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), 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; Meijs et al., 1996; Lopez et al., 1997; Meijs 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 (Wihovsky 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 HMGRCoA 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-
tants would allow degradation of Hmg2p in the presence of drugs such as lovastatin, an inhibitor of HMG-R, that normally slow degradation by decreasing production of the signal. By our model (see Fig. 1), H mg2p 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 E R 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 controls the effect of Hmg2p degradation through acting on Ca sup 2- levels in the E R.

Materials and Methods

Materials

Restriction enzymes, V ent DNA polymerase and T 4 DNA ligase were obtained from New England Biolabs. Lovastatin, L 659,699 and zaragic acid were generously provided by Dr. James Bergerstrom (Merrck, Rahway, N J). R o48-8071 was a gift from Dr. Olivier Morand (F. Hoffman-LaRoche). The 9E10 cell culture supernatant was produced in our lab from cells (CRL 1729; Amercan Type Culture Collection) grown in RPM 1640 culture medium (GIBCO BRL) with 10% fetal calf serum and supplements. 12CA S anti-HA antibody was obtained from Dr. Don R o (U C Berkeley, Berkeley, C A). A 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 pR H 468 (integrating) expressed 1myc-Hmg2p from a GAPDH promoter. pR H 468 was constructed by removing the Ncol-A attl segment containing part of the URA3 open reading frame from pR H 423 (integrating, URA3; Hampton and Bhakta, 1997). The H mg2p-GFP reporter protein was expressed from plasmid pR H 680 (integrating, E U2) or pR H 469 (integrating, URA3; Gardner et al., 1998). pH 680 was constructed from pR H 469 by replacing the A attl-A pal segment of the URA3 gene in pR H 469 with the A attl-A pal fragment from pS512 containing the LEU2 gene (Sikorski and Hieter, 1989). Hmg1p-GFP was expressed from pH 475 (integrating, URA3, HMG1::GFP). pR H 550 was constructed by integrative transformation of pH 532 with pH 475 (integrating, URA3, H MG1::GFP). pR H 1205 was constructed by replacing the formation of pH 791 with the BamH1-Heml fragment from pCS186. A corresponding Cod1p strain (R H 1232), was constructed by integrative transformation of pH 791 with linearized pCS186 to produce a strain with the cod1-1 LEU2 disruption allele in tandem with functional COD1. R H 2201, R H 2202, R H 2203, and R H 2204 were constructed by transforming pH 469 into CS601A, CS601B, CS601C, and CS601D, respectively (Suzuki and Shimma, 1999).

The COD1 paralogue Y OR 291w was deleted by transformation of the haploid strain R H 791 with a KanMX disruption cassette with 40bp flanks homologous to the Y OR 291w 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 Carousal® 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 reduce blue light. A nalysis of H mg2p-GFP fluorescence by flow microfluorimetry was performed on a Becton Dickinson FACS calibur® flow microfluorimeter and Cell Quest software. Strains were typically grown into early log phase in minimal media. A tet 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 CaCl2 or other salt ~12 h before the addition of mevalonate pathway inhibitors. In 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, MgCl2 or CaCl2 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
plasmid pRH 379 (2µ, URA 3) that expressed HA epitope-tagged ubiquitin from the GAPDH1 promoter. Ubiquitination of Hmg2p was assayed by immunoprecipitation of Hmg2p followed by immunoblotting with 12CA 5 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).

**Table I. Yeast Strains**

| Strain | Genotype |
|--------|----------|
| RHY468 | MATa hmg2::HIS3 ura3-52 LEU2 |
| RHY541 | MATa 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::LEU2 |
| RHY658 | MATa 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 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP |
| RHY792 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP |
| RHY811 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1 |
| RHY812 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1 |
| RHY871 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP |
| RHY872 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP |
| RHY880 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP hrd1::URA3 |
| RHY910 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1 |
| RHY911 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1 |
| RHY1056 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1 ubc7::URA3 |
| RHY1076 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1 ubc7::URA3 |
| RHY1077 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1 ubc7::URA3 |
| RHY1127 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1 hrd1::URA3 |
| RHY1128 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1 hrd1::URA3 |
| RHY1202 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG2::GFP cod1-1 LEU2 |
| RHY1203 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::K6RHMG2::GFP cod1-1 |
| RHY1205 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::K6RHMG2::GFP |
| RHY1473 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::6MYC HMG2::GFP cod1-1 |
| RHY1475 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::6MYC HMG2::GFP |
| RHY1882 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG1::GFP |
| RHY1883 | MATa hmg2::HIS3 1mycHMG2 ura3-52::URA3::HMG1::GFP |
| RHY2201 | MATa ura3-52::URA3::HMG2::GFP |
| RHY2202 | MATa pmr1::LEU2 ura3-52::URA3::HMG2::GFP |
| RHY2203 | MATa spf1::TRP1 ura3-52::URA3::HMG2::GFP |
| RHY2204 | MATa spf1::TRP1 pmr1::LEU2 ura3-52::URA3::HMG2::GFP |

**Genetic Analysis**

Cod1-1 candidates were crossed to the wild-type strain RHY 542, to analyze segregation of the mutant phenotype. All mutants were recessive and belonged to a single complementation group.

The wild-type CO D1 was cloned by plasmid complementation of a cod-1 mutant with a URA3 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 RHY 811 (cod1-1) with plasmids pRH 810 and pRH 811 that contained only the COD1 coding region rescued the Cod+ phenotype. Linkage of SPF1 to cod1 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. D-lactate liquid cultures of RHY 791 or RHY 811 were 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 HMG Co-A reductase, 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-
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 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
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 HMG1 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 CodΔ cells. In similar experiments, we found that the degradation rate of the regulated 1myc-Hmg2p did not differ substantially between wild-type and CodΔ cells in the absence of mevalonate pathway inhibitors (data not shown). Clearly, cod1Δ 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 CodΔ 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 luminal 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-

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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.
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 Ca2+ homeostasis, though in distinctly different ways.

**Manipulating Ca2+ 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 mutant and wild-type strains. Incubation of a cod1 deletion mutant in 200 mM CaCl2 partially restored regulation of Hmg2p-GFP (Fig. 8). No other ions similarly tested (MnCl2, CaCl2, KCl, or NaCl) restored regulation of Hmg2p-GFP degradation. The high concentration of CaCl2 also caused a drop in the pH of the growth media, but lowering the pH of the media with HCl instead of CaCl2 failed to produce any effect (data not shown).

We also tested the effect of Ca2+ depletion on regulation of Hmg2p stability in wild-type cells by treatment with EGTA, a chelator of divalent ions with high Ca2+ 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 CaCl2, but not MgCl2. These experiments with EGTA were consistent with a role for CaCl2 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 Ca2+ 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 Ca2+-transporter. The Cod1 phenotype is reversed by addition of Ca2+ to the growth medium of mutant cells, and no other divalent ions tested could do this. Furthermore, treatment of wild-type cells with the Ca2+-preferring chelator EGTA caused aberrant regulation that was similar to the Cod1- phenotype, and specifically reversed by calcium.
This connection between calcium and HMG R regulation is especially intriguing given that regulated degradation of HMG R 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; Miggs et al., 1996). Similarly, cod1-1 mutants cannot respond to the FPP-derived signal for H mg2p 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 parologue, Y OR 291w, had no observable effect on yeast growth or H mg2p 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 H mg2p stability. Changes to the ER environment in a cod1 mutant might alter H mg2p 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 H mg2p 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 luminal protein CPY* (Duer et al., 1998). In contrast to the effect of pmr1Δ on CPY* degradation, pmr1Δ had no effect on H mg2p 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 H mg2p 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 H mg2p, and so would score as a cod candidate, like cod1-1. However, >300,000 mutantized colonies of the parent strain were screened yet only alleles (39) of COD1 were recovered. Thus, it may be that the mechanism of H mg2p regulation does not involve protection factors. A tentatively, 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 HMG R is a viable one. Integration of the COD1 gene’s function into the scenario of H mg2p regulation and ER function will be an important aspect of completing the picture of HMG R regulated degradation, in yeast and most likely in other eukaryotes as well.

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