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 Ca\textsuperscript{2+}/Mn\textsuperscript{2+} 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\textsuperscript{*}; Wolf and Fink, 1975) and mutant proteinase A (PrA\textsuperscript{*}; 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 fit into this category. Instead, DER5 was allelic to PMR1. PMR1 encodes a Ca\textsuperscript{2+}/Mn\textsuperscript{2+} 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...
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 (Hmg)1p 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 metabolic 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

### Table 1. Strains used in this study

<table>
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<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<td>P. Walter, UCSF</td>
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</tr>
</tbody>
</table>
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 DNA polymerase) of pRS315 (Sikorski and Heiter, 1989) as a 4580-base pair SpeI/SpeI fragment to generate the complementing clone pSM1. A C-terminal HA-epitope tag was introduced in 3 steps: Purification of a Sali/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’-GCCAATTCATATCCACCTGGTCG-3’) S2 (5’-CATAAAAAGCCATGCTTAGAGGCAATCTT-3’) followed by digestion with NotI; and purification of the vector pDN413 bearing a single HA-epitope tag followed the ACT1 terminator in pRS315 cleaved with SacI and NotI. 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’-GGGTTGACGATTCCATATTGC-3’; S4: 5’-GGGTTAATCTTATTTAGTACG-3’). The fragments were digested with SacI and SpeI, respectively, and were inserted into pB5 SK(+)l. Ligation of the two fragments created an internal Smal site. The HIS3 gene from pRS303 (Sikorski and Heiter, 1989) was inserted into the Smal 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 µg/ml) and lacking uracil, and were screened for the reversal of the red, nonsegregating 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/SFP1, ECM1, BUD16, and YEL028W). Digestion of p82-R2 with Clal followed by religation generates a plasmid (p82-R2ΔCla) containing COD1/SFP1 as the only intact open reading frame. p82-R2ΔCla was found to complement all mutant phenotypes of per5Δ and the cod1 null strain.

Cell Labeling and Immunoprecipitation

Typically, 2 A600 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 OD600 of 0.5–0.9 and were treated with 2.5 µg/ml tunicamycin for 60 min to induce the UPR. Formaldehyde (EM grade; Polysciences, Inc., Warrington, PA) was then added directly to the media to 3.2% 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 added 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 (5% 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 568 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 µg/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 × 10⁸) 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]mannose (21 Ci/mmol; Moravek 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.

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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 (Cod1pHA, 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 Cod1pHA. As shown in Figure 1, Cod1pHA 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 (Rosasane et al., 1999). Instead, the pattern is characteristic of the ER. This assertion was confirmed by perfect coincident

![Figure 1. Intracellular localization of Cod1pHA.](image-url)
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 Δhac1 (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, Cod1pHA synthesis is elevated 3.3- and 3.8-fold after 60 and 120 min of tunicamycin treatment, respectively. Under stress, although Cod1p and Pmr1p are localized to distinct 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-

**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 by metabolic pulse-chase analysis. As shown in Figure 3A, the delay of CPY* degradation by metabolic pulse-chase analysis. As shown in Figure 3A, the delay of CPY* degradation by metabolic pulse-chase analysis. As shown in Figure 3A, the delay of CPY* degradation by metabolic pulse-chase analysis. As shown in Figure 3A, the delay of CPY* degradation by metabolic pulse-chase analysis. As shown in Figure 3A, the delay of CPY* degradation by metabolic pulse-chase analysis.

![Figure 2. COD1 expression is regulated by the unfolded protein response. (A) Wild-type (W303a) and UPR-deficient (Δhac1: JC408) cells were mock treated or incubated with 2.5 μg/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 [32P]-labeled probes against COD1, KAR2 (positive control), and ACT1 (loading control) and was visualized by autoradiography. (B) Wild-type cells expressing Cod1pHA 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 [35S]met/cys and detergent lysates were prepared. Cod1pHA, 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”*) and nonglycoylated (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.](image_url)
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 Ca2+ 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 Man8GlcNAc2 N-glycan, whereas Hmg2p (Cronin et al., 1998) and Ste6-166p (Figure 3B) are 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.
It was more surprising to uncover its role in Man\(_9\)GlcNAc\(_2\) chain glycosylation that are consistent with its localization. et al. pmr1 enzymes that modify protein-linked oligosaccharides (Durr

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\(_n\)GlcNAc\(_2\) to Man\(_n\)GlcNAc\(_2\) 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 (“mCPY,” 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 of the untrimmed Man\(_n\)GlcNAc\(_2\) 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\(_n\)GlcNAc\(_2\) to Man\(_n\)GlcNAc\(_2\) 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\(_n\)GlcNAc\(_2\) (wild-type) and Man\(_n\)GlcNAc\(_2\) (Δmns1) 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
extent, in Δpmr1 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 Δcod1Δpmr1 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 Δcod1Δpmr1 double mutant (t_1/2 26 min) and to a lesser extent in the Δcod1 and Δpmr1 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 5. COD1 and PMR1 are required for the modification of oligosaccharide chains in the Golgi apparatus. Wild-type (W303), Δcod1 (SMY8), Δpmr1 (SMY33), and Δcod1Δpmr1 (SMY36) cells were pulse-labeled with [35S]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 Δmns1 (YG746) were pulse-labeled for 10 min with [35S]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.
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 (Catté and Goffeau, 1996). Common to all P-type ATPases is the formation of a phosphorylated aspartyl-intermediate during the reaction cycle (Catté 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 molecular identity of the ion transported by Cod1p is 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 Δcod1 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-associated 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$^{2+}$ $\Delta$ 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-
linked oligosaccharides, we determined that Man\textsubscript{9}GlcNAc\textsubscript{2} is responsible for this processing step, ER mannosidase I, requires Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} levels as was shown for a pmr1 strain. As CPY\textsuperscript{*} 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 sst3-7 allele can be suppressed by inactivating nonessential genes involved in ERAD (Jakob et al., 2001). The Sst3 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\textsuperscript{*}; Figure 4) but also of a membrane-spanning mutant glycoprotein (sst3-7p) indicates the importance of Ca\textsuperscript{2+} 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 luminal Mr\textsuperscript{2+}, 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 procP with 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, \textDelta pmr1 \textDelta ire1 mutants are viable and \textDelta pmr1 mutants do not constitutively activate the UPR, suggesting that critical ER functions are not as compromised as in \textDelta 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 \textDelta 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 \textDelta pmr1 than in a \textDelta 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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