THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 44, Issue of October 29, pp. 31671–31678, 1999

Printed in U.S.A.

A Highly Conserved Signal Controls Degradation of 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) Reductase in Eukaryotes*

(Received for publication, July 15, 1999, and in revised form, August 16, 1999)

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Sterol synthesis by the mevalonate pathway is modulated, in part, through feedback-regulated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR). In both mammals and yeast, a non-sterol isoprenoid signal positively regulates the rate of HMGR degradation. To define more precisely the molecule that serves as the source of this signal, we have conducted both pharmacological and genetic manipulations of the mevalonate pathway in yeast. We now demonstrate that farnesyl diphosphate (FPP) is the source of the positive signal for Hmg2p degradation in yeast. This FPP-derived signal does not act by altering the endoplasmic reticulum degradation machinery in general. Rather, the FPP-derived signal specifically modulates Hmg2p stability. In mammalian cells, an FPP-derived molecule also serves as a positive signal for HMGR degradation. Thus, both yeast and mammalian cells employ the same strategy for regulation of HMGR degradation, perhaps by conserved molecular processes.

The mevalonate pathway is responsible for the biosynthesis of numerous essential molecules including prenyl groups, coenzyme Q, dolichol, and sterols such as cholesterol (1). 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR)[1] is a key enzyme of the pathway and is rate-determining for cholesterol synthesis in mammals (2, 3). The mevalonate pathway is modulated in large part by feedback control of the amount of HMGR protein (1), and a significant portion of HMGR feedback control occurs through regulation of HMGR degradation (4–6). HMGR is an integral endoplasmic reticulum (ER) membrane protein, and its degradation occurs without exit from the ER (6–8). The non-catalytic, N-terminal transmembrane anchor of HMGR is both necessary and sufficient for regulated degradation (6, 8–10). When there is abundant synthesis of pathway products, HMGR degradation is fast, and steady-state levels of the protein tend to be low. Conversely, when synthesis of pathway products is reduced, for instance when a patient is given the HMGR inhibitor lovastatin, HMGR degradation is slowed, and steady-state levels of the protein tend to increase. However, neither the identity of the mevalonate-derived signal nor the mechanism by which this signal is coupled to HMGR degradation is known.

In order to discover and understand the mechanisms of HMGR-regulated degradation, we have been studying the process in the yeast *Saccharomyces cerevisiae* (6, 11, 12). Our earlier work has shown that the yeast HMGR isozyme Hmg2p is degraded in a regulated manner with many similarities to the analogous process in mammals. Through the use of genetic selections and screens, we have been able to identify genes required for the degradation of Hmg2p, called *HRD* genes (11), and genes required for normal regulation of Hmg2p degradation.[2]

Concurrently with these screens, we have been studying the nature of the mevalonate-derived signals that control Hmg2p stability. Hmg2p degradation is regulated by an unknown signal from the mevalonate pathway (Fig. 1*a*). Inhibiting early pathway enzymes, such as HMGR itself or HMG-CoA synthase, decreases the rate of Hmg2p degradation (6). These early pathway blocks decrease the availability of a downstream signal for degradation. Conversely, inhibiting the enzyme squalene synthase, which is downstream of HMGR, stimulates degradation and ubiquitination of Hmg2p (12). Furthermore, the degradation-enhancing effect of squalene synthase inhibition is abolished by simultaneous inhibition of HMG-CoA synthase or HMGR.

These pharmacological studies imply that the signal for Hmg2p degradation is a pathway molecule between mevalonate and squalene (Fig. 1*a*). The most reasonable candidate for this signal is farnesyl diphosphate (FPP) or an off-pathway FPP derivative. The idea that FPP, or a derivative, is a positive signal for Hmg2p degradation is particularly interesting since there is accumulating evidence from *in vitro* and *in vivo* studies that farnesol, an FPP-derivative, is a signal for regulation of mammalian HMGR stability (13–17).

We have now tested the hypothesis that FPP provides a molecular signal for control of Hmg2p stability using unique genetic opportunities available in yeast. In conjunction with pharmacological and biochemical approaches, we have constructed yeast strains that allowed either overexpression or down-regulation of specific mevalonate pathway genes (Fig. 1*b*). Our results indicated that FPP was indeed a source of a signal for Hmg2p degradation in yeast, demonstrating that there is striking conservation for this mode of HMGR regulation among eukaryotes.

EXPERIMENTAL PROCEDURES

Materials and Reagents—All enzymes were obtained from New England Biolabs (Beverly, MA). Chemical reagents were obtained from Sigma. Lovastatin and zaragozic acid were generously donated by Merck. Terbinafine was commercially obtained as a 1% Lamisil® solution from Novartis (East Hanover, NJ). ECL™ chemiluminescence immunodetection reagents were from Amersham Pharmacia Biotech. The anti-Myc 9E10 antibody was used as a cell culture supernatant

* This work was supported by National Institutes of Health Grant DK5199601 (to R. Y. H.) and a Searle scholarship (to R. Y. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; FPP, farnesyl diphosphate; ER, endoplasmic reticulum; ZA, zaragozic acid; Lov, lovastatin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; kb, kilobase pair; HSV, herpes simplex virus; FACS, fluorescence-activated cell sorter; wt, wild type; HA, hemagglutinin; GFP, green fluorescent protein.

[2] S. Cronin and R. Hampton, manuscript in preparation.

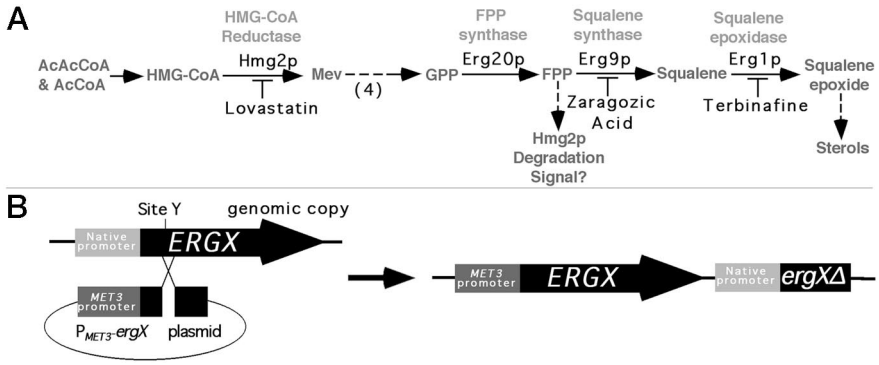

FIG. 1. **Manipulation of the mevalonate pathway.** *A,* representation of the mevalonate (*Mev*) pathway indicating key enzymes and their inhibitors. *B*, construction of a *MET3* promoter-regulated allele by "promoter switching." A mevalonate pathway gene, *ERGX*, was placed under control of the *MET3* promoter through targeted integration of a promoter-switch plasmid at the *ERGX* genomic locus. The plasmid consisted of a non-functional, 5′ portion of the *ERGX* gene placed behind the *MET3* promoter (P_{MET3}). Integration resulted in a functional copy of the *ERGX* gene placed under control of the *MET3* promoter and a non-functional, deleted copy behind the native promoter.

obtained by growing the 9E10 hybridoma (ATCC CRL 1729) in RPMI 1640 culture medium (Life Technologies, Inc.) with 10% fetal calf serum. The anti-HA antibody was an ascites fluid obtained from Babco (Berkeley, CA). The anti-HSV-Tag antibody was obtained from Novagen (Madison, WI). Affinity-purified goat anti-mouse horseradish peroxidase-conjugated antiserum was obtained from Sigma.

Recombinant DNA and Molecular Cloning—PCR was performed as described previously (18). The genes encoding squalene synthase (*ERG9*), farnesyl-diphosphate synthase (*ERG20*), and squalene epoxidase (*ERG1*) were PCR-amplified from yeast strain RHY623 genomic DNA (18), isolated by the Winston method (19), using separate primers that contained *Pst*I and *Bam*HI sites in the upstream primers and *Nhe*I and *Sal*I sites in the downstream primers. The amplified *ERG9* and *ERG20* genes were cloned between the *Pst*I and *Sal*I sites in pRH423 (12), thereby placed under control of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) promoter (P_{GAPDH}) (20). The squalene synthase-containing plasmid was named pRH440, and the farnesyl-diphosphate synthase-containing plasmid was named pRH830.

Squalene synthase (*ERG9*) was tagged at the C terminus with the HSV epitope sequence (21). The plasmid containing an HSV-tagged squalene synthase expressed from the GAPDH promoter was made by annealing primers that encoded the HSV epitope sequence (QPELAPEDPED) and cloning the resulting DNA fragment between the *Nhe*I and *Sal*I sites in pRH440 to yield pRH442 (*ERG9*-HSV). The plasmid to tag *ERG9* at its genomic locus with HSV was made by digesting pRH442 with *Mun*I, and the 5.4-kb vector fragment was reclosed to yield pRH885. The remaining portion of *erg9* included codons 208–446 and the 3′ HSV sequence.

Plasmids that allowed expression of the genomic copy of either *ERG9*, *ERG20,* or *ERG1* from the *MET3* promoter (P_{MET3}) (22, 23) were constructed as follows: pRH442 was digested with *Eco*RI, and pRH448 was digested with *Kpn*I. Each vector was reclosed with the insert removed. The *erg9* vector was named pRH948, and the *erg20* vector was named pRH950. The *MET3* promoter was cloned into pRH948 and pRH950 by digesting each plasmid with *Ssp*I and *Pst*I and replacing the insert with the *MET3* promoter containing *Ssp*I-*Pst*I fragment from pHAM8 obtained from Dr. Harry Mountain (Staffordshire, UK). The P_{MET3}-*erg9* plasmid was named pRH973 and the P_{MET3}-*erg20* plasmid was named pRH975. A PCR product containing the *ERG1* coding region was digested with *Bam*HI and *Pvu*II. The 840-base pair fragment was then cloned between the *Bam*HI and *Hpa*I sites in pRH973, and the resulting plasmid was named pRH1204.

The plasmid to delete *HRD1* was constructed as follows. A 1.45-kb *Bgl*II-*Xho*I fragment from pUG6 (24), which contained the *kanMX* expression module (25), replaced the 1.43-kb *Bgl*II-*Sal*I fragment in pRH507, which contained the *HRD1* gene (11). The resulting plasmid was named pRH1122.

Strains and Media—Escherichia coli DH5α strains were grown at 37 °C in LB + amp (100 μg/ml). Yeast strains were grown at 30 °C in minimal medium supplemented with glucose and the appropriate amino acids, as described (6). The lithium acetate method was used to transform yeast with plasmid DNA (26).

Yeast strain RHY871 (*a his3Δ200 lys2-801 ade2-101 leu2 ura3-52::LEU2::hmg2-GFP met2 hmg1::LYS2 hmg2::HIS3::1MYC-HMG2*) was the parent strain for transformation of plasmids containing GAPDH-expressed mevalonate pathway genes. RHY871 co-expressed 1Myc-Hmg2p (12) and the autofluorescent Hmg2p-GFP (27). Each integrating plasmid was introduced by targeted integration at the *Stu*I site of the *ura3-52* genomic locus. Yeast transformants were selected for Ura^+ prototrophy.

Yeast strain RHY1326 (*a his3Δ200 lys2-801 ade2-101 leu2 ura3-52::LEU2::hmg2-GFP MET2 hmg1::LYS2 hmg2::HIS3::1MYC-HMG2*) was made by transforming RHY871 with a functional *MET2* gene fragment from pGMET (28), followed by selection for Met^+ prototrophy. RHY1326 and RHY1462 (*a his3Δ200 lys2-801 ade2-101 leu2::6myc-hmg2-GFP::LEU2 ura3-52::6MYC-HMG2 MET2 hmg1::LYS2 hmg2::HIS3*) were the parent strains for transformation of all plasmids containing P_{MET3}-expressed mevalonate pathway genes. Plasmid pRH973 (P_{MET3}-*erg9*) was introduced by targeted integration at the *Hpa*I site of *ERG9*. Plasmid pRH975 (P_{MET3}-*erg20*) was introduced by targeted integration at the *Hin*dIII site of *ERG20*. Plasmid pRH1204 (P_{MET3}-*erg1*) was introduced by targeted integration at the *Age*I site of *ERG1*. Yeast transformants were selected for Ura^+ prototrophy.

Biochemical Assays—Cycloheximide chase assays and log phase steady-state assays were performed as described previously (18).

Methionine chase assays were performed as follows. Cells were grown to early log phase with an absorbance (A_{600}) of 0.01. Methionine was added to a final concentration of 2 mM, and the cells grown at 30 °C for 15 h. Cells were then either used for the cycloheximide chase assay as described above or for the FACS analysis described below.

Hmg2p ubiquitination assays were performed as described previously.[3] Strains were transformed with pRH1100,[3] which expressed a triple HA epitope-tagged ubiquitin from the constitutive GAPDH promoter. Transformants were selected for Ade^+ prototrophy. Hmg2p ubiquitination was assayed by immunoprecipitation of 1Myc-Hmg2p and then immunoblotting the precipitate for covalently linked HA-ubiquitin.

Flow Cytometry (FACS Analysis)—FACS analysis was performed as described previously (18). Living cells were analyzed by flow microfluorimetry using a FACScalibur™ (Becton Dickinson, Palo Alto, CA) flow microfluorimeter with settings for fluorescein-labeled antibody analysis. Histograms were produced from 10,000 individual cells and were plotted with log fluorescence (arbitrary units) on the horizontal axis and cell number on the vertical axis.

RESULTS

Zaragozic Acid Increased Hmg2p Degradation by Squalene Synthase Inhibition—We previously discovered that addition of zaragozic acid (ZA), a potent inhibitor of squalene synthase (30) (Fig. 1*a*), to yeast cells stimulated degradation of Hmg2p (12). Squalene synthase inhibition would be expected to cause a build-up of farnesyl diphosphate (FPP), the substrate of the enzyme, implicating FPP as a source of the positive signal for Hmg2p degradation. However, it was possible that the effect of ZA was the result of some action of the drug. We addressed this by testing the effect of ZA on Hmg2p degradation in a strain that overexpressed squalene synthase. If ZA was inducing Hmg2p degradation through squalene synthase inhibition, then cells expressing increased levels of squalene synthase should require increased amounts of ZA to cause the same degree of degradation.

We placed the squalene synthase gene (*ERG9*) under control of the constitutive GAPDH promoter, P_{GAPDH} (20), and transformed the P_{GAPDH}-*ERG9* construct into yeast cells. P_{GAPDH}-*ERG9* was present as a single integrated copy through targeted insertion of an integrating vector. The strain expressing a

[3] Gardner, R. G., and Hampton, R. Y. (1999) *EMBO J.,* in press.

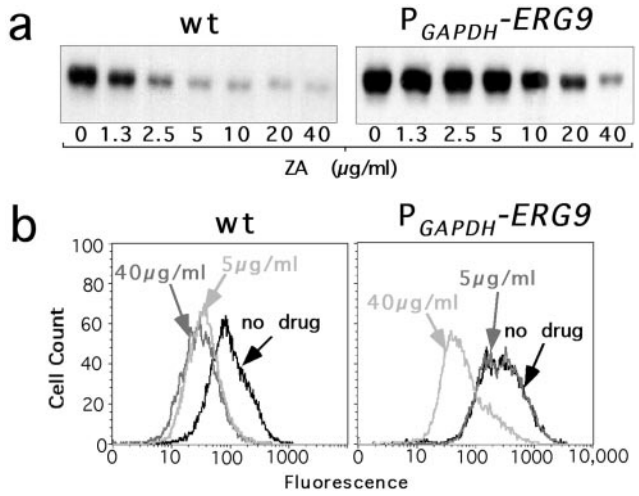

FIG. 2. **Squalene synthase overexpression increased the concentration of ZA required for Hmg2p degradation.** *a*, effect of ZA, which hastens Hmg2p degradation, on 1Myc-Hmg2p steady-state levels in a wild-type strain (*wt*) or in an otherwise isogenic strain carrying a single integrant of the P_{GAPDH}-*ERG9* allele (P_{GAPDH}-*ERG9*). Strains were treated for 4 h with the indicated concentrations of ZA and then subjected to immunoblotting to determine 1Myc-Hmg2p steady-state levels. In all figures, 1Myc-Hmg2p immunoreactivity was detected with the anti-Myc 9E10 antibody. *b*, effect of ZA on Hmg2p-GFP steady-state levels in a wild-type strain (*wt*) or in the strain carrying a single integrant of the P_{GAPDH}-*ERG9* allele (P_{GAPDH}-*ERG9*). Cells were grown 4 h in the presence of the indicated concentrations of ZA and then analyzed by flow cytometry.

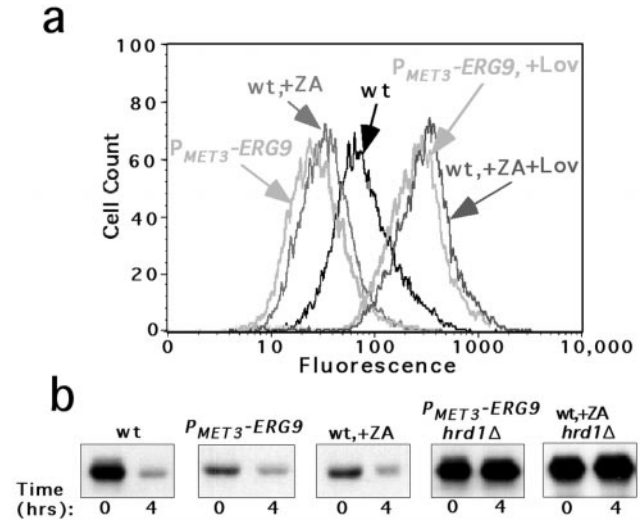

FIG. 3. **Squalene synthase down-regulation decreased Hmg2p levels.** *a*, effect of squalene synthase down-regulation on Hmg2p-GFP steady-state levels. Cells expressing squalene synthase from either the wild-type promoter (*wt*) or the *MET3* promoter (P_{MET3}-*ERG9*) were grown for 15 h at 30 °C in the presence of 2 mM methionine. ZA (+*ZA*, 10 μg/ml final concentration) or lovastatin (+*Lov*, 25 μg/ml final concentration) was added to the appropriate cultures, and all cultures were grown an additional 4 h at 30 °C. Hmg2p-GFP fluorescence was analyzed by flow cytometry. *b*, increased 1Myc-Hmg2p degradation as a result of squalene synthase down-regulation was *HRD1*-dependent. Cycloheximide chase assay of strains expressing squalene synthase from the wild-type promoter (*wt*) or from the *MET3* promoter (P_{MET3}-*ERG9*) in the presence of a normal *HRD1* gene or the *hrd1*Δ allele (P_{MET3}-*ERG9, hrd1*Δ, and *wt, hrd1*Δ). Cells were grown for 15 h at 30 °C in the presence of 2 mM methionine, and then cycloheximide was added to 50 μg/ml final concentration. After addition of cycloheximide, lysates were prepared at the indicated times and immunoblotted to determine Hmg2p levels.

single, integrated copy of P_{GAPDH}-*ERG9* was 8-fold more resistant to the growth-slowing effect of ZA than a wild-type strain, consistent with approximately 8-fold higher squalene synthase levels in the P_{GAPDH}-*ERG9* strain than the wild-type strain, as measured by Western blot of HSV-tagged versions of squalene synthase.[4]

We then tested if the Hmg2p degradation-enhancing effect of ZA required higher doses upon squalene synthase overexpression. The yeast strains used above also co-expressed two versions of Hmg2p, 1Myc-Hmg2p and the fluorescence reporter protein Hmg2p-GFP which have identical degradation behaviors as normal Hmg2p (12, 18). Cells containing a single, integrated copy of P_{GAPDH}-*ERG9* required 8-fold more ZA to decrease 1Myc-Hmg2p and Hmg2p-GFP steady-state levels as was required for wild-type cells (Fig. 2, *a* and *b*, 40 μg/ml for P_{GAPDH}-*ERG9 versus* 5 μg/ml for wt), consistent with the 8-fold overexpression of squalene synthase. These results suggested that the mechanism for ZA-induced degradation of Hmg2p was through squalene synthase inhibition.

Squalene Synthase Levels Determined the Degree of Hmg2p Degradation—The above results indicated that ZA altered Hmg2p degradation by decreasing squalene synthase activity. In that case, genetic down-regulation of the squalene synthase gene (*ERG9*) should also increase Hmg2p degradation. Because mevalonate pathway enzymes are essential for yeast viability, a null allele of *ERG9* in yeast results in cell death (32, 33). Therefore, we made a conditional allele of squalene synthase by placing the wild-type *ERG9* gene under control of the *MET3* promoter (22), which is repressed by the presence of high extracellular concentrations (>0.5 mM) of methionine (23). We constructed a "promoter-switch" plasmid that contained a truncated version of *erg9* placed behind the *MET3* promoter. Targeted integration of this plasmid into the *ERG9* locus resulted in the creation of a single, functional copy of *ERG9* under control of the regulated *MET3* promoter (P_{MET3}) (Fig. 1*b*). This plasmid was used to transform a methionine prototroph yeast strain to allow growth in any concentration of methionine. The strain also co-expressed 1Myc-Hmg2p and Hmg2p-GFP allowing a complete characterization of Hmg2p degradation. When grown in the absence of methionine, normal regulated Hmg2p degradation was observed in the yeast strain that expressed squalene synthase from the *MET3* promoter (P_{MET3}-*ERG9*).[4]

Genetic down-regulation of squalene synthase enhanced Hmg2p degradation in a manner identical to inhibition with ZA. After 15 h growth in 2 mM methionine, Hmg2p degradation in the P_{MET3}-*ERG9* strain was increased. This was indicated by a lower steady-state level of Hmg2p-GFP and 1Myc-Hmg2p in the P_{MET3}-*ERG9* strain compared with the wild-type strain (Fig. 3, *a* and *b*, P_{MET3}-*ERG9 versus wt*). The effect of down-regulation was similar to that in the wild-type strain after 15 h incubation in the presence of ZA (P_{MET3}-*ERG9* compared with *wt,+ZA*). Hmg2p-GFP in the P_{MET3}-*ERG9* was stabilized by the addition of lovastatin to a similar degree as the stabilization of Hmg2p-GFP by lovastatin addition in the wild-type strain preincubated with ZA (P_{MET3}-*ERG9*, +*Lov* compared with *wt,+ZA+Lov*), indicating that enhanced Hmg2p degradation caused by squalene synthase down-regulation or ZA addition was still regulated by the mevalonate pathway.

It was possible that enhanced Hmg2p degradation was the result of cell death inadvertently caused by squalene synthase down-regulation rather than the build-up of a positive signal for degradation. Cells containing the P_{MET3}-*ERG9* allele ceased to grow after 15 h incubation in 2 mM methionine (6 doublings) (see below, Fig. 8*b*). However, when the P_{MET3}-*ERG9* cells from this 15-h time point were transferred to media without methionine, they retained the same plating efficiency[4] and growth curve as identically treated wild-type *ERG9* cells (see below,

[4] R. Gardner and R. Hampton, unpublished observations.

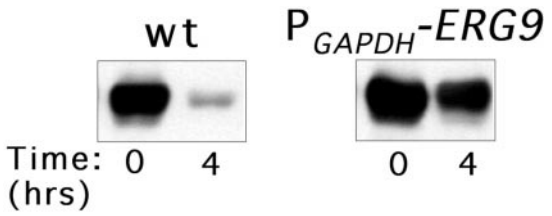

FIG. 4. **Squalene synthase overexpression decreased Hmg2p degradation.** Effect of squalene synthase overexpression on 1Myc-Hmg2p degradation. Otherwise identical strains expressing squalene synthase from the wild-type promoter (*wt*) or from a single, integrated allele with the *GAPDH* promoter (P_{GAPDH}-*ERG9*) were subjected to a cycloheximide chase assay. Lysates for each indicated time point after addition of cycloheximide were made and immunoblotted to determine Hmg2p levels.

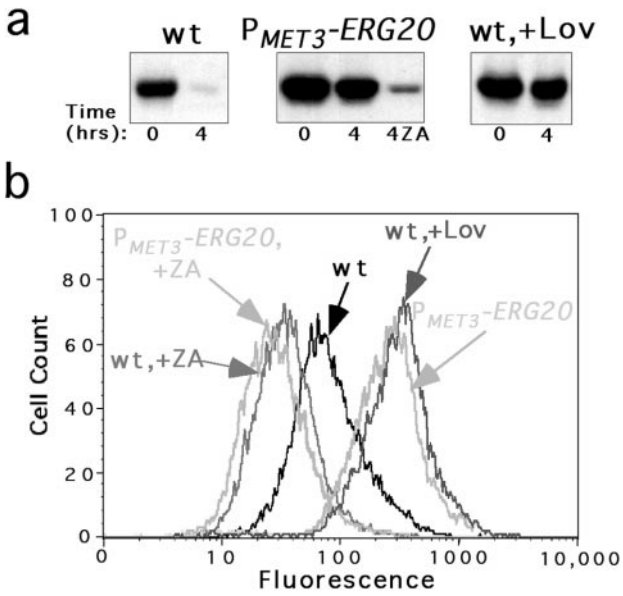

FIG. 5. **FPP synthase down-regulation decreased Hmg2p degradation.** *a,* effect of FPP synthase down-regulation on 1Myc-Hmg2p degradation. Otherwise identical strains expressing FPP synthase from the wild-type promoter (*wt*) or from the *MET3* promoter (P_{MET3}-*ERG20*) were grown for 15 h at 30 °C in the presence of 2 mM methionine and then subjected to a cycloheximide chase assay. Lysates for each indicated time point were made and immunoblotted to determine Hmg2p levels. ZA (10 μg/ml final concentration) was added to the indicated sample (*4ZA*) at the same time as addition of cycloheximide. Lovastatin (50 μg/ml final concentration) was added to the indicated wild-type strain (*wt,+Lov*) at the same time as addition of methionine. *b,* effect of FPP synthase down-regulation on Hmg2p-GFP steady-state levels. Cells expressing FPP synthase from either the wild-type promoter (*wt*) or the *MET3* promoter (P_{MET3}-*ERG20*) were grown for 15 h at 30 °C in the presence of 2 mM methionine. ZA (+*ZA*, 10 μg/ml final concentration) or lovastatin (+*Lov*, 25 μg/ml final concentration) was added to the appropriate cultures, and all cultures were grown an additional 4 h at 30 °C. Hmg2p-GFP fluorescence was analyzed by flow cytometry.

Fig. 8*b*), indicating that these cells were still viable.

The enhanced Hmg2p degradation by ZA addition or squalene synthase down-regulation required the *HRD* gene-encoded proteins. Enhanced degradation by squalene synthase or ZA addition was completely eliminated by the presence of the *hrd1*Δ allele (Fig. 3*b,* P_{MET3}-*ERG9, hrd1*Δ, and *wt +ZA, hrd1*Δ), which normally stabilizes Hmg2p (11). This indicated that the degradation-enhancing effect of squalene synthase down-regulation or ZA addition required a functional *HRD* pathway and was not due to aberrant degradation by an alternate pathway. Furthermore, identical steady-state levels of 1Myc-Hmg2p in the wild-type, *hrd1*Δ strain and the P_{MET3}-*ERG9, hrd1*Δ strain indicated that the lower steady-state levels of Hmg2p in the P_{MET3}-*ERG9* strain were due only to enhanced degradation and not reduced translation efficiency.

Additionally, we observed that overexpression of squalene synthase stabilized Hmg2p. In the strains previously described in Fig. 2, which overexpressed squalene synthase by the presence of the P_{GAPDH}-*ERG9* allele, both versions of Hmg2p were significantly stabilized. This was observed as both an increase in the steady-state level of 1Myc-Hmg2p (Fig. 4, P_{GAPDH}-*ERG9 versus wt*) and Hmg2p-GFP,[4] and as a decrease in 1Myc-Hmg2p degradation when squalene synthase was overexpressed (Fig. 4, P_{GAPDH}-*ERG9 versus wt*). Thus, squalene synthase overexpression had the opposite effect on Hmg2p degradation as squalene synthase down-regulation.

Down-regulation of Farnesyl-diphosphate Synthase Stabilized Hmg2p—The above studies implicated the substrate of squalene synthase, FPP, as a central molecule in the regulation of Hmg2p stability. Manipulation of squalene synthase predicted to increase FPP levels hastened Hmg2p degradation, whereas manipulation of squalene synthase predicted to decrease FPP levels slowed Hmg2p degradation. We wanted to test further the hypothesis that FPP was the source of the positive signal for Hmg2p degradation, by eliminating FPP production. This could be accomplished by inhibition of farnesyl-diphosphate synthase (FPP synthase), which generates FPP as a product. Unfortunately, no drugs are currently available that inhibit yeast FPP synthase *in vivo*. Therefore, we again used a genetic approach to lower FPP synthase production. As with the other enzymes of the mevalonate pathway, yeast cells that contain a null allele of FPP synthase are not viable (34), so we generated a conditional allele of the FPP synthase coding region (*ERG20*) that resulted in the wild-type *ERG20* gene placed under control of the *MET3* promoter, similar to *ERG9* described above. When grown in the absence of methionine, normal regulated Hmg2p degradation was observed in the yeast strain that expressed FPP synthase from the *MET3* promoter (P_{MET3}-*ERG20*).[4]

In contrast to enhanced Hmg2p degradation caused by squalene synthase down-regulation, FPP synthase down-regulation resulted in stabilization of Hmg2p. When the P_{MET3}-*ERG20* strain was grown 15 h in 2 mM methionine, Hmg2p was exceedingly stable, as indicated by both a higher steady-state level and decreased degradation of 1Myc-Hmg2p in the P_{MET3}-*ERG20* strain compared with the wild-type strain (Fig. 5*a,* P_{MET3}-*ERG20 versus wt*). Similarly, Hmg2p-GFP steady-state levels were increased dramatically in the P_{MET3}-*ERG20* strain (Fig. 5*b*, P_{MET3}-*ERG20 versus wt*), and this effect was mimicked by growth of the wild-type strain in the presence of lovastatin (P_{MET3}-*ERG20* compared with *wt,+Lov*), which acts to slow Hmg2p degradation (6). The effect of FPP synthase down-regulation was reversed by the presence of ZA in the degradation assay (Fig. 5*a*, P_{MET3}-*ERG20*, *4ZA lane*), and in Hmg2p-GFP steady-state fluorescence (Fig. 5*b*, P_{MET3}-*ERG20*,+*ZA* compared with *wt*,+*ZA*), indicating that regulated degradation of Hmg2p was still operative. Although the methionine treatment halted the growth of the P_{MET3}-*ERG20* strain after 15 h (see below, Fig. 8*b*), if these cells were then transferred to media that did not contain methionine, they retained a similar plating efficiency[4] and growth curve as the wild-type *ERG20* control cells (see below, Fig. 8*b*), indicating that Hmg2p stabilization was not a result of inadvertent cell death.

Thus, Hmg2p stabilization was caused by FPP synthase down-regulation, further strengthening the model that FPP was the source of a positive signal for Hmg2p degradation.

FPP Synthase Down-regulation Also Blocked Hmg2p Ubiquitination—The covalent attachment of ubiquitin is a critical and regulated step in Hmg2p degradation (12). Because FPP synthase down-regulation stabilized Hmg2p, we also deter-

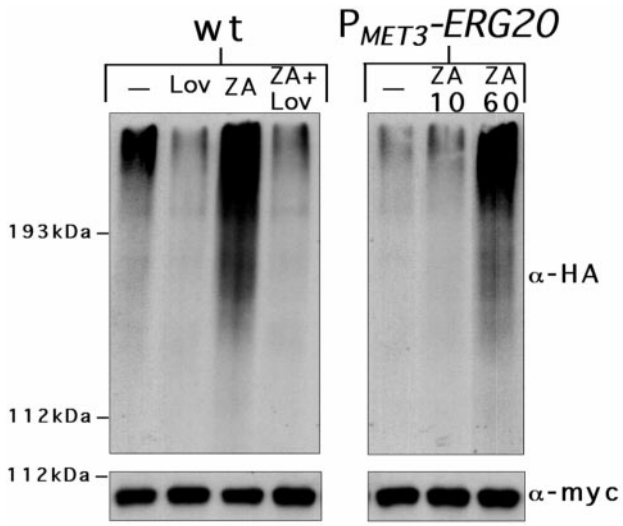

FIG. 6. **FPP synthase down-regulation decreased Hmg2p ubiquitination.** Regulated ubiquitination of 1Myc-Hmg2p in cells expressing FPP synthase from the wild-type promoter (*wt*) or the *MET3* promoter (P_{MET3}-*ERG20*). Cells were grown 15 h at 30 °C in the presence of 2 mM methionine. Ubiquitination assays were performed in the presence of no drug (−), 25 μg/ml lovastatin (*Lov*), or 10 μg/ml ZA (*ZA*). For the wild-type strain, lovastatin was added 30 min prior to cell lysis, and ZA was added 10 min prior to cell lysis. For the P_{MET3}-*ERG20* strain, ZA was added either 10 min (*ZA10*) or 60 min (*ZA60*) prior to cell lysis. *Upper panels* are the result of anti-HA (*α-HA*) immunoblotting for covalently linked HA-tagged ubiquitin. *Lower panels* are the result of parallel immunoblotting an aliquot (1/8 total volume) of the same immunoprecipitates with the 9E10 anti-Myc antibody (*α-myc*) to assess total immunoprecipitated Hmg2p.

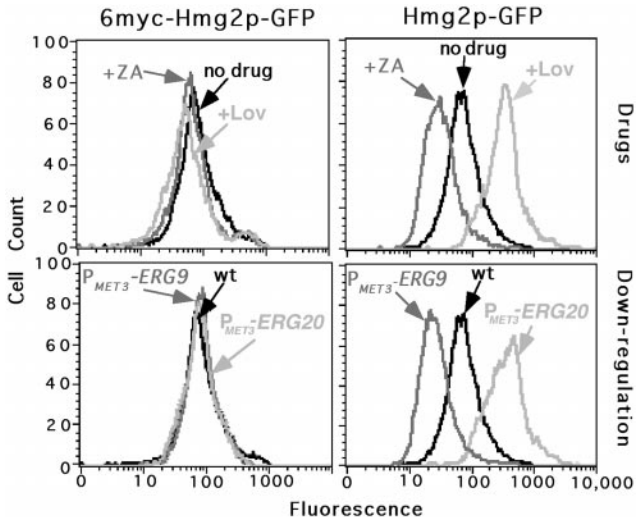

FIG. 7. **Conditional alleles of squalene synthase and FPP synthase altered regulation of Hmg2p degradation.** *Upper panels*, cells expressing either unregulated 6Myc-Hmg2p-GFP or normally regulated Hmg2p-GFP were treated with either no drug, 10 μg/ml ZA (*ZA*), or 25 μg/ml lovastatin (*Lov*) and grown for 4 h at 30 °C. *Bottom panels,* cells from the *upper panel* expressing either squalene synthase from the *MET3* promoter (P_{MET3}-*ERG9*), FPP synthase from the *MET3* promoter (P_{MET3}-*ERG20*), or both from their respective wild-type promoters (*wt*) were grown for 15 h at 30 °C in the presence of 2 mM methionine. 6Myc-Hmg2p-GFP or Hmg2p-GFP steady-state fluorescence was analyzed by flow cytometry.

mined its effect on Hmg2p ubiquitination. To assay Hmg2p ubiquitination, strains containing the appropriate wild-type or regulated alleles of the FPP synthase gene were transformed with plasmids that expressed HA epitope-tagged ubiquitin.[3] Hmg2p ubiquitination was assayed by immunoprecipitation of 1Myc-Hmg2p, followed by anti-HA immunoblotting to detect covalently attached HA-ubiquitin.[3] Regulation of Hmg2p ubiquitination was assessed by performing each assay in the presence of lovastatin (Lov), which decreases ubiquitination, or zaragozic acid (ZA), which increases ubiquitination (12) (Fig. 6, *wt*).

Down-regulation of FPP synthase caused a drastic decrease in the level of Hmg2p ubiquitination (Fig. 6, P_{MET3}-*ERG20 versus* wt, no drug lanes). This effect was similar to the addition of lovastatin to the wild-type strain during the ubiquitination assay (*wt, Lov lane*). Furthermore, the addition of ZA for 10 min during the ubiquitination assay, which normally increases Hmg2p ubiquitination, had no effect on Hmg2p ubiquitination in the FPP synthase down-regulated strain (*wt ZA lane versus* P_{MET3}-*ERG20*, *ZA10 lane*). This effect of FPP synthase down-regulation on ZA action was identical to addition of lovastatin to the wild-type cells (P_{MET3}-*ERG20*, *ZA10 lane* compared with *wt, ZA+Lov lane*). However, addition of ZA for 1 h did increase Hmg2p ubiquitination in the FPP synthase down-regulated strain similar to that in wild-type strain (P_{MET3}-*ERG20*, *ZA60 lane* compared with *wt ZA lane*). Thus, pharmacological and genetic manipulations that altered the cellular levels of FPP correspondingly altered Hmg2p ubiquitination in a manner consistent with its role as a positive degradation signal.

Genetic Manipulations Did Not Alter General ER Degradation—The above genetic experiments were consistent with FPP being the source of a positive signal for Hmg2p degradation. However, the results could also be explained by effects on the machinery for ER degradation, rather than on the signal controlling Hmg2p degradation. To test this, we examined if these genetic manipulations altered the degradation of 6Myc-Hmg2p, a previously described unregulated mutant of Hmg2p (11). Degradation of 6Myc-Hmg2p requires the same degradation pathway as normal Hmg2p (11), but 6Myc-Hmg2p degradation is not regulated by signals from the mevalonate pathway. Thus, if down-regulation of mevalonate pathway enzymes altered only the regulatory signal for Hmg2p degradation, then 6Myc-Hmg2p degradation should be unaffected.

The effect of squalene synthase or FPP synthase down-regulation on 6Myc-Hmg2p degradation was determined in a strain that was isogenic to the wild-type strain described above, except that unregulated 6Myc-Hmg2p and 6Myc-Hmg2p-GFP were co-expressed rather than 1Myc-Hmg2p and Hmg2p-GFP. As shown in the *top left panel* of Fig. 7, 6Myc-Hmg2p-GFP steady-state levels were unaffected by addition of drugs, such as lovastatin or ZA, which normally altered the steady-state levels of Hmg2p-GFP (*top right panel*). Similarly, down-regulation of squalene synthase or FPP synthase had no effect on the steady-state levels of the unregulated 6Myc-Hmg2p-GFP (*bottom left panel*), whereas these perturbations appropriately altered the steady-state levels of the regulated Hmg2p-GFP (*bottom right panel*). The co-expressed 6Myc-Hmg2p was similarly unresponsive to the same pharmacological or genetic manipulations that affected 1Myc-Hmg2p or the optical Hmg2p-GFP reporter.[4] These results indicated that squalene synthase and FPP synthase down-regulation affected the regulation of Hmg2p stability and not the process of ER degradation.

Squalene Epoxidase Down-regulation Did Not Alter Hmg2p Degradation—The previous experiments provided strong evidence that FPP levels within the cell controlled the rate of Hmg2p degradation. However, it was not clear if this feature was unique to FPP or if other downstream products could also affect Hmg2p degradation. Squalene epoxidase is the next enzyme after squalene synthase in the pathway and is responsible for the formation of squalene epoxide from squalene (Fig. 1*a*). We wondered if altering squalene epoxidase levels would

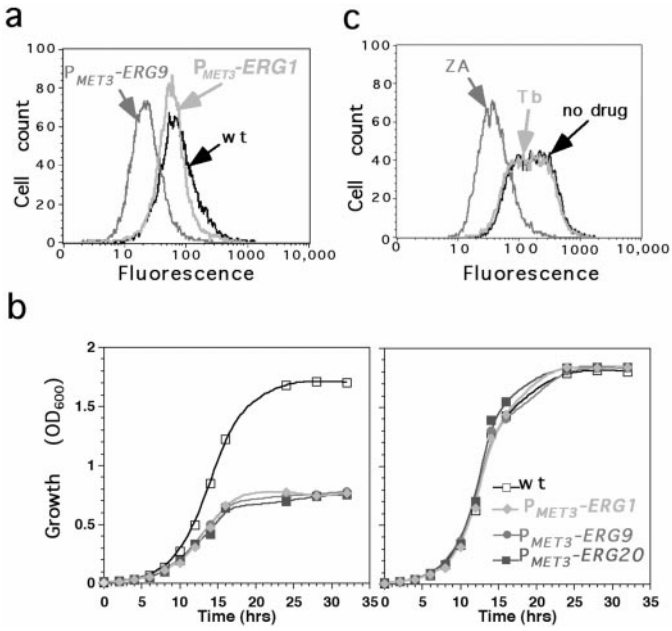

FIG. 8. **Squalene epoxidase down-regulation or inhibition had no effect on Hmg2p-GFP steady-state levels.** *a*, effect of squalene epoxidase down-regulation on Hmg2p steady-state levels. Cells expressing squalene epoxidase from either the wild-type promoter (*wt*) or the *MET3* promoter (P_{MET3}-*ERG1*) or squalene synthase from the *MET3* promoter (P_{MET3}-*ERG9*) were grown for 15 h at 30 °C in the presence of 2 mM methionine. Hmg2p-GFP fluorescence was analyzed by flow cytometry. *b*, *left panel,* cells containing either the conditional alleles of squalene synthase (P_{MET3}-*ERG9*), FPP synthase (P_{MET3}-*ERG20*), or squalene epoxidase (P_{MET3}-*ERG1*) were grown to mid-log phase (A_{600} approximately 0.5) in media that did not contain methionine. New cultures containing 2 mM methionine were inoculated from these previously grown cultures to an initial A_{600} of 0.01 and then incubated for 32 h at 30 °C. The new A_{600} for these cultures was measured at the indicated time points and plotted *versus* time. Growth of each strain was compared with the wild-type strain (*wt*). *Right panel,* new cultures, which contained no methionine, were inoculated from the previous 15-h methionine-grown cultures to an initial A_{600} of 0.01 and then incubated for 32 h at 30 °C. The new A_{600} for these cultures was measured at the indicated time points and plotted *versus* time. *c*, effect of terbinafine (*Tb*), a squalene epoxidase inhibitor, on Hmg2p-GFP steady-state levels. Cells were treated with either no drug, 40 μg/ml terbinafine (*Tb*), or 10 μg/ml ZA (*ZA*) and grown for 4 h at 30 °C. Hmg2p-GFP steady-state fluorescence was analyzed by flow cytometry.

have any effect on Hmg2p degradation. Therefore, we generated a *MET3*-regulated allele of the squalene epoxidase gene (*ERG1*), similar to the *ERG9* and *ERG20* alleles described above. When grown in the absence of methionine, normal regulated Hmg2p degradation was observed in the yeast strain that expressed squalene epoxidase from the *MET3* promoter (P_{MET3}-*ERG1*).[4]

Unlike the other manipulations above, squalene epoxidase down-regulation had no effect on Hmg2p degradation. When grown in methionine, the P_{MET3}-*ERG1* strain and the wild-type strain had identical Hmg2p-GFP steady-state levels (Fig. 8*a*, P_{MET3}-*ERG1* compared with *wt*), and 1Myc-Hmg2p degradation.[4] This was in contrast to squalene synthase down-regulation (see above and Fig. 8*a*, P_{MET3}-*ERG1 versus* P_{MET3}-*ERG9*), which had the expected effects in the same experiment.

It was possible that squalene epoxidase down-regulation had no effect on Hmg2p degradation because the conditional allele was not correctly functioning. However, cells carrying the ineffective P_{MET3}-*ERG1* allele showed identical methionine-dependent growth retardation as the other alleles that affected degradation (Fig. 8*b*). Thus, the conditional allele of squalene epoxidase was correctly integrated and down-regulated. To confirm further that a block at squalene epoxidase did not affect Hmg2p degradation, we treated the wild-type strain with terbinafine, a specific inhibitor of fungal squalene epoxidase (35–38). No concentration of terbinafine had any observable effect on Hmg2p-GFP steady-state levels (Fig. 8*c, +Tb* compared with *no drug*) or 1Myc-Hmg2p degradation,[4] despite examining the effect with lethal doses of the drug. Together these results indicated that FPP was the primary, if not the only, source of the positive signal for Hmg2p degradation.

DISCUSSION

HMGR degradation is regulated by signals generated downstream in the mevalonate pathway. The identity of these signals and the mechanism by which they control HMGR degradation is currently not known. To understand better the nature of this regulatory mechanism, we have examined the regulated degradation of the yeast HMGR isozyme Hmg2p through genetic and pharmacological manipulation of the mevalonate pathway.

The results detailed in this work strongly implicated the mevalonate pathway product FPP as the source of the positive signal for Hmg2p degradation. ZA addition or squalene synthase down-regulation, both predicted to increase FPP levels in the cell, increased Hmg2p degradation. Conversely, addition of lovastatin, FPP synthase down-regulation, or squalene synthase overexpression, all predicted to decrease FPP levels in the cell, stabilized Hmg2p. Identical Hmg2p steady-state levels in degradation-deficient strains containing either normal or down-regulated squalene synthase levels indicated that FPP levels affected only Hmg2p degradation.

Utility of the MET3 Promoter—In order to examine the molecular signals for Hmg2p degradation, we required a way to manipulate the mevalonate pathway at any enzymatic step. Drugs that inhibit some of the pathway enzymes are available, such as lovastatin and ZA. However, there are no currently available drugs that inhibit enzymes between HMGR and squalene synthase. Furthermore, because the products of the mevalonate pathway are essential for yeast viability, null alleles of mevalonate pathway genes result in cell death. Therefore, we required a way to alter the level or activity of mevalonate pathway enzymes in a controllable, yet viable, manner.

To accomplish this, we manipulated the levels of key mevalonate pathway enzymes by placing them under the control of a regulated promoter. We chose the *MET3* promoter (22), which can be repressed by incubation of cells in high concentrations of methionine (>0.5 mM) (23). In all cases, the conditional alleles were similar to null alleles in that cells carrying the conditional alleles, when continuously incubated in methionine, were unable to grow after a few doublings. However, unlike cells with null alleles, cells carrying the conditional alleles could be induced to grow by transferring them to media containing no methionine. This type of genetic manipulation provided a facile way to alter the expression of target genes and could be used, in theory, to create regulated alleles of any yeast gene.

FPP as the Source of the Signal for Hmg2p Degradation—Manipulation of FPP levels by both pharmacological and genetic means resulted in the expected changes to Hmg2p degradation consistent with FPP, or a derivative, as a positive signal for Hmg2p degradation. The idea that intracellular levels of FPP, or a derivative, serve to modulate Hmg2p degradation in yeast paralleled similar observations in mammalian cells. It has been proposed that degradation of mammalian HMGR is regulated by the intracellular levels of farnesol, a derivative of FPP (13–17). It may also be the case that farnesol is the positive signal for Hmg2p degradation in yeast, and we are currently in the process of examining whether FPP or farnesol regulates Hmg2p degradation.

One way to distinguish between FPP and farnesol as the

regulatory signal would be by elimination of the pyrophosphatase activity required to convert FPP to farnesol (17). Recently, two pyrophosphatases, *LPP1* and *DPP1*, that appear to convert FPP to farnesol *in vitro* have been described (39). Null alleles of the genes encoding these enzymes have been made in yeast, and the cells are viable. We examined Hmg2p degradation in these strains and found that the absence of Lpp1p and Dpp1p had no effect on Hmg2p regulated degradation.[4] This could mean that either FPP, not farnesol, is the signal for HMGR degradation in yeast or that these enzymes are not solely responsible for the conversion of FPP to farnesol *in vivo*. In either case, it is apparent that FPP serves as the source of a signal for HMGR degradation in both mammals and yeast.

In mammalian cells, there is an additional sterol signal that acts to modulate HMGR degradation. The addition of sterols to mammalian cells results in increased degradation of HMGR (4, 5). However, this downstream signal from sterols does not accelerate HMGR degradation in the absence of the upstream FPP-derived signal (4, 17), indicating that sterols provide an additional positive signal for HMGR degradation that works only in conjunction with the FPP-derived signal. In this study, our results demonstrated that no other pathway product downstream of FPP was required for Hmg2p degradation in yeast. That is inhibition of squalene epoxidase activity or down-regulation of squalene epoxidase had no stabilizing effect on Hmg2p degradation. However, yeast may contain abundant stores of sterols, and so the existence of other signals cannot be ruled out by these negative results. We are further exploring, by both genetic and pharmacological means, whether an additional signal downstream of squalene can act in conjunction with FPP to hasten Hmg2p degradation.

Mechanisms by Which FPP Regulates Hmg2p Degradation—The identification of FPP, or a derivative, as the positive signal for HMGR degradation in yeast leads to several models for regulation of Hmg2p degradation. The simplest model for the regulatory mechanism is that the FPP-derived signal acts as an allosteric regulator and physically binds to the transmembrane domain of Hmg2p, altering its susceptibility to degradation. Alternatively, the FPP-derived molecule could modify or interact with a separate effector protein that alters Hmg2p stability. The effector could act to promote or prevent Hmg2p degradation, as either of these possibilities have precedent in cellular degradation (40, 41). It is also possible that the FPP-derived signal directly affects the structure of the ER membrane. By this model, the Hmg2p transmembrane domain would respond to the altered membrane by becoming increasingly susceptible to degradation. In this regard, it is interesting that the distance from the ER surface of key residues in the Hmg2p transmembrane is critical in regulation of Hmg2p stability.[3]

We are currently exploring the mechanism by which FPP acts to regulate Hmg2p degradation. It is clear that both yeast and mammalian cells measure FPP production as a means to control HMGR degradation. This conservation in signaling strategy, together with the similar location and machinery required for HMGR degradation, indicates that the mechanism for regulation of HMGR degradation may also be conserved among eukaryotes.

A final and important note should be made regarding a recent study on a fascinating side effect of the HMGR inhibitor lovastatin. It has been demonstrated that the closed β-lactone ring form of this particular statin can directly inhibit the proteasome (29). At first thought, it might be reasonable to conclude that this action is responsible for the stabilizing effects of lovastatin on Hmg2p, which is degraded by the proteasome (11). However, several observations show quite clearly that all actions of lovastatin we have observed were due to altered signal production and not proteasome inhibition. Degradation of 6Myc-Hmg2p, which is proteasome-dependent, is unaffected by doses of lovastatin that slow Hmg2p degradation (Ref. 11 and Fig. 7). Furthermore, the pathway inhibitor L659,699, which inhibits HMG-CoA synthase but contains no lactone moiety, is equally effective at stabilizing Hmg2p as lovastatin (12). Third, genetic manipulations of HMG-CoA synthase (6), or FPP synthase (this work), which slow production of the degradation signals but do not involve drugs, have identical actions to lovastatin on Hmg2p degradation but not 6Myc-Hmg2p degradation. Finally, lovastatin decreases ubiquitination of Hmg2p (Ref. 12 and this study), an effect opposite of that observed when the proteasome is compromised or inhibited (11). Although proteasome inhibition by the closed ring form of lovastatin is interesting and perhaps important clinically, it plays no obvious or important role in the actions of this drug on regulating Hmg2p stability in yeast and is most likely not involved in mammalian HMGR degradation, for similar reasons.

Acknowledgments—We gratefully thank Merck for the generous gifts of zaragozic acid and lovastatin, Dr. Paul Sternweis for the *lpp1*Δ/*dpp1*Δ strains, Dr. Harry Mountain for the *MET2* and *MET3* plasmids, and Dr. Robert Rickert for the use of the FACScalibur™ flow microfluorimeter and software.

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