Regulation of HMG-CoA Reductase Degradation Requires the P-Type ATPase Cod1p/Spf1p

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Abstract. The integral ER membrane protein HMG-CoA reductase (HMGR) is a key enzyme of the mevalonate pathway from which sterols and other essential molecules are produced. HMGR degradation occurs in the ER and is regulated by mevalonate-derived signals. Little is known about the mechanisms responsible for regulating HMGR degradation. The yeast Hmg2p isozyme of HMGR undergoes regulated degradation in a manner very similar to mammalian HMGR, allowing us to isolate mutants deficient in regulating Hmg2p stability. We call these mutants *cod* mutants for the control of HMG-CoA reductase degradation. With this screen, we have identified the first gene of this class, *COD1*, which encodes a P-type ATPase and is identical to *SPF1*. Our data suggested that Cod1p is a calcium transporter required for regulating Hmg2p degradation. This role for Cod1p is distinctly different from that of the well-characterized Ca^{2+} P-type ATPase Pmr1p which is neither required for Hmg2p degradation nor its control. The identification of Cod1p is especially intriguing in light of the role Ca^{2+} plays in the regulated degradation of mammalian HMGR.

Key words: hydroxymethylglutaryl CoA reductase • Ca^{2+}-transporting ATPase • ubiquitin • endoplasmic reticulum • *Saccharomyces cerevisiae*

Introduction

The ER resident, integral membrane protein, hydroxymethylglutaryl-coenzyme A reductase (HMGR)[1], catalyzes the first committed step of the mevalonate pathway from which sterols and other essential isoprenoids are produced. HMGR is subject to numerous modes of regulation, including feedback control of HMGR stability (Edwards et al., 1983; Chun et al., 1990; Goldstein and Brown, 1990). Increased production of mevalonate pathway products causes an increase in the degradation rate of HMGR and a lowered steady-state level of the protein (Edwards et al., 1983). Conversely, decreased production of pathway products causes a decrease in the degradation rate of HMGR and an elevated steady-state level of the protein. The mevalonate-derived molecules that control the stability of HMGR are poorly defined, and the mechanisms by which these signals control HMGR stability remain unknown (Roitelman and Simoni, 1992; Keller et al., 1996; Meigs et al., 1996; Lopez et al., 1997; Meigs and Simoni, 1997).

The yeast HMGR isozyme Hmg2p undergoes regulated degradation with many similarities to the mammalian enzyme, including control by a signal derived from the mevalonate pathway product farnesyl pyrophosphate FPP (Hampton and Rine, 1994; Hampton and Bhakta, 1997; Gardner and Hampton 1999a). We have identified genes required for the degradation of Hmg2p, referred to as *HRD* genes (pronounced "herd", for HMG-CoA reductase degradation). The *HRD* genes are also required for the degradation of numerous other proteins, none of which are regulated by the mevalonate pathway (Wilhovsky et al., 2000; Hiller et al., 1996; Bordallo et al., 1998; Galan et al., 1998; Plemper et al., 1998).

Our studies suggest that regulation of Hmg2p stability does not occur by modulation of the *HRD*-encoded degradation machinery (Hampton et al., 1996a; Gardner and Hampton, 1999b). Rather, we posit that separate genes are required for specifically regulating degradation (see Fig. 1). We refer to these regulatory genes as *COD* genes, for the control of HMG-CoA reductase degradation. *cod* mutants deficient in regulation of Hmg2p degradation could fall into two phenotypic classes. Those in which Hmg2p is constitutively stable even when the degradation machinery is intact, and those in which Hmg2p is constitutively degraded even when degradation signals are low. This second class of mutants is the subject of this work. These mu-

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[1]*Abbreviations used in this paper:* FPP, farnesyl pyrophosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMGR, HMG-CoA reductase.

tants would allow degradation of Hmg2p in the presence of drugs such as lovastatin, an inhibitor of HMGR, that normally slow degradation by decreasing production of the signal. By our model (see Fig. 1), Hmg2p degradation in this type of *cod* mutant should still be blocked by *hrd* mutations (see Fig. 1).

We have isolated the first *cod* mutant and cloned the relevant gene, *COD1*. The *cod1-1* mutant failed to properly regulate Hmg2p degradation but did not generally affect the degradation of ER proteins. *COD1* was identical to *SPF1*, a gene previously identified in an unrelated screen (Suzuki and Shimma, 1999). Cod1p belongs to a large family of P-type ATPases involved in ion transport. From the results presented below, we hypothesize that Cod1p effects the control of Hmg2p degradation through acting on Ca^{2+} levels in the ER.

Materials and Methods

Materials

Restriction enzymes, Vent DNA polymerase and T4 DNA ligase were obtained from New England Biolabs. Lovastatin, L659,699 and zaragozic acid were generously provided by Dr. James Bergstrom (Merck, Rahway, NJ). Ro48-8071 was a gift from Dr. Olivier Morand (F. Hoffman-LaRoche). The 9E10 cell culture supernatant was produced in our lab from cells (CRL 1729; American Type Culture Collection) grown in RPMI1640 culture medium (GIBCO BRL) with 10% fetal calf serum and supplements. 12CA5 anti-HA antibody was obtained from Dr. Don Rio (UC Berkeley, Berkeley, CA). Affinity-purified HRP-conjugated goat anti–mouse antibodies were purchased from Sigma. ECL chemiluminescence immunodetection reagents were from Amersham. All other chemical reagents were obtained from Sigma or Fisher.

Plasmid Construction and DNA Manipulation

Plasmid pRH468 (integrating) expressed 1myc-Hmg2p from a GAPDH promoter. pRH468 was constructed by removing the NcoI-AatII fragment containing part of the *URA3* open reading frame from pRH423 (integrating, *URA3*; Hampton and Bhakta, 1997). The Hmg2p-GFP reporter protein was expressed from plasmid pRH680 (integrating, *LEU2*) or pRH469 (integrating, *URA3*; Gardner et al., 1998). pRH680 was constructed from pRH469 by replacing the AatII-ApaI segment of the *URA3* gene in pRH469 with the AatII-ApaI fragment from pRS312 containing the *LEU*2 gene (Sikorski and Hieter, 1989). Hmg1p-GFP was expressed from pRH475 (integrating, *URA3*; Gardner et al., 1998).

Plasmid pRH397 (2μ, *URA3*) expressed HA-tagged ubiquitin from the GAPDH promoter and was constructed as follows. The HA-ubiquitin coding region was excised from YEp112 (Ellison and Hochstrasser, 1991) with EcoRI-ClaI and cloned into the same sites in Bluescript KS II to make pRH381. The HA-ubiquitin coding region was then cloned as a BamHI-ClaI fragment into the same sites of pRH98-3 (2μ, *URA3*; Donald et al., 1997) to yield pRH397.

Plasmids pRH810 (ARS/CEN, *LEU2*) and pRH811 (2μ, *LEU2*) expressed *COD1*. *COD1* was amplified from yeast genomic DNA by PCR along with 300 bp of flanking sequence. The PCR product was cloned into the vector pCR2.1 (Invitrogen). The BamHI fragment containing *COD1* was subcloned into YEp13 (2μ, *LEU2*; Broach et al., 1979) to generate pRH811 or into pRS315 (ARS/CEN, *LEU2*; Sikorski and Hieter, 1989) to generate pRH810.

pCS186, used for disruption of the *COD1/SPF1* open reading frame, was provided by Chise Suzuki (National Food Research Institute, Tsukuba, Japan; Suzuki and Shimma, 1999).

Yeast Culture and Strains

Yeast strains were grown in minimal media (Difco Yeast Nitrogen Base without Amino Acids) with glucose and the appropriate supplements as described previously, except that leucine supplementation was increased to 60 mg/liter (Hampton and Rine, 1994). Experiments were performed in minimal media at 30°C unless otherwise noted. For experiments with EGTA, strains were grown in YNB without $CaCl_2$ from Bio 101, Inc. buffered with potassium MES to pH 6.5. Yeast was transformed with plasmid DNA using the LiOAC method (Ito et al., 1983). The parental genotype of all of the yeast strains used in this study was *MATa ade2-101, his3*-Δ200, lys2-801, met, hmg1::LYS, hmg2::HIS3, ura3-52, and leu2Δ. Only distinguishing mutations are listed in Table I and discussed below.

RHY541, the parent strain for the COD screen, expressed the reporter proteins 1myc-Hmg2p and Hmg2p-GFP. RHY541 was constructed from the mevalonate auxotrophic strain RHY468 by integration at the *hmg2::HIS3* locus of plasmid pRH468 expressing 1myc-Hmg2p and selection for mevalonate prototrophy. The resulting strain was then transformed with pRH469 cut at the BsgI site and selected for uracil prototrophy to give the RHY541 parent. Various genotypes of *cod1-1* were constructed by crossing with appropriate isogenic strains and isolation of haploid progeny.

RHY1127 (*cod1-1/hrd1Δ*) was constructed by crossing RHY911 (*cod1-1*) with an isogenic strain with *hrd1Δ*::URA3, followed by sporulation and recovery of RHY1127 as a meiotic segregant. RHY1076 (*cod1-1, ubc7Δ*) was made by crossing RHY911 to RHY1056 (*ubc7Δ*). The *ubc7Δ::URA3* allele carried by RHY1056 was made by PCR disruption with *HIS3* and subsequent replacement of *HIS3* with *URA3* with pHU10 (Cross, 1997).

RHY1473 (*cod1-1*) and RHY1475 (*COD1*) expressed Hmg1p-GFP from the GAPDH promoter. They were constructed in a cross of RHY911 to RHY550 (*ura3-52::URA3::HMG1::GFP*). RHY550 was constructed by integrative transformation of RHY532 with pRH475 (integrating, *URA3, HMG1::GFP*).

RHY1203 (*cod1-1*) expressing K6R-Hmg2p-GFP was constructed by plating RHY811 on 5-fluoro-orotic acid to remove pRH469, and subsequent transformation with pRH671 (integrating, *K6RHMG2::GFP, URA3*; Gardner and Hampton, 1999a). The isogenic Cod^+ strain RHY1205 was recovered from a cross of RHY1203 to RHY872.

SPF1/COD1 was disrupted by transformation of RHY791 with the BamHI-NheI fragment from pCS186. A corresponding Cod^+ strain (RHY1232), was constructed by integrative transformation of RHY791 with linearized pCS186 to produce a strain with the *cod1::LEU2* disruption allele in tandem with functional *COD1*. RHY2201, RHY2202, RHY2203, and RHY2204 were constructed by transforming pRH469 into CS601A, CS601B, CS601C, and CS601D, respectively (Suzuki and Shimma, 1999).

The *COD1* paralogue YOR291w was deleted by transformation of the haploid strain RHY791 with a KanMX disruption cassette with 40-bp flanks homologous to the YOR291w locus (Wach et al., 1994). Disruption was confirmed by PCR.

Optical Assays

The optical techniques used in this study are described in full detail elsewhere (Cronin and Hampton, 1999). For assaying colony fluorescence, a Kodak Carousel® 4400 slide projector with a 488-nm narrow bandpass filter (Omega Optical) placed in the slot for slides provided illumination. Fluorescence of colonies was assessed visually with a long bandpass filter (Kodak Wratten No. 12) to remove blue light.

Analysis of Hmg2p-GFP fluorescence by flow microfluorimetry was performed on a Becton Dickinson FACScalibur® flow microfluorimeter and Cell Quest software. Strains were typically grown into early log phase in minimal media. After addition of drugs, cultures were incubated 4 h before analysis. Data from 10,000 cells were used for each histogram. In flow microfluorimetry experiments testing the effects of ions, cultures were incubated in exogenous $CaCl_2$ or other salt ~12 h before the addition of mevalonate pathway inhibitors. For ion chelation experiments, EGTA was added to a final concentration of 780 μM in cultures diluted to an optical density of 0.001 at 600 nm ~12 h before the addition of mevalonate pathway inhibitors. When indicated, $MgCl_2$ or $CaCl_2$ was added to EGTA-treated cultures to a concentration of 1 mM simultaneously with mevalonate pathway inhibitors, 4 h before analysis. For analysis by fluorescence microscopy, cultures grown as described for flow microfluorimetry were viewed using a Nikon Optiphot II microscope with a B2-A filter.

Cycloheximide Chase

To analyze regulated degradation directly, a cycloheximide chase followed by lysis and immunoblotting was used as described previously (Gardner et al., 1998).

Ubiquitination Assay

To aid in detection of ubiquitin, the strains tested were transformed with

Table I. Yeast Strains

Strain	Genotype
RHY468	*MAT**a** hmg2::HIS3 ura3-52 LEU2*
RHY541	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP LEU2*
RHY542	*MATa hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP LEU2*
RHY555	*MATa hmg2::HIS3 ura3-52::6MYC HMG2 hrd1-1 LEU2*
RHY658	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP LEU2 cod1-1*
RHY660	*MATa hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP LEU2 cod1-1*
RHY791	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP*
RHY792	*MATa hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP*
RHY811	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1*
RHY812	*MATa hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1*
RHY871	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP*
RHY872	*MATa hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP*
RHY880	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP hrd1Δ::URA3*
RHY910	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP cod1-1*
RHY911	*MATa hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP cod1-1*
RHY1056	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP ubc7Δ::URA3*
RHY1076	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP cod1-1 ubc7Δ::URA3*
RHY1077	*MATa hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP cod1-1 ubc7Δ::URA3*
RHY1127	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP cod1-1 hrd1Δ::URA3*
RHY1128	*MATa hmg2::HIS3::1mycHMG2 ura3-52:LEU2::HMG2::GFP cod1-1 hrd1Δ::URA3*
RHY1202	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG2::GFP cod1Δ::LEU2*
RHY1203	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::URA3::K6RHMG2::GFP cod1-1*
RHY1205	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::URA3::K6RHMG2::GFP*
RHY1473	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG1::GFP cod1-1*
RHY1475	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::URA3::HMG1::GFP*
RHY1882	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::URA3::6MYC HMG2::GFP*
RHY1883	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::URA3::6MYC HMG2::GFP cod1Δ::LEU2*
RHY2201	*MAT**a** ura3-52::URA3::HMG2::GFP*
RHY2202	*MATa pmr1Δ::LEU2 ura3-52::URA3::HMG2::GFP*
RHY2203	*MAT**a** spf1Δ::TRP1 ura3-52::URA3::HMG2::GFP*
RHY2204	*MATa spf1Δ::TRP1 pmr1Δ::LEU2 ura3-52::URA3::HMG2::GFP*

plasmid pRH379 (2μ, *URA3*) that expressed HA epitope-tagged ubiquitin from the GAPDH promoter. Ubiquitination of Hmg2p was assayed by immunoprecipitation of Hmg2p followed by immunoblotting with 12CA5 anti-HA antibody to detect covalently linked ubiquitin and with 9E10 anti-MYC antibody to detect immunoprecipitated 1myc-Hmg2p, as described previously (Gardner et al., 1998; Gardner and Hampton, 1999b).

Mutagenesis and COD Screen

EMS (methane sulfonic acid ethyl ester) mutagenesis was based on the protocol of Lawrence (1991). Mutagenized RHY541 cells (75% killing by EMS) were sequentially evaluated for four regulatory phenotypes. The mutagenized cells were plated onto minimal solid medium supplemented with 30 mg/liter adenine sulfate, 30 mg/liter methionine, and 12.5 μg/ml lovastatin. The wild-type strain is normally bright when plated on this medium due to lovastatin-induced stabilization of Hmg2p-GFP. After 3 d, colonies were screened visually for low fluorescence compared with surrounding colonies. Candidate *cod* mutants with low fluorescence were isolated, rescreened for the optical phenotype, and then assayed for growth sensitivity to 200 μg/ml lovastatin by replica plating. Patches of candidate mutants were grown on YPD plates, then replica plated onto a YPD master plate. The master plate was immediately replica plated onto minimal plates with 200 μg/ml lovastatin and then onto minimal plates without lovastatin. This second dilution plating step insured low, uniform density of test patches on the lovastatin plates. Candidates that were sensitive to 200 μg/ml lovastatin were isolated from the YPD master plate. Dark, lovastatin-sensitive candidates recovered from the no-drug master were next tested directly for ability to regulate Hmg2p-GFP levels using flow microfluorimetry as described above. Finally, candidates exhibiting poor regulation of Hmg2p-GFP levels were assayed for regulation of 1myc-Hmg2p using the cycloheximide chase assay.

Genetic Analysis

Cod^- candidates were crossed to the wild-type strain RHY542, to analyze segregation of the mutant phenotype. All mutants were recessive and belonged to a single complementation group.

The wild-type *COD1* was cloned by plasmid complementation of a *cod1-1* mutant with a *URA3*, ARS-CEN library (Rose et al., 1987). Ura^+ transformants were tested for restoration of Cod^+ phenotype using the screening assays described above.

Plasmids recovered from the revertants were retested by transformation into the mutant strain. Insert flanks were sequenced and the sequences were compared with the *Saccharomyces* Genome Database. Transformation of RHY811 (*cod1-1*) with plasmids pRH810 and pRH811 that contained only the *COD1* coding region rescued the Cod^- phenotype. Linkage of *SPF1* to *cod-1* was tested genetically by crossing a Leu^-*cod1-1* strain to a strain with *LEU2* (from pCS186) integrated in tandem with functional *COD1* allele at the *COD1* locus. The resulting diploid was sporulated to confirm anti-segregation of the Cod^- and Leu^+ phenotypes in the haploid progeny.

Growth Curves

Susceptibility of *cod1-1* to the mevalonate pathway inhibitors lovastatin, L659,699, zaragozic acid and Ro48-8071 was tested in minimal media. Dilute liquid cultures of RHY791 or RHY811 used to serially dilute the tested agent. The resulting cultures were incubated at 30°C and measured at various times for optical density at 600 nm.

Results

The COD Screen

We designed a screen to identify *cod* mutants that could not slow degradation of Hmg2p when degradation signals from the mevalonate pathway were low. Specifically, we isolated mutants that failed to stabilize Hmg2p upon treat-

ment with lovastatin, an inhibitor of HMG-CoA reductase that lowers these signals (Hampton and Rine, 1994; Gardner and Hampton, 1999a). We identified these *cod* mutants by scoring phenotypes resulting from the regulation of two distinct versions of Hmg2p: Hmg2p-GFP, a noncatalytic optical reporter of degradation, and 1myc-Hmg2p, an enzymatically active, epitope-tagged version of Hmg2p.

The optical reporter protein Hmg2p-GFP undergoes normal, regulated degradation that can be observed by examining cellular fluorescence by microscopy or flow microfluorimetry (Hampton et al., 1996b; Cronin and Hampton, 1999). Hmg2p-GFP was expressed from the constitutive glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter so that changes in the steady-state level of the protein and the resultant cellular fluorescence depended solely on changes in its degradation rate. Hmg2p-GFP expressed in wild-type cells is stabilized when the cells are treated with lovastatin, which lowers degradation signals from the mevalonate pathway by inhibiting HMGR, leading to increased cellular fluorescence (Cronin and Hampton, 1999; Gardner and Hampton, 1999a). A *cod* mutant unable to stabilize Hmg2p-GFP in response to lowered regulatory signals would fail to increase fluorescence in the presence of lovastatin. To score such mutants, we used our previously described GFP colony fluorescence assay (Cronin and Hampton, 1999). Wild-type colonies expressing Hmg2p-GFP fluoresced brightly when plated on media containing a small dose of lovastatin (12.5 μg/ml), whereas *cod* mutants remained dark even in the presence of lovastatin.

A second, independent phenotype of poor Hmg2p regulation was also scored. We have shown that strains expressing only a poorly stabilized cis mutant of Hmg2p are much more sensitive to lovastatin than otherwise isogenic strains expressing normally regulated Hmg2p (Hampton et al., 1996a; Gardner et al., 1998). Thus, constitutive degradation of normal Hmg2p caused by a trans *cod* mutation would similarly be expected to render the mutant hypersensitive to lovastatin, compared with an isogenic wild-type strain. The parent strain for the COD screen expressed the normally regulated 1myc-Hmg2p from a constitutive promoter as its only source of HMGR activity (Gardner et al., 1998). Misregulation of 1myc-Hmg2p by a *cod* mutant was scored as hypersensitivity to lovastatin.

We combined the optical and pharmacological assays described above using a strain coexpressing Hmg2p-GFP and 1myc-Hmg2p. A successful *cod* candidate would be dark when plated on a low dose of lovastatin and be hypersensitive to the toxic effects of higher doses of lovastatin. By screening for both phenotypes (see Materials and Methods), we were able to rule out cis mutants of either reporter, as well as trans mutants that affected processes other than regulation of Hmg2p degradation. For example, a mutant unable to stabilize Hmg2p-GFP upon lovastatin treatment due to impermeability to lovastatin would be dark, but would be resistant to lovastatin as opposed to hypersensitive, and so would fail as a *cod* candidate.

COD1 Was Required for the Regulated Degradation of Hmg2p

A total of >300,000 colonies were screened, from which we isolated *cod1-1* and 38 other members of the same complementation group. In all assays the *cod1-1* mutant was defective in regulating Hmg2p-GFP degradation. In wild-type cells, inhibition of the mevalonate pathway with lovastatin stabilized Hmg2p-GFP resulting in increased fluorescence which was seen by microscopy and flow microfluorimetry (Fig. 2, A and B). In contrast, addition of lovastatin hardly increased the fluorescence of *cod1-1* mutant cells even though the concentration (25 μg/ml) used in these experiments was >10 times that normally needed to cause a maximal stabilization of Hmg2p-GFP (Gardner et al., 1998; Cronin, S., unpublished observations). Unlike lovastatin, addition of the squalene synthase inhibitor zaragozic acid increases degradation of Hmg2p-GFP by increasing a signal derived from the mevalonate pathway product farnesyl pyrophosphate (Hampton and Bhakta, 1997; Gardner et al., 1998; Gardner and Hampton, 1999a). Hmg2p regulation in *cod1-1* mutants was also unresponsive to zaragozic acid when compared with wild-type cells (Fig. 2 B).

The *cod1-1* mutant was hypersensitive to lovastatin, indicating that the coexpressed 1myc-Hmg2p was also misregulated. *cod1-1* rendered cells 10 times more sensitive to lovastatin than the isogenic wild-type cells (Fig. 2 C). In contrast, the growth sensitivity of *cod1-1* mutants to inhibitors of other pathway enzymes including HMG-CoA synthase (L659,699), squalene synthase (zaragozic acid), or oxidosqualene-lanosterol cyclase (Ro48-8071) remained unchanged (Figs. 1 A and 2 C, and data not shown). These results suggested that *cod1-1* hypersensitivity to lovastatin was due to misregulation of Hmg2p rather than any general effects on pathway enzymes or other pleiotropic actions of the *cod1-1* mutation.

We directly tested the ability of the *cod1-1* mutant to regulate Hmg2p degradation with cycloheximide chase assays. In these experiments, protein synthesis was blocked at time zero by the addition of cycloheximide and degra-

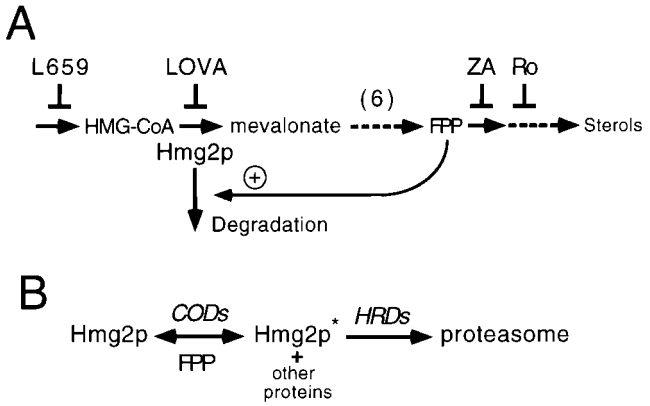

Figure 1. Regulation of Hmg2p degradation by the mevalonate pathway. (A) Increased production of FPP promotes Hmg2p degradation. Relevant pathway intermediates are shown. Drugs are shown above the enzymatic step that is inhibited (L659, L659,699; LOVA, lovastatin; ZA, zaragozic acid; Ro, Ro48-8071). Dotted arrows indicate multi-enzyme steps. (B) A model for genes that control of Hmg2p degradation. Entry of Hmg2p into the *HRD*-encoded ER degradation pathway is controlled by *COD* genes in response to an FPP-derived signal.

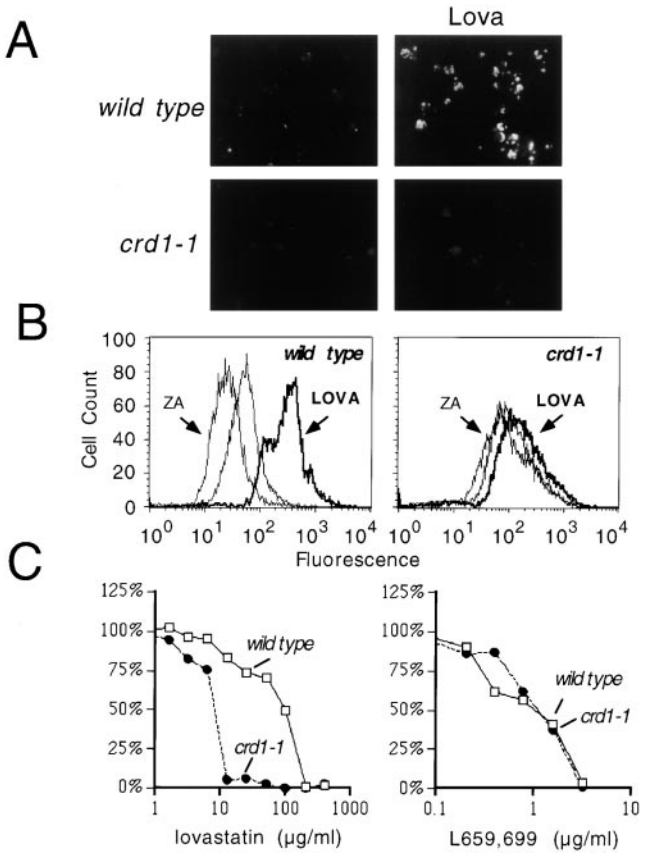

Figure 2. cod1-1 prevented regulation of Hmg2p-GFP level and caused lovastatin hypersensitivity. (A) Regulation of Hmg2p-GFP stability in wild-type or *cod1-1* cells. Early log phase cultures were allowed to grow for 4 h in the presence or absence of 25 μg/ml lovastatin (LOVA). Each panel shows equivalent numbers of cells, photographed with identical optical settings. (B) Regulation of Hmg2p-GFP level examined by flow microfluorimetry. Cultures of wild-type or *cod1-1* cells were grown for 4 h without drugs or in the presence of lovastatin or zaragozic acid (ZA) as indicated and then subjected to flow microfluorimetry. Each histogram represents 10,000 cells. (C) Growth arrest of wild-type and *cod1-1* cells by mevalonate pathway inhibitors. Low density ($OD_{600} < 0.01$) cultures were allowed to grow for 2.5 d in the indicated concentrations of lovastatin or L659,699. Final OD_{600} readings were taken for each concentration and plotted as percent of the appropriate untreated culture. Representative experiments are shown.

dation was allowed to proceed. 1myc-Hmg2p level was determined by immunoblotting at various times to assess degradation. Addition of lovastatin drastically slows the degradation of 1myc-Hmg2p in wild-type cells (Hampton and Rine, 1994; Fig. 3), whereas in *cod1-1* lovastatin had little or no effect.

COD1 Was Required for Regulation of Hmg2p Ubiquitination

Ubiquitination is required for Hmg2p degradation and is regulated in response to the same stimuli that control Hmg2p stability. For example, treatment with zaragozic acid increases Hmg2p ubiquitination and this effect is blocked by simultaneous treatment with inhibitors of

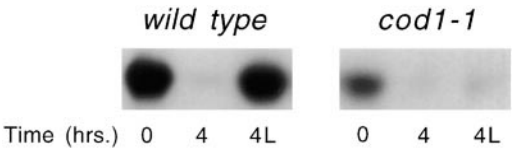

Figure 3. cod1-1 disrupted regulation of 1myc-Hmg2p degradation. Wild-type or *cod1-1* cells were examined for degradation of 1myc-Hmg2p by addition of cycloheximide at time 0, followed by lysis at the indicated time (0 or 4 hours), and anti-myc immunoblotting. Lovastatin (L) was added to the indicated samples at the same time as cycloheximide.

upstream pathway enzymes such as HMG-CoA synthase (Hampton and Bhakta, 1997; Gardner et al., 1998). We examined the regulation of Hmg2p ubiquitination in *cod1-1* mutants. Hmg2p ubiquitination was assayed by coimmunoprecipitation using strains expressing HA epitope-tagged ubiquitin (Gardner et al., 1998). Hmg2p was immunoprecipitated with polyclonal antibodies, then immunoblotted for HA immunoreactivity to evaluate ubiquitination or for MYC immunoreactivity to evaluate the total amount of Hmg2p precipitated. In wild-type cells ubiquitination of Hmg2p was regulated as previously reported: brief treatment with zaragozic acid increased ubiquitination of Hmg2p, and this effect of zaragozic acid was blocked by pretreatment with the HMG-CoA synthase inhibitor L659,699 (see Fig. 4; Hampton and Bhakta, 1997). In contrast, Hmg2p ubiquitination was uniformly higher in the *cod1-1* mutant, and was unaffected by the addition of either zaragozic acid or L659,699, consistent with the constitutive, unregulated degradation of the two Hmg2p reporters.

ER Degradation Mutants Stabilized Hmg2p in cod1-1

We have proposed a model in which the *COD* genes regulating Hmg2p degradation are distinct from the genes encoding the ER degradation machinery (Fig. 1). This model predicts that the unregulated degradation of Hmg2p in a *cod1-1* mutant would still be blocked in *hrd* mutants, which are deficient in Hmg2p degradation. To test this model, we constructed strains with the *cod1-1* mutation and null mutations in *HRD1* or *UBC7*, each are essential

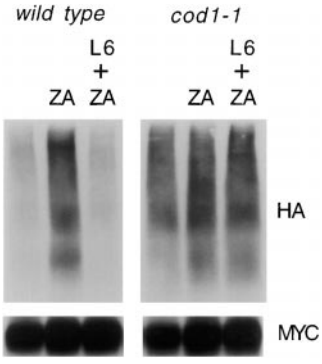

Figure 4. Ubiquitination of Hmg2p was unregulated in *cod1-1* cells. Wild-type or *cod1-1* cells expressing HA-tagged ubiquitin were grown in the presence or absence of the indicated drugs. L659,699 (L6) was added 30 min before lysis and zaragozic acid (ZA) was added 10 min before lysis. The cultures were lysed, Hmg2p was immunoprecipitated with antibodies against Hmg2p and then immunoblotted following SDS-PAGE. Ubiquitinated Hmg2p was detected by anti-HA immunoblotting, and total Hmg2p was detected by anti-myc immunoblotting.

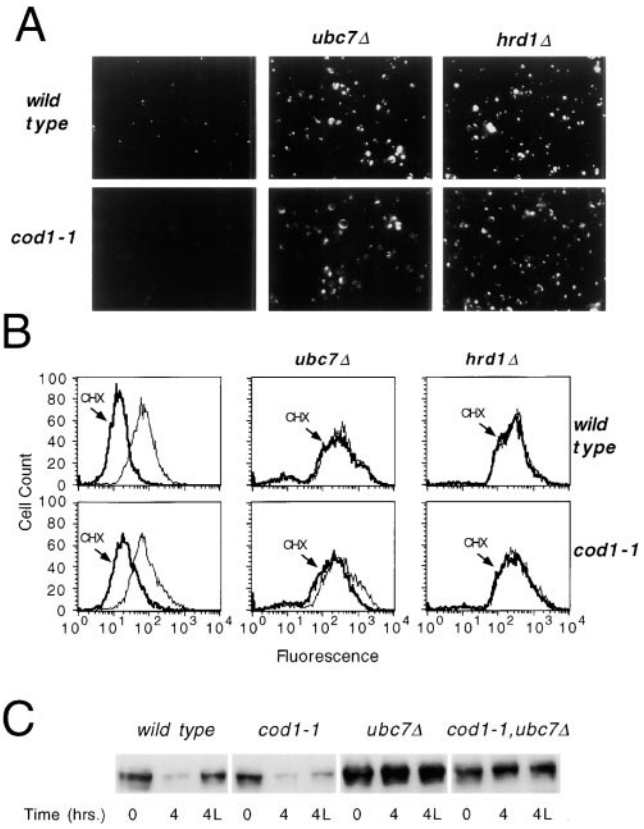

Figure 5. Hmg2p in *cod1-1* was stabilized by ER degradation mutants. (A) Hmg2p-GFP fluorescence of wild-type or mutant cells was examined by fluorescence microscopy. Cells in the top row of panels are wild-type for *COD1* and cells in the bottom row have the *cod1-1* mutation. Cells in the middle and right columns have *ubc7Δ* or *hrd1Δ* null alleles, respectively. Equivalent numbers of cells were photographed in each panel using identical settings. (B) Degradation of Hmg2p-GFP was examined by flow microfluorimetry in wild-type or *cod1-1* cells with or without *ubc7Δ* or *hrd1Δ* as labeled. Cycloheximide (CHX) was added to the indicated cultures 4 h before analysis. (C) 1myc-Hmg2p degradation in wild-type, *cod1-1*, *ubc7Δ*, and *cod1-1/ubc7Δ* cells was assayed by cycloheximide chase. After addition of cycloheximide samples were lysed at 0 or 4 h followed by SDS-PAGE and immunoblotting of the lysates. Lovastatin (L) was added to the indicated samples at the same time as cycloheximide.

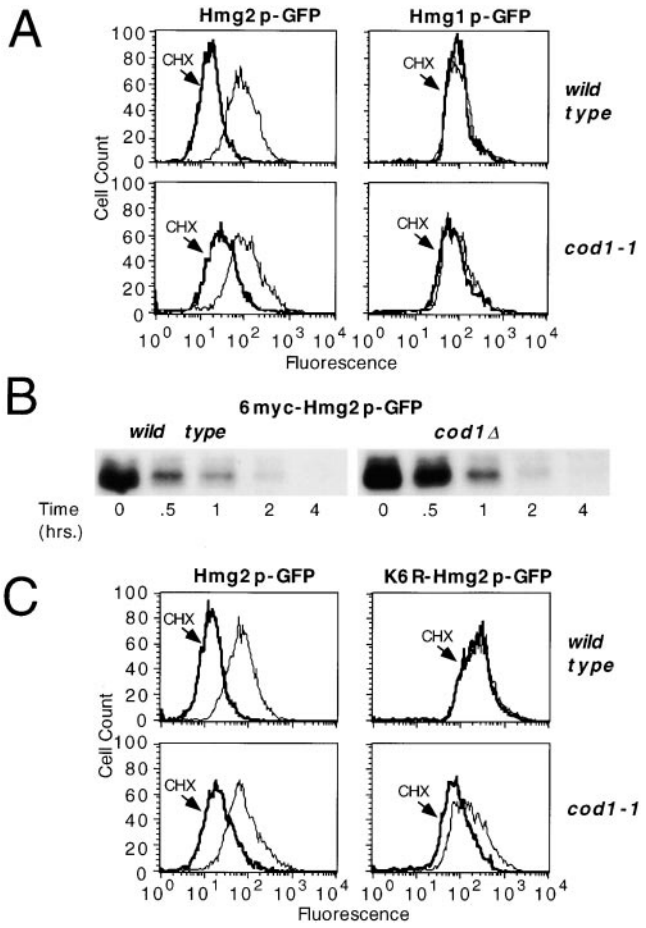

Figure 6. The stability of other ER membrane proteins in Cod$^-$ cells. (A) Wild-type and *cod1-1* cells expressing either Hmg2p-GFP or Hmg1p-GFP were assayed by flow microfluorimetry for loss of fluorescence after 4-h incubation in the presence or absence of cycloheximide (CHX). (B) The stability of 6myc-Hmg2p-GFP in wild-type and *cod1Δ* cells was assayed by cycloheximide chase. After addition of cycloheximide samples were lysed at various times followed by SDS-PAGE and immunoblotting of the lysates. (C) The K6R mutation enhances Hmg2p-GFP stability in *cod1-1*. Wild-type and *cod1-1* cells expressing either Hmg2p-GFP or the K6R variant of Hmg2p-GFP were assayed by flow microfluorimetry for loss of fluorescence after 4-h incubation in the presence or absence of cycloheximide (CHX).

for Hmg2p degradation (Hampton and Bhakta, 1997; Hampton et al., 1996a). Deletion of either *UBC7* or *HRD1* greatly increased Hmg2p-GFP fluorescence in both wild-type and *cod1-1* mutants as measured by microscopy (Fig. 5 A) or flow microfluorimetry (Fig. 5 B). The *hrd1Δ* and *ubc7Δ* null mutations completely blocked the constitutive degradation caused by the *cod1-1* mutation, such that fluorescence histograms of the double mutants were superimposable with the histograms of the *hrd1Δ* and *ubc7Δ* single mutants (Fig. 5 B). The stability of Hmg2p-GFP in each strain was directly tested by addition of cycloheximide followed by flow microfluorimetry to evaluate loss of cellular fluorescence due to Hmg2p-GFP degradation. This loss of fluorescence was completely inhibited by the presence of *ubc7Δ* (middle panels) or *hrd1Δ* (right panels). *ubc7Δ* (Fig. 5 C) or *hrd1Δ* (data not shown) also blocked degradation of 1myc-Hmg2p.

Degradation of Other ER Proteins in cod1-1

The *cod1-1* mutation removed regulation of Hmg2p, rendering its degradation constitutive. We wanted to determine if *cod1-1* mutation generally altered the stability of ER proteins. Accordingly, we examined the effect of the *cod1-1* mutation on the stable, ER localized Hmg1p-GFP reporter protein derived from the HMGR isozyme Hmg1p (Hampton and Rine, 1994; Hampton et al., 1996b; Gardner et al., 1998). In both wild-type and *cod1-1* strains Hmg1p-GFP remained stable during the 4 h of an optical cycloheximide chase (Fig. 6 A). Hmg1p-GFP was stable in the *cod1-1* mutant under all conditions tested for Hmg2p-GFP indicating that the *cod1-1* mutation does not promote degradation of this normally stable ER protein.

We also tested the effect of *COD1* mutation on the stability of a short-lived, unregulated variant of Hmg2p, 6myc-Hmg2p-GFP (Hampton et al., 1996a), by cycloheximide chase (Fig. 6 B). In both wild-type and *cod1Δ* cells, 6myc-Hmg2p-GFP was degraded rapidly with an estimated half-life of less than an hour, though it appeared to be slightly more abundant in the Cod$^-$ cells. In similar experiments, we found that the degradation rate of the regulated 1myc-Hmg2p did not differ substantially between wild-type and Cod$^-$ cells in the absence of mevalonate pathway inhibitors (data not shown). Clearly, *cod1* mutation did not generally affect the stability of ER proteins. Rather, it specifically affected the feedback regulation of Hmg2p degradation by signals from the mevalonate pathway.

Recognition of cis Determinants for Hmg2p-regulated Degradation in cod1-1

Recently, we have shown that the regulated degradation of Hmg2p is critically dependent on two lysines in the transmembrane region of the protein. Replacement of either lysine 6 or lysine 357 of Hmg2p with arginine (or any other amino acid) strongly stabilizes Hmg2p or Hmg2p-GFP (Gardner and Hampton 1999b). Furthermore, the function of these lysines is extremely sensitive to alterations in Hmg2p structure. Since these lysines play a critical role in regulation of stability, we wondered if they would still function in a *cod1-1* background. In both *cod1-1* and wild-type genetic backgrounds, the K6R mutant of Hmg2p-GFP was more stable than wild-type Hmg2p, though some degradation is apparent in the *cod1-1* mutant (Fig. 6 C). The same result was seen with the K357R replacement, and with the double mutant (data not shown). These data indicated that the degradation of Hmg2p in a *cod1-1* mutant was still largely dependent on the same cis determinants that are necessary for regulating Hmg2p degradation in a wild-type cell. However, the incomplete stability of the K6R mutant in *cod1-1* suggested that Cod1p has a subtle effect on the structure of Hmg2p or the recognition of its distributed degron (Gardner and Hampton, 1999b).

COD1 Encoded a P-Type ATPase

The wild-type *COD1* gene was isolated by plasmid library complementation of the *cod1-1* mutation and was shown by linkage analysis to be YEL031w, previously isolated as *SPF1* (sensitivity to *Pichia farinosa*). We deleted *COD1* in a haploid strain and the null mutant was viable. In all assays for regulation of Hmg2p degradation, the *cod1Δ* mutant behaved exactly as the *cod1-1* mutant (Fig. 7, data not shown). Additionally, overexpression of *COD1* from a 2μ plasmid failed to produce any observable change in regulation of Hmg2p levels.

COD1 belongs to a large family of genes encoding P-type ATPases that actively transport various ions across membranes (Catty et al., 1997; Axelsen and Palmgren, 1998). The yeast genome encodes 16 members of this family with the uncharacterized open reading frame YOR291w being most similar to Cod1p (26% identity, 44% similarity across 1,124 amino acids of homology). Deletion of YOR291w failed to produce any obvious phenotypes or affect regulation of Hmg2p degradation. Double mutants carrying deletions of both YOR291w and *COD1* were viable and retained the Cod$^-$ phenotype without any enhancement or suppression (data not shown). Evidently, despite the high homology between the two proteins, only Cod1p functioned in Hmg2p regulation.

The more distantly related P-type ATPase Pmr1p (22% identity, 39% similarity across 733 amino acids of homology) has recently been implicated in the degradation of the misfolded ER lumenal protein CPY*. Deletion of *PMR1* prevents the degradation of CPY* by the ubiquitin proteasome pathway (Duerr et al., 1998). We examined the effect of *pmr1Δ* on Hmg2p-GFP degradation and found that Hmg2p-GFP stability was identical in an isogenic series including wild-type, *pmr1Δ*, *cod1Δ*, and *pmr1Δ*/*cod1Δ* strains (Fig. 7 A).

We tested the effects of *pmr1Δ* on Hmg2p-GFP regulation. In the wild-type H2071 genetic background used in these studies, Hmg2p-GFP degradation was relatively slow, but could be hastened by the addition of zaragozic acid (Fig. 7 B). While the response of Hmg2p-GFP to zaragozic acid was severely blunted in *cod1Δ*, Hmg2p-

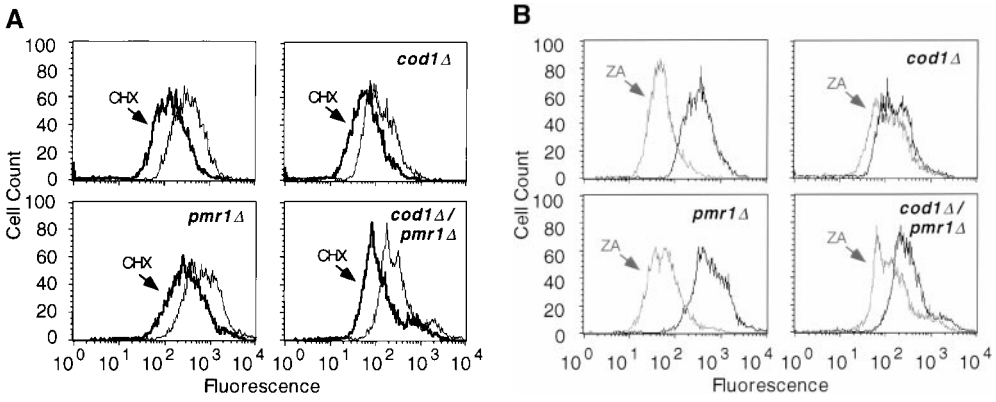

Figure 7. Deletion of *PMR1* did not affect the regulated degradation of Hmg2p-GFP. (A) Stability of Hmg2p-GFP in wild-type, *cod1Δ*, *pmr1Δ*, and *cod1Δ*/*pmr1Δ* cells. Early log phase cultures were grown for 4 h in the presence or absence of cycloheximide (CHX) and subjected to flow microfluorimetry. (B) Regulation of Hmg2p-GFP degradation in wild-type, *cod1Δ*, *pmr1Δ*, and *cod1Δ*/*pmr1Δ* cells. Early log phase cultures were grown for 4 h in the presence or absence of zaragozic acid (ZA) and subjected to flow microfluorimetry.

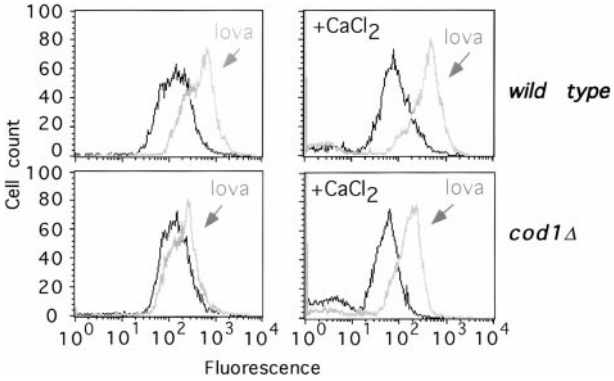

Figure 8. $CaCl_2$ restored control of degradation to *cod1-1*. Wild-type or *cod1Δ* cells were grown overnight in the presence or absence of 200 mM $CaCl_2$. Lovastatin (lova) was added to the indicated cultures 4 h before analysis by flow microfluorimetry.

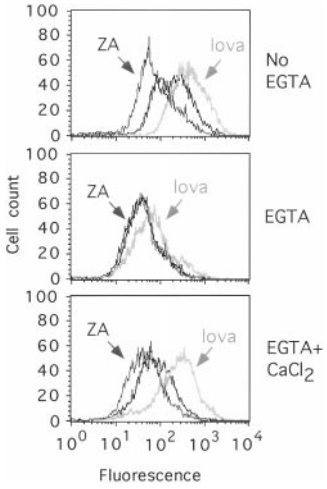

Figure 9. EGTA blunted regulation of Hmg2p-GFP levels in a Ca^{2+}-dependent manner. Cells expressing Hmg2p-GFP were assayed for regulation of fluorescence after overnight growth in the presence or absence 780 μM EGTA. Lovastatin (lova), zaragozic acid (ZA), and/or $CaCl_2$ were added 4 h before analysis.

GFP degradation was regulated normally in *pmr1Δ*. Interestingly, the defective regulation of Hmg2p seen in *cod1Δ* was partially *suppressed* by simultaneous deletion of *PMR1* suggesting that Pmr1p and Cod1p may both play a role in Ca^{2+} homeostasis, though in distinctly different ways.

Manipulating Ca^{2+} Affected Regulation of Hmg2p Stability

Phenotypic defects in some yeast P-type ATPase mutants can be overcome or exacerbated by manipulating the concentration of ions in the growth media (Duerr et al., 1998; Suzuki and Shimma, 1999). We examined the effect of such manipulations on the *cod1-1* mutant and wild-type strains. Incubation of a *cod1* deletion mutant in 200 mM $CaCl_2$ partially restored regulation of Hmg2p-GFP (Fig. 8). No other ions similarly tested ($MnCl_2$, $CaCl_2$, KCl, or NaCl) restored regulation of Hmg2p-GFP degradation. The high concentration of $CaCl_2$ also caused a drop in the pH of the growth media, but lowering the pH of the media with HCl instead of $CaCl_2$ failed to produce any effect (data not shown).

We also tested the effect of Ca^{2+} depletion on regulation of Hmg2p stability in wild-type cells by treatment with EGTA, a chelator of divalent ions with high Ca^{2+} specificity (Fig. 9). Overnight treatment with a sub-lethal concentration of EGTA blunted the regulatory responses to both lovastatin and zaragozic acid when compared with untreated cells. These effects of EGTA treatment were overcome by addition of $CaCl_2$, but not $MgCl_2$. These experiments with EGTA were consistent with a role for $CaCl_2$ in the regulation of Hmg2p degradation. However, EGTA treatment of wild-type cultures did not fully mimic the Cod^- phenotype and significantly reduced growth (data not shown) indicating that the effects of *COD1* were more specific for Hmg2p degradation than those caused by gross Ca^{2+} depletion.

Discussion

The striking cis and trans specificity of Hmg2p stability regulation led us to posit that this process involves a separate set of genes referred to as *COD* genes. In this work, we have isolated the first member of this class of genes. We focused our search on *cod* mutants that always degrade Hmg2p even when signals for degradation are low. By our model (Fig. 1), Hmg2p degradation in such a *cod* mutant would still be halted by mutations in genes encoding the degradation machinery, such as a *hrd1* mutant.

The resulting mutant, *cod1-1*, had the desired phenotype: Hmg2p and Hmg2p-GFP each undergo constitutive degradation that is largely refractory to regulatory signals. Despite the lack of regulation, Hmg2p in the *cod1* mutant was degraded at roughly the same rate as in the wild-type under normal growth conditions. Importantly, the constitutively degraded Hmg2p in a *cod1* mutant was strongly stabilized by the simultaneous presence of a *hrd1* or *ubc7* mutant, showing that indeed regulation can be uncoupled from degradation. The *cod1* mutant did not globally alter the stability of ER proteins, since *cod1* mutation neither destabilized the normally stable Hmg1p-GFP nor altered the degradation rate of the misfolded, constitutively degraded 6myc-Hmg2p-GFP.

The degradation of Hmg2p can be slowed with drugs that block early in the mevalonate pathway or hastened by inhibition of squalene synthase with zaragozic acid (Hampton and Rine, 1994; Hampton and Bhakta, 1997; Gardner and Hampton, 1999a). Both actions arise from alteration of the mevalonate-derived molecule farnesyl pyrophosphate (FPP; Gardner and Hampton, 1999a). The *cod1* mutants showed strongly blunted responses to both lovastatin and zaragozic acid. Thus, *COD1* is required for coupling the rate of Hmg2p degradation to levels of the FPP-derived signal.

Cod1p is a P-type ATPase. Members of this widely conserved family function in ATP-dependent pumping of ions across biological membranes. The ion specificity of a given P-type ATPase can not yet be determined from sequence information alone. However, our studies indicate that Cod1p may be a Ca^{2+} transporter. The *cod1* phenotype is reversed by addition of Ca^{2+} to the growth medium of mutant cells, and no other divalent ions tested could do this. Furthermore, treatment of wild-type cells with the Ca^{2+}-preferring chelator EGTA caused aberrant regulation that was similar to the $Cod1^-$ phenotype, and specifically reversed by calcium.

This connection between calcium and HMGR regulation is especially intriguing given that regulated degradation of HMGR in mammals is similarly sensitive to perturbations of cellular calcium (Roitelman et al., 1991; Roitelman and Simoni, 1992). In mammalian cells Ca^{2+} deprivation specifically inhibits the action of the degradation signal derived from FPP (Roitelman and Simoni, 1992; Meigs et al., 1996). Similarly, *cod1-1* mutants cannot respond to the FPP-derived signal for Hmg2p degradation.

Cod1p appeared to have a fairly specific function. The *COD1* gene is not essential and the viable *cod1Δ* null mutant has a phenotype identical to that of the *cod1-1* allele. A null mutation in *COD1*'s closest paralogue, YOR291w, had no observable effect on yeast growth or Hmg2p regulation, alone or in combination with the *cod1Δ* null. Our ongoing studies have localized the Cod1p protein to the ER (Cronin, S.R., and R.Y. Hampton, manuscript in preparation), and our current model is that Cod1p is a Ca^{2+} transporter that is important for establishing a lumenal environment appropriate for control of Hmg2p stability. Changes to the ER environment in a *cod1* mutant might alter Hmg2p stability by affecting the presentation of the highly specific structural determinants needed for regulated degradation (Gardner and Hampton, 1999b). Alternatively, Cod1p activity may be critical for the function of trans factors regulating Hmg2p degradation.

A model in which Cod1p functions in the ER might explain some of the other phenotypes reported for *cod1* mutants. *COD1* was previously identified as *SPF1* in an apparently unrelated screen for mutants resistant to a killer toxin. Other phenotypes reported for *spf1Δ* null mutants include defective glycosylation of invertase, resistance to vanadate, and sensitivity to hygromycin and calcofluor white (Suzuki and Shimma, 1999). The sensitivity to hygromycin and calcofluor white, indicating defective cell wall synthesis, and the defective glycosylation of invertase all support a role for Cod1p in maintaining the lumenal environment of the ER, an environment thought to be controlled principally by *PMR1*.

The Golgi-localized P-type ATPase Pmr1p is thought to play a major role in maintaining Ca^{2+} levels in the secretory pathway (Strayle et al., 1999). *PMR1* is required for numerous ER functions and has recently been identified as *DER5*, a gene necessary for the degradation of the misfolded lumenal protein CPY* (Duerr et al., 1998). In contrast to the effect of *pmr1Δ* on CPY* degradation, *pmr1Δ* had no effect on Hmg2p degradation or its feedback regulation. Thus, Pmr1p and Cod1p appear to have distinct roles, at least in this ER function. There are metazoan P-type ATPase family members of unknown function that have higher similarity to *COD1* than to *PMR1*. It is tempting to speculate that they may have similar, specialized functions in a variety of organisms.

We currently do not know the mechanism of regulated stability, and one possibility is that there are proteins that specifically protect Hmg2p from degradation when degradation signals are lowered. If such protection factors exist, they would be particularly important both in the basic understanding of regulated ER degradation, and as possible targets for cholesterol lowering drugs. Loss of a protection factor by mutation would cause signal-independent, constitutive degradation of Hmg2p, and so would score as a *cod* candidate, like *cod1-1*. However, >300,000 mutagenized colonies of the parent strain were screened yet only alleles (39) of *COD1* were recovered. Thus, it may be that the mechanism of Hmg2p regulation does not involve protection factors. Alternatively, it is possible that this version of the COD screen was biased towards recovery of *COD1* alleles.

In summary, the above work demonstrates that the genetic approach to understanding regulated degradation of HMGR is a viable one. Integration of the *COD1* gene's function into the scenario of Hmg2p regulation and ER function will be an important aspect of completing the picture of HMGR regulated degradation, in yeast and most likely in other eukaryotes as well.

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