JCB Article

# Cod1p/Spf1p is a P-type ATPase involved in ER function and $Ca^{2+}$ homeostasis

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The internal environment of the ER is regulated to accommodate essential cellular processes, yet our understanding of this regulation remains incomplete. Cod1p/Spf1p belongs to the widely conserved, uncharacterized type V branch of P-type ATPases, a large family of ion pumps. Our previous work suggested Cod1p may function in the ER. Consistent with this hypothesis, we localized Cod1p to the ER membrane. The *cod1Δ* mutant disrupted cellular calcium homeostasis, causing increased transcription of calcium-regulated genes and a synergistic increase in cellular calcium when paired with disruption of the Golgi apparatus–localized $Ca^{2+}$ pump Pmr1p. Deletion of *COD1* also impaired ER function, causing constitutive activation of the unfolded protein response, hypersensitivity to the glycosylation inhibitor tunicamycin, and synthetic lethality with deletion of the unfolded protein response regulator *HAC1*. Expression of the *Drosophila melanogaster* homologue of Cod1p complemented the *cod1Δ* mutant. Finally, we demonstrated the ATPase activity of the purified protein. This study provides the first biochemical characterization of a type V P-type ATPase, implicates Cod1p in ER function and ion homeostasis, and indicates that these functions are conserved among Cod1p's metazoan homologues.

## Introduction

The ER is the site of essential cellular processes that require stringent regulation of lumenal ion levels. The ER also serves as a source of releasable $Ca^{2+}$ that can be mobilized for various cellular demands. Consequently, the ion levels of the ER are under continuous, dynamic control in order to serve these essential functions. The ion levels of many membrane compartments are maintained by the action of a superfamily of ATP-dependent ion pumps known as P-type ATPases (Axelsen and Palmgren, 1998; Scarborough, 1999). These proteins catalyze ATP-dependent active transport of ions across numerous cellular membranes. The P-type ATPases that have been characterized are highly specific, transporting only a single ion, or sometimes two related ones. However, there are a large number of P-type ATPases identified solely by sequence, having unknown function and specificity, that await assignment of biochemical and physiological functions. In mammals, one mechanism of ER ion regulation occurs through the action of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA)* pumps, which are localized to the ER and belong to the type IIa, calcium-specific subfamily of P-type ATPases (Carafoli and Brini, 2000; Axelsen and Palmgren, 2001). Although clearly important in animals, SERCA pumps are absent in other organisms, such as fungi, that nevertheless control ER ion content (Catty et al., 1997; Axelsen and Palmgren, 1998). Thus, it is likely that a more widely conserved P-type ATPase is responsible for ER ion maintenance in these organisms, and perhaps play an important, yet until now unsuspected, role in mammals as well.

Because fungi do not posses SERCA ATPases, the mechanisms by which calcium is supplied to the yeast ER had remained enigmatic. The absence of SERCA pumps led several groups to suggest that the yeast ER is supplied with calcium solely by the action of the Golgi-localized pump Pmr1p (Duerr et al., 1998; Marchi et al., 1999; Strayle et al., 1999). This model is reasonable because *pmr1Δ* mutants have a number of phenotypes related to the function of the ER (Duerr et al., 1998; Strayle et al., 1999). Like SERCA, Pmr1p belongs to the type IIa family of P-type ATPases and some phenotypes of *pmr1Δ* mutants can be complemented by expression of SERCA1a or related ATPases (Liang et al.,

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*Abbreviations used in this paper: GFP, green fluorescent protein; HA, hemagglutinin; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; ICP-OES, inductively coupled plasma optical emission spectrometry; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; UPR, unfolded protein response.

Key words: ion transport; magnesium; protein folding; endoplasmic reticulum; *Saccharomyces cerevisiae*

1997; Duerr et al., 1998; Talla et al., 1998; Degand et al., 1999). Pmr1p has been shown to transport both calcium and manganese, but how the Golgi apparatus–localized Pmr1p supplies calcium to the ER is unclear (Mandal et al., 2000; Wei et al., 2000).

We and others have recently identified another P-type ATPase in yeast, Cod1p/Spf1p, that adds a new dimension to the regulation of ER ion supply (Suzuki and Shimma, 1999; Cronin et al., 2000). Cod1p belongs to the type V subfamily of P-type ATPases that are broadly conserved in eukaryotes but for which no ionic substrates have yet been identified (Axelsen and Palmgren, 1998). Close homologues of Cod1p are present in *Drosophila melanogaster*, *Caenorhabditis elegans*, *Dictyostelium discoideum*, *Arabidopsis thaliana*, and humans (Costanzo et al., 2000). Cod1p is the only member of the type V subfamily for which any phenotypic information is known. We identified *COD1* in a genetic screen for mutants incapable of regulating the degradation of the ER membrane protein Hmg2p (Cronin et al., 2000). The involvement of Cod1p in the degradation of an ER protein as well as other ER phenotypes implies that Cod1p functions in the ER (Suzuki and Shimma, 1999; Cronin et al., 2000). Furthermore, the *cod1* mutant phenotype suggests a role for Cod1p in calcium regulation because the mutant phenotype could be partially suppressed by exogenous calcium (Cronin et al., 2000). These observations led us to propose that Cod1p functions to supply $Ca^{2+}$ to the ER (Cronin et al., 2000).

In this paper, we directly examined the role of Cod1p in ER function and calcium regulation in vivo and the biochemical requirements for Cod1p ATPase activity in vitro. We have now demonstrated that Cod1p localized to the ER by both immunofluorescence and subcellular fractionation, and have provided phenotypic evidence supporting a role for Cod1p in ER function and cellular calcium regulation. Further, we have distinguished phenotypically the roles of Cod1p and Pmr1p in ER function and quality control. Finally, we purified the Cod1 protein and directly demonstrated the ATPase activity of Cod1p in vitro. Taken together, our data indicated that Cod1p plays a significant role in ER function and cellular $Ca^{2+}$ homeostasis that is not equivalent to, nor redundant with, the role of Pmr1p or other P-type ATPases. Moreover, the presence of Cod1p homologues in the genomes of all sequenced metazoans suggested that the function of Cod1p is likely to be more widely conserved in eukaryotes than that of more restricted, but better known, pumps such as the SERCA ATPases.

## Results

### Cod1p is localized to the ER

Many of the phenotypes of the *cod1* mutants suggested that Cod1p functions in the ER (Suzuki and Shimma, 1999; Cronin et al., 2000). Accordingly, we determined the localization of Cod1p by both immunofluorescence microscopy and density gradient fractionation (Fig. 1). To facilitate localization, we constructed the *3MYC::COD1* allele of *COD1* expressing a protein with three myc epitopes fused to the amino terminus of the protein. 3myc–Cod1p was expressed from the unmodified *COD1* promoter in single copy, preventing aberrant localization due to overexpression.

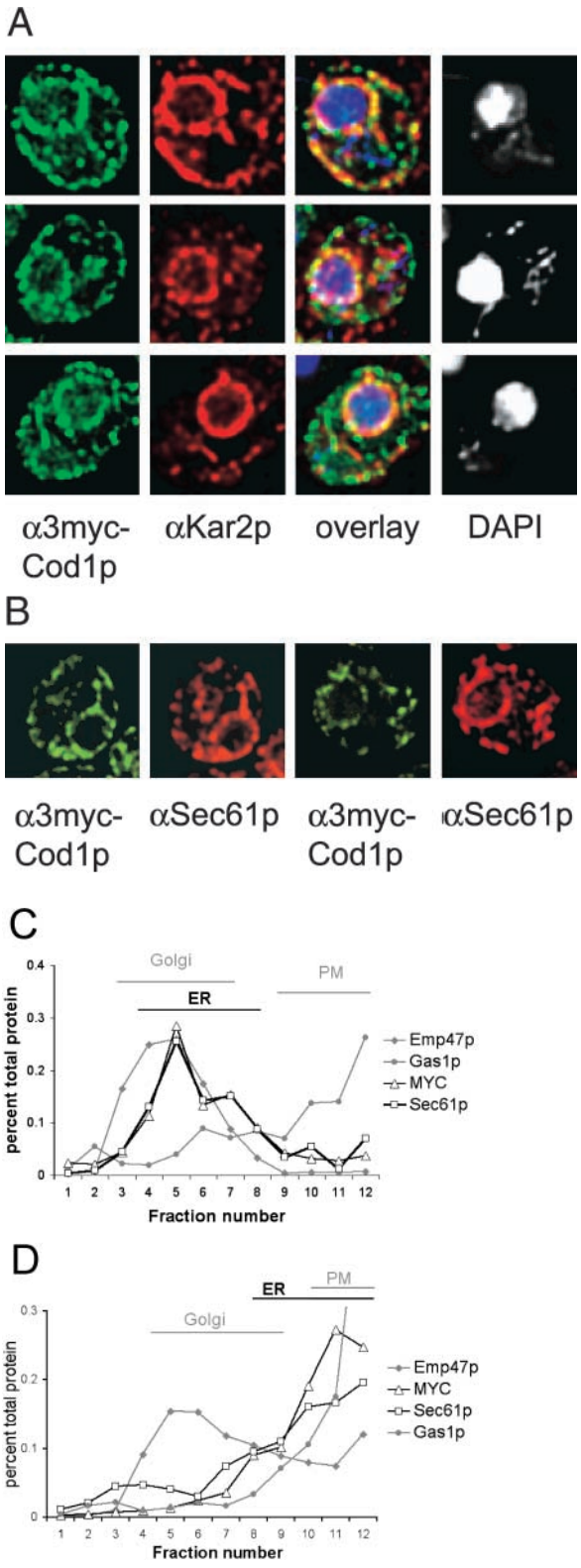


Figure 1. **Cod1p is localized to the endoplasmic reticulum.** Comparison of the distribution of 3myc–Cod1p with the distribution of the ER proteins Kar2p (A) and Sec61p (B) by immunofluorescence as described in the Materials and methods. Yeast lysates prepared in the presence of EDTA (C) or magnesium (D) were fractionated on a 10–60% linear sucrose gradient. Protein from each fraction was separated by SDS-PAGE, transferred to nitrocellulose, and probed with the appropriate antibodies.

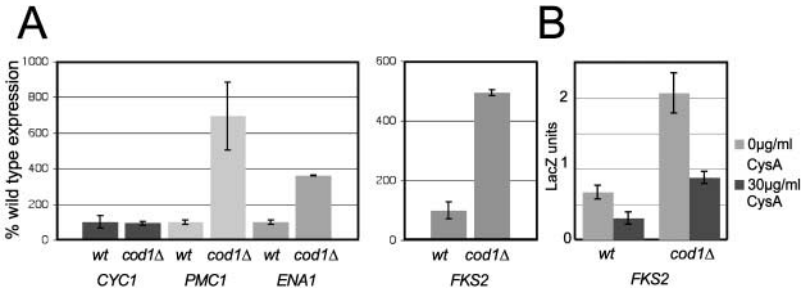


Figure 2. **Expression of calcium-responsive genes is induced in *cod1Δ*.** (A) Log phase cultures of wild-type (RHY1708) and *cod1Δ* cells (RHY1709) expressing β-galactosidase reporters were grown in YPD and assayed for β-galactosidase activity as described in the Materials and methods. (B) Log phase cultures of wild-type and *cod1Δ* cells were grown overnight in the presence or absence of 30 μg/ml of cyclosporin A and assayed for β-galactosidase activity. Representative experiments are shown.

Strains expressing 3myc–Cod1p are phenotypically wild type in all ways tested (unpublished data). The pattern of staining for 3myc–Cod1p was typical for ER localization, with a distinct ring around the nucleus and additional staining near the cell periphery. The distribution of Cod1p, though slightly more extensive, overlapped considerably with the distribution of Kar2p, a protein residing in the lumen of the ER (Fig. 1 A) (Rose et al., 1989; Preuss et al., 1991). We also saw the same pattern of perinuclear and peripheral staining with Sec61p, a component of the ER translocation apparatus (Fig. 1 B) (Stirling et al., 1992).

We independently tested the localization of 3myc–Cod1p by fractionation of intracellular membranes on sucrose density gradients in the presence of EDTA or magnesium as described in the Materials and methods (Fig. 1, C and D). A characteristic of ER microsomes is that their buoyancy in a sucrose gradient depends on the presence or absence of magnesium in the gradient, allowing unambiguous assignment of a membrane protein to the ER by testing the behavior of the protein under both conditions (Roberg et al., 1997). The distribution of 3myc–Cod1p in all gradients closely overlapped the distribution of the ER-localized Sec61p. When fractions were prepared with 2 mM $MgCl_2$, the ER containing 3myc–Cod1p and Sec61p cofractionated with the plasma membrane marker Gas1p (Doering and Schekman, 1996) and separated from the Golgi apparatus–derived membranes labeled by Emp47 (Schröder et al., 1995). When fractions were prepared with EDTA, 3myc–Cod1p and Sec61p cofractionated with Emp47p and separated from Gas1p, thus confirming that the fractions containing 3myc–Cod1p were derived from the ER.

## Deletion of Cod1p induced expression of calcium-regulated genes

Calcium concentration within the cytosol and secretory pathway regulates the expression of a number of genes, and mutations that perturb cellular calcium induce compensatory changes in gene expression (Garrett-Engele et al., 1995; Matheos et al., 1997; Locke et al., 2000). For instance, deletion of *PMR1* produces a five- to sevenfold increase in the transcription of *PMC1*, a vacuolar calcium ATPase (Marchi et al., 1999, Locke et al., 2000). We investigated the effect of *cod1* deletion on the expression of *FKS2*, *PMC1*, and *ENA1,* genes known to be regulated in response to calcium concentration, using previously described β-galactosidase reporter plasmids (Fig. 2, A and B) (Matheos et al., 1997; Locke et al., 2000). The expression of each of the three calcium-activated reporters was three- to sevenfold higher in the *cod1Δ* mutant than in wild-type cells grown under normal laboratory conditions and in the absence of any external calcium stimulus. This effect of *cod1Δ* was similar to that reported earlier for *pmr1Δ* (Locke et al., 2000). Importantly, transient treatment with 200 mM $CaCl_2$ induced β-galactosidase activity to the same level both in wild-type and *cod1Δ* cells, indicating that deletion of *COD1* did not interfere with the ability of the reporter constructs to respond to calcium stimulation (unpublished data). Likewise, treatment of cultures with the calcineurin inhibitor cyclosporin A diminished expression of the reporter genes, indicating that the increased expression in *cod1Δ* was largely calceneurin dependent (Fig. 2 B; unpublished data). A portion of the increase may be calcineurin independent, as the treatment did not reduce expression to the same level as in wild type. The increased β-galactosidase activity in the *cod1Δ* mutants was specific to the calcium-regulated reporters. Expression of the *CYC1*–β-galactosidase reporter, which is not responsive to calcium (Matheos et al., 1997), did not differ significantly between wild-type and *cod1Δ* strains (Fig. 2 A).

## *cod1Δ* mutants are specifically hypersensitive to extracellular calcium

Hypersensitivity to an extracellular ion can indicate a defect in intracellular transport of that ion. For example, mutants defective in the calcium and manganese pump Pmr1p are hypersensitive to the presence of calcium and manganese in the growth medium (Lapinskas et al., 1995; Wei et al., 2000) (Fig. 3 A). Thus, we tested the ability of the *cod1Δ* null mutant to grow in high levels of extracellular calcium or other cations. We found that *cod1Δ* mutants had over twice the sensitivity to calcium as wild-type cells, sensitivity equivalent to that of *pmr1Δ* mutants. The combined deletion of both *cod1* and *pmr1* further increased sensitivity to extracellular calcium to four times that of wild-type cells. The defect was specific to calcium, as *cod1Δ* mutants were no more sensitive to other divalent ions than wild-type cells (unpublished data). For example, deletion of *COD1* did not affect manganese sensitivity, which was greatly increased in *pmr1Δ* strains, and the hypersensitivity of a *pmr1Δ* null mutant to manganese was not further exacerbated by a *cod1Δ* allele (Fig. 3 A).

## Role of Cod1p in maintaining total cellular calcium level

The increase in expression of calcium-regulated genes and hypersensitivity to calcium suggested that Cod1p might influence total cellular ion content under some conditions. For example, deletion of *PMR1* results in increased cellular calcium and increased cellular manganese (Lapinskas et al., 1995) (Fig. 3 B). We examined the level of a number of ions in wild-type and *cod1Δ* cultures by inductively coupled

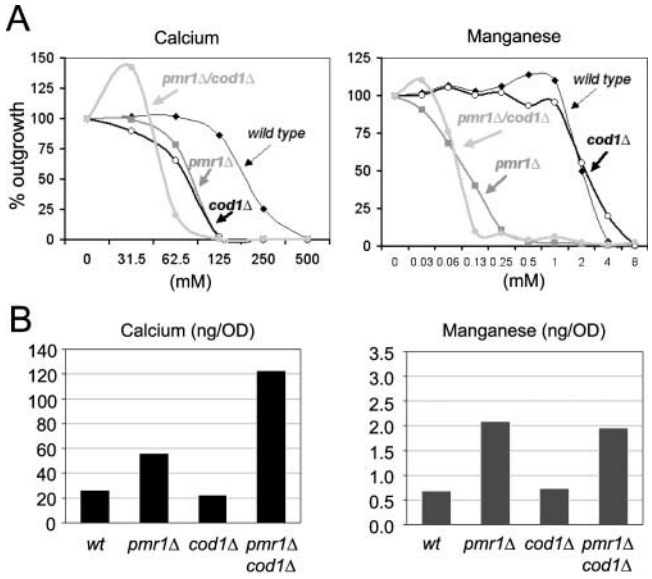


Figure 3. ***cod1Δ* display phenotypes specific to calcium but not to manganese.** (A) Growth arrest of wild-type, *cod1Δ*, *pmr1Δ*, and *cod1Δ/pmr1Δ* cells (CS601A, CS601B, CS601C, and CS601D) by $CaCl_2$ or $MnCl_2$. Low-density cultures ($OD_{600} < .01$) were grown in liquid YPD, pH 5.5, in the presence of the indicated concentrations of added $CaCl_2$ or $MnCl_2$ for 2.5 d. Growth is plotted as a percentage of the outgrowth of the untreated culture. (B) Deletion of *PMR1* and *COD1* causes a synergistic increase in total cellular calcium, but not manganese. Cultures were grown overnight in YPD, pH 5.5, to an optical density of approximately one and then processed as described in the Materials and methods. Representative experiments are shown.

plasma optical emission spectrometry (ICP-OES) as described in the Materials and methods (Fig. 3 B). Deletion of *COD1* alone did not affect cellular calcium levels. However, deletion of both *COD1* and *PMR1* produced a synergistic increase in intracellular calcium compared with *cod1Δ* or *pmr1Δ* alone. This synergistic effect was specific to calcium: although *pmr1Δ* cells also displayed increased manganese levels, the combined deletion of *cod1* and *pmr1* did not further increase total cellular manganese (Fig. 3 B). Deletion of *COD1* either alone or in the presence of *pmr1Δ* had no effect on the total cellular concentration of magnesium, iron, zinc, copper, or any other ion detected in yeast cultures, demonstrating that the effect of *cod1Δ* was specific.

## Cod1p is necessary for normal ER function

The localization of Cod1p to the ER and the previously reported phenotypes of the *cod1* mutants suggested that Cod1p plays a major role in the function of the ER. We tested the sensitivity of *cod* mutants to the reducing agent DTT and the glycosylation inhibitor tunicamycin, both of which (through distinct mechanisms) alter protein folding in the ER (Fig. 4 A). *cod1-1* cells had twice the sensitivity to tunicamycin as wild-type cells, but maintained normal sensitivity to DTT. Treatment of wild-type cells with either agent provokes the accumulation of unfolded proteins in the ER, inducing the unfolded protein response (UPR) (Frand and Kaiser, 1998; Pollard et al., 1998). We measured the unfolded protein response with a UPR reporter plasmid (4xUPRE–green fluorescent protein [GFP]) consisting of four unfolded protein response elements

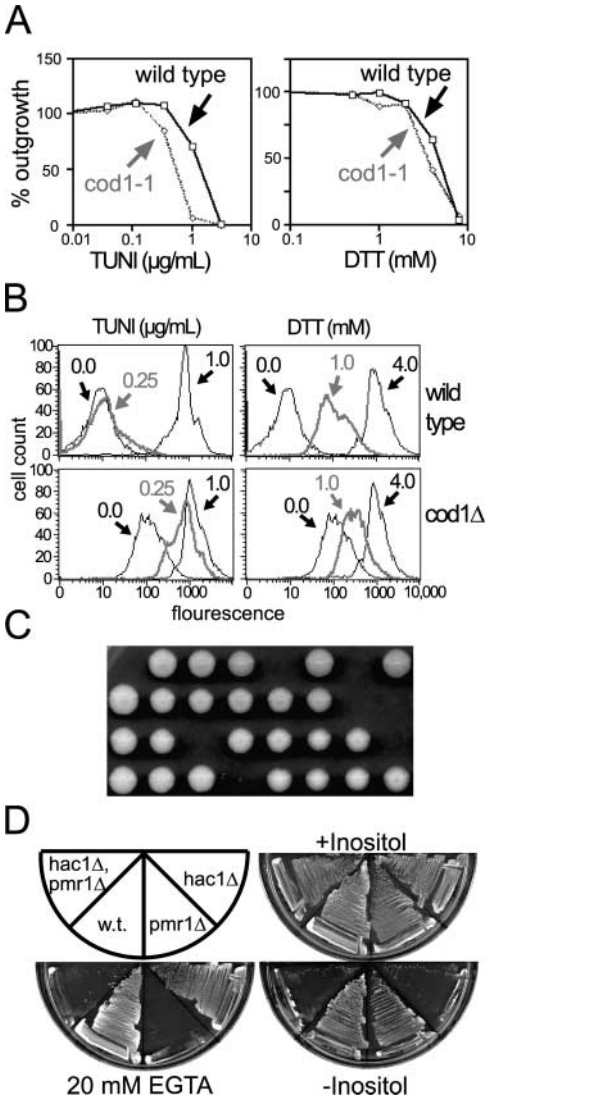


Figure 4. **Deletion of *COD1* produces phenotypes indicating the presence of misfolded proteins in the ER.** (A) Growth arrest of wild-type (RHY791) and *cod1-1* (RHY811) cells by tunicamycin or DTT. Low-density cultures ($OD_{600} < 0.01$) were grown in minimal medium in the presence of the indicated concentrations of added tunicamycin or DTT. Growth is plotted as a percentage of the outgrowth of the untreated culture. (B) Wild-type (RHY1708) and *cod1Δ* (RHY1709) cells transformed with a plasmid expressing GFP from four unfolded protein response elements were treated for 4 h with the indicated concentrations of tunicamycin or DTT and analyzed by flow microfluorimetry. Each histogram represents 10,000 cells. (C) *cod1-1* null mutants (RHY910) were mated to *hac1Δ* mutants (RHY1664). The diploids were sporulated and dissected on YPD plates. (D) Wild-type, *hac1Δ*, *pmr1Δ*, and *hac1Δ/pmr1Δ* cells (MYY290, RHY1884, RHY2179, and RHY2277) were streaked onto minimal media lacking inositol or containing 20 mM EGTA.

driving expression of the GFP coding region (4xUPRE; Fig. 4 B; Pollard et al., 1998). Strikingly, the GFP reporter in the *cod1Δ* mutant was constitutively expressed at 10 times the level of expression in wild-type cells in the absence of any additional stimulus. Furthermore, in *cod1Δ*, maximum expression of the UPR reporter required only half the dose of tunicamycin (2 μg/ml) needed to stimulate maximum expression in wild-type cells. As expected from the growth sensitivity experiments, the *cod1Δ* mutant displayed wild-type sensitivity to DTT in the unfolded

protein response. Maximal induction of the reporter protein required 2 mM DTT in both wild-type and *cod1Δ*.

## Inactivation of the UPR is synthetically lethal in a *cod1* null

The unfolded protein response is mediated by the transcription factor Hac1p (Cox and Walter, 1996; Nikawa et al., 1996). We tested the necessity of the UPR in the *cod1* mutant by crossing a *cod1-1* null mutant to a *hac1Δ* mutant (Fig. 4 C). Sporulation of the diploid and dissection of 22 of the resulting ascospores revealed the synthetic lethality of *hac1Δ cod1-1* double mutants. We confirmed this result using *cod1Δ::Kan*$^R$ and *hac1Δ::URA3* alleles. In 19 tetrads, no colonies with the Ura$^+$, Kan$^R$ phenotype were observed. We obtained identical results using *cod1Δ::HIS3* and *hac1Δ::URA3* null alleles in the unrelated MYY290 genetic background (14 tetrads analyzed). Microcolonies, three to five cells in diameter, were seen by microscopy on the dissection plate, but these did not grow further even after prolonged incubation. The presence of the microcolonies indicated that the *cod1Δ, hac1Δ* spores were capable of germinating but incapable of sustained growth. In contrast, double mutants of *hac1* and YOR291w, the closest yeast homologue to *COD1*, were viable (unpublished data).

*pmr1* mutants have phenotypes indicative of a perturbed UPR, including hypersensitivity to DTT and tunicamycin, inositol auxotrophy, and increased expression of the ER chaperone Kar2p (Duerr et al., 1998). We examined the viability of a *pmr1Δ hac1Δ* double mutant in the same genetic background in which *cod1Δ hac1Δ* cells were inviable (Fig. 4 D). Surprisingly, the *pmr1Δ hac1Δ* double mutants were viable. The *pmr1Δ hac1Δ* double mutant retained phenotypes of both parent strains: hypersensitivity to EGTA (*pmr1Δ*) and inositol auxotrophy (*hac1Δ*). The *cod1Δ* mutant was prototrophic for inositol in all strains tested (unpublished data). Thus, although Cod1p and Pmr1p had synthetic effects on whole cell calcium that implied shared functions, they also clearly had distinct, nonoverlapping ER functions.

## Regulated degradation of Hmg2p does not require the UPR

The constitutive activation of the UPR and the constitutive degradation of Hmg2p in the *cod1Δ* mutant raised the possibility that UPR played a role in regulating Hmg2p degradation (*cod1Δ* mutants cannot regulate the degradation of Hmg2p (Cronin et al., 2000). In wild-type cells, treatment with lovastatin (a 3-hydroxy-3-methylglutaryl coenzyme A reductase [HMGR] inhibitor) slows Hmg2p degradation and raises Hmg2p levels (Hampton and Rine, 1994). This regulation can be measured by flow cytometry using the Hmg2p–GFP reporter protein (Hampton et al., 1996; Gardner and Hampton, 1999). We tested the effect of UPR activation on the regulated degradation of the Hmg2p–GFP by treatment with the glycosylation inhibitor tunicamycin (Fig. 5 A). Hmg2p–GFP degradation in cells treated with 4 μg/ml tunicamycin (a dose that fully induces the UPR) still responded to treatment with lovastatin in the same manner as cells grown without tunicamycin. In fact, Hmg2p–GFP degradation was moderately slowed by tunicamycin treatment. Thus, the unregulated, constitutive degradation of Hmg2p present in *cod1* mutants cannot be attributed to activation of the unfolded protein response or to defective glycosylation. We deleted *HAC1* to determine if the unfolded protein response was required for regulation of Hmg2p degradation (Fig. 5 B). The regulated degradation of Hmg2p–GFP in *hac1Δ* cells treated with lovastatin was indistinguishable from that seen in wild-type cells under the same treatment despite reports that some degradation substrates require the UPR for ER-associated degradation.

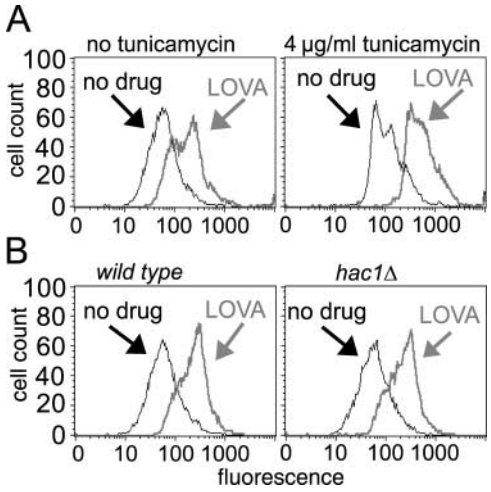


Figure 5. **Glycosylation and the UPR are not required for regulating Hmg2p–GFP degradation.** (A) Tunicamycin does not affect the stabilization of Hmg2p–GFP by lovastatin. Early log phase cultures were grown for 4 h in 4 μg/ml tunicamycin and the presence or absence of 25 μg/ml lovastatin and subjected to flow cytometry. (B) Hmg2p–GFP regulation does not require *HAC1*. Early log phase cultures of wild-type (RHY872) or *HAC1Δ* (RHY1664) cells were grown for 4 h in the presence or absence of 25 μg/ml lovastatin and subjected to flow cytometry.

## The role of Cod1p is functionally conserved between yeast and *D. melanogaster*

Genes closely homologous to *COD1* have been identified in several metazoans including humans. These putative orthologues are more similar to *COD1* than *COD1* is to its closest yeast homologue, YOR291w. For example, *D. melanogaster* expresses a gene GH06032, which we refer to as *dCOD1,* with 40% identity and 58% similarity in the protein sequence to Cod1p (Costanzo et al., 2000). We constructed a modified version of *dCOD1* for expression in yeast, encoding dCod1p with a hemagglutinin (HA) epitope tag fused to the amino terminus. We expressed HA–dCod1p from several different promoters in the *cod1Δ* mutant to test its ability to complement the mutant phenotype. *cod1Δ* mutants cannot regulate the degradation of the HMGR isozyme Hmg2p (Cronin et al., 2000) (Fig. 6). This defect in the regulated degradation of Hmg2p causes *cod1Δ* mutants to be hypersensitive to the HMGR inhibitor lovastatin in the appropriate genetic background. Expression of *dCOD1* from the strong *TDH3* or *PMA1* promoters rescued growth of *cod1Δ* mutants on lovastatin, whereas expression of *dCOD1* from the weaker *COD1* promoter was not sufficient to restore lovastatin resistance (Fig. 6). As expected from the reversal of drug sensitivity, *dCOD1* also restored regulation of Hmg2p degradation, as measured by flow cytometry using the Hmg2p–GFP reporter protein (Fig. 6 B).

Complementation of *cod1Δ* phenotypes was specific for P-type ATPases closely related to Cod1p. We tested the abil-

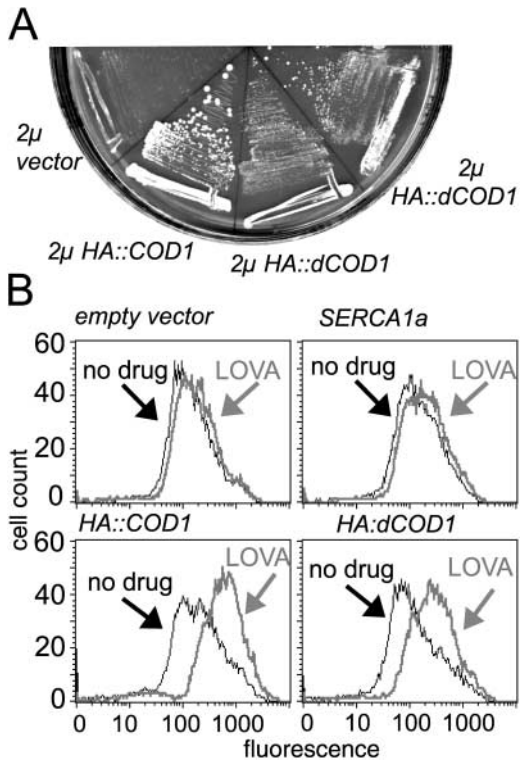


Figure 6. **Functional conservation of Cod1 between *S. cerevisiae* and *D. melanogaster*.** (A) Lovastatin sensitivity of *cod1Δ* cells (RHY1401) transformed with a high copy plasmid expressing nothing, $P_{TDH3}$-*HA::COD1*, or $P_{TDH3}$-*HA::dCOD1*. Cultures were streaked onto plates containing 200 μg/ml lovastatin and incubated at 30°C. (B) Regulation of Hmg2p–GFP degradation in *cod1-1* cells (RHY911) expressing the indicated P-type ATPases expressed from the *PMA1* promoter. Early log phase cultures were grown for 4 h in the presence or absence of 25 μg/ml lovastatin and subjected to flow cytometry.

ity of the type IIa P-type ATPases SERCA1a and *ACA3* (from *A. thaliana*) to complement the *cod1* mutant phenotype with respect to Hmg2p–GFP degradation. Both of these genes complement the *pmr1* mutant phenotype, but neither could complement the *cod1* mutant phenotype, nor could overexpression of *PMR1* (Fig. 6 B; unpublished data). Similarly, overexpression of *HA::COD1* or *HA::dCOD1* could not complement the sensitivity of *pmr1Δ* mutants to EGTA, suggesting that the roles of Cod1p and Pmr1p in ER function do not overlap significantly (unpublished data). The lack of cross-complementation also raises the possibility that the similar effects of *pmr1* and *cod1* on total cellular calcium may arise through different mechanisms.

## Biochemical characterization of Cod1p

To directly study the biochemistry of Cod1p, we constructed a gene that expressed a modified version of the Cod1 protein with nine histidine residues and an HA epitope tag inserted just after the amino-terminal methionine. The plasmid complemented the *cod1Δ* mutant phenotype (Fig. 7). Cells expressing the histidine-tagged Cod1 protein from the native *COD1* promoter at single copy or the much stronger *TDH3* promoter from a 2μ plasmid were wild type for regulating Hmg2p–GFP degradation (Fig. 7; unpublished data). We also constructed a derivative of this plasmid expressing the mutant D487N-

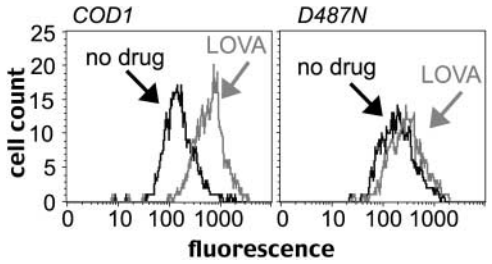


Figure 7. **The D487N mutation inactivates Cod1p.** Regulation of Hmg2p–GFP in *cod1Δ* cells (RHY2681) transformed with a plasmid expressing $P_{TDH3}$-9HIS–HA–COD1 (COD1) or $P_{TDH3}$-9HIS–HA–COD1 with the D487N mutation (D487N). Lovastatin was added at 25 μg/ml and cultures were grown at 30°C for 4 h after the addition of lovastatin.

Cod1p. In this mutant protein, the aspartyl phosphorylation site strictly conserved in all P-type ATPases was replaced by an asparagine residue. The plasmid expressing the D487N-Cod1p was unable to complement the *cod1Δ* null phenotype as expected from previous studies (Fig. 7; Suzuki, 2001).

We purified wild-type histidine-tagged Cod1p and the nonfunctional D487N protein using nickel affinity chromatography as described in detail in the Materials and methods section (Fig. 8, A and B). The purification yielded a single major band corresponding to the tagged Cod1p. Minor products detected in the elutions were the same in the purifications of both wild-type and the D487N mutant proteins.

We tested the purified proteins for the ability to hydrolyze ATP using a sensitive radiochemical assay described previously (Bais, 1975; Ghosh et al., 1990; Mandal et al., 2000). The assay measures the free inorganic phosphate produced from [$\gamma^{32}$-P]ATP, taking advantage of the fact that activated charcoal will sequester unhydrolyzed ATP but not the liberated $^{32}$Pi. Wild-type Cod1p hydrolyzed ATP in a time-dependent manner, with free phosphate increasing linearly with time (Fig. 8 C). In contrast, the mutant D487N protein produced no detectable ATPase activity (Fig. 8 C). The reaction catalyzed by the eluted Cod1p was dependent on the concentration of ATP and could be modeled using Michaelis-Menten kinetics (Fig. 8 D). In the presence of 5 mM magnesium, Cod1p exhibited a $K_m$ of ~15 μM for ATP hydrolysis and a $V_{max}$ of ~150 nmol Pi/min/mg. As with other P-type ATPases, the activity of Cod1p was inhibited by 500 μM vanadate (unpublished data).

It has previously been demonstrated that the ATPase activity of P-type pumps can be stimulated by the presence of its ionic substrates. Thus, stimulation of ATPase activity by an ion normally indicates that the ion is transported by the ATPase. For example, the ATPase activity of the Golgi apparatus–localized Pmr1p is stimulated with calcium and manganese, the substrates it transports (Mandal et al., 2000). Accordingly, we tested calcium to see if it would stimulate the ATPase activity of Cod1p (Fig. 9, A and B). Surprisingly, calcium failed to increase the ATPase activity of Cod1p. In fact, higher concentrations of calcium (>10 μM free calcium) inhibited the activity of Cod1p (Fig. 9, A and B). We next tested various biologically relevant cations with the aim of identifying which ion was the substrate of Cod1p. The range of ions tested included $Ca^{2+}$, $Sr^{2+}$, $Ba^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, and $Cd^{2+}$, none of which stimulated activity above the level seen

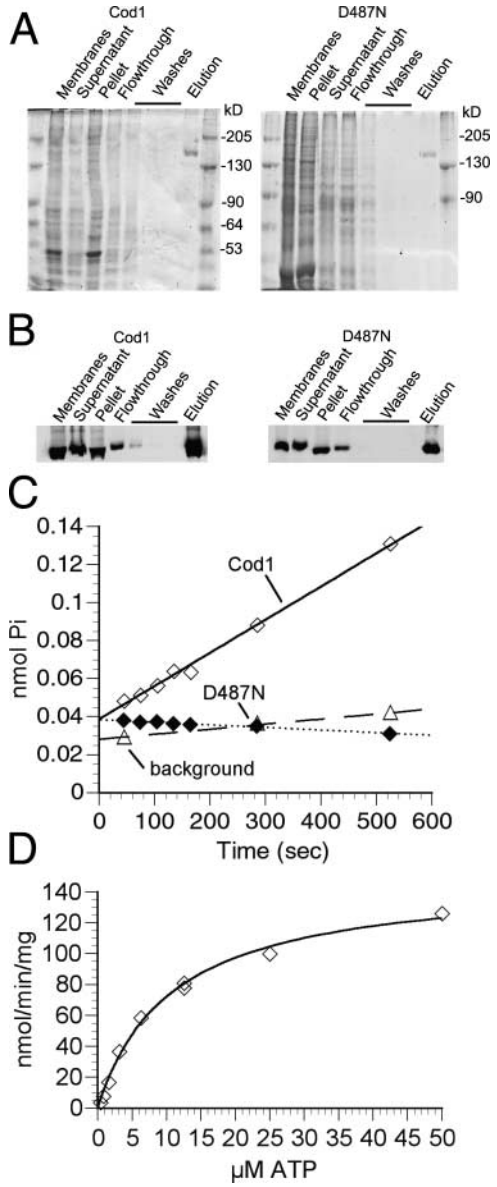


Figure 8. **Purification and ATPase activity of wild-type and D487N versions of 9HIS–HA–Cod1p.** (A) SDS-PAGE stained with Gelcode blue. (B) Western blots with anti-HA antibodies. (C) 0.015 μg of histidine-tagged Cod1p (◇), D487N (◆), or nothing (△) were incubated in 50 mM Hepes/TRIS, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, and 50 μM $\gamma^{32}$-labeled ATP for the indicated times. (D) 0.015 μg of histidine-tagged Cod1p (◇) was incubated for 5 min in 50 mM Hepes/TRIS, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, and the indicated amounts of $\gamma^{32}$-labeled ATP. Representative experiments are shown.

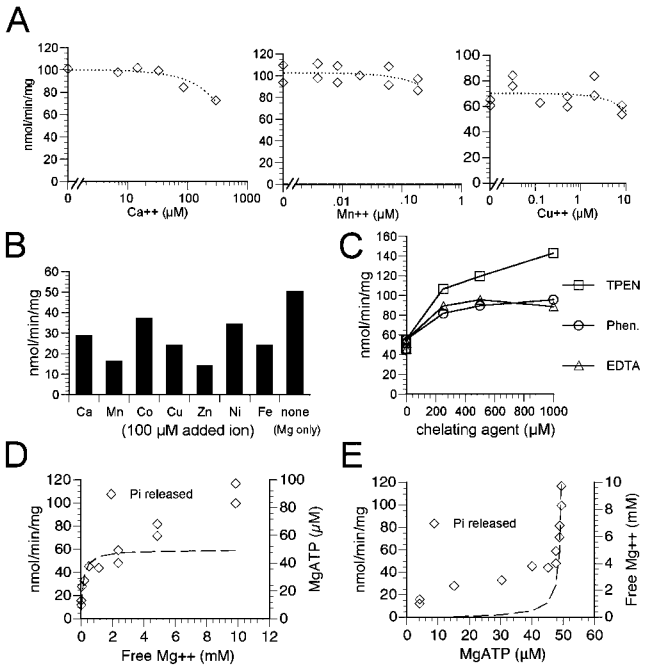


Figure 9. **Effect of ions on Cod1p.** (A) 0.135 μg of histidine-tagged Cod1p was incubated in 1 mM EDTA, 50 mM Hepes/TRIS, 100 mM KCl, 10 mM $MgCl_2$, 50 μM [$\gamma^{32}$]ATP, and increasing amounts of $CaCl_2$, $MnCl_2$, or $CuSO_4$. Experiments with $CuSO_4$ were done without EDTA. Results are plotted with respect to the calculated free concentration of the indicated ion. (B) 0.15 μg of histidine-tagged Cod1p was incubated in 50 mM Hepes/TRIS, 100 mM KCl, 5 mM $MgCl_2$, and 50 μM $\gamma^{32}$-labeled ATP for 5 min with the addition of 100 μM of the indicated ions, or nothing. (C) 0.15 μg of Cod1p was incubated in 50 mM Hepes/TRIS, 100 mM KCl, 5 mM $MgCl_2$, and 50 μM $\gamma^{32}$-labeled ATP for 5 min in the presence of the indicated concentrations of TPEN, 1,10-phenanthroline (Phen), or EDTA. (D) 0.015 μg of Cod1p was incubated for 5 min in 50 mM Hepes/TRIS, 100 mM KCl, 1 mM EDTA, 50 μM $\gamma^{32}$-labeled ATP, and increasing concentrations of $MgCl_2$. The ATPase activity of Cod1p (◇) is plotted with respect to the concentration of free $Mg^{2+}$ on the primary y axis. The calculated concentration of MgATP with respect to the concentration of free $Mg^{2+}$ is plotted as a dotted line on the secondary y axis. (E) The same data as in D, plotted on the primary y axis with respect to the calculated concentration of MgATP. The calculated concentration of free $Mg^{2+}$ is plotted with respect to the calculated concentration of MgATP as a dotted line. Representative experiments are shown.

with magnesium alone (Fig. 9, A and B; unpublished data). Replacing the $K^+$ in the assays with $Li^+$, $Na^+$, $Rb^+$, or $Cs^+$ did not affect the activity of the protein (unpublished data). To ensure that trace amounts of any possible contaminating cations were not stimulating the activity of Cod1p, we assayed the ATPase activity in the presence of increasing concentrations of the chelators TPEN, 1,10-phenanthroline, and EDTA (Fig. 9 C). TPEN and 1,10-phenanthroline have been used to remove metals such as zinc from metalloenzymes (Arslan et al., 1985; Dawson et al., 1986; Bays et al., 2001). None of the chelators diminished the activity of Cod1p; rather, the chelating agents all stimulated the activity of the enzyme, suggesting that heavy metals may inhibit the activity of the enzyme. The only ion for which Cod1p showed any dependence was $Mg^{2+}$.

$Mg^{2+}$ in the form of an MgATP complex is required for all characterized P-type ATPases. Studies of other P-type ATPases have shown a requirement for additional free $Mg^{2+}$ in order to obtain maximal activity (Skou, 1974; Ahlers, 1981; Plesner and Plesner, 1981; Brooker and Slayman, 1983). In these studies, maximal stimulation of the ATPase was usually observed when the total concentration of magnesium was one to three times the total concentration of ATP. We investigated the dependence of Cod1p on $Mg^{2+}$ by varying the concentration of added $MgCl_2$ while keeping a total ATP concentration of 50 μM (Fig. 9, D and E). Because of the large excess of $Mg^{2+}$ relative to the concentration of ATP, the MgATP concentration was nearly constant above 1.25 mM. Cod1p activity increased up to 10 mM $Mg^{2+}$, after which it began to decline slowly (Fig.

9, D and E; unpublished data). The increase in Cod1p activity between 1.25 and 10 mM indicated a dependence on free $Mg^{2+}$, because in this range, the concentration of MgATP remained constant. However, the increase in total activity due to free magnesium was on the order of twofold at maximum.

## Discussion

The regulation of ions in the ER is of central importance to the cell, given the numerous functions of this organelle. However, the only known supplier of ions to the ER was the Golgi apparatus–localized Pmr1p, which has been shown to influence the concentration of calcium in the ER (Strayle et al., 1999). Our previous work and that of others raised the possibility that Cod1p might play an important role with regard to ER ion regulation (Suzuki and Shimma, 1999; Cronin et al., 2000). In this work, we show that Cod1p is localized to, and centrally involved in, the function of the ER and in calcium regulation, and demonstrate the biochemical activity of Cod1p. Our cross-complementation studies, along with the broad conservation of Cod1p among metazoans, indicate that Cod1p shares similar functions in a broad range of organisms. Thus, it appears that Cod1p and its orthologues play a unique role in the functioning of the ER, quite distinct from other known P-type ATPases.

Our studies clearly show that Cod1p is a functionally important ER protein. Unique among yeast P-type ATPases, Cod1p localized to the ER membrane, as measured by immunofluorescence and direct biochemical analysis. The Cod1p subcellular localization is consistent with the functions and phenotypes of this ion pump. Our study and other studies had previously demonstrated phenotypes in ER-associated processes such as N-linked glycosylation and regulation of HMGR stability (Suzuki and Shimma, 1999; Cronin et al., 2000). The experiments above showed that Cod1p was critical in maintaining the proper ER folding environment and was required for viability of mutants deficient in the UPR, presumably due to its role in maintaining ER ion balance, as discussed below. Furthermore, at least in our strains, Cod1p had a more important role in this regard than Pmr1p, because the same synthetic lethality with *hac1Δ* was not observed with a *pmr1Δ* null. Taken together, our localization and phenotypic studies showed Cod1p to be a critical and conserved participant in ER function, with numerous phenotypes that arise from its action on ER physiology.

The identity of Cod1p as a P-type ATPase and its importance in the normal functioning of the ER suggests that it functions to control the balance of one or more ions in the lumen of the ER. The idea that this ion might be calcium first arose from our previous observations of *cod1* suppression by manipulating exogenous calcium (Cronin et al., 2000). In this work, we further examined the role of Cod1p in calcium maintenance in a variety of ways. Increased expression of calcineurin-related genes can indicate depletion of secretory pathway calcium store, and a *cod1Δ* null showed the expected increase in expression of the calcineurin-activated genes *PMC1*, *ENA1*, and *FKS2* (Cunningham and Fink, 1996; Matheos et al., 1997). In this assay, it is interesting to note that the magnitude of the effect of *COD1* inactivation was similar to that reported for *pmr1Δ* nulls (Locke et al., 2000). Similarly, the specific growth hypersensitivity of *cod1Δ* strains to calcium chloride is reminiscent of the growth sensitivities of mutants lacking calcium-specific P-type ATPases. For example, deletion of the vacuolar calcium ATPase *PMC1* results in calcium hypersensitivity, and deletion of the Golgi apparatus ATPase *PMR1* leads to calcium and manganese hypersensitivity (Cunningham and Fink, 1994a; Lapinskas et al., 1996; Wei et al., 2000). Finally, the synergistic effect of *cod1Δ* and *pmr1Δ* on total cellular calcium, but no other ions, also pointed to a role for *COD1* in maintaining cellular calcium homeostasis.

Although the calcium-related phenotypes were suggestive of direct involvement in calcium transport, our cross-complementation experiments and biochemical data suggested that Cod1p may influence the ER in a different manner. The known $Ca^{2+}$–transporting ATPases group together by sequence homology (type II) and are generally capable of substituting for each other's function in vivo. This property has been used extensively to clone new $Ca^{2+}$ pumps and aid in the characterization of $Ca^{2+}$ specificity of pumps cloned by other means (Liang et al., 1997; Harper et al., 1998; Talla et al., 1998; Degand et al., 1999). For example, *SERCA1a* from rabbit, *ECA1* from *A. thaliana*, and *SMA1* from *Schistosoma mansoni* all restore some functions to yeast mutants lacking the genes encoding the endogenous $Ca^{2+}$ pumps Pmr1p and Pmc1p (Liang et al., 1997; Talla et al., 1998; Degand et al., 1999). It should be noted that the ability of exogenous pumps to complement yeast mutants does not strictly depend on the localization of the exogenous pump. Thus, functions of the Golgi apparatus–localized Pmr1p and the vacuolar Pmc1p can be substituted by the exogenous SERCA1 pump localized to the ER in yeast (Degand et al., 1999). In contrast to the apparent facility with which these pumps replace each other, neither SERCA nor *ACA2*, nor overexpressed *PMR1*, could suppress phenotypic defects of the *cod1Δ* null mutant. Similarly, overexpression of Cod1p did not suppress the EGTA sensitivity of *pmr1Δ*. Thus, if Cod1p contributes to the ER by directly pumping calcium, it must be doing so in a manner distinct from the previously known type II calcium pumps.

To further examine whether or not Cod1p was directly involved in calcium transport, we purified the Cod1 protein to identify the biochemical requirements for Cod1p ATPase activity in vitro. P-type ATPases exhibit enhanced activity in the presence of their substrates, such that the ATPase activity of SERCA1 is stimulated by calcium, the sodium/potassium ATPase by sodium and potassium (Koenderink et al., 2000), Pmr1p by manganese and calcium (Mandal et al., 2000), and the Menkes protein by copper (Voskoboinik et al., 2001). Surprisingly, calcium was not required for the biochemical activity of Cod1p, and at higher concentrations ($>100$ μM), $Ca^{2+}$ inhibited activity. In fact, in the apparent absence of any substrate, Cod1p hydrolyzed ATP at a rate similar to that of other P-type ATPases. For example, Pmr1 has a Vmax of near 200 nmol Pi/min/mg in the presence of its preferred substrates $Ca^{2+}$ and $Mn^{2+}$ (Mandal et al., 2000), whereas Cod1p exhibits a Vmax for ATP hydrolysis of ~150 nmol Pi/min/mg in reactions containing solely the eluted product, magnesium, potassium, and micromolar quantities of sodium. The metal chelators EDTA, TPEN, and 1,10-phenanthroline each activated (or de-inhibited?) Cod1p activity when used at concentrations that should have greatly reduced

the free concentration of any divalent ions in the assay with the exception of magnesium. Cod1p appears to be acting quite differently from known P-type pumps.

A possible explanation is that the in vitro ATPase activity demonstrated by Cod1p is simply uncoupled from pumping activity. This might occur if the factors coupling Cod1p to a specific ion or ions were lost during the purification of the enzyme. This model suggests that the inability of the *Drosophila* homologue of Cod1p to complement the *cod*Δ mutant at low copy was due to poor interaction of dCod1p with the required factors in yeast. A requirement for specificity-determining factors also leaves open the possibility for a direct role for Cod1p in calcium transport. Such coupling factors would be quite unexpected. The ATPase activity of some pumps bas been uncoupled from transport by cis mutations, and several mammalian P-type ATPases have β subunits. However none of the β subunits appear to modify the substrate specificity of the enzyme. Additionally, although we have been able to measure calcium transport by Pmr1p, Pmc1p, rabbit SERCA, and other $Ca^{2+}$-specific ATPases in yeast microsomes (Sorin et al., 1997; Marchi et al., 1999; Ton et al., 2002), we have been unable to detect calcium transport by Cod1p-containing microsomes (unpublished data).

Another possibility is that magnesium is a substrate for Cod1p. P-type ATPases in general need free magnesium in order to hydrolyze ATP. The concentration of free magnesium needed for activation depends on the concentration of ATP present, such that generally the ratio of magnesium to ATP needed falls within the range of 1–3 mol magnesium per mol ATP (Ahlers, 1981; Plesner and Plesner, 1981; Brooker and Slayman, 1983; Skou, 1974). In the case of Cod1p, we obtained the greatest activity at a relatively high concentration of 10 mM magnesium, a concentration 200 times the concentration of ATP in the assay mix. A role for Cod1p in $Mg^{2+}$ is attractive given the biological importance of magnesium. At present, magnesium supply and regulation in yeast are poorly understood and information about the intracellular distribution of magnesium is scarce (Beeler et al., 1997). ATPases in the lumen of the ER, such as Kar2, and other enzymes within the secretory pathway would presumably need magnesium in the lumen. It would seem fitting that yeast cells would have a way to transport magnesium into the lumen of the ER. Although the high level of magnesium needed for the activity of Cod1p does not demonstrate that Cod1p pumps magnesium, it suggests magnesium may be a good starting point for further biochemical study.

A third possibility is that Cod1p transports nonmetallic substrate. Recently, several groups have gathered evidence that the type IV group of P-type ATPases are involved in the translocation of aminophospholipids necessary for the maintenance of membrane lipid asymmetry (Tang et al., 1996; Chen et al., 1999; Ding et al., 2000; Gomes et al., 2000), though this idea has been challenged (Siegmund et al., 1998; Marx et al., 1999). The type IV ATPases that have been studied, including the Golgi apparatus–localized yeast protein Drs2p, are apparently specific for phosphatidylserine. Yeast lacking *DRS2* are defective in translocating a fluorescently labeled phosphatidylserine, a defect that can be complemented by expression of the *A. thaliana ALA1*, and the ATPase activity of bovine ATPase II is stimulated by phosphatidylserine (Tang et al., 1996; Ding et al., 2000; Gomes et al., 2000). Although the type V pumps, such as Cod1p, are most homologous to $Ca^{2+}$-transporting ATPases, when the sequence comparisons are restricted to certain core domains, the type V pumps are more similar to the type IV ATPases than to other members of the P-type ATPase family (Axelsen and Palmgren, 1998, 2001). The similar features of core domain sequences between the type IV and V groups suggest that an investigation of nonmetallic substrates for Cod1p may be fruitful.

Understanding the role of Cod1p has significance for the understanding of the possible functions of the other type V ATPases. Genes predicted to encode type V ATPases are present in all of the eukaryotic genomes that have been sequenced to date, yet none of the other members of the type V family are known other than by sequence homology. A number of genes have been identified, including one in humans, which are predicted to encode proteins with a high degree of homology to Cod1p (>35% identity and 55% similarity; Costanzo et al., 2000). Our demonstration that one of these, the *D. melanogaster* dCod1p, rescues the *cod1*Δ mutant phenotype indicated experimentally that the function of these proteins is conserved and these proteins are orthologous to Cod1p. More distant homologues of *COD1* in the type V subgroup are even lesser known and likely have other functions. YOR291w, the second yeast type V ATPase has less homology to *COD1* than *dCOD1* (Costanzo et al., 2000). Deletion of YOR291w produces none of the phenotypes displayed by *cod1*Δ mutants (this paper; Cronin et al., 2000). In fact, we have been unable to detect any phenotypes associated with this gene (unpublished data). Thus, the degree of sequence similarity (or disparity) to Cod1p appears to be a useful predictor of function.

In conclusion, we have greatly extended the phenotypic characterization of Cod1p, defining its role in calcium regulation and ER function, and we have provided the first biochemical exploration of Cod1p. This study provides the foundation for further biochemical studies of Cod1p that will likely illuminate the obscurity surrounding ion regulation and enhance our understanding of the widely conserved, yet little known, type V group of P-type ATPases.

## Materials and methods

### Materials

Restriction enzymes, Vent DNA polymerase, and T4 DNA ligase were obtained from New England Biolabs, Inc. The 9E10 cell culture supernatant was produced in our lab from cells (American Type Culture Collection accession no. CRL 1729) grown in RPMI1640 culture medium (Life Technologies) with 10% FCS and supplements. HA.11 anti-HA antibody was obtained from BAbCo. CY5-conjugated anti–rabbit antibody was a gift from Dr. Kit Pogliano (UCSD). Affinity-purified HRP-conjugated goat anti–mouse antibodies were purchased from Sigma-Aldrich. ECL chemiluminescence immunodetection reagents were from Amersham Pharmacia Biotech. *Escherichia coli* total lipid extract was obtained from Avanti Polar Lipids, Inc. All other chemical reagents were obtained from Sigma-Aldrich or Fisher Scientific.

### Plasmid construction

pRH1112 (CEN, *LEU2, HA::COD1*) was constructed from pRH810 by using synthetic overlap extension to add sequence coding for an HA epitope tag to the 5′ end of *COD1*, just after the start codon. pRH1312 was constructed from pRH1112 by replacing the native promoter of *COD1* between SacI and SphI sites with a PCR-amplified *TDH3* (glyceraldehyde 3-phosphate dehydrogenase) promoter.

Plasmid pOT2-GH06032 containing the *dCOD1* cDNA was purchased from Research Genetics. pRH1388 (CEN, *LEU2, HA::dCOD1*) expressing HA–dCod1p from the *COD1* promoter was constructed as follows. Primers

Table I. **Yeast strains**

| Strain | Genotype | Reference |
|---|---|---|
| RHY791 | *MATa hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP* | Cronin et al., 2000 |
| RHY792 | *MATα hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP* | Cronin et al., 2000 |
| RHY811 | *MATa hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1* | Cronin et al., 2000 |
| RHY812 | *MATα hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1* | Cronin et al., 2000 |
| RHY871 | *MATa hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP* | Cronin et al., 2000 |
| RHY872 | *MATα hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP* | Cronin et al., 2000 |
| RHY910 | *MATa hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP cod1-1* | Cronin et al., 2000 |
| RHY911 | *MATα hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP cod1-1* | Cronin et al., 2000 |
| RHY1647 | *MATα HMG2 ura3-52 hac1Δ::URA3* | This study |
| RHY1401 | *MATα hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP cod1Δ::KanMX* | This study |
| RHY1402 | *MATα hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP cod1Δ::LEU2* | This study |
| RHY1664 | *MATα hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP hac1Δ::URA3* | This study |
| RHY1722 | *MATa HMG2 ura3-52 cod1Δ::KanMX* | This study |
| RHY1708 | *MATa hmg2::HIS3::1mycHMG2 ura3-52* | This study |
| RHY1709 | *MATa hmg2::HIS3::1mycHMG2 ura3-52 cod1Δ::LEU2* | This study |
| RHY1870 | *MATα HMG2 ura3-52 3myc::COD1* | This study |
| MYY290 | *MATa ura3, his3, leu2* | Smith and Yaffe, 1991 |
| MYY291 | *MATα ura3, his3, leu2* | Smith and Yaffe, 1991 |
| RHY1884 | *MATa ura3, his3, hac1Δ::URA3* | This study |
| RHY2179 | *MATα ura3, his3, leu2, pmr1Δ::HIS3* | This study |
| RHY2277 | *MATa ura3, his3, hac1Δ::URA3, pmr1Δ::HIS* | This study |
| RHY2681 | *MATα hmg2::HIS3::1mycHMG2 ura3-52::LEU2::HMG2::GFP cod1Δ::KanMX* | This study |
| CS601A | *MATa ura3-52::URA3* | Suzuki and Shimma, 1999 |
| CS601B | *MATα pmr1Δ::LEU2 ura3-52* | Suzuki and Shimma, 1999 |
| CS601C | *MATa spf1Δ::TRP1 ura3-52* | Suzuki and Shimma, 1999 |
| CS601D | *MATα spf1Δ::TRP1 pmr1Δ::LEU2 ura3-52* | Suzuki and Shimma, 1999 |

oRH1328 and oRH1329 were used to amplify the proximal part of the *dCOD1* ORF before the AgeI site, adding sequence coding for an HA tag just after the start codon. This PCR product was cut with AatII and AgeI and ligated with the BglII–AatII fragment of pRH1112 and the AgeI–BglII fragment of pOT2-GH06032. The *HA–dCOD1* ORF was subsequently transferred to a number of plasmids including pRH1389 (2μ *LEU2* $P_{TDH3}$-*HA::dCOD1*) pRH1431 (2μ *URA3* $P_{PMA1}$-*HA::dCOd1*) using AatII and BglII.

pRH1432 expressed 9HIS–HA–Cod1p from the *TDH3* promoter on a high copy plasmid. Synthetic overlap extension was used to add sequence coding for nine histidine residues to the 5′ end of $P_{TDH3}$-*HA::COD1*, just after the start codon and before the sequence coding for the HA epitope tag. Oligonucleotide primers oRH1079, oRH1082, oRH1354, and oRH1355 were used to amplify from the template plasmid pRH1331 (2μ, *LEU2,* $P_{TDH3}$-*HA::COD1*). The resulting PCR product was subcloned into pRH1332 (2μ, *URA3*), using BsiWI and EagI to yield pRH1432. Plasmid pRH1478 expressed the D487N mutant of 9HIS–HA–Cod1p from the *TDH3* promoter. It was constructed by removing the BstEII–BstEII fragment coding for the D487N mutation from pCS210 and placing it into the same sites of pRH1432.

## Yeast culture and strains

Yeast strains were grown in minimal media (yeast nitrogen base without amino acids; Difco) with glucose and the appropriate supplements as described previously, except that leucine supplementation was increased to 60 mg/liter. Experiments were performed in minimal media at 30°C unless otherwise noted. For protein purification, yeast strains were grown in minimal media with glucose supplemented with 1 g/liter adenine, 1 g/liter methionine, 1.5 g/liter leucine, 1.5 g/liter lysine, 1 g/liter histidine, and 20 g/liter casamino acids (Difco). Crosses and transformations were done using standard techniques.

The 3myc–Cod1p allele expressed in RHY1870 (Table I) was constructed using a PCR epitope-tagging method (Schneider et al., 1995). *URA3* and the flanking myc epitope tags from pMPY–3xMYC were amplified with primers oRH1190 and oRH1191. The primers added sequences homologous to the 5′ end of *COD1*. Yeast were transformed with the PCR product and selected for acquisition of uracil prototrophy. Transformants were screened by the fluorescence plate assay for the $Cod^-$ phenotype. $Cod^-$, $Ura^+$ candidates were grown in YPD to allow "pop-out" of *URA3* mediated by the direct repeats of the myc epitope tag followed by counterselection for loss of *URA3* on media containing 5-fluoroorotic acid. The resulting $Ura^-$, $Cod^+$ candidates were then tested for expression of the 3myc–Cod1p by western analysis.

The *cod1Δ::KanMX* allele in RHY1722 (*HMG2, cod1Δ::KanMX*) was constructed by PCR-mediated disruption of the *cod1Δ::LEU2* allele from RHY1402, which was derived from a cross of RHY792 and RHY1202 (Cronin et al., 2000). The resulting strain (RHY1401) was crossed to RHY795 (*HMG2, ura3-52*). Sporulation and dissection yielded RHY1722.

RHY1708 (1MYC::HMG2) and RHY1709 (1MYC::HMG2, cod1Δ::LEU2) were used for β-galactosidase assays. They were constructed by treatment of RHY791 and RHY1202 (Cronin et al., 2000) with 5-fluoroorotic acid. The β-galactosidase reporter plasmids were transformed into the strains using standard techniques.

*hac1Δ* strains were constructed as follows. RHY1647 (*hac1Δ::URA3, COD1*) and RHY1664 (*hac1Δ::URA3, COD1*) were constructed by transforming RHY534 and RHY872, respectively, with the BamHI fragment of pHAKO (Cox and Walter, 1996). RHY534 is a diploid version of JRY1159 (Hampton and Rine, 1994). RHY1884 was constructed by transforming MYY290 (Smith and Yaffe, 1991) with the BamHI fragment of pHAKO (Cox and Walter, 1996). RHY2179 (*pmr1Δ::HIS3*) was constructed by transforming MYY291 (Smith and Yaffe, 1991) with the AatII–AatII fragment from pL127-6 (Cunningham and Fink, 1994b). RHY2277 was isolated from a cross of RHY2179 and RHY1884.

## Growth curves

Susceptibility of mutants to DTT, tunicamycin, $MnCl_2$, and other divalent cations was tested in minimal media. Susceptibility to $MnCl_2$, $CaCl_2$, and EGTA was tested in liquid YPD buffered with succinate to pH 5.5. Low-density liquid cultures of wild-type or mutant cells were used to serially dilute the tested agent. The resulting cultures were incubated and measured for optical density at 600 nm.

## Immunofluorescence microscopy

Immunofluorescence was performed essentially as described in detail previously (Rossanese et al., 1999), except that the cells were adhered to a multiwell slide instead of a coverslip. Images were captured using a DeltaVision confocal microscope and processed with the accompanying software.

## β-Galactosidase assays

β-Galactosidase assays were performed as previously described (Guarente, 1983), except that freeze–thawing in liquid nitrogen was used to lyse cells.

## Subcellular fractionation

Fractionation was performed as described by Roberg et al., 1997. Protein from each fraction was separated by SDS-PAGE, transferred to nitrocellulose, probed with the appropriate antibodies, and detected by chemiluminescence using the ECL system. NIH Image 1.6 was used to determine signal intensity.

### Flow cytometry

Analysis of GFP fluorescence in living cells by flow microfluorimetry was performed on a Becton Dickinson FACScalibur® flow microfluorimeter using settings appropriate for GFP. Strains were typically grown into early log phase in minimal media. After addition of drugs, cultures were allowed to incubate 4 h before analysis.

### ICP-OES

The total amount of calcium and other ions in whole cells and subcellular fractions was determined by ICP-OES. To determine the total ion content of whole cells, cells were grown in YPD, pH 5.5, to $OD_{600}$ of 1.5 ml of the culture was collected by vacuum filtration onto 0.4-μm filters. The cells were then washed with 5 ml deionized water. The filters were transferred to microcentrifuge tubes and digested in 500 μl of 33% nitric acid overnight at 65°C. Samples were diluted with 3 ml of deionized water before analysis using a PerkinElmer Optima 3000XL spectrometer and software.

### Solubilization and purification of histidine-tagged Cod1p

9HIS–HA–Cod1p was prepared by detergent extraction of the protein from ER membranes followed by nickel affinity chromatography. ER membranes were prepared by sucrose gradient centrifugation. Before harvesting, cells were treated with 10 mM sodium azide. The cells were washed in 10 mM sodium azide, 5 mM Tris, pH 7.5, and then resuspended in lysis buffer (4 M sorbitol, 1 mM EDTA, 0.1 M $K_xH_xPO_4$, pH 7.5) plus protease inhibitors (10 mM PMSF, 2 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) so that the packed yeast cells made up one third of the volume. The cells lysed with four 30-s pulses with 1-min intervals in a Bead Beater® (BioSpec Products, Inc.). The crude lysates was clarified by centrifugation at 350 *g* in a Sorvall SS-34 rotor. The clarified lysates were loaded onto a sucrose step gradient and centrifuged for 2 h at 174,000 *g* in a Beckman Coulter SW28 rotor. ER membranes containing 9HIS–HA–Cod1p were collected from the interface between 30% and 54% sucrose. The collected membranes were diluted with an equal volume of P&R buffer (0.5 M sucrose, 10 mM MES/KOH, pH 6, 150 mM potassium chloride) with protease inhibitors (1 mM PMSF, 2 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). The membranes were pelleted by centrifugation for 1 h at 174,000 *g*. The pelleted membranes were resuspended in P&R buffer with protease inhibitors and stored at −90°C in 110-μl aliquots. Protein concentration in the membranes was measured using the BCA assay (Pierce Chemical Co.).

Detergent extracts of the membranes were prepared as follows. 10 mg of membrane was suspended in 10 ml of S-buffer (20 mM Hepes/Tris, pH 7.0, 20% glycerol, 0.5% *E. coli* lipid extract, 6 mM β-mercaptoethanol, 10 mM imidazole, protease inhibitors, and 1.5% n-octyl-β-D-glucopyranoside) and allowed to shake gently at 4°C for 2.5 h. The resulting suspension was centrifuged at 100,000 *g* for 1 h to pellet insoluble material.

The detergent-solubilized 9HIS–HA–Cod1p was then purified by nickel affinity chromatography. 250 μl of NTA-Ni agarose beads (QIAGEN) per milliliter of solubilized membranes was washed twice with deionized water and then twice with S-buffer. The equilibrated beads were added to the detergent extract and gently shaken at 4°C for 30 min. The extract with beads was then transferred to 2 ml Micro Bio-Spin columns (Bio-Rad Laboratories). Unbound material was allowed to flow through by gravity elution. Each column was washed twice with 1 ml of S-buffer plus 200 mM NaCl, and the column was washed a third time with S-buffer. 9HIS–HA–Cod1p was eluted after a 5-min incubation with 0.2 ml per column of S-buffer plus 300 mM imidazole. The eluted material was immediately frozen in a dry ice–ethanol bath and stored at −90°C in 60-μl aliquots.

### Assaying ATPase activity

ATPase activity was measured using the radiometric assay described previously (Bais, 1975; Mandal et al., 2000) with some modifications. Assays were done in a 50-μl volume and generally contained 50 mM Hepes/Tris, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA. [$\gamma^{32}$P]ATP (30 Ci/mmol; Amersham Pharmacia Biotech) was diluted 10-fold with 1 mM NaATP to the final concentration desired. Variations in the assay conditions are noted in the figures. The concentrations of free ions and MgATP complexes were calculated using WINMAXC v2.05 (Bers et al., 1994) with supplementary constants from Data for Biochemical Research (Dawson et al., 1986). EDTA was excluded from experiments determining the effect of transition metals on Cod1p, because the high affinity of EDTA (and other chelators) for transition metals prevented accurate determination of the free ion within the desired range. Solutions were checked for contaminating ions by ICP-OES. Vanadate was prepared by the method of Gordon (1991).

The authors thank members of the Hampton and Rao labs for assistance with various parts of the project. The authors thank Dr. Chise Suzuki (National Food Research Institute, Tsukuba, Japan) for providing material prior to publication, Dr. Kyle Cunningham (Johns Hopkins University), Dr. Hans Rudolph (Universitat Stuttgart, Stuttgart, Germany), Dr. Peter Walter (UCSF), Dr. Jeff Harper (The Scripps Research Institute, La Jolla, CA), and Dr. Patrice Catty (Commissariat à l'Energie Atomique, Grenoble, France) for providing plasmids and strains, Dr. Jeff Harper, Karina Quon, and Kevin Walda (Scripps Institute for Oceanography, La Jolla, CA) for assistance with ICP-OES, Kit Pogliano and Marc Sharp for use of the Deltavision microscope, and Dr. Robert Rickert (UCSD) for use of the FACScalibur® flow microfluorimeter and software.

Submitted: 11 March 2002
Revised: 18 April 2002
Accepted: 18 April 2002

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