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 NH_2-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)[1] 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., 1983*a*,*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_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_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_2-terminal region

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1. *Abbreviations used in this paper*: CHX, cycloheximide; CPY, carboxypeptidase Y; GPD, 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.

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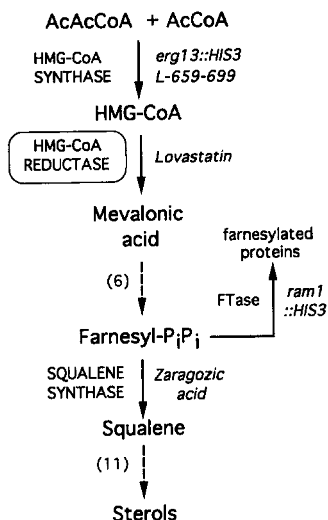

Figure 1. The mevalonate pathway. Only the enzymatic steps directly relevant to this work are depicted in the figure. The name of the enzyme responsible for a reaction is shown to the left of the arrow. The genetic or pharmacological block used to inhibit that step is shown in italics to the right of 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 (1990) and Parks (1978).

couples the mevalonate pathway to the ER degradation apparatus is entirely unclear. It is also not known how HMG-R degradation is selectively modulated in the presence of other proteins with unaltered stabilities.

The yeast *Saccharomyces cerevisiae* possesses two very similar isozymes of HMG-R, Hmg1p and Hmg2p, encoded by the *HMG1* and *HMG2* genes, respectively (Basson et al., 1988). Either isoform can fulfill the essential requirement for HMG-R activity (Basson et al., 1986). There are intriguing similarities between yeast and mammalian HMG-R. Each yeast enzyme is an integral ER membrane protein, with the same general sequence plan as the animal cell enzyme: a COOH-terminal catalytic domain connected by a linker domain to an NH_2-terminal hydrophobic region responsible for ER localization (Wright et al., 1988; Basson et al., 1988). The catalytic domains of the yeast isozymes are each very similar to the mammalian catalytic domain (65% sequence identity, 25% conservative replacements). Expression of either the human or hamster HMG-R gene can rescue otherwise inviable yeast *hmg1 hmg2* double mutants (Basson et al., 1988). Furthermore, mild overexpression (10-fold) of isozyme Hmg1p in yeast causes the proliferation of the membrane within which Hmg1p resides, resulting in the stacked perinuclear structures named karmellae (Wright et al., 1988). This effect of Hmg1p overexpression is analogous to the proliferation of ER membranes observed in mammalian cells that overexpress HMG-R due to pharmacological (Chin et al., 1982) or genetic (Wright et al., 1990) manipulations, or in some tissues that naturally produce abundant sterols (Sisson and Fahrenbach, 1967). This noncatalytic function has also been conserved between yeast and mammals. Yeast isozyme Hmg1p, upon expression in animal cells, can proliferate the ER, and human HMG-R, upon expression in yeast, can cause karmellae formation (Wright et al., 1990). However, clear differences exist between the yeast and animal cell versions of HMG-R. The NH_2-terminal of the yeast isozymes have been proposed to have seven transmembrane spans (Sengstag et al., 1990) whereas mammalian HMG-R appears to have eight (Olender and Simon, 1992; Roitelman et al., 1992). Furthermore, although the yeast NH_2-termini are similar to each other (>50% sequence identity) they bear no sequence resemblance to mammalian NH_2-terminal regions, which are conserved amongst themselves (Liscum et al., 1985; Luskey and Stevens, 1985) and some other metazoans (Chin et al., 1982; Woodward et al., 1988).

The similarities between yeast and animal cell HMG-R, along with indications that yeast may regulate HMG-R levels in response to changes in isoprenoid synthesis, led us to investigate the degradation of yeast HMG-R, and whether the stability of this enzyme was regulated. Our data indicated that *S. cerevisiae* offers unique opportunities to unravel mechanisms of mevalonate signalling in eukaryotic cells, as well as the enzymology and function of the ER protein degradation pathway.

Materials and Methods

Materials and Reagents

PMSF, leupeptin, pepstatin A, TPCK, cycloheximide, cysteine HCl, methionine, Tween 20, mevalonic acid lactone, and aprotinin were from Sigma Chemical (St. Louis, MO). [^{35}S]methionine- and [^{35}S]cysteine-containing Tran^{35}S-label™ was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). ECL™ chemiluminescence immunodetection reagents, [1-^{14}C] sodium acetate, and [^{125}I]-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 β-galactosidase–Hmg1p 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-Hmg1p-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 JRY527 (*HMG1 HMG2*) (Wright et al., 1988) was the parent for all strains in these experiments. Strain JRY1159 (*hmg1::LYS2 HMG2*) was derived from JRY527 by one step gene replacement of the *HMG1* gene with a *hmg1::LYS2* disruption fragment, and JRY1160 was similarly derived by disruption of the *HMG2* gene with an *hmg2::HIS3* fragment (Basson et al., 1986). Strain JRY1266, which expresses only *HMG2*, was made by transforming strain JRY1159 with a 2-μ, *URA3* plasmid containing a 4.9-kb yeast genomic fragment carrying the *HMG2* gene (pJR360 [Wright et al., 1988]). Strain JRY1596 (*hmg1::LYS2 HMG2*) with only a single genomic copy of *HMG2*, strain JRY1595 (*HMG1 hmg2::HIS3*), with only a single genomic copy of *HMG1*, and strain JRY1593 (*hmg1::LYS2 hmg2::HIS3*), with both HMG-R genes disrupted (and consequently a mevalonate auxotroph), were all obtained by sporulation of the diploid produced from the rare mating of the otherwise isogenic strains JRY1159 with JRY1160 (Basson et al., 1988). All haploid spores from this cross 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 JRY1595 (*HMG1 hmg2::HIS3*) with a 2-μ, *URA3* plasmid containing an ∼8-kb, *HMG1*-bearing yeast genomic fragment (Basson et al., 1986). Strain RHY46-4 (*hmg1::LYS2 HMG2 erg13::HIS3*) was made from JRY1596 (*hmg1::LYS2 HMG2*) by one step gene replacement (Rothstein, 1991) of the native *ERG13* (HMG-CoA synthase) gene with an *erg13::HIS3* disruption

Table I. Yeast Strains

Strain	Description
JRY1159	*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	*ram1::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 + pJR59 (*HMG1, URA3,* 2-μ YEp24 derivative)
JRY527	*HMG1 HMG2 ura3-52 his3Δ200 ade2-101 lys2-801 met*
RHY131-17	JRY527 + pCS4 (*HMG1-TMR::SUC2::HIS4c, URA3,* 2-μ YEp352 derivative)
RHY131-4	JRY527 + pCS17 (*HMG2-TMR::SUC2::HIS4c, URA3,* 2-μ YEp352 derivative)

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. 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 reselected 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 PrA activity by the APE color reaction (Jones, 1977), resulting in RWY60 (*pep4::HISG*). Strain RHY106-1 (*hmg1::LYS2 HMG2 pep4::HISG*) was derived from RWY60 by one step gene replacement of the *HMG1* locus with an *hmg1:LYS2* disruption fragment (Basson et al., 1986). Lys2$^+$ isolates were then screened for loss of Hmg1p expression using Hmg1p-specific anti-peptide antibodies by immunoblotting (see below). Strain RHY106-12 is strain RHY106-1 transformed with the 2-μ, *URA3*, *HMG2* plasmid pJR360. Strain RHY183 (*hmg1::LYS2 hmg2HIS3::URA3::pGPD-HMG2*), that only expressed the *HMG2* gene from the heterologous glyceraldehyde 3 phosphate dehydrogenase (GPD) promoter, was made from mevalonate auxotroph JRY1593 (*hmg1::LYS2 hmg2::HIS3*) by directed integration of a *URA3* plasmid containing the *HMG2*-coding region driven by the GPD promoter (pRH144-2) at the BamHI site in the *HMG2*-coding region of the *hmg2::HIS3* allele. After transformation of JRY1593 with BamHI-cut pRH144-2, Lys$^+$ His$^+$ Ura$^+$ mevalonate prototrophs were selected. One isolate was checked for correct integration by Southern blotting as well as cosegregation of the *URA3* and *HIS3* genes. Strain RHY223-1 (*hmg1::LYS2 hmg2::HIS3 ura3-52::pGPD-HMG1::URA3*), that expressed only the *HMG1* gene from the GPD promoter, was made in the same manner from JRY1593 by integration (at the *ura3-52* StuI site) of a *URA3* plasmid containing the *HMG1*-coding region driven by the GPD promoter (pRH105-25). Strain RHY131-4 and RHY131-17 were strain JRY527 (*HMG1 HMG2*) transformed with pCS4 or pCS17, respectively, and therefore expressed tripartite fusion proteins consisting of either the Hmg1p (pCS17) or Hmg2p (pCS4) transmembrane region fused to a Suc2-His4c fusion protein (Sengstag et al., 1990). RHY171, with a disruption of the *RAM1* C_{15} farnesyl transferase gene (Schafer et al., 1989), *ram1::HIS3*, was made in JRY1596 (*hmg1::LYS2 HMG2*) by one-step gene replacement of the *RAM1* allele with a *ram1::HIS3* fragment as described (Schafer et al., 1989), followed by transformation of an isolate with the 2-μ, *URA3*, *HMG2* plasmid pJR360. Strain RHY184-1 (*hmg1::LYS2 HMG2 sec18-1*), was made by replacing the wild type *SEC18* gene with the temperature-sensitive *sec18-1* allele by two step gene replacement, using the *URA3*, *sec18-1* plasmid pTSH1 as described (Eakle et al., 1988). The temperature sensitivity of the resulting strain, RHY184-1, can be corrected by introduction of the wild-type *SEC18* allele on a plasmid, and shows the expected defect (Graham and Emr, 1991) in the processing of carboxypeptidase Y (CPY) at the nonpermissive temperature (data not shown). RHY184-1 was then transformed with pJR360 to give strain RHY184.

All yeast strains were grown in yeast minimal medium with rotary aeration at 30°C unless otherwise indicated. Yeast minimal medium (YM): 2% glucose, 0.67% Bacto-yeast nitrogen base (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, XL1 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 phage for oligonucleotide-directed mutagenesis was an isolate of M13KO7 (Vieira and Messing, 1987).

Recombinant DNA Methods

pJR59 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 YIplac211 (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 pG-1 (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/SalI 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*, *pGPD-HMG2*), to allow expression of the *HMG2* reading frame from the GPD promoter. Analogous integrating plasmid pRH105-25, with the *HMG1* reading frame cloned into pRH98-2 for the GPD promoter-driven expression of *HMG1*, was made by cloning the 3.4-kb *HMG1* fragment (with no 5′ untranslated region) from pJR429 (Basson et al., 1988) into the SalI site of pRH98-2. pCS17 and pCS4, containing tripartite fusion genes consisting of either the *HMG1* (pCS4) or *HMG2* (pCS17) NH_2-terminal transmembrane region coding region fused inframe to a portion of the yeast invertase gene (*SUC2*) and a COOH-terminal fragment of histidinol dehydrogenase (*HIS4*) (see Fig. 11) were made as described (Sengstag et al., 1990).

Immunoprecipitation

The technique employed for immunoprecipitation of yeast proteins is similar to that described by Sengstag et al. (1990). Cells from logarithmic phase cultures (OD_{600} 0.5–1.1) grown in supplemented YM were resuspended in

fresh supplemented YM and pulse labeled with Tran^{35}S-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 1–4 OD_{600} U/ml, and then incubated at 30°C. Equal volume samples were removed at various times, and prepared for immunoprecipitation: cells were washed once with YM (without glucose) + 0.1% NaN_3 + 5 mM PMSF, and the cell pellet was overlayed with 75 μL of SUTE (1% SDS, 8 M 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 + PI: 15 mM NaH_2PO_4, pH 7.5, 150 mM NaCl, 2% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 0.02% NaN_3 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 10 mM Tris (pH 7.5), 50 mM NaCl, aspirated to dryness, overlaid with 30 μl of 2 × urea sample buffer (USB: 8 M 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 manufacturer 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% NaN_3/5 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 placed 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 electrophoretically transblotted (Burnette, 1981) onto two stacked pieces of nitrocellulose. The front piece (touching the gel) was used for the immunoblot: immediately after transblotting, the front nitrocellulose was placed in TBS (10 mM Tris, pH 8.0, 150 mM NaCl) with 0.05% Tween 20, 0.1% NaN_3, 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 blots were exposed on Kodak X-Omat AR film. In figure 8 c, [^{125}I]-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 −70°C. The second (back) piece of nitrocellulose was stained for total protein with India ink by swirling the blot in TBST buffer with ink (100 μl/100 ml).

The above immunoblotting procedure was used to study the stability of the entire HMG-R pool by measuring the level of HMG-R immunoreactivity at various times after blocking protein synthesis with cycloheximide (CHX). This procedure is called a cycloheximide chase and was performed as follows: a culture of logarithmic phase cells (OD_{600} 0.5–1.0) were resuspended in fresh, supplemented YM medium, to a cell density of ∼0.5 OD_{600} U/ml, and the cultures were incubated in a shaking water bath at 30°C for 15–30 min before initiating the experiment. The decay period was begun by addition of CHX to a final concentration of 50 μg/ml, along with indicated drugs. The CHX-treated cultures were then further incubated at 30°C, and samples of equal volume (usually ∼3–4 ml) were withdrawn at various times for analysis by immunoblotting as described.

SDS-PAGE

SDS-PAGE was accomplished with a Hoefer Mighty Small® (Hoefer Sci. Instrs., San Francisco, CA) electrophoresis apparatus, using a procedure similar to that described (Laemmli et al., 1970). The lower running gel was a 6%/0.16% acrylamide/bisacrylamide mix, with 0.38 M Tris base, pH 8.8, and 0.1% SDS. The upper stacking gel was a 4%/0.11% acrylamide/Bisacrylamide mix with 0.125 M Tris base, pH 6.8, and 0.1% SDS. Gels were 1.5-mm thick, and were run at 20–30 mA for 2–4 h.

Measurement of Sterol Synthesis

Sterol synthesis was measured by the incorporation of [1-^{14}C]acetate into ergosterol as described (Parks et al., 1985). Labeling was initiated by addition of 20 μCi [1-^{14}C]acetate (sodium salt) to cells suspensions (∼2 OD_{600} U in 1–2 ml). Labeling was allowed to proceed at 30°C. Labeled cells were then pelleted, resuspended in 0.5 ml 0.1 M HCl, and steamed for 20 min at 95°C. Cells were then washed twice with distilled H_2O, and resuspended in 67 μl MeOH and 33 μl H_2O. 100 μl of glass beads were then added to the suspension and the mixture was lysed by vortexing for 3 min. Next, 1 ml of MeOH and 0.5 ml 60% (wt/vol) aqueous KOH was added directly to the lysed cell–bead slurry, and the mixture was refluxed at ∼70°C for 2 h in order to saponify the lipids. The saponified mixture was extracted with 3 × 1 ml hexane. The hexane phase was evaporated under a stream of N_2 (g) and the resultant lipids were resuspended in a small volume of hexane and applied to a Silica gel 60A TLC plate, with fluorescence indicator (Whattman Int. Ltd., Maidstone, England). The plate was developed in benzene/ethyl acetate (5:1), dried and subjected to both autoradiography and phosphorimager 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

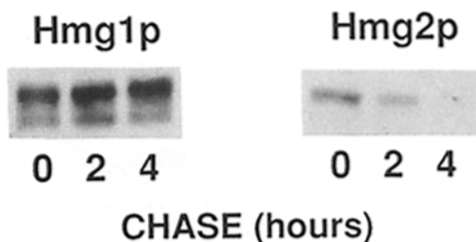

Figure 2. Different stabilities of the two yeast HMG-R isozymes, Hmglp, and Hmg2p. The stability of HMG-R in strains expressing only Hmglp (RHY194) or only Hmg2p (JRY1266) was measured by the pulse–chase and immunoprecipitation protocols described in Materials and Methods. The samples from strain RHY194 (*Hmglp*) are shown in the left set and those from strain JRY1266 (*Hmg2p*) are shown in the right set.

Hmg1p (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 Hmg1p-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 Hmg1p isozyme was quite stable, with essentially no degradation in a 4 h experiment. Hmg1p 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 *pep4*Δ strain, confirming the presence of the *pep4*Δ 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 *sec18-1* 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 *sec18-1* 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 *sec18-1* mutation. By this criterion, the secretory pathway was not required of the degradation of Hmg2p. The efficacy of the trafficking block in the *sec18-1* 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 *sec18-1* 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

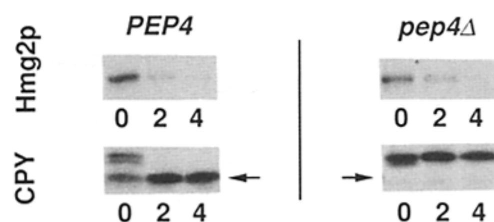

Figure 3. Vacuolar proteases were not required for Hmg2p degradation. The stability of Hmg2p in a *PEP4* strain (JRY1266, *left*) and an isogenic *pep4::hisG* strain (RHY 106-12, *right*) was compared by pulse–chase and immunoprecipitation analysis. The results of the Hmg2p immunoprecipitations are shown for each strain at the indicated chase times in the upper part of each panel (*Hmg2p*). As a control, the lysates from the Hmg2p immunoprecipitations (collected after the first centrifugation of protein A–Sepharose) were subjected to a second round of immunoprecipitation with an antiserum against carboxypeptidase Y (*CPY*, PrC) in order to insure that the *pep4::hisG* strain (RHY106-12) was indeed deficient in *PEP4* function, as reflected in the inability to produce the mature vacuolar form (*arrow*) of *CPY*.

Figure 4. *SEC18* gene function was not required for the degradation of the Hmg2p. The degradation of Hmg2p in a *SEC18* strain (JRY1266, *left*) and an isogenic *sec18-1* strain (RHY184, *right*) were compared in a pulse–chase experiment. The function of the *sec18-1* gene product is lost in less than 5 min (Graham and Emr, 1991) upon shift to 37°C, but is normally functional at 23°C. Cells of either strain were grown and pulse labeled at 23°C. The chase period was conducted at 37°C.

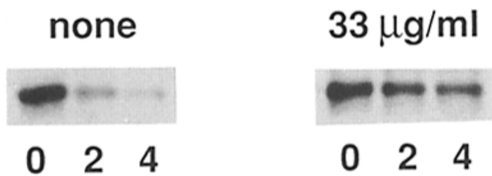

Figure 5. Lovastatin treatment stabilized Hmg2p. The stability of Hmg2p was assessed in the presence (*right*) or absence (*left*) of the HMG-R inhibitor lovastatin. Cells expressing only Hmg2p (JRY1266) were pulse labeled in the presence or absence of 43 μg/ml lovastatin. The chase period was then initiated by addition of cys-HCl/met (0.25 M/0.25 M) stock solution to the labeling mixture, resulting in a final chase period concentration of lovastatin of 33 μg/ml in the treated culture. This experiment was run along with the experiment depicted in Fig. 3, and the no lovastatin panel (*left*) is a different autoradiographic exposure of the left panel (*PEP4*) in Fig. 3.

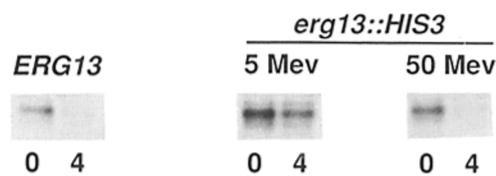

Figure 6. Mevalonate control of Hmg2p isozyme stability. In order to examine the effect of mevalonic acid levels on Hmg2p stability, a strain that expressed only Hmg2p and had a disruption of the HMG-CoA synthase gene (*ergl3::HIS3*, RHY46-42, mevalonate auxotroph), was subjected to pulse-chase analysis in the presence of high (50 mg/ml) or low (5 mg/ml) mevalonic acid lactone (*Mev*). *ergl3::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 *ERGl3* strain (JRY1266) was subjected to the same protocol, with the chase conducted at 5 mg/ml Mev (*left*, *ERGl3*).

nonpermissive temperature for the time period of the protocol.

The independence of Hmg2p degradation from vacuolar function or secretory traffic indicated the existence of an ER membrane protein degradation pathway in yeast that was similar to that described in mammals (Klausner and Sitia, 1990; Bonifacino and Lippincott, 1991).

Degradation of Hmg2p Was Regulated by Signals from the Mevalonate Pathways

In mammalian cells the degradation of HMG-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 by drugs (Edwards et al., 1983) or mutations (Panini et al., 1992) results in stabilization of HMG-R, contributing to an increased steady state level of enzyme. Since the Hmg2p protein was rapidly degraded in a fashion similar to the mammalian protein, we examined if the stability of Hmg2p was subject to feedback regulation.

If the rapid degradation of Hmg2p in the previous experiments depended upon the level of HMG-R activity, then decreased activity would be expected to result in stabilization of Hmg2p. Therefore we used a competitive inhibitor of HMG-R (lovastatin) to determine whether decreasing the flux through the mevalonate pathway had any effect on degradation of Hmg2p. In cells treated with lovastatin the half-life of Hmg2p was drastically increased, from ~1 h to greater than 4 h (Fig. 5). The same results were obtained if the lovastatin is added to the cells at the beginning of the chase period (e.g., Fig. 7, 8, and 9) with no preincubation. Thus, the effect of lovastatin was rapid. The concentration of lovastatin used in these experiments had no effect on the viability of the cells during the time of the experiment, and was 10-fold below that needed to cause overt cytotoxicity. These results established that inhibition of HMG-R activity resulted in 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 in the yeast gene (*ERGl3*) that encodes HMG-CoA synthase (RHY46-42). This enzyme provides HMG-R with its substrate HMG-CoA (Fig. 1). Consequently *ergl3::HIS3* strains are totally dependent on exogenous mevalonate for growth. By altering the exogenous concentration of mevalonate, the flux through the mevalonate pathway can be modulated without recourse to drugs that bind HMG-R. The *ergl3::HIS3* strain was grown in a high concentration of mevalonic acid (50 mg/ml) for several days, pulse labeled, and then chased in either 50 or 5 mg/ml mevalonic acid. For comparison, the otherwise isogenic wild-type *ERGl3* strain (JRY1266) was chased in the presence of 5 mg/ml mevalonate as well (Fig. 6). The degradation of Hmg2p in the *ergl3::HIS3* strain (RHY46-42) in the high concentration of mevalonate was similar to that of the isogenic *ERGl3* strain (Fig. 6, *right* versus *left*). In contrast, the *ergl3::HIS3* cells in a low concentration of mevalonate exhibited markedly increased stability of Hmg2p (*middle*). These results established that mevalonate or a molecule derived from mevalonate was used to regulate HMG-R degradation in yeast. Stabilization of Hmg2p was similarly observed in wild-type cells that were treated with an inhibitor of HMG-CoA synthase (L-659,699) (Greenspan et al., 1987), at the time of chase (data not shown). Thus, three separate methods of reducing flux through the mevalonate pathway all resulted in marked stabilization of yeast Hmg2p.

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-

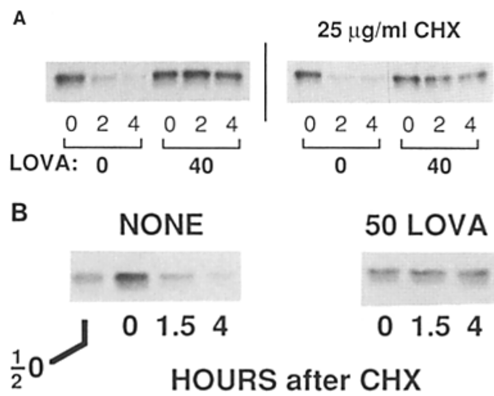

Figure 7. Protein synthesis was not required for regulated degradation of the Hmg2p. (*A*) Effect of lovastatin on the degradation of Hmg2p was examined by pulse-chase analysis in the presence (*right*) or absence (*left*) of 25 µg/ml CHX. Hmg2p-expressing cells (JRY1266) were pulse labeled and then 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).

creased 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, nor 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

Figure 8. Regulated degradation of Hmg2p expressed from an unregulated promoter. (*A*) A strain that expressed the *HMG2* gene exclusively from an integrated copy of the *HMG2* gene under the control of the GPD promoter (RHY183; *hmg1::LYS2 hmg2::HIS3::URA3::pGPD-HMG2*), was analyzed for Hmg2p stability by pulse-chase experiment. (*B*) The effect of lovastatin on the stability of the entire pool of Hmg2p in RHY183 was measured by cycloheximide chase. The procedure used was identical to that described in Fig. 7 *B*. (*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 [^{125}I]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 actu-

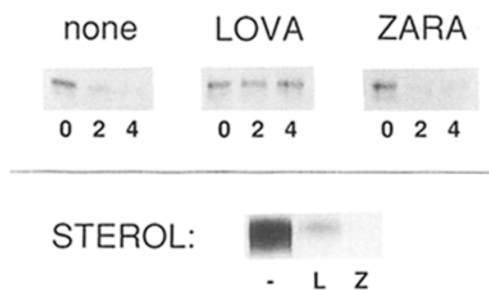

Figure 9. Effect of mevalonate pathway inhibitors on the degradation of Hmg2p. Cultures (8 OD U in 6 ml) of Hmg2p-expressing cells (JRY1266) were incubated for 15 min at 30°C with no drug (*none*), 62.5 μg/ml lovastatin (*LOVA*), or 62.5 μg/ml zaragozic acid (*ZARA*) in supplemented YM medium. 1.5-ml of each culture were then removed to separate tubes for labeling of sterols with [1-^{14}C] acetate as described in Materials and Methods. The remainder of each culture was subjected to pulse-chase analysis as above. The acetate-labeled cultures also received chase medium in the same manner as the pulse-chase cultures, so that in both experiments the final drug concentrations were 50 μg/ml, and all incubations occurred in the presence of excess methionine and cysteine. The upper panel depicts the results of the pulse-chase experiment. The numbers under each lane are the hours after the beginning of the chase period. The lower panel shows the results of the parallel [^{14}C]acetate-labeling experiment. The autoradiograph of the ergosterol band (*STEROL*) from the TLC plate is shown for the cultures with no drug (−), lovastatin (*L*), and zaragozic acid (*Z*). The inhibition of sterol labeling compared to the no drug control was 85% for lovastatin and 98% for zaragozic acid, as measured by phosphorimager analysis.

ally 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 were used to monitor flux through the mevalonate pathway for regulating Hmg2p degradation.

A Prenylated Protein May Be Involved in the Degradation of Hmg2p

The mevalonate pathway provides the cell with prenyl groups that are covalently added to a variety of proteins (reviewed in Schafer and Rine, 1992; Clarke, 1992). These modifications are required for the function of a variety of proteins including those that can function as signaling molecules or modulators of membrane traffic. If a prenylated protein were required for rapid degradation of Hmg2p, then 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 *RAM1* gene (*ram1::HIS3*), which encodes the β subunit of the yeast farnesyl transferase (Schafer et al., 1989; Schafer and Rine, 1992). Strains with the *ram1::HIS3* allele are deficient in protein farnesylation (Schafer et al., 1990). The cells are temperature

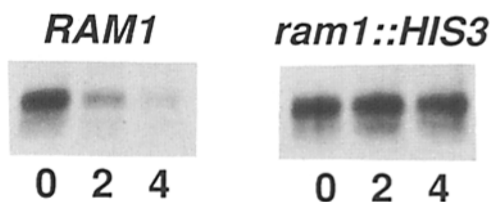

Figure 10. Effect of a disruption of the *RAM1* farnesyltransferase gene on the stability of Hmg2p. A strain expressing only Hmg2p and wild-type for the *RAM1* gene (JRY1266) and an isogenic strain with the *ram1::HIS3* disruption (RHY171) were compared for Hmg2p stability by pulse–chase analysis. Both 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.

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 the *ram1::HIS3* (RHY171) and an isogenic *RAM1* strain (JRY1266) by a pulse–chase experiment. *RAM1* (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 (*RAM1* ∼1 h, *ram1::HIS3* ∼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 NH_2-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 fates were determined by the distinct noncatalytic NH_2-terminal regions of each protein. In order to test in isolation the role of the NH_2-terminal 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* NH_2-terminal transmembrane region (*left*) was, like the parent protein, quite stable. In contrast, the transmembrane region from *HMG2* imparted rapid degradation to an other-

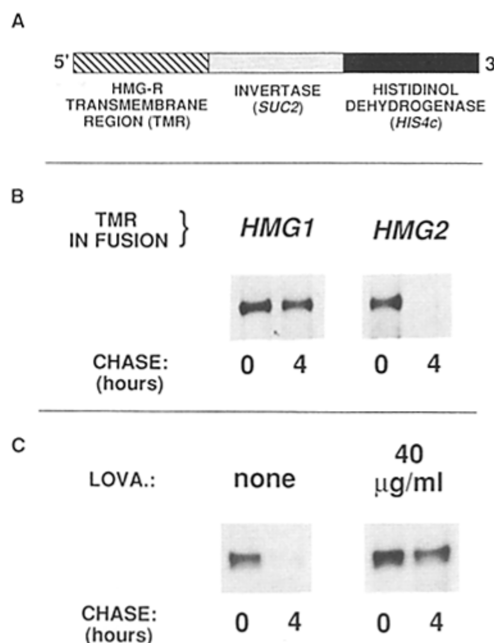

Figure 11. The noncatalytic NH_2-terminal regions of the yeast HMG-R determine stability. (*A*) Schematic of the fusion gene construct used to assess the role of the NH_2-terminal transmembrane region (*TMR*) in the stability of a given isozyme. The fusion gene coding region is depicted. Either the *HMG1* 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-μ 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 μg/ml final concentration of lovastatin present in the chase medium.

wise 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 μg/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

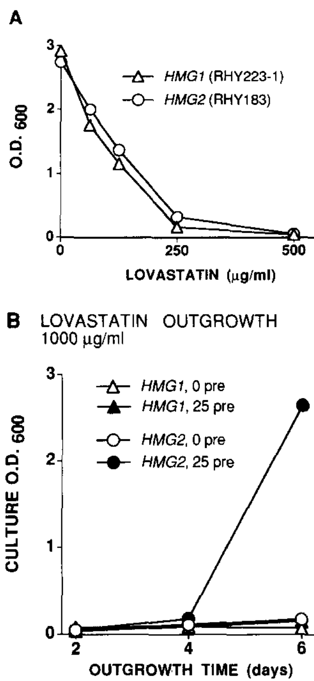

Figure 12. The effect of lovastatin preincubation of Hmglp- or Hmg2p-expressing cells on subsequent survival in a high concentration of lovastatin. Strains expressing only *HMGl* or *HMG2*, from the GPD promoter (RHY223-1 or RHY183, respectively), were compared in studies of sensitivity to lovastatin. 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} ~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 of lovastatin on subsequent outgrowth at a high dose. The same two strains were grown in supplemented YM containing 0 (*open symbols*) or 25 μg/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 ~0.05. As expected from the growth curve in *A*, the effect of the 25 μg/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 μg/ml lovastatin and incubated at 30°C. At the indicated times the OD_{600} of each culture was measured.

of half-life regulation from the contributions of altered gene expression to the physiological effects, we used strains in which the relevant isozyme was expressed from the unregulated GPD promoter. Strains were constructed that had either the *HMGl*-coding region (integrated at the *ura3-52* locus; RHY223-1) or *HMG2*-coding region (integrated at the *hmg2::HIS3* locus; RHY183) under control of the GPD promoter. In these two strains the steady state levels of HMG-R as directly measured by enzyme assay (data not shown) or as reflected in the IC_{50} for growth inhibition by lovastatin (Fig. 12 *a*) were quite similar. The reason for the similarity in HMG-R levels, considering that Hmglp isozyme is stable and the Hmg2p isozyme is unstable, has not yet been explored and may simply reflect better expression of *HMG2* from this promoter in the integrated locus. The important point is that the similarity in enzyme levels allowed meaningful comparison of the physiology of these two strains.

Since Hmg2p was increased by pretreatment with low doses of lovastatin (Fig. 8), one might expect, by analogy to the heat shock paradigm (Nicolet and Craig, 1991), that such a pretreatment would render cells better able to grow out from a high dose of the drug by virtue of having elevated levels of HMG-R. If the enhanced resistance involved suppressing degradation of Hmg2p, then the effect should be specific for that isozyme, because Hmglp degradation is unaffected by lovastatin. The strains expressing only Hmglp (RHY223-1; *pGPD-HMGl*) or Hmg2p (RHY183; *pGPD-HMG2*) were grown in 0 or 25 μg/ml lovastatin for 12 h. The 25 μg/ml pretreatment dose was a small one, and had equal, slight effects on the growth of cells of either strain (Fig. 12 *a*). The pretreated cells were then transferred to a high concentration of lovastatin (1,000 μg/ml), and monitored for outgrowth (Fig. 12 *b*). The cells that expressed Hmg2p showed a marked effect of lovastatin pretreatment on the ability to grow out at the high concentration (*open circles* versus *closed circles*). In contrast, cells that expressed Hmglp failed to show any enhancement of outgrowth rate caused by lovastatin pretreatment (*open triangles* versus *closed triangles*). It is important to stress that the initial response to lovastatin, reflected in a minor (~25%) slowing of growth during the 12 h pretreatment, was identical in the two strains. Only the subsequent response to the second, high concentration of lovastatin distinguished the two strains. Thus, the two isozymes could impart easily observable differences in cell physiology, presumably due to differences in the feedback regulation of protein stability.

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 isozymes of yeast had distinctly different posttranslational 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 Pep4p 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 (Pep4p), 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 lysosome.

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 zaragozic 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 *ram1::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 *ram1::HIS3* mutation with caution. In cells grown at 23°C (the permissive temperature for *ram1* strains) the steady state level of the Hmg2p in the *ram1::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 *ram1::HIS3* strain gradually dropped to that of the *RAM1* strain. Nevertheless, the degradation rate of the Hmg2p remained slow in the *ram1::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 *ram1::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 known 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::His4c fusion protein (with only the NH_2-terminal half of the Hmg2p protein) is not affected by the *ram1::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 nonproteinaceous intermediate of the mevalonate pathway. The role of a prenylated protein in Hmg2p degradation is indicated by the experiments with the *raml::HIS3* strains. However, we believe that a farnesylated protein can not be the only necessary condition of rapid degradation of Hmg2p. Rather, there also appears to be a requirement for a nonproteinaceous 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 (Repko 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 nonproteinaceous mevalonate-derived molecule (or molecules) is a neccessary 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 isozyme 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 *HMGl* gene is regulated by the mevalonate pathway as well, at the level of modulated translation (Dimster-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 NH_2-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 *HMGl* 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 isozyme-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 NH_2-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 NH_2-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 NH_2 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 NH_2 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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