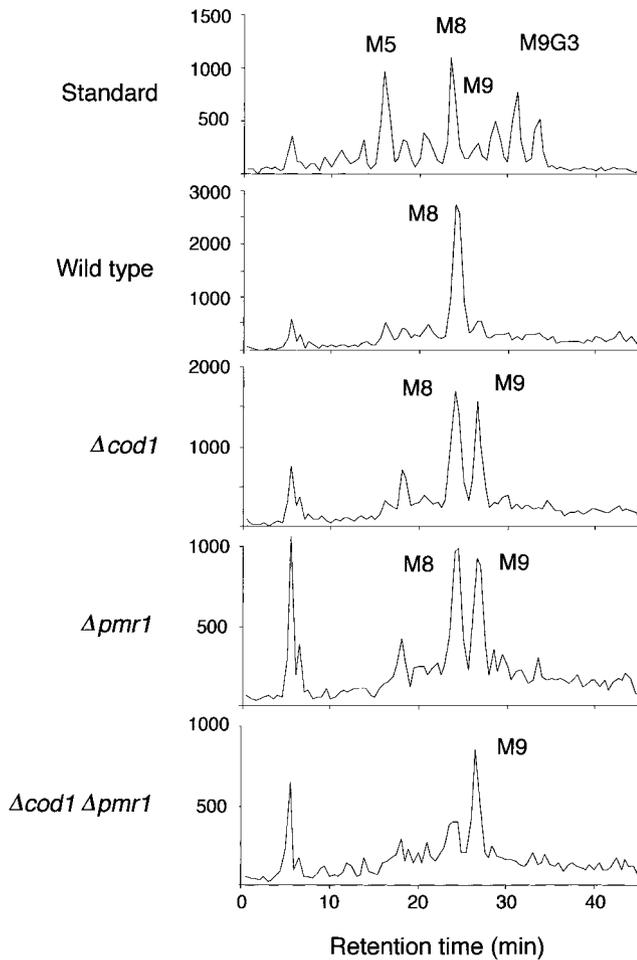


Figure 4. Processing of *N*-linked oligosaccharides in $\Delta cod1$, $\Delta pmr1$ single mutants, and $\Delta cod1 \Delta pmr1$ double mutants is impaired. *N*-linked oligosaccharides of wild type (W303), $\Delta cod1$ (SMY8), $\Delta pmr1$ (SMY33), and $\Delta cod1 \Delta pmr1$ (SMY36) were in vivo labeled with [3 H]mannose for 12 min at 30°C. After extraction of lipid-linked oligosaccharides, *N*-linked oligosaccharides were released by *N*-glycosidase F-digest and were separated by high-performance liquid chromatography. Oligosaccharides of known structure were used as standard.

Modification of Oligosaccharide Chains in the Golgi Requires the Combined Functions of COD1 and PMR1

Pmr1p helps maintain Mn^{2+} homeostasis needed for Golgi enzymes that modify protein-linked oligosaccharides (Durr *et al.*, 1998). As such, *pmr1* mutants exhibit defects in outer chain glycosylation that are consistent with its localization. It was more surprising to uncover its role in $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ oligosaccharide trimming because this occurs exclusively in the ER. Because *Cod1p* is also needed for this activity, we wondered whether this ER protein is also needed for Golgi modification of carbohydrates. To address this question, we compared the maturation of endogenous cargo proteins *Gas1p* and *CPY*. The mature forms of these proteins are localized at the plasma membrane and vacuole,

respectively (Fankhauser and Conzelmann, 1991; Van Den Hazel *et al.*, 1996). During transit through the Golgi apparatus, their carbohydrate chains are extended, and the modifications can be observed by characteristic decreases in gel mobility. After a 10-min pulse, *CPY* migrates in a polyacrylamide gel in two forms: a faster P1 form (ER) and a slower migrating P2 form (Golgi) (Figure 5A, lane 1, “-Endo H”). The shift in mobility to the P2 form is reduced in the $\Delta cod1$ and $\Delta pmr1$ single mutants and is abolished in the double mutant (Figure 5A, lanes 2 through 4, “-Endo H”). The alterations reflect defects in Golgi carbohydrate processing because mature *CPY* (“m*CPY*,” proteolytically processed in the vacuole) generated after the chase maintain the mobility differences (Figure 5A, “-Endo H,” lanes 5 through 8), and removal of *N*-linked sugars eliminates these differences (Figure 5A, “+Endo H”). A similar pattern emerged for the plasma membrane protein *Gas1p*. *Gas1p* differs from *CPY* as it is modified by both *N*- and *O*-linked sugars that are extended in the Golgi. After a 10-min pulse, the *Gas1p* ER forms from each strain migrate identically, indicating that *O*-mannosylation and core *N*-linked oligosaccharide addition are unaffected (Figure 5B, lanes 1 through 4, “-Endo H”). Although a small amount of the Golgi-modified forms was apparent after the pulse, a 30-min chase was applied to complete the processing. In each case, *Gas1p* was processed into a slower migrating Golgi form. However, *Gas1p* is modified only partially in $\Delta cod1$, even less efficiently in $\Delta pmr1$, and is most compromised in the $\Delta cod1 \Delta pmr1$ double mutant (Figure 5B, lanes 5 through 8, “-Endo H”). The defect is not exclusive to *N*-linked sugars because Endo H digestion does not completely eliminate the mobility differences, indicating a defect in the extension of *O*-mannosylated residues (Figure 5B, lanes 5 through 8, “+Endo H”).

Because a significant fraction of the protein-linked oligosaccharides are of the untrimmed $Man_9GlcNAc_2$ form in each mutant, we wondered whether the defects in outer chain glycosylation are simply due to this species being a poor substrate for Golgi-modifying enzymes. For this question, we analyzed the processing of *CPY* and *Gas1p* in a strain deleted of the *MNS1* gene (coding for the ER mannosidase). Trimming of $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ oligosaccharides in the ER is abolished in this strain (Figure 5C, lane P; visualized by slight changes of electrophoretic mobility of *CPY* and *Gas1p*) (Puccia *et al.*, 1993). As shown in Figure 5C, both proteins are processed in the Golgi, indistinguishably from wild type, confirming that $Man_8GlcNAc_2$ (wild-type) and $Man_9GlcNAc_2$ ($\Delta mns1$) carbohydrates can be extended normally in the Golgi (Puccia *et al.*, 1993).

PMR1 and COD1 Are Required for the Normal Transport of Cargo Proteins

A strain lacking *PMR1* was previously observed as defective in the trafficking of *CPY* and chitinase, suggesting that proper Ca^{2+} and Mn^{2+} levels in the ER and/or Golgi are important for vesicular transport (Durr *et al.*, 1998). If *Cod1p* works with *Pmr1p* to maintain luminal homeostasis, we expect to observe similar trafficking defects in $\Delta cod1$ cells that are exacerbated in the double mutant. Metabolic pulse-chase experiments were performed to monitor the transport of two endogenous cargo proteins, *Gas1p* and *CPY*. As shown in Figure 6A, processing of *Gas1p* to the Golgi form was delayed in $\Delta cod1$ cells ($t_{1/2}$ 18 min) and, to a lesser

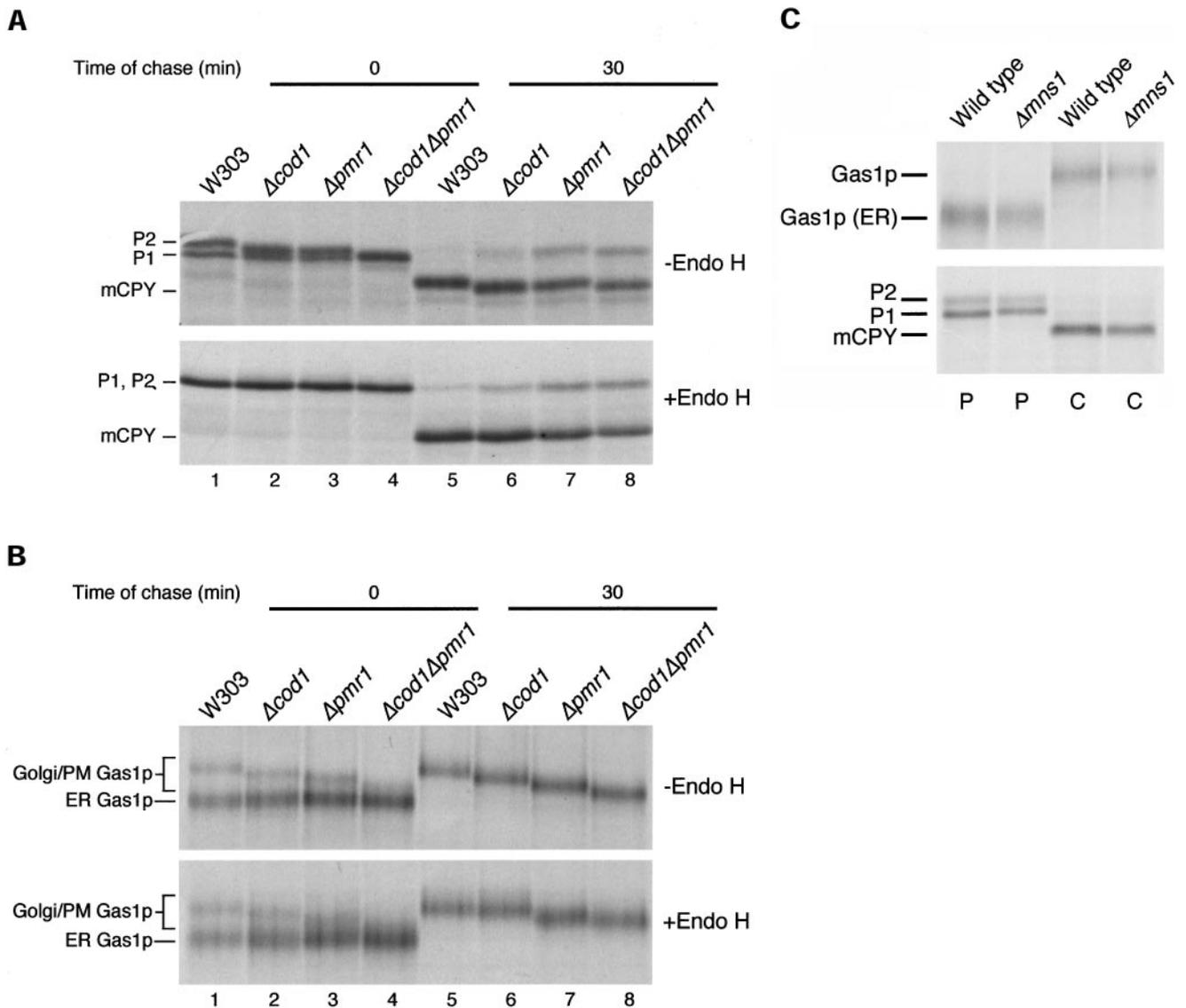
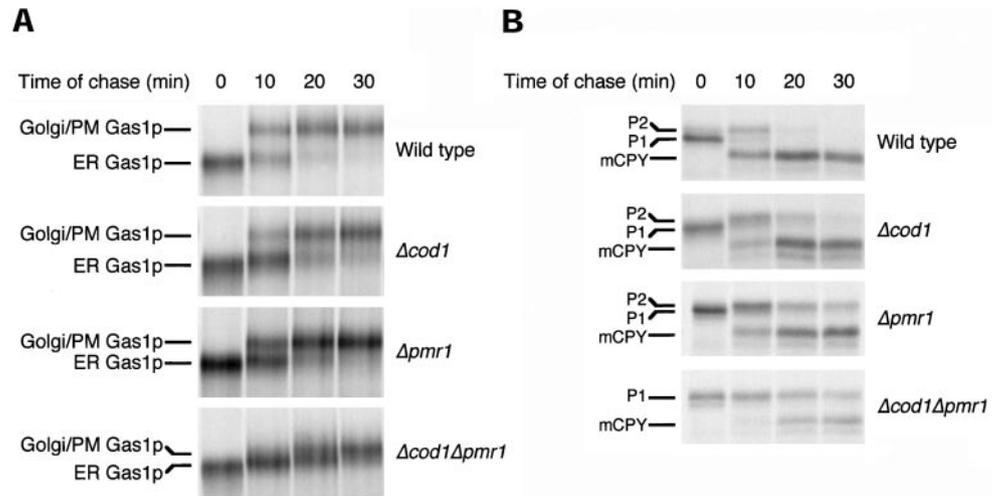


Figure 5. COD1 and PMR1 are required for the modification of oligosaccharide chains in the Golgi apparatus. Wild-type (W303), $\Delta cod1$ (SMY8), $\Delta pmr1$ (SMY33), and $\Delta cod1\Delta pmr1$ (SMY36) cells were pulse-labeled with [35 S]met/cys for 10 min and were chased for 0 and 30 min. Carboxypeptidase Y (CPY) and Gas1p were immunoprecipitated from detergent lysates and the antibody/antigen/resin complexes were divided into two aliquots. Eluted proteins were either mock treated or digested with endoglycosidase H for 1 h. Proteins were separated by SDS-PAGE and were visualized by autoradiography. (A) CPY, untreated (top panel); deglycosylated CPY (bottom panel). "P1" and "P2" indicate the positions of the ER and Golgi forms, respectively. "mCPY" indicates the position of vacuolar processed mature CPY. (B) Gas1p, untreated (top panel); deglycosylated (*N*-linked carbohydrates only) Gas1p. "Golgi/PM Gas1p" indicates the position of mature form of Gas1p. (C) Wild type (W303) and $\Delta mns1$ (YG746) were pulse-labeled for 10 min with [35 S]met/cys and were chased for 0 min (P) or 30 min (C). CPY and Gas1p were immunoprecipitated and analyzed as described for A and B. The positions of intermediate and mature forms of the proteins are indicated as in A and B.

extent, in $\Delta pmr1$ cells ($t_{1/2}$ 13 min) when compared with wild type ($t_{1/2}$ 10 min). These data are consistent with an ER-to-Golgi transport defect. For the $\Delta cod1\Delta pmr1$ mutant, the two Gas1p glycoforms were not resolvable, therefore we were unable to quantify the extent of the transport defect (Figure 6A, bottom). However, it was possible to analyze the trafficking of CPY in this strain. Protein trafficking from the ER to vacuole was monitored through the processing of proCPY (Figure 6B,

"P1 and P2") to the mature vacuolar form (Figure 6B, "mCPY"). The maturation of CPY was found most disrupted in the $\Delta cod1\Delta pmr1$ double mutant ($t_{1/2}$ 26 min) and to a lesser extent in the $\Delta cod1$ and $\Delta pmr1$ mutants ($t_{1/2}$, 15 and 18 min, respectively) as compared with wild type ($t_{1/2}$ 9 min). The requirement for Cod1p and Pmr1p in the vesicular transport of cargo proteins provides an additional line of evidence of their interdependence in maintaining organelle homeostasis.

Figure 6. Vesicular transport of cargo proteins is delayed in *COD1* and *PMR1* mutants. Wild-type (W303), $\Delta cod1$ (SMY8), $\Delta pmr1$ (SMY33), and $\Delta cod1\Delta pmr1$ (SMY36) cells were pulse-labeled with [35 S]met/cys for 5 min and were chased for 0, 10, 20, and 30 min. Gas1p (A) and CPY (B) were immunoprecipitated from detergent lysates (each time point normalized by trichloroacetic acid-precipitable counts), separated by SDS-PAGE, and visualized by autoradiography and quantified by phosphorimager analysis (see text). The positions of the ER ("ER Gas1p") and mature ("Golgi/PM Gas1p") forms of Gas1p and ER ("P1"), Golgi ("P2"), and mature ("mCPY") forms of CPY are indicated.



DISCUSSION

Through our efforts to understand the physiology of the unfolded protein response, a variety of functions were uncovered that are monitored and/or regulated by the pathway. Using a genetic approach, we identified genes affecting *N*- and *O*-linked glycosylation, protein translocation, folding, glycosylphosphatidylinositol addition, quality control, and ERAD (Ng *et al.*, 2000 and our unpublished data). Together, these account for most requisite functions used for ER protein maturation. The discovery of *COD1* expanded the breadth of our analysis because it likely functions to maintain ion homeostasis of the luminal environment.

Cod1p belongs to the P-type ATPase family of enzymes that primarily catalyze the ATP-dependent transport of ions across membranes (Catty and Goffeau, 1996). Common to all P-type ATPases is the formation of a phosphorylated aspartyl-intermediate during the reaction cycle (Catty *et al.*, 1997), a residue that was recently shown to be important for suppressing the SMKT-resistant phenotype of *cod1/spf1* mutants (Suzuki and Shimma, 1999). Based on primary sequence, Cod1p has been placed in the type V subfamily of these enzymes (Axelsen and Palmgren, 1998). Although the molecules transported by Cod1p are not yet conclusively determined, recent evidence from Hampton and colleagues suggest that calcium might be among them (Cronin *et al.*, 2000, 2002). Our data showing functions requiring luminal Mn^{2+} and Ca^{2+} to be compromised in $\Delta cod1$ cells support that view. In mammals, type IIa P-type ATPases called sarco [endoplasmic] reticulum calcium ATPase (SERCA) pumps are responsible for the maintenance of ER luminal Ca^{2+} . However, SERCA pumps are absent from a large number of eukaryotic organisms, including fungi and plants. In yeast, two calcium ATPases, Pmr1p and Pmc1p, were previously identified. Pmr1p belongs to the family of type IIa P-type ATPases, but exhibits properties that are distinct from those of the SERCA pumps. The Golgi-localized Pmr1p was shown to also transport Mn^{2+} (Durr *et al.*, 1998). The other calcium P-type ATPase found in yeast is the vacuolar Pmc1p, which is closely related to the mammalian plasma membrane P-type ATPase (Cunningham and Fink, 1994). The yeast vacuole accumulates >95% of the total cell-asso-

ciated calcium (Eilam *et al.*, 1985). Strains lacking both calcium pumps are not viable (Cunningham and Fink, 1994).

Due to the lack of any SERCA pumps in yeast, it was suggested that *PMR1* is responsible for maintaining the supply of calcium to the ER. This hypothesis was supported by the observations that CPY* degradation is inhibited in *PMR1* mutants, and the expression of the rabbit SERCA1a pump can abrogate low Ca^{2+} and EGTA sensitivity in *pmr1* null cells (Durr *et al.*, 1998). In addition, the measurement of free Ca^{2+} in the ER revealed a 50% decrease in *pmr1* null mutants (Strayle *et al.*, 1999). Taken together, these studies demonstrate an important role for Pmr1p in maintaining ER Ca^{2+} homeostasis, but did not rule out the possibility of other transporters.

The *COD1* gene was initially identified as *SPF1*; mutations in this gene result in resistance to *Pichia farinosa* killer toxin (Suzuki and Shimma, 1999). A noted phenotype of *SPF1* mutants was expression of underglycosylated invertase although the precise nature of the defect was unclear. In an independent genetic study, *COD1* was discovered for its involvement in regulating the degradation of Hmg2p (Cronin *et al.*, 2000). Although its role in Hmg2p regulation is not yet understood, adjustment of Ca^{2+} concentrations in the media partially restored regulation in a *COD1*-deficient strain, whereas Ca^{2+} depletion in the media of wild-type cultures was disruptive. In addition, a *cod1Δ* mutant activates calcium responsive genes and strongly increases intracellular calcium levels when combined with a *PMR1* deletion (Cronin *et al.*, 2002). Taken together with our results, the data implicate a requirement for *COD1* in Ca^{2+} homeostasis. To identify the ion(s) transported by Cod1p, Hampton and coworkers used a biochemical approach that took advantage of the substrate-coupled ATPase activity of most P-type ATPases (Cronin *et al.*, 2002). Surprisingly, neither Ca^{2+} nor Mn^{2+} stimulated the ATPase activity of purified Cod1p. Although their results do not rule out these ions as substrates, they raised the possibility of accessory factors or substrates of Cod1p yet to be determined.

Our study extends and integrates observations of previous studies of the *COD1* and *PMR1* genes. We show that *COD1* mutants share several phenotypes with a strain de-

leted of *PMR1*, raising the possibility that the two genes perform similar functions even as they are localized to distinct compartments. In ERAD, both genes are needed for the degradation of CPY*, but are dispensable for Ste6-166p and Hmg2p. As these are all substrates of ERAD, the seemingly contradictory observation could be explained by a common defect in oligosaccharide processing. By analyzing protein-linked oligosaccharides, we determined that Man₉GlcNAc₂ to Man₈GlcNAc₂ carbohydrate trimming is compromised in strains lacking either or both transporters. The enzyme responsible for this processing step, ER mannosidase I, requires Ca²⁺ for activity (Vallee *et al.*, 2000). As the effect on trimming is nearly identical when either gene is lacking (Figure 4), it seems likely that loss of *COD1* compromises ER Ca²⁺ levels as was shown for a *pmr1* strain. As CPY* degradation requires N-glycan trimming (Knop *et al.*, 1996; Jakob *et al.*, 1998) and neither Ste6-166p nor Hmg2p have this requirement (Figure 3B and C.A. Jakob, unpublished data), it likely account for most, if not all, of the ERAD phenotype. The trimming defect is most severe in the double mutant, suggesting that Cod1p functions independently of Pmr1p rather than as a factor that regulates Pmr1p activity. In addition, a second-site suppression screen to identify further genes involved in protein degradation was performed. The screening procedure was based on the observation that the temperature-sensitive growth phenotype of the *stt3-7* allele can be suppressed by inactivating nonessential genes involved in ERAD (Jakob *et al.*, 2001). The Stt3 protein, an essential subunit of the oligosaccharyltransferase complex, is N-glycosylated and spans the ER membrane at least 10 to 12 times. In this genetic screen, multiple mutant alleles of the *COD1* gene were isolated (R. Szathmary and C.A. Jakob, unpublished data). The fact that inactivation of *COD1* not only reduced the degradation of a soluble glycoprotein (CPY*; Figure 4) but also of a membrane-spanning mutant glycoprotein (*stt3-7p*) indicates the importance of Ca²⁺ homeostasis in efficient degradation of glycoproteins.

In the Golgi apparatus, Pmr1p is needed for correct outer chain processing of carbohydrate chains (Durr *et al.*, 1998). This requirement is attributed to the maintenance of luminal Mn²⁺, a cofactor of the processing enzymes. Surprisingly, we found that *COD1* mutants are similarly defective in this function despite its ER localization (Figure 5). This is the reciprocal relationship to *PMR1* and ER carbohydrate processing. Furthermore, cells lacking both genes are the most compromised and are entirely ineffective in converting proCPY from the ER P1 form to the Golgi P2 form. From these data, we conclude that luminal homeostasis of each compartment is dependent, not only on its own resident transporter, but also on the transporter of the other organelle. The importance of the partnership is underscored by the exacerbation of functional phenotypes as well as severely impaired growth in the double mutant. Despite the extent of the defects, they are specific because other ER functions, including the transfer of oligosaccharides to asparagine side chains and protein import, are unaffected even in the double mutant (Figure 5).

Despite phenotypic similarities between *COD1* and *PMR1* mutants, there are important differences. We identified *COD1* through a synthetic lethality screen with *IRE1*, a key component of the unfolded protein response. Activation of the UPR is believed to alleviate disequilibrium caused by ER

stress. Loss of *COD1* function leads to the constitutive activation of the UPR (Ng *et al.*, 2000). This phenotype is consistent with our data that ER functions are perturbed. We also demonstrated that *COD1* is part of the UPR program because its expression is induced through the pathway during ER stress (Figure 2). By contrast, $\Delta pmr1 \Delta ire1$ mutants are viable and $\Delta pmr1$ mutants do not constitutively activate the UPR, suggesting that critical ER functions are not as compromised as in $\Delta cod1$ mutants (Durr *et al.*, 1998 and our unpublished data). Consistent with this view, the regulation of Hmg2p degradation in the ER is disrupted in $\Delta cod1$ strains, but is unaffected in strains lacking *PMR1* (Cronin *et al.*, 2000). In addition, a recent report showed that mutants lacking *COD1* exhibit defects in membrane protein orientation, whereas a strain lacking *PMR1* was unaffected (Tipper and Harley, 2002). Conversely, the Golgi-localized modification of carbohydrates is more compromised in $\Delta pmr1$ than in a $\Delta cod1$ mutant (Figure 5). These data show that although both proteins are needed to maintain homeostasis of the ER/Golgi membrane system, each is less dispensable for their respective organelles.

Our study reveals a functional partnership of two related but distinctly localized proteins in maintaining the luminal homeostasis of two organelle systems. It was previously shown that one of these proteins, Pmr1p, is needed for ER function, although localized primarily in the Golgi (Durr *et al.*, 1998). The extensive exchange of luminal contents through anterograde and retrograde transport can explain how disequilibrium of one compartment can affect the other. The reciprocal relationship with the ER-localized Cod1p provides another facet of this homeostatic mechanism. Although our studies support a role of Cod1p as part of the UPR regulatory program in maintaining the ER, future work will focus on how both genes are coordinately regulated to maintain homeostasis in the ER/Golgi membrane system.

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