Regulated Degradation of HMG-CoA Reductase, an Integral Membrane Protein of the Endoplasmic Reticulum, in Yeast

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Abstract. Numerous integral membrane proteins are degraded in the mammalian ER. HMG-CoA reductase (HMG-R), a key enzyme in the mevalonate pathway by which isoprenoids and sterols are synthesized, is one substrate of ER degradation. The degradation of HMG-R is modulated by feedback signals from the mevalonate pathway. We investigated the role of regulated degradation of the two isozymes of HMG-R, Hmglp and Hmg2p, in the physiology of Saccharomyces cerevisiae. Hmglp was quite stable, whereas Hmg2p was rapidly degraded. Degradation of Hmg2p proceeded independently of vacuolar proteases or secretory traffic, indicating that Hmg2p degradation occurred at the ER. Hmg2p stability was strongly affected by modulation of the mevalonate pathway through pharmacological or genetic means. Decreased mevalonate pathway flux resulted in decreased degradation of Hmg2p. One signal for degradation of Hmg2p was a nonsterol, mevalonate-derived molecule produced before the synthesis of squalene. Genetic evidence indicated that a farnesylated protein may also be necessary for Hmg2p degradation. Studies with reporter genes demonstrated that the stability of each isozyme was determined by its noncatalytic NH2-terminal domain. Our data show that ER protein degradation is widely conserved among eukaryotes, and that feedback control of HMG-R degradation is an ancient paradigm of regulation.

In addition to its role in secretion, the ER is now recognized as an organelle in which proteins are degraded (Bienkowski, 1983; Chun et al., 1990; Klausner and Sitia, 1990; Bonifacino and Lippincott, 1991; Meigs and Simoni, 1992; Tsuji et al., 1992; Wikstrom and Lodish, 1992). The hallmark of ER protein degradation is its independence of both lysosomal enzymes and exit from the ER. Both soluble and integral membrane proteins are degraded in the ER. The physiological functions of ER degradation are poorly understood, but may include "metabolic proofreading" of misfolded and mutant proteins. However, a sizeable portion of correctly made protein is also constitutively degraded by this route (Bienkowski, 1983). It is currently not known whether ER degradation of the various substrates occurs by a single mechanism or by multiple mechanisms.

One clear function of ER protein degradation in mammalian cells has been revealed in the study of the ER-resident, integral membrane protein 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) \textsuperscript{3} reductase (HMG-R), which appears to be a substrate of this degradation pathway (Chun et al., 1990; Meigs and Simoni, 1992). HMG-R is the rate-limiting enzyme in the biosynthesis of sterols and other isoprenoids of the mevalonate pathway (Fig. 1). The steady state levels of HMG-R are tightly regulated by the cell through coordinate modulation of synthesis and degradation rates of HMG-R, in order to balance production of isoprenoids with cellular needs (Nakanishi et al., 1988; Goldstein and Brown, 1990). Thus, when flux through the mevalonate pathway is high, the degradation of HMG-R is fast. Conversely, when flux through the mevalonate pathway is low, as when a person is treated with the HMG-R inhibitor lovastatin, the degradation rate is slowed. In this way the half-life of HMG-R in cultured cells can vary between 40 min and >10 h (Edwards et al., 1983a,b). The molecular signals that affect degradation of HMG-R are not known. It appears that both early pathway products and sterols can modulate protein degradation by acting together or independently (Panini et al., 1992; Roitelman and Simoni, 1992).

Mammalian HMG-R has two distinct structural domains: a COOH-terminal catalytic region connected by a linker to an NH\textsubscript{2}-terminal region that anchors the enzyme to the ER membrane by virtue of its multiple membrane-spanning domains (Liscum et al., 1985; Luskey and Stevens, 1985). The NH\textsubscript{2}-terminal region is not required for catalysis, but is required for regulated ER degradation of the native enzyme (Nakanishi et al., 1988), or of fusion proteins bearing this region (Chun et al., 1990). How the NH\textsubscript{2}-terminal region

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1. Abbreviations used in this paper: CHX, cycloheximide; CPY, carboxypeptidase Y; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMG-R, HMG-CoA reductase; TPCK, tosyl-phenylalanine chloromethyl ketone; YM, yeast minimal medium.
The mevalonate pathway. Only the enzymatic steps directly relevant to this work are depicted in the figure. The name of the enzyme responsible for a reaction is shown to the left of the arrow. The genetic or pharmacological block used to inhibit that step is shown in italics to the right of the arrow. Dotted arrows represent multi-enzyme steps, with the number of reactions indicated in parentheses to the left of such arrows. Adapted from Goldstein and Brown (1978).

**Materials and Methods**

**Materials and Reagents**

PMSE, leupeptin, peptatin A, TPCK, cycloheximide, cysteine HCl, methionine, Tween 20, mevalonic acid lactone, and aprotinin were from Sigma Chemical (St. Louis, MO). [3H]methylamine- and [35S]cysteine-containing Trans-3-Sulfonium [TM] was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). ECL™ chemiluminescence immunodetection reagents, [1-14C]sodium acetate, and [125I]-protein A were from Amersham Corp. (Arlington Heights, IL). Lovastatin and zaragozic acid were generously provided by A. Alberts and J. Bergstrom (Merck and Co., Rahway, NJ). Lovastatin was prepared for use by hydrolysis of an 85 mg/ml solution in EtOH with 0.2 M NaOH at 65°C for 40 min, followed by addition of 1 M Tris HCl pH 8.0, and adjustment of pH to yield a final stock solution of 25 mg/ml lovastatin, 20 mM Tris, HCl pH 8.0, in 25% EtOH. Zaragozic acid (also called squalestatin 1 [Baxter et al., 1992]) was directly dissolved in DMSO to a concentration of 12.5 mg/ml. Both drug stocks were stored at -20°C. Horseradish peroxidase-conjugated donkey anti-rabbit IgG and ECL immunodetection reagents were from Amersham Corp. Rabbit antiserum to a B-galactosidase-Hmglp catalytic domain fusion protein was prepared and affinity purified with the fusion antigen as previously described (Wright et al., 1988). This antiserum immunoprecipitated both isoforms of yeast HMG-R. Rabbit anti-carboxypeptidase Y (CPY) antiserum was provided by P. Herman and by R. Schekman (University of California, Berkeley, CA). Affinity-purified anti-invertase antiserum was provided by R. Schekman. Rabbit anti-Hmglp-specific antibodies, generated by immunizing rabbits with a synthetic COOH-terminal 15-amino acid peptide, were produced and provided by R. Wright (University of Washington, Seattle, WA).

**Strains and Media**

Yeast strains used herein are summarized in Table I. Strain JRY1527 (HMG1 HMG2) (Wright et al., 1988) was the parent for all strains in these experiments. Strain JRY1159 (hmgI::LYS2 HMG2) was derived from JRY527 by one step gene replacement of the HMG1 gene with a hmgI::LYS2 disruption fragment, and JRY1160 was similarly derived by disruption of the HMG2 gene with an hmg2::HIS3 fragment (Basson et al., 1986). Strain JRY1256, which expresses only HMG2, was made by transforming strain JRY1159 with a 2-u, URA3 plasmid containing a 4.9-kb yeast genomic fragment carrying the HMG2 gene (tJR350 [Wright et al., 1988]). Strain JRY1596 (hmgI::LYS2 HMG2) with only a single genomic copy of HMG2, strain JRY1593 (hmgI::HIS3), with only a single genomic copy of HMG1, and strain JRY193 (hmgI::LYS2 hmg2::HIS3), with both HMG1 and HMG2 genes disrupted (and consequently a mevalonate auxotroph), were all obtained by sporation of the diploid produced from the rare mating of the otherwise isogenic strains JRY1159 with JRY1160 (Basson et al., 1988). All haploid spores from these crosses are thus isogenic with the parents, and with strain JRY527, except for the relevant HMG-R loci. Strain RHY194, which expresses only the HMG1 isozyme, was obtained by transforming strain JRY1595 (HMG1 hmg2::HIS3) with a 2-u, URA3 plasmid containing an ~8-kb, HMG1-bearing yeast genomic fragment (Basson et al., 1986). Strain RHY194-4 (hmgI::LYS2 HMG2 erg13::HIS3) was made from JRY1596 (hmgI::LYS2 HMG2) by one step gene replacement (Rothstein, 1991) of the native ERG13 (HMG-CoA synthase) gene with an erg13::HIS3 disruption.

**Figure 1.** The mevalonate pathway. Only the enzymatic steps directly relevant to this work are depicted in the figure. The name of the enzyme responsible for a reaction is shown to the left of the arrow. The genetic or pharmacological block used to inhibit that step is shown in italics to the right of the arrow. Dotted arrows represent multi-enzyme steps, with the number of reactions indicated in parentheses to the left of such arrows. Adapted from Goldstein and Brown (1978).
gene as described (Schafer et al., 1989). Integrants were mevalonate auxotrophs with lys+his+ phenotype. Strain RHY46-42 was made by transformation of a resultant auxotroph with the 2-μ, URA3, HMG2 plasmid pJR360. Strain RWY60 (pep4::hisG), provided by R. W. Wright (University of Washington) was produced from strain JRY527 (HMG1 HMG2) by one step gene replacement of the entire PEP4 coding region with a "disrupter" insertion hisG::URA3::hisG (Alani et al., 1987). After selection of integrants on ura- medium, purified Ura - isolates were rescreened for loss of the Ura - phenotype by growth on 5-FOA (Boeke et al., 1987). Ura - isolates from the FOA selection were then screened for the loss of the PEP4 activity by the APE color reaction (Jones, 1977), resulting in RWY60. Strains of bacteria used for DNA manipulations were DH5α, XLI Blue, and CJ236 (Kunkel et al., 1987). Bacteria were grown in LB broth: 1% Bacto-tryptone (Difco Laboratories, Detroit, MI) and indicated amino acid supplements. Final concentrations of supplements were: Lys (30 mg/l), His (20 mg/l), Ade (20 mg/l), Met (20 mg/l), and Ura (20 mg/l). Yeast were transformed with DNA using the LiOAc method as described (Ito et al., 1983).

Strains of bacteria used for DNA manipulations were DH5α, XLI Blue, and CJ236 (Kunkel et al., 1987). Bacteria were grown in LB broth: 1% Bacto-tryptone (Difco Laboratories, Detroit, MI), 0.5% NaCl, 0.5% Bacto-yeast extract (Difco). Helper plasmid for oligonucleotide-directed mutagenesis was an isolate of M13K07 (Vieira and Messing, 1987).

### Recombinant DNA Methods

pJR360 and pJR360 are 2-μ, URA3 plasmids with genomic fragments bearing the HMG1 or HMG2 genes, respectively, and were described previously (Basson et al., 1986; Wright et al., 1988). pRH98-2 was an integrating vector that allows expression of reading frames from the yeast GPD promoter (Bitter and Egan, 1984). pRH98-2 was derived from the parent vector Yplac111 (integrating, URA3) (Gietz and Sugino, 1988) as follows: the unique BamHI site was removed by cleavage, filling in with Klenow fragment and blunt end ligation. A ~1.5-kb HindIII/XbaI fragment from pCI (Schena et al., 1991), provided by K. Yamamoto, University of California, San Francisco, CA), bearing the GPD promoter and the PGK terminator with a BamHI/SalI cloning site between the two control regions, was then cloned into the HindIII/XbaI sites of the modified parent, resulting in pRH98-2. Positions -6 through -1 of the HMG2 gene on the 4.9-kb genomic fragment from pJR360 were converted into a PstI site with a mutagenic 29-base oligonucleotide used as a primer as described (Kunkel et al., 1987). The resulting novel 3.4-kb PstI fragment bearing the HMG2 reading frame was then cloned into the BamHI/SalI sites of pRH98-2 by ligation in the presence of a single stranded BamHI/PstI adaptor (NEB), resulting in pRH144-2 (integrating URA3, HMG2-PGD-HMG2), to allow expression of HMG2 reading frame from the GPD promoter. Analogous integrating plasmid pRH105-25, with the HMG1 coding region driven by the GPD promoter (pRH105-25), was assembled by directed cloning into the PstI site of pRH98-2. Positions -6 through -1 of the HMG1 gene on the 4.9-kb genomic fragment from pJR360 were converted into a PstI site with a mutagenic 29-base oligonucleotide used as a primer as described (Kunkel et al., 1987). The resulting novel 3.4-kb PstI fragment bearing the HMG1 reading frame was then cloned into the BamHI/SalI sites of pRH98-2 by ligation in the presence of a single stranded BamHI/PstI adaptor (NEB), resulting in pRH144-2 (integrating URA3, HMG2-PGD-HMG2), to allow expression of HMG1 reading frame from the GPD promoter. Analogous integrating plasmid pRH105-25, with the HMG1 coding region driven by the GPD promoter, was made by cloning the 3.4-kb HMG1 fragment (with no 5' untranslated region) from pJR360 into a PstI site with a mutagenic 29-base oligonucleotide used as a primer as described (Kunkel et al., 1987).

### Immunoprecipitation

The technique employed for immunoprecipitation of yeast proteins is similar to that described by Sengstag et al. (1990). Cells from logarithmic phase cultures (OD600 0.5-1.1) grown in supplemented YM were resuspended in

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**Table 1. Yeast Strains**

| Strain     | Description                                                                                                                                 |
|------------|-------------------------------------------------------------------------------------------------------------------------------------------|
| JRY159     | hmg1::LYS2 HMG2 ura3-52 his3Δ200 ade2-101 lys2-801 met                                                                                   |
| JRY1266    | hmg1::LYS2 HMG2 ura3-52 his3Δ200 ade2-101 lys2-801 met + pJR360 (HMG2, URA3, 2-μ pSey8 derivative)                                        |
| RHY106-12  | pep4::hisG hmg1::LYS2 HMG2 ura3-52 his3Δ200 ade2-101 lys2-801 met + pJR360                                                             |
| RHY184     | sec18-1 hmg1::LYS2 HMG2 ura3-52 his3Δ200 ade2-101 lys2-801 met + pJR360                                                              |
| RHY46-12   | erg13::HIS3 hmg1::LYS2 HMG2 ura3-52 his3Δ200 ade2-101 lys2-801 met + pJR360                                                           |
| RHY171     | raml3::HIS3 hmg1::LYS2 HMG2 ura3-52 his3Δ200 ade2-101 lys2-801 met + pJR360                                                           |
| JRY1593    | hmg1::LYS2 hmg2::HIS3 ura3-52 his3Δ200 ade2-101 lys2-801 met                                                                           |
| RHY183     | hmg1::LYS2 hmg2::HIS3::URA3::pGPD-HMG2 ura3-52 his3Δ200 ade2-101 lys2-801 met                                                         |
| RHY223-1   | hmg1::LYS2 hmg2::HIS3 ura3-52::pGPD-HMG1::URA3 his3Δ200 ade2-101 lys2801 met                                                        |
| JRY1590    | HMG1 hmg2::HIS3 ura3-52 his3Δ200 ade2-101 lys2-801 met                                                                              |
| RHY194     | JRY1590 + pJR39 (HMG1, URA3, 2-μ YEp24 derivative)                                                                                     |
| RHY527     | HMG1 HMG2 ura3-52 his3Δ200 ade2-101 lys2-801 met                                                                                       |
| RHY131-17  | pCS4 (HMG1-TMR::SUC2::HIS4c, URA3, 2-μ YEp552 derivative)                                                                               |
| RHY131-4   | pCS17 (HMG2-TMR::SUC2::HIS4c, URA3, 2-μ YEp552 derivative)                                                                               |
fresh supplemented YM and pulse labeled with Tran35S-label™ for 10 min, usually at 30°C. To initiate the chase period, labeled cell suspension was added to supplemented YM medium containing sufficient cysteine–HCl and methionine to yield final concentrations of 50 mM of each (chase medium) and a cell density of 1.5 OD600 U/ml, and the cultures were incubated at 30°C. OD600 volume samples were removed at various times, and prepared for immunoprecipitation: cells were washed once with YM (without glucose) + 0.1% NaNO2 + 5 mM PMSF, and the cell pellet was overlaid with 75 μL of SUTE (1% SDS, 8 mM urea, 10 mM Tris base, pH 7.5, 10 mM EDTA) with the following protease inhibitors added from concentrated stock solutions immediately prior to use: 5 mM PMSF, 50 μg/ml leupeptin, 50 μg/ml pepstatin A, 50 μg/ml TPCK, and 20 μg/ml aprotinin. 100 μl of 0.5-mm glass beads were then added and the tube was vortexed at maximum speed for 1.5 min. The lysed slurry was incubated at 65°C for 10 min, and then 400 μl of immunoprecipitation buffer with protease inhibitors (IPB; IPB + PI: 13 mM NaH2PO4 pH 7.5, 150 mM NaCl, 2% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 0.02% NaN3 with the following protease inhibitors: 1.0 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml TPCK, and 4 μg/ml aprotinin) was added to the slurry. The total liquid lysate from the tube was removed, clarified by 5-min centrifugation and kept on ice until the end of an experiment. To immunoprecipitate the proteins under study, 10 μl of polyclonal anti–β-galactosidase-Hmglp antiserum (Wright et al., 1988) (or other antiserum as indicated) was added to the lysate which was then incubated at room temperature for 5 min. The sample was next centrifuged for 5 min and the supernatant was incubated at 4°C for 12-15 h. A 50-μl vol of protein A-Sepharose CL-4B slurry (10% wt/vol equilibrated and defined in IPB; Pharmacia Diagnostics Inc., Fairfield, NJ) was then added, and the incubation was continued for 2 h at room temperature. The beads were then washed by centrifugation and resuspension with IPB followed by PBS (pH 7.5). 50 μM NaCl was aspirated to dryness, overlaid with 30 μl of 2 × urea sample buffer (USB: 8 mM urea, 4% SDS, 10% β-mercaptoethanol, 0.125 M Tris, pH 6.8) and incubated at 65°C for 10 min, A 20-μl sample of the USB was then loaded onto an SDS-PAGE gel (see below) and separated by electrophoresis at 20-30 mA at room temperature. Gels were then treated with Amplify (Amersham Corp.) as specified by the manufacturers and autoradiographed on Kodak X-Omat film at −80°C. Occasionally dried gels were analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). All centrifugation steps in the immunoprecipitation protocol were performed at 15,000 g.

**Immunoblotting**

Whole cell lysates for immunoblot analysis were prepared as follows: cells from an experimental culture were washed once with YM (no glucose) 0.1% NaNO2 + 5 mM PMSF and resuspended in 100 μl SUTE + PI at pH 6.8 (versus 8.0 used in the immunoprecipitation protocol). 100 μl of acid-washed 0.5 mm glass beads were added and the mixture was vortexed at maximal speed for 1.5 min. A 100-μl vol of USB was then added and the mixture was incubated at 65°C for 10 min. The liquid lysate was then removed from the glass beads and clarified by 5-min centrifugation. Samples of lysate were loaded onto an SDS-PAGE gel and separated by electrophoresis (see below). Proteins in the SDS-PAGE gel were then electrophotographically transferred (Burnette, 1981) onto two stacked pieces of nitrocellulose. The front piece (touching the gel) was used for the immunoblot: immediately after transferring, the front nitrocellulose was placed in TBS (10 mM Tris, pH 8.0, 150 mM NaCl) with 0.02% Tween 20, 0.1% NaN3, and 5% Carnation nonfat dried milk (TBSTM) for 2 h at room temperature. The blot was next incubated with a 1/2,000 dilution of affinity purified anti–β-galactosidase-Hmglp antiserum in TBSTM (with 2% milk) at 4°C overnight or for 3 h at room temperature. The nitrocellulose was then washed, treated with HRP–donkey anti–rabbit antiserum, washed, and developed with the Amersham ECL™ chemiluminescent detection reagents as per the manufacturers instructions. The secondary antibody treatment and all washes were in TBS with 0.05% Tween 20 (TBST) solution. Chemiluminescent exposure was performed on Kodak X-OMAT AR film. In figure 8, [125I]–protein A was used as a secondary detection reagent. In this instance the blot was incubated in ~2 μCi of labeled protein A in 10 ml TBST, followed by washing and autoradiography at 30°C. The CL-dB slurry (10% wt/vol equilibrated to dryness and applied to a Silica gel 60 TLC plate, with fluorescence indicator (Whatman Int. Ltd., Maidstone, England). The plate was developed in benzene/ethyl acetate (5:1), dried and subjected to both autoradiography and phosphorimagery analysis for quantitation. In this protocol, ergosterol esters are completely saponified (>98%) to free ergosterol. The ergosterol was located on the plate both by blockade of fluorescence at 300 nm illumination, and by migration of a standard.

**Results**

**The Hmglp and Hmg2p Isozymes Had Different Stabilities**

In wild type S. cerevisiae, both isozymes of HMG-R are simultaneously expressed from their respective genes. In order to investigate the posttranslational fate of each isozyme of HMG-R in S. cerevisiae, strains were used that each expressed a single isozyme. The strain that expressed only

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Hmglp (RHY194) had a disruption of the HMG2 gene (hmg2::HIS3) and carried a 2-μ plasmid with the HMG1 gene (pJR59). Conversely, the strain that only expressed Hmg2p (JRY1266), had a disruption of the HMG1 gene (hmg1::LYS2) and carried a 2-μ plasmid with the HMG2 gene (pJR360). The use of 2-μ plasmids in these strains allowed for better signal in immunoprecipitation experiments, and insured that the flux through the mevalonate pathway would be high. The total amount of HMG-R in the Hmglp-producing strain is about 10 times that in wild type, and in the Hmg2p-producing strain, the HMG-R activity is about four times that of wild type. At these levels of expression, the HMG-R proteins could not be detected with silver staining on a polyacrylamide gel (R. Wright and J. Rine, unpublished observation), and thus represented a minor component of the total protein.

By making use of polyclonal antiserum that cross-reacted with either isozyme in a pulse–chase protocol, the stability of each protein was assessed (Fig. 2). The Hmglp isozyme was quite stable, with essentially no degradation in a 4 h experiment. Hmglp was stable at all levels of expression tested, including the level produced by a single genomic copy of HMG1 (data not shown). In contrast, the Hmg2p isozyme was rapidly degraded with a characteristic half-life on the order of 50–60 min. The rapid degradation of Hmg2p was quantitatively similar over a range of expression levels, although measurement of Hmg2p degradation from a single genomic copy of HMG2 was below the limit of detection.

**Hmg2p Degradation Did Not Require Vacuolar Proteases**

The yeast vacuole is analogous to the mammalian lysosome. It is the site of degradation of numerous proteins, including integral membrane proteins such as the mating factor receptors Ste2p and Ste3p (Davis et al., 1993), and the Golgi-resident protease Kex2p (Wilcox et al., 1992). Yeast strains with disruptions of the PEP4 gene show dramatic stabilization of proteins that are degraded in the vacuole. This stabilization by pep4 mutations provides a diagnostic measure of vacuole-dependent degradation. A strain with a complete disruption of the PEP4 reading frame (RHY106-12; pep4::hisG), but otherwise isogenic to the Hmg2p-expressing strain used in Fig. 2 (JRY1266), was used to evaluate the involvement of vacuolar proteases in Hmg2p stability. Degradation of the Hmg2p was similar in the presence or absence of a functional PEP4 gene (Fig. 3, top). As a control measure of vacuolar function, the vacuolar enzyme carboxypeptidase Y (CPY), which depends upon PEP4 for its posttranslational processing, was also immunoprecipitated from the same lysates. The processing of CPY to the mature vacuolar form (Fig. 3, bottom arrow) was completely deficient in the pep4Δ strain, confirming the presence of the pep4Δ phenotype in strain RHY106-12. Thus, PEP4 deletion had no effect on the efficient degradation of Hmg2p, indicating that vacuolar proteases are not involved.

**The Secretory Pathway Was Not Required for the Degradation of Hmg2p**

The role of the secretory pathway in the degradation of Hmg2p was assessed to determine whether this process required exit from the ER. Mutations in SEC18 block transport of both soluble (Graham and Emr, 1991) and integral membrane proteins (Roberts et al., 1989) from the ER to the cis–Golgi compartment. The role of SEC18 in Hmg2p turnover was evaluated in a strain with the sec18-I temperature-sensitive mutation. In cells harboring this mutation, a shift to a nonpermissive temperature causes a rapid (less than 5 min) cessation of traffic out of the ER (Graham and Emr, 1991). The stability of Hmg2p was compared in a SEC18 strain (JRY1266) and an isogenic sec18-l strain (RHY184-12). Cells were pulse labeled at the permissive temperature (23°C) and then shifted to the nonpermissive temperature (37°C) at the time of chase (Fig. 4).

The degradation of Hmg2p at the nonpermissive temperature was unaffected by the presence of the sec18-I mutation. By this criterion, the secretory pathway was not required of the degradation of Hmg2p. The efficacy of the trafficking block in the sec18-I strain was confirmed in the experimental lysates by immunoprecipitation of CPY. As reported previously, the conversion of CPY from its ER form to later species was blocked in the sec18-I strain for the entire time of the experiment (Graham and Emr, 1991) (data not shown). The viability of the cells was unaffected by incubation at the
Degradation of Hmg2p Was Regulated by Signals from the Mevalonate Pathways

In mammalian cells the degradation of Hmg2p–R is regulated by signals from the mevalonate pathway, but the identity of the signals has remained elusive. This regulation of protein degradation is one component of the multi-level regulation of HMG-R activity (Goldstein and Brown, 1990). Thus, decreased flux through the mevalonate pathway caused the stabilization of Hmg2p, and suggested that decreasing flux through the mevalonate pathway caused the stabilization.

One limitation in the interpretation of this experiment was that lovastatin binds to HMG-R. Thus, the stabilization of Hmg2p might reflect effects of lovastatin binding on Hmg2p conformation. To determine whether stabilization of Hmg2p was due to a regulatory signal or to binding of the inhibitor, an independent method of modulating mevalonate availability was used during a pulse-chase experiment. A strain expressing only Hmg2p was constructed that had a disruption of the yeast gene (ERG13), which encodes HMG-CoA synthase (Greenspan et al., 1987). Consequently, strain (JRY1266) was subjected to pulse-chase analysis in the presence of high (50 mg/ml) or low (5 mg/ml) mevalonic acid lactone (Mev). These results established that inhibition of HMG-CoA synthase gene (erg3::HIS3, RHY46-42, mevalonate auxotroph), was subjected to pulse–chase analysis in the presence of high (50 mg/ml) or low (5 mg/ml) mevalonic acid lactone (Mev). The erg3::HIS3 strain RHY46-42 was grown several times to saturation over a 3-d period (by repetitive dilution) in supplemented YM with 50 mg/ml Mev. This culture was then diluted into the same medium and allowed to grow into mid logarithmic phase. Cells were then pulse labeled and chased in the presence of either 5 mg/ml Mev (5 Mev) or 50 mg/ml Mev (50 Mev). Samples from 0 and 4-h chase time were analyzed by immunoprecipitation. For comparison, the isogenic ERG13 strain (JRY1266) was subjected to the same protocol, with the chase conducted at 5 mg/ml Mev (left, ERG13).

Perturbation of the Mevalonate Pathway Acted Directly on the Stability of the Hmg2p

The regulation of HMG-R levels in mammalian cells occurs at several biochemical levels (Nakanishi et al., 1988). De-
Figure 7. Protein synthesis was not required for regulated degradation of Hmg2p. (A) Effect of lovastatin on the degradation of Hmg2p was examined by pulse-chase analysis in the presence (right) or absence (left) of 25 μg/ml CHX. Hmg2p-expressing cells (JRY1266) were pulse labeled and then placed in chase medium with appropriate additions to yield 0 or 33 μg/ml lovastatin in the presence or absence of 25 μg/ml CHX, as indicated. (B) The cycloheximide chase experiment. The effect of lovastatin on the stability of the entire Hmg2p pool was examined by immunoblot analysis at various times after treatment with cycloheximide. Logarithmically growing cells expressing Hmg2p (JRY1266) were resuspended in fresh supplemented YM and incubated for 30 min at 30°C. CHX was then added to the culture (50 μg/ml) and the cells were immediately divided into two flasks with (right) or without (left) lovastatin (50 μg/ml final). At the indicated times during the subsequent 30°C incubation equal volumes of each culture were analyzed for Hmg2p by immunoblotting. Except for the leftmost lane (1/2 0), all of the lanes are the result of loading ~0.25 OD equivalents of cells. The leftmost lane in the figure is the result of loading one-half of the amount of lysate used in the 0 h, no lovastatin lane (0, second from the left) in order to provide a visual gauge of 50% degradation. Staining of the second blot (see Materials and Methods) for total protein by India ink revealed that all of the experimental time point lanes (2–7) had identical protein loads (data not shown).

Increased flux through the mevalonate pathway results in increased transcription of HMG-R message, increased translation of those messages, and enhanced stability of the protein. In yeast, it appears that at least a subset of these regulatory circuits exist. Thus it was possible that the effect of lovastatin on Hmg2p degradation was caused by increased HMG2 gene expression, coupled with a concomitant secondary effect on Hmg2p stability due to saturation of the degradation machinery, or other mechanisms. We used two independent methods to evaluate the potential involvement of changes in HMG2 gene expression on the regulation of Hmg2p half-life. In one control, protein synthesis was inhibited to block de novo synthesis of Hmg2p during measurement of stability. In a second control, the HMG2 gene was placed under the control of a different promoter to eliminate potential effects mediated by the endogenous promoter region.

The protein synthesis inhibitor CHX was used to test rigorously if ongoing translation had any role in stabilization of Hmg2p by lovastatin. Cells expressing only Hmg2p (JRY1266) were pulse labeled and then added to chase medium containing either 0 or 25 μg/ml CHX, in the presence or absence of 40 μg/ml lovastatin. The presence of CHX during the chase period had no effect on the degradation of Hmg2p, and addition of lovastatin stabilized Hmg2p in the presence or absence of CHX (Fig. 7 a). Control experiments established that the concentration of CHX used in these experiments (25 μg/ml) arrests >95% of protein synthesis within 2 min. Thus, the degradation of Hmg2p, and the regulation of this process by altering mevalonate availability, can each occur in the absence of protein synthesis.

Since CHX did not affect the degradation of Hmg2p, nor the ability of lovastatin to attenuate the rate of degradation, the drug was used to examine the stability of the entire pool of Hmg2p by immunoblotting. This experiment would reveal whether the behavior of the newly synthesized Hmg2p accurately reflected the dynamics of the entire pool. Cells were treated with CHX in the presence or absence of lovastatin and sampled periodically for immunoblot analysis of total Hmg2p. This procedure was termed a cycloheximide chase. As expected from the pulse–chase experiment in Fig. 7 a, addition of CHX to the cultures in the absence of lovastatin caused a time-dependent loss of Hmg2p protein (Fig. 7 b, lanes 1–4). Comparison with the first lane (loaded with 50% of 0 h sample) revealed that the 1.5 h signal (lane 3) was less than 50% of the initial signal, and indicated that the entire pool decayed at a similar rate to a pulse-labeled sample. When lovastatin was added along with the CHX at the beginning of the experiment, stabilization of the entire pool occurred (lanes 5–8). Taken together, these results demonstrated that ongoing protein synthesis was not required for the degradation of Hmg2p, but for the modulation of half-life by altered flux through the mevalonate pathway. Thus the effect of altered flux on Hmg2p degradation was a direct effect, and not a secondary effect due to altered gene expression. The results also demonstrated that the entire Hmg2p pool was subject to the same degradation rate and regulation as a pulse-labeled sample.

In order to examine regulated degradation in isolation from other possible effects on the promoter region, the HMG2 coding region was cloned adjacent to the constitutive GPD promoter (Schena et al., 1991) into an integrating (Yip) expression vector. This plasmid (pRH144-2) was integrated into the genome of an HMG-R-deficient strain (JRY1593; hmg1::LYS2 hmg2::HIS3) in order to create a strain that expressed Hmg2p from a single copy coding region driven by the GPD promoter (RHY183; hmg1::LYS2 hmg2::HIS3::URA3::pGPD::HMG2). The Hmg2p produced by this strain (RHY183) was rapidly degraded and subject to modulation by lovastatin (50 μg/ml), as measured by either pulse–chase (Fig. 8 a) or cycloheximide chase (Fig. 8 b) procedures. When this strain was grown in the same concentration of lovastatin in the absence of CHX, the steady state level of Hmg2p increased (Fig. 8 c). Dilution of immunoblotted samples indicated that induction of Hmg2p caused by lovastatin was ~5 fold. Induction of Hmg2p steady state level by lovastatin was also observed with a strain that expressed Hmg2p from the GAL1,10 heterologous promoter (data not shown). Thus, these studies provide a second line of evidence that the stabilization of Hmg2p by limited flux through the mevalonate pathway was a direct effect on the degradation of the protein. The maximal effect of lovastatin on Hmg2p stability in either pulse–chase or whole pool experiments was observed at a concentration (50 μg/ml) well below that which caused overt toxicity in these strains.

A strain bearing an integrated copy of HMG1 driven by the same promoter showed no alteration in steady state levels

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ting, using [35S]protein A and autoradiography as the detection method. The technique was identical to that described in Fig. 7B. (C) Effect of lovastatin on the steady state levels of Hmg2p in RHY183. Cultures of RHY183 were grown from an initial OD of ~0.05 in supplemented YM in the presence (right) or absence (left) of 50 μg/ml lovastatin for 15 h at 30°C. Hmg2p was then analyzed by immunoblotting, using [35S]protein A and autoradiography as the detection method. For each sample, 20 μl of the lysate (1) and 20 μl of a 1:5 dilution of the lysate (1/5) were loaded to allow approximate quantitation of the induction. Total protein transfer was verified by India ink staining and was the same for each sample at a given dilution.

When incubated with lovastatin, nor any decay in protein upon treatment with CHX (data not shown). This result was consistent with the pulse–chase experiments (Fig. 2) that show Hmg1p to be stable, and establishes the specificity of Hmg2p degradation in these procedures.

Sterols Were Not Involved in the Regulation of Yeast Hmg2p Degradation

In mammalian cells, the degradation of HMG-R is controlled by both sterol signals and nonsterol signals from the mevalonate pathway. The response of Hmg2p to limited production of mevalonic acid might reflect alteration of either a sterol or a nonsterol regulator, since both would be depleted in the experiments described above. In order to test the possibility that sterols were involved in the regulation of Hmg2p, we employed the drug zaragozic acid, a potent, specific inhibitor of yeast and animal cell squalene synthase (Fig. 1) (Baxter et al., 1992; Bergstrom et al., 1993; Bergstrom, J. D., personal communication). If sterols or other molecules formed from squalene were signals of abundant isoprenoid production with respect to Hmg2p degradation, then treatment with zaragozic acid should lead to depletion of the signal for degradation. In that case, zaragozic acid would slow the degradation of Hmg2p. In fact, zaragozic acid at high concentrations had no effect on the degradation of Hmg2p. A comparison between the effect of zaragozic acid or lovastatin on Hmg2p stability in an Hmg2p-producing strain (JRY1266) is shown in Fig. 9. Lovastatin strongly stabilized the protein, whereas the zaragozic acid had no effect. This difference in the effects did not reflect a difference in the efficacy of the two drugs. This was demonstrated by concomitantly measuring the effect of each agent on the biosynthesis of sterols by incorporation of radioactive acetate. In the experiment shown, the zaragozic acid was actually more effective in blocking the production of ergosterol than was the lovastatin. The lack of any effect of zaragozic acid on the degradation of Hmg2p has been repeated in numerous variations of the experiment shown. Neither long preincubations (2 h) nor use of higher concentrations (150 μg/ml) of the drug had any effect on Hmg2p degradation (data not shown). These results indicated that neither squalene levels nor the levels of a later product of the mevalonate pathway abundance could affect prenylation of this protein, and thus affect Hmg2p stability. A prediction of this model was that the loss of ability to prenylate the modulator should result in stabilization of Hmg2p. In order to examine this possibility, a strain (RHY171) was constructed that expressed only Hmg2p and had a disruption of the RAMI gene (raml::HIS3), which encodes the β subunit of the yeast farnesyl transferase (Schafer et al., 1989; Schafer and Rine, 1992). Strains with the raml::HIS3 allele are deficient in protein farnesylation (Schafer et al., 1990). The cells are temperature...
sensitive for growth at 37°C, and have numerous phenotypic characteristics that reflect the loss of farnesyl transferase activity. The stability of Hmg2p was compared in a pulse-chase experiment. RAMI (Fig. 10, left lane) cells showed the expected rapid degradation of Hmg2p. In contrast, the cells with the ram1::HIS3 had a clear stabilization of Hmg2p (Fig. 10, left lane). When the same strains were examined in the cycloheximide chase experiment, the difference in half life was also evident, but not as dramatic as in the pulse-chase experiment (RAMI \( \sim 1 \) h, ram1::HIS3 \( \sim 3.5 \) h, data not shown).

**Stability of Each HMG-R Isozyme Was Determined by the Transmembrane Region of the Molecule**

Like the mammalian protein, each HMG-R isozyme consists of a COOH-terminal catalytic region, and an NH2-terminal transmembrane region that allows anchoring in the resident membrane (Basson et al., 1988; Wright et al., 1988, 1990). In animal cells, the noncatalytic parts of the reductase molecule are responsible for the ER degradation of the protein, and will impart regulated ER degradation to appropriate fusion proteins (Chun et al., 1990). Because the yeast HMG-R isozymes have drastically different stabilities, we tested whether the differing posttranslational fate were determined by the distinct noncatalytic NH2-terminal regions of each protein. In order to test in isolation the role of the NH2-terminal transmembrane portion of HMG-R in determining posttranslational fate, two fusion genes were constructed. The fusion genes consisted of either the HMG1 or HMG2 transmembrane portion coding region (codons 1-523 of HMG1 and codons 1-522 of HMG2) fused in-frame to a portion of the invertase (SUC2)-coding region, fused in turn to the 3' end of the histidinol dehydrogenase-coding region (HIS4c) (Sengstag et al., 1990) (Fig. 11 a). These fusion genes thus produced proteins with either the HMG1 (pCS4) or the HMG2 (pCS17) transmembrane region fused to an identical Suc2::His4c fusion protein. When strains expressing these chimeric proteins were subjected to pulse–chase analysis, the fusion proteins faithfully reflected the stability of the authentic enzymes from which the transmembrane region came (Fig. 11 b). The fusion protein bearing the HMG1 NH2-terminal transmembrane region (left) was, like the parent protein, quite stable. In contrast, the transmembrane region from HMG2 imparted rapid degradation to an otherwise identical fusion protein (right). The degradation of the HMG2-related fusion was, also like the parent isozyme, unaffected by the pep4::hisG disruption (data not shown). The degradation of the Hmg2::Suc2::His4c fusion protein was sensitive to lovastatin. Addition of 40 \( \mu \)g/ml of the drug caused dramatic slowing of degradation. These results showed that the regulated degradation of Hmg2p is imparted by the noncatalytic region of the protein. Curiously, the stability of the Hmg2::Suc2::His4c fusion protein was unaffected by disruption of the RAM1 farnesyl transferase (ram1::HIS3) (data not shown).

**Different Physiological Effects of Hmg1p and Hmg2p**

The different behaviors of the two yeast HMG-R isozymes raised the question of whether or not these differences might be reflected in different physiological responses of the cells bearing individual isozymes. To separate the contributions

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*Figure 10.* Effect of a disruption of the RAM1 farnesyltransferase gene on the stability of Hmg2p. A strain expressing only Hmg2p and wild-type for the RAM1 gene (JRY1266) and an isogenic strain with the ram1::HIS3 disruption (RHY171) were compared for Hmg2p stability by pulse–chase analysis. Both strains were grown at 23°C to mid log phase, resuspended in fresh medium, and allowed to incubate at 30°C for 15 min. The pulse labeling and chase steps were then performed at 30°C.

*Figure 11.* The noncatalytic NH2-terminal regions of the yeast HMG-R determine stability. (A) Schematic of the fusion gene construct used to assess the role of the NH2-terminal transmembrane region (TMR) in the stability of a given isozyme. The fusion gene coding region is depicted. Either the HMG1 or HMG2 TMR-coding region (striped) was fused to identical invertase (SUC2, lightly shaded)-histidinol dehydrogenase (HIS4c, darkly shaded) reporter gene as described (Sengstag et al., 1990). Either reading frame was expressed from a 2- \( \mu \) plasmid (parent YEp352; HMG1 TMR plasmid: pCS4, HMG2 TMR plasmid: pCS17). The invertase region allows immunoprecipitation of either resultant protein with anti-invertase antibodies. (B) Stability of fusion proteins resulting from the expression of the fusion genes described in A. Strains expressing either the fusion gene with the HMG1 TMR (RHY134-4, HMG1) or the HMG2 TMR (RHY134-17, HMG2) were subjected to pulse–chase analysis and immunoprecipitation using affinity-purified polyclonal anti-invertase antiserum to precipitate each 170-kD fusion protein. (C) Effect of lovastatin on the stability of the rapidly degraded fusion protein encoded by the HMG2 TMR-bearing fusion gene. A pulse–chase experiment was performed as in B, with or without 40 \( \mu \)g/ml final concentration of lovastatin present in the chase medium.
Figure 12. The effect of lovastatin preincubation of Hmglp- or Hmg2p-expressing cells on subsequent survival in a high concentration of lovastatin. Strains expressing only HMG1 or HMG2, from the GPD promoter (RHY223-1 or RHY183, respectively), were compared in studies of sensitivity to lovastatin. These two strains have very similar steady state levels of HMG-R activity. (A) Effect of lovastatin on the outgrowth of cultures of either strain. Supplemented YM medium samples with the indicated concentrations of lovastatin were inoculated (initial OD$_{600}$ $\sim$0.04) from overnight cultures of either the Hmglp-producing strain (RHY223-1, triangles) or the Hmg2p-producing strain RHY183 (HMG2, circles). The cultures were incubated at 30°C for 14 h and outgrowth was assessed by OD$_{600}$ measurement. (B) Effect of preincubation with a small dose ofLovastatin on subsequent outgrowth at a high dose. The same two strains were grown in supplemented YM containing 0 (open symbols) or 25 $\mu$g/ml (closed symbols) of lovastatin for 12 h at 30°C. The preincubation cultures were inoculated with cells grown from single colonies for 24 h in the same medium, and the starting OD$_{600}$ was $\sim$0.05. As expected from the growth curve in A, the effect of the 25 $\mu$g/ml preincubation concentration on the two strains was identical (RHY223-1: 25% growth inhibition as compared to no drug, RHY183: 24% inhibition). Equal amounts of cells from each of the four preincubation cultures were then added to supplemented YM containing 1,000 $\mu$g/mlLovastatin and incubated at 30°C. At the indicated times the OD$_{600}$ of each culture was measured.

Discussion

The regulated degradation of HMG-R involves two poorly understood processes of biological and medical interest: the ER degradation of membrane proteins, and the signaling pathway that cells use to measure and modulate flux through the mevalonate pathway. We have discovered regulated degradation of yeast HMG-R that was strikingly similar to the same process in mammalian cells. Thus, yeast will provide a tractable way to study the underlying mechanisms of regulated ER protein degradation.

The two isoforms of yeast had distinctly different post-translational fates. Hmglp was quite stable under all conditions tested. In contrast, Hmg2p was degraded at different rates depending on the flux through the mevalonate pathway. The effect of lowered mevalonate pathway flux on Hmg2p stability was direct, with the entire Hmg2p pool subject to regulated degradation. These observations established the existence of feedback control of HMG-R stability in yeast. Because the degradation of Hmg2p had the hallmarks of ER protein turnover, these results indicated the existence of a general degradation pathway for ER membrane proteins in this organism.

The molecular signals that couple the mevalonate pathway to Hmg2p degradation appeared to be intermediates in the mevalonate pathway that lie between mevalonic acid and squalene, and may include a prenyl group on a modulatory protein. Studies with chimeric proteins indicated that the stability of each isozyme was determined by its noncatalytic NH$_2$-terminal region. The rapid degradation of a fusion protein bearing the HMG2 noncatalytic region was also regulated by altering mevalonate production. Finally, we demonstrated experimental differences in cell physiology between yeast strains that express one or the other isozyme of HMG-R indicating that the observed differences in regulation can be physiologically relevant.
ER Degradation in Yeast

We have concluded that the Hmg2p isozyme was degraded in the ER from two lines of evidence: (a) the stability of the protein was unaffected by loss of the pleiotropic master vacuolar protease Psp4p and (b) Hmg2p stability was unaffected by loss of SEC18 function.

Vacuolar protease activity is strongly attenuated in strains with a disruption of the PEP4 gene, resulting in depressed levels of the three major proteases, PrA (Psp4p), PrB, and PrC (CPY) that are as low as those in strains with disruptions of each of the three genes (Woolford et al., 1993). Since Hmg2p degradation was unaffected by a complete deletion of the PEP4 gene (Fig. 3, pep4::hisG), it was clear that these activities were not important in Hmg2p stability. This result was in stark contrast to the effects of pep4 mutations on the degradation of membrane proteins known to be processed in the vacuole (Wilcox et al., 1992; Davis et al., 1993). In the case of the a-factor receptor (Ste3p), for instance, a loss of function in PEP4 results in a change in the half-life from >15 min to >4 h (Davis et al., 1993). The independence of the degradation of the HMG2 protein from vacuolar function is similar to degradation of HMG-R in mammalian cells (Nakanishi et al., 1988), which is independent of the lyosome.

Experiments with a sec18-1 strain showed that the degradation of Hmg2p occurred in the absence of SEC18 gene function. The lack of a role for SEC18 in Hmg2p degradation provided independent confirmation that the vacuole was not involved because the movement of both soluble and membrane-bound proteins from the ER to the vacuole requires the function of SEC18 (Roberts et al., 1989; Graham and Emr, 1991). The absence of a requirement for SEC18 function in the degradation of Hmg2p also indicated that movement of Hmg2p from the ER to distal compartments of the secretory pathway was not required for degradation. It is still formally possible that Hmg2p is brought to the vacuole (or some other degradative compartment) by a novel and independent trafficking pathway. However, this alternate transport pathway would have to be coupled with an alternate PEP4-independent vacuolar degradation machinery. Therefore, the simpler interpretation, consistent with findings on HMG-R in mammalian cells, is that Hmg2p is degraded in (or at) the ER itself. These data established the existence of an ER degradation pathway for native Hmg2p and most likely for other integral ER membrane proteins in yeast.

Mevalonate Signaling Pathway in Yeast

Our experiments demonstrated that the degradation of Hmg2p was coupled to the mevalonate pathway. Treatment of cells with lovastatin caused a rapid stabilization of Hmg2p. Further experiments using a mevalonate auxotrophic strain with a disruption of the HMG-CoA synthase gene (erg13::HIS3) or the inhibitor of HMG-CoA synthase L-659,699 indicated that the effect of lovastatin was due to altered production of mevalonate pathway products that serve as regulatory signals for HMG-R degradation. These results excluded concern that the ability of lovastatin to bind to HMG-R somehow altered its susceptibility to degradation. Experiments with inhibitors of protein synthesis and heterologous promoter fusions demonstrated that the action of lovastatin on the degradation of Hmg2p was a primary effect on the half-life of the protein, and not a secondary effect due to elevation of Hmg2p levels brought about by other mechanisms.

What sort of signaling molecules are involved in the communication between the mevalonate pathway and the degradation of Hmg2p? Lovastatin (Fig. 5) or HMG-CoA synthase inhibitor L-659-699, or low ambient levels of mevalonic acid in a mevalonate auxotroph (Fig. 6), all caused rapid stabilization of Hmg2p. In contrast, the squalene synthase inhibitor zaragociz acid had no effect on Hmg2p degradation. Therefore, the signaling molecule(s) appears to be made before the synthesis of squalene, but after or at the production of mevalonic acid. The regulated degradation of mammalian HMG-R appears to have a sterol-independent and -dependent component (Goldstein and Brown, 1990; Panini et al., 1992; Roitelman and Simoni, 1992). It may be that yeast possesses only the sterol-independent component of HMG-R stability control.

Studies with the raml::HIS3 mutation indicated that a farnesylated protein played a role in the degradation of Hmg2p. A disruption of the RAM1 farnesyl transferase gene clearly stabilized Hmg2p. Coupling the degradation of Hmg2p to the mevalonate pathway by attachment of a mevalonate pathway product to a protein involved in the degradation process would be an appealing mechanism for regulating degradation (Goldstein and Brown, 1990; Edwards et al., 1992). Since prenylated proteins play numerous roles in the control of cellular signaling processes and membrane dynamics, it is possible to imagine several ways that a prenylated protein could be involved in the regulation of Hmg2p half-life. At this point, however, we must interpret these results with the raml::HIS3 mutation with caution. In cells grown at 23°C (the permissive temperature for raml) strains) the steady state level of the Hmg2p in the raml::HIS3 was higher than the level of Hmg2p in the corresponding RAM1 strain, as expected from the observed stabilization. However, when the cells were then shifted to the semi-permissive temperature 30°C (at which the pulse-chase experiments are performed) the Hmg2p steady state level in the raml::HIS3 strain gradually dropped to that of the RAM1 strain. Nevertheless, the degradation rate of the Hmg2p remained slow in the raml::HIS3 strain at 30°C. Therefore it would appear that at 30°C there are compensatory effects on the production or processing of Hmg2p in the raml::HIS3 background that compensate for the decreased turnover of the protein.

Since the loss of RAM1 function is a strong, global perturbation of cell physiology, we do not yet know how direct the connection between farnesylation and Hmg2p degradation is. The most direct connection would be if the Hmg2p protein were itself farnesylated, and thus rendered competent for degradation. The (stable) Hmg1p protein has a "Caax" box at its COOH terminus, and is thus a candidate for prenylation, although there is no direct evidence of its prenylation. In contrast, the Hmg2p protein has no such putative farnesylation site. Thus it seems unlikely that the effect of the RAM1 farnesyl transferase is through modification of Hmg2p itself, although this will be directly examined in the future. It is interesting that the degradation of the Hmg2p::Suc2::I-Iis4c fusion protein (with only the NH2-terminal half of the Hmg2p protein) is not affected by the raml::HIS3 mutation. Thus, perhaps there are determinants in the COOH-terminal part of the Hmg2p protein necessary for the sensitivity of degradation to farnesyl transferase activity. However, a
“Caax” box farnesylation site is unlikely to be such a determinant. Another possibility that must also be considered is that the Hmg2p::Suc2::His4c fusion protein is degraded by a different, but similar, degradation mechanism. The resolution of these differences between degradation substrates will be resolved by a genetic analysis of the underlying degradation machinery, as well as a molecular biological analysis of the cis determinants for regulated turnover of Hmg2p and related proteins.

Taken together, our data suggest that the stability of Hmg2p is controlled by both a prenylated protein and by a nonproteineaceous intermediate of the mevalonate pathway. The role of a prenylated protein in Hmg2p degradation is indicated by the experiments with the ram1::HIS3 strains. However, we believe that a farnesylated protein cannot be the only necessary condition of rapid degradation of Hmg2p. Rather, there also appears to be a requirement for a nonproteineaceous mevalonate product for rapid turnover of Hmg2p. This conclusion results from our observation that regulated degradation of Hmg2p occurred during treatment with the drug CHX, in the absence of protein synthesis. The reasoning for the existence of a second mevalonate-derived signal different from a prenylated protein is as follows: prenylation of proteins appears to be cotranslational and irreversible (Reptko and Maltese, 1989; Philips et al., 1993). Thus, a change in the degree of prenylation of a regulator protein would occur by continued synthesis of the protein in conditions of lowered prenyl group availability, resulting in an altered function of that protein. If a high degree of prenylation of the regulator protein were the only necessary condition for rapid Hmg2p degradation, then modulation of Hmg2p stability by lovastatin would require continued protein synthesis in order to allow the buildup of the unprenylated regulator. However, our experiments with CHX showed that lovastatin slowed the degradation of Hmg2p in the absence of protein synthesis, ruling out the simplest model in which a prenylated protein is the only measure of pathway flux. Instead, it appears that a nonproteineaceous mevalonate-derived molecule (or molecules) is a necessary determinant of pathway product abundance, and a farnesylated protein may be an ancillary factor for fully regulated degradation of native Hmg2p.

The Function of Two Reductase Isozymes

Only the Hmg2p isoyme was subjected to rapid, regulated degradation. Hmglp, in contrast, was extremely stable in all conditions tested. Other studies in our laboratory, however, indicate that the HMG1 gene is regulated by the mevalonate pathway as well, at the level of modulated translation (Dinster-Denk, D., M. Thorsness, and J. Rine, manuscript submitted for publication). Thus, our current view is that yeast, like the mammalian cell, controls the total activity of HMG-R at multiple biochemical levels. However, in contrast to mammals, there are two isozymes of reductase, and they are regulated differently. How these differences relate to the cellular physiology and biology of the yeast remain to be discovered by further studies. It is interesting to note that multiple, independently regulated isozymes of HMG-R appear to be common in plants (Choi et al., 1992; Chye et al., 1992), indicating that the evolutionary theme of HMG-R multiplicity is widespread.

Either HMG-R isozyme can supply the enzyme activity required for viability in S. cerevisiae (Basson et al., 1986). In fact, either catalytic domain, expressed without transmembrane sequences, can similarly provide the required catalytic activity (Hampton, R., and J. Rine, unpublished observations). These data raise the issue of why yeast would evolve two HMG-R isozymes. It would appear that the evolutionary selection for two isozymes may lie in the different regulatory functions imparted by the noncatalytic NH2-terminal regions of the two proteins. One role for the distinct regulation of Hmg2p may be in anaerobiosis. When oxygen availability decreases, the expression of HMG1 decreases and the expression of HMG2 increases (Thorsness et al., 1989; Casey et al., 1992). Anaerobic yeast cells may require tighter control over the mevalonate pathway, as would be provided by the regulated degradation of Hmg2p. This requirement for tighter control could lie in the inability of anaerobic cells to synthesize sterols, due to the lack of molecular oxygen necessary for sterol synthesis. In this circumstance there can be a buildup of early mevalonate pathway products, some of which can be growth inhibitory (Cuthbert and Lipsky, 1991). Thus, regulated degradation of Hmg2p, which we have shown responds to pathway signals before sterol synthesis, provides a natural way to measure and limit the production of early pathway products.

The two transmembrane regions of the yeast reductase isozymes display other differences in biological activity. Hmglp resides in the perinuclear ER membrane, whereas Hmg2p is found in the peripheral ER membrane (Wright et al., 1988). This difference in cellular localization may allow isoyme-specific compartmentalization of mevalonate production (Casey et al., 1992). Furthermore, the Hmglp transmembrane region can cause the proliferation of its resident membrane, forming the stacked membrane structures known as karmellae (Wright et al., 1988, 1990). Mammalian HMG-R also causes dramatic proliferation of its resident membrane (Chin et al., 1982), and thus the mammalian and yeast NH2-termini of HMG-R contain both degradative and membrane proliferative information. Whereas a single mammalian protein has the sequence information for both of these processes, it appears that the yeast has divided the information between the two isozymes. Studies are currently in progress to define the determinants of the NH2-termini responsible for the various behaviors of each yeast isozyme. The human enzyme, when expressed heterologously in yeast, retains both of these biological actions: karmellae are expressed (Wright et al., 1990) and the human protein is degraded rapidly in a vacuole-independent manner (Hampton, R., and J. Rine, unpublished observation).

A remarkable aspect of the similarity between the yeast and mammalian enzymes vis-a-vis regulated degradation is the apparent lack of any sequence similarity between the NH2 termini of these species. Thus, communication between the mevalonate pathway and ER degradation of HMG-R has been preserved between yeast and mammals in the face of no obvious sequence similarities between the relevant regions of the target proteins. Is there cryptic information in common between the human and yeast NH2 termini? Perhaps these regions have diverged to the extent that only key motifs have been preserved, which have so far escaped recognition. Alternatively, it is possible that similar tertiary structures, formed by the distinct primary sequences, determine this function. The availability of two very similar proteins with distinct degradative behavior (Hmglp and Hmg2p)
along with two very distinct proteins with similar degradative behavior (Hmg2p and human HMG-R) will allow molecular mapping experiments to resolve this intriguing question.

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