Geranylgeranyl Pyrophosphate Is a Potent Regulator of HRD-dependent 3-Hydroxy-3-methylglutaryl-CoA Reductase Degradation in Yeast* **

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3-Hydroxy-3-methylglutaryl (HMG)-CoA reductase (HMGR), the rate-limiting enzyme of sterol synthesis, undergoes feedback-regulated endoplasmic reticulum degradation in both mammals and yeast. The yeast Hmg2p isozyme is subject to ubiquitin-mediated endoplasmic reticulum degradation by the HRD pathway. We had previously shown that alterations in cellular levels of the 15-carbon sterol pathway intermediate farnesyl pyrophosphate (FPP) cause increased Hmg2p ubiquitination and degradation. We now present evidence that the FPP-derived, 20-carbon molecule geranylgeranyl pyrophosphate (GGPP) is a potent endogenous regulator of Hmg2p degradation. This work was launched by the unexpected observation that GGPP addition directly to living yeast cultures caused high potency and specific stimulation of Hmg2p degradation. This effect of GGPP was not recapitulated by FPP, GGOH, or related isoprenoids. GGPP-caused Hmg2p degradation met all the criteria for the previously characterized endogenous signal. The action of added GGPP did not require production of endogenous sterol molecules, indicating that it did not act by causing the build-up of an endogenous pathway signal. Manipulation of endogenous GGPP by several means showed that naturally made GGPP controls Hmg2p stability. Analysis of the action of GGPP indicated that the molecule works upstream of retrotranslocation and can directly alter the structure of Hmg2p. We propose that GGPP is the FPP-derived regulator of Hmg2p ubiquitination. Intriguingly, the sterol-dependent degradation of mammalian HMGR is similarly stimulated by the addition of GGOH to intact cells, implying that a dependence on 20-carbon geranylgeranyl signals may be a common conserved feature of HMGR regulation that may lead to highly specific therapeutic approaches for modulation of HMGR.

The sterol or mevalonate pathway is highly conserved and essential in eukaryotes. Sterol synthesis entails stepwise construction of 5-carbon isoprenes from acetyl CoA, followed by condensations, cyclizations, and modifications to form the wide array of sterol and non-sterol products produced by this versatile biosynthetic route (see Ref. 1 and Fig. 1). The first committed step of the pathway is catalyzed by HMG-CoA reductase (HMGR), catalyzing mevalonic acid formation from HMG-CoA. HMGR undergoes multivalent regulation, including feedback regulated degradation that is conserved from yeast to mammals (2). When sterol pathway flux is high, HMGR degradation is fast, and steady state levels tend to be low. When sterol pathway flux is slowed, the degradation rate is slowed, and HMGR levels tend to rise. In this way, the amount of HMGR is altered to meet changing cellular demand for sterol pathway products. The HMGR molecule consists of a globular C-terminal catalytic domain, connected to a multispanning ER anchor. The N-terminal multispanning transmembrane domain is responsible for regulated degradation, whereas the catalytic region can function autonomously and is not subject to regulation when freed from its ER anchor. Conversely, the N-terminal transmembrane region of HMGR is sufficient for feedback regulated degradation, allowing fusion of reporter genes such as lacZ or GFP to facilitate biochemical and genetic analysis of this process.

Feedback regulation of HMGR stability is observed in both mammals and yeast. As an avenue toward understanding the mechanistic details of HMGR-regulated degradation, we have been studying this process in Saccharomyces cerevisiae, using a combination of techniques made facile in this guide organism (2). Yeast expresses two isozymes of HMGR, Hmg1p and Hmg2p; Hmg1p is quite stable, whereas Hmg2p undergoes sterol pathway-regulated degradation. Hmg2p degradation depends on its multispanning N-terminal anchor, with a half-life that varies between ~10 min and many hours depending on the level of degradation signal from the sterol pathway. Hmg2p degradation proceeds by the HRD (HMG-CoA reductase degradation) pathway (2–4), mediated by the Hrd1p ubiquitin ligase and a variety of other factors. The HRD pathway is also a principle pathway of ER-associated degradation, responsible for the destruction of numerous misfolded and damaged proteins in the ER lumen or membrane. Thus, regulated Hmg2p
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degradation occurs by signals from the sterol pathway promoting Hmg2p entry into the HRD quality control pathway, possibly by selective misfolding of the normal Hmg2p molecule to a structure that is recognized as a misfolded protein by the HRD apparatus.

In our analyses of the sterol pathway signals that accelerate Hmg2p degradation, we discovered that altering cellular levels of 15-carbon farnesyl pyrophosphate (FPP) causes striking changes in Hmg2p stability (5). Increasing FPP by lowering the activity of the FPP-utilizing enzyme squalene synthase (SS), either by pharmaceutical or genetic means, causes increased ubiquitination and degradation of Hmg2p. Conversely, lowering cellular FPP levels either by upstream inhibition of its production or by overexpression of SS slows degradation of Hmg2p. Thus, we have long thought that the signal was FPP or an FPP-derived isoprenoid. Direct studies on the structure of Hmg2p using limited proteolysis and thermal denaturation assays indicated that isoprenoids can directly alter the structure of Hmg2p, causing a reversible misfolding of the protein that might make it more susceptible to the HRD pathway (6, 7). Because the most effective lipid in these assays was farnesol (FOH), derived from FPP, we speculated that either farnesol was the FPP-derived regulator or that the FPP itself employed this feature of its structure to accelerate Hmg2p degradation.

Recently, in an attempt to directly stimulate Hmg2p degradation, as opposed to using drugs or mutants to alter the endogenous, FPP-derived signal, we tested the effect of directly adding various pathway molecules to intact cells of the highly permeable pdr5Δ null strain. The ability to directly stimulate Hmg2p degradation would allow more refined analysis of both the signal and the process of regulated HRD-dependent Hmg2p degradation. To our surprise, we found a highly specific potent effect of the extended 20-carbon geranylgeranyl pyrophosphate (GGPP), which is a natural derivative of FPP, but no effect of any other lipid tested including the primary candidate FPP. GGPP caused specific stimulation of Hmg2p ubiquitination and degradation. In fact, GGPP was equally effective in normal yeast without the pdr5Δ mutation, apparently crossing the intact yeast membrane and cell wall despite its large size and high charge. Moving forward from this serendipitous observation, we have conducted a detailed analysis of the root of this effect and have concluded from the studies below that endogenously synthesized GGPP is in all likelihood the physiological, FPP-derived regulator of Hmg2p degradation. A clear but poorly understood role for geranylgeranyl lipids in regulated degradation of mammalian HMGBl has been reported (8), thus providing an intriguing new connection between HMGBl regulation in yeast and mammals. In addition, this non-sterol mechanism of stability regulation may direct us to new approaches to modulating HMGBl for clinical benefit.

**EXPERIMENTAL PROCEDURES**

Reagents—Isoprenoids were purchased from Sigma-Aldrich. Lovastatin and zaragozic acid were gifts from Merck.

Strains and Media—Strains were derived from the S288C-derivative genetic background except for strains obtained from other laboratories. Yeast were grown at 30 °C with aeration as described previously (3) or at 25 and 37 °C where indicated. The cultures were logarithmically grown in minimal medium with 2% glucose and appropriate amino acid supplements to an A600 of <0.35. Standard yeast techniques were used to introduce plasmids, prepare gene deletions, and incorporate mutant alleles. The yeast strains are listed in supplemental Table S1. RHY1015 was previously described (5). The btsΔ null strain was made by PCR-mediated homologous recombination of the nourseothricin gene (NatR) flanked by 50 base pairs of homology of the BTS1 coding region. Null was confirmed by cold temperature sensitivity at 17 °C and PCR. All of the primer sequences are available upon request. The geranylgeranyl transferase type I subunit mutant allele bet2-1 strain and its corresponding wild type were provided by Susan Ferro-Novick (University of California, San Diego) (9), and the strain carrying the type II subunit mutant allele cdc43-2 was provided by Jasper Rine (University of California, Berkeley) (10). The cdc43-2 allele was complemented by the expression of CDC43 on a CEN plasmid to serve as the control strain. This plasmid was constructed by subcloning CDC43 from YEplp(43)2 (also received from the laboratory of J. Rine) into pRS415. Complementation was confirmed by growth at the restrictive temperature of 37 °C. The plasmid carrying the GAL1 promoter-driven BTS1 was a gift from Seiichi Matsuda (Rice University, Houston, TX). Plasmids YEps6PN and YEps6G53E, expressing MRS6 and mutant mrsG53E, respectively, were provided by Antonella Ragnini-Wilson (University of Tor Vergata, Rome, Italy) and described previously (11, 12).

Flow Cytometry—Flow cytometry was carried out as previously described (13, 14). Yeast grown in minimal medium with 2% glucose and appropriate amino acids into log phase (A600 < 0.2) were incubated with mevalonate pathway isoprenoid intermediates (typically 11 μM unless indicated differently), zaragozic acid (10 μg/ml), lovastatin (25 μg/ml), vehicle (for GGPP and other isoprenoids, this consisted of addition of the same volume of the solution 3 parts 1 mM ammonia and 7 parts methanol), or cycloheximide (50 μg/ml) for the times indicated. The BD Biosciences FACScalibur flow cytometer measured the individual fluorescence of 10,000 cells. CellQuest software was used to analyze the data and plotted fluorescence vs. cell count histograms.

Whole Cell Lysates—Preparation of whole cell lysates was previously described (15). After each time indicated with GGPP (11 μM) or vehicle incubation, the cells were pelleted (1 optical density equivalent) and resuspended in 100 μl of SUME (1% SDS, 8 μM urea, 10 mM MOPS, pH 6.8, 10 mM EDTA) with the following protease inhibitors (PIs): 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin hemisulfate, 76 μM pepstatin A, and 142 μM TPCK. 100 μl of acid-washed glass beads were added to suspension, and the cells were broken by vortexing three times for 1-min intervals with 1-min intervals on ice between each vortexing. Following the addition of 100 μl of 2× urea sample buffer (2× USB: 8 μl urea, 4% SDS, 200 mM dithiothreitol, 125 mM Tris, pH 6.8), the slurry was incubated at 50 °C for 10 min. The lysates were clarified by centrifugation for 5 min at 14,000 × g. The proteins were resolved by 8% SDS-PAGE and transferred to nitrocellulose membrane blots. 5% nonfat-dried milk in Tris-buffered saline with Tween (TBS-T: 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) was used to block blots,
and 2% nonfat dried milk in TBS-T was used in antibody incubations. Hmg2p-GFP was detected with anti-GFP (BD Biosciences, San Jose, CA) and 1myc-Hmg2p-GFP with anti-Myc 9E-10 antibody (hybridoma from ATCC (Manassas, VA)). Goat anti-mouse conjugated with hors eradish peroxidase (Jackson ImmunoResearch, West Grove, PA) recognized primary antibodies. Western Lightning chemiluminescence reagents (PerkinElmer Life Sciences) were used for immunodetection.

**In Vivo Ubiquitination**—Yeast cells were grown in minimum medium to an optical density of 0.3 optical density/ml. The cells were pelleted at 2500 rpm and resuspended with 4 ml of fresh medium. Either 4 μl of Me6SO or ZA was added or 20 μl of vehicle or GGPP (final concentration, 11 mM) and incubated for 10 min before lysis. The cells were pelleted at 3500 rpm and resuspended in 200 μl of SUME with the following PIs: 1 mM phenylmethylsulfonyl fluoride, 260 mM 4–(2-aminoethyl) benzensulfonyl fluoride hydrochloride, 100 mM leupeptin hemisulfate, 76 mM pepstatin A, 5 mM 6-aminocaproic acid, 5 mM benzamidine, 142 mM TPCK, and 5 mM N-ethylmaleimide. Glass lysis beads were added to meniscus. The tubes were vortexed six times for 1-min intervals with incubations on ice for 1 min in between. The liquid was removed from the beads and placed in a fresh tube. The beads were washed with 600 μl of IPB (15 mM Na2HPO4, 150 mM NaCl, 2% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 10 mM EDTA, pH 7.5) with PIs and N-ethylmaleimide and combined with initial lysate. The lysis suspension was cleared of unbroken cells and large debris by centrifugation at 14,000 × g for 5 min. The supernatant was transferred to a fresh tube, to which 15 μl of anti-GFP was added. The immunoprecipitation was incubated for 5 min on ice and then subjected to centrifugation for 5 min at 14,000 × g. The supernatant was transferred to a fresh tube, and the immune-precipitation was incubated overnight at 4 °C. Protein A-Sepharose was added and incubated for an additional 2 h. The protein-bound beads were washed once with IPB and once with IPW (50 mM NaCl, 10 mM Tris, pH 7.5). The beads were aspirated to dryness, and the proteins were eluted with 55 μl of 2× USB. The proteins were separated by SDS-PAGE (8%) and transferred to nitrocellulose membranes. After membrane autoclav ing (16), ubiquitinated Hmg2p-GFP was detected with monoclonal anti-ubiquitin (Zymed Laboratories Inc., South San Francisco, CA). The levels of Hmg2p-GFP were detected with monoclonal anti-GFP.

**Protease Protection Assay**—The microsomes were prepared from the strain expressing the MycL-Hmg2p-GFP for trypsin digestion as previously described (7). The microsomes were resuspended in Xl buffer (1.2 M sorbitol, 5 mM EDTA, 0.1 M KH2PO4/K2HPO4, final pH 7.5). Vehicle, farnesol (200 mM), or GGPP (20 μM) was added prior to incubation of 150 μg/ml trypsin (Sigma-Aldrich) for 0, 2, and 10 min. An equal volume of 2× USB was added to stop the reactions. The samples were then separated by 14% SDS-PAGE, and immunoblots were detected with anti-Myc 9E-10 antibody.

**In Vitro Ubiquitination**—The assay was carried out as previously described (17) with the exception that microsome donor strains do not express HRD1 from the TDH3 promoter. Briefly, microsomes from a ubc7Δ null yeast strain expressing 3HA-Hrd1p from the HRD1 promoter and Hmg2p-GFP (WT or NR1) from the TDH3 promoter were prepared by glass bead lysis. 25 A600 units of log phase cells were lysed with 500 μl of membrane fractionation buffer (MF: 20 mM Tris, pH 7.5, 100 mM NaCl, 300 mM sorbitol) with same PIs as described under “In Vivo Ubiquitination.” At 4 °C, the cells were lysed using hand vortexing six times for 1-min intervals with 1-min intervals on ice between each vortexing. The lysate was collected and pooled with two bead rinses with MF to give the crude microsomal lysate and cleared of cellular debris. The microsomes were pelleted at 14,000 × g for 45 min at 4 °C and then resuspended in B88 buffer (20 mM Hepes, pH 6.8, 250 mM sorbitol, 150 mM KOAc, 5 mM MgOAc, 1 mM dithiothreitol) with PIs to a final concentration of 0.3 optical density equivalent units/μl. Cytosol was prepared from a hrd1Δ ubc7Δ double null control strain and its overexpressing Ubc7p counterpart in a similar manner as the Schekman lab (18). 500 optical density equivalents of cells were pelleted, rinsed twice with water, rinsed once with B88 buffer, and resuspended in 500 μl of B88 buffer with PIs. The resuspended cells were transferred to a chilled mortar containing liquid nitrogen and ground with a pestle to form a frozen powder. 1 mM ATP from a 500 mM stock solution in H2O, pH 7, was added to thawed cytosol. This crude cytosol lysate was centrifuged at 3,000 × g for 5 min to remove large debris, and then the resulting supernatant was centrifuged at 20,000 × g for 15 min. The lysate was further clarified by ultracentrifugation at 100,000 × g for 1 h. The cytosol concentrations were determined by Bradford reagent and adjusted with B88 to 20 mg/ml. MG-132 (Sigma-Aldrich) was added to a final concentration of 300 μM to microsome and cytosol preparations.

One in vitro ubiquitination reaction consisted of 10 μl of microsomes and 12 μl of cytosol. ATP (500 mM stock) was added to each reaction to a final concentration of 30 mM. The reactions were incubated for 1 h at 30 °C. The assay was stopped by solubilization with 200 μl of SUME with PIs and 5 mM N-ethylmaleimide. 600 μl of IPB with PIs and N-ethylmaleimide was added and followed by the addition of 15 μl of rabbit polyclonal anti-GFP. Immunoprecipitation, separation by SDS-PAGE, and detection of unmodified and ubiquitinated Hmg2p-GFP were carried out as described above under “In Vivo Ubiquitination.”

**RESULTS**

**Direct Addition of GGPP to Yeast Cell Cultures Enhances Hmg2p Degradation**—Our earlier studies indicated that a molecule derived from FPP is the principle signal for HRD-dependent degradation of Hmg2p (5). Those studies depended on the indirect production of the FPP-derived signal by genetic or pharmacological means. For example, the addition of the squale sene synthase inhibitor ZA causes build-up of the FPP substrate derived from FPP is the principle signal for HRD-depend ent degradation of Hmg2p. We explored whether the addition of various isoprenoids to yeast cell cultures could directly stimulate Hmg2p.

We tested a variety of isoprene molecules formed along or from the sterol pathway including our original candidate molecule FPP, the 10-carbon precursor geranyl pyrophosphate, the two 5-carbon precursors dimethylallyl pyrophosphate and iso-

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![Diagram of isoprenoids and signaling pathways](http://www.jbc.org)

**FIGURE 1.** Key isoprenoids of the mevalonate pathway.

Pentenyl pyrophosphate, and the 20-carbon GGPP, which is derived from FPP (Fig. 1). To facilitate our tests, we employed the normally regulated, noncatalytic reporter Hmg2p-GFP (5, 20), consisting of the eight-spanning N-terminal transmembrane anchor followed by GFP. Hmg2p-GFP undergoes normally regulated degradation that can be monitored biochemically or by in vivo flow cytometry of whole cell GFP. Log phase cells expressing Hmg2p-GFP were treated by direct addition of various test molecules to the culture medium for 1 h, followed by flow cytometry to evaluate changes in steady state levels caused by enhanced degradation.

Of the molecules tested, GGPP alone had an effect on Hmg2p-GFP levels, and this effect was quite strong (Fig. 2A). In contrast, even very high concentrations of FPP did not affect Hmg2p-GFP levels (data not shown), despite its clear role as a precursor of the in vivo signal. Because GGPP is formed in vivo from FPP and isopentenyl pyrophosphate (Fig. 1), we also tested adding this pair in combination, but they similarly had no effect, and squalene, which lies downstream of FPP along the sterol pathway (Fig. 1) (5), was also without effect, as expected (data not shown).

This unique action of 20-carbon GGPP was reminiscent of stimulated degradation of mammalian HMGCR caused by the direct addition of nonphosphorylated geranylgeraniol (GGOH) to cultured cells (8). Nevertheless, a range of GGOH concentrations added to yeast cultures up to 50 times that of the lowest maximally effective GGPP concentration did not alter levels of Hmg2p-GFP (Fig. 2B). Although the 15-carbon alcohol FOH derived from FPP had strong effects in our in vitro structural assays (7), FOH is highly toxic to yeast cells and thus could not be tested over the time scales normally used. However, short term studies of FOH in yeast indicate that this molecule was similarly without effect (data not shown). Thus, only GGPP had a direct effect on Hmg2p, and a striking one, prompting further investigation of this action.

The concentration dependence of GGPP was determined (Fig. 2, C and D). Logarithmically growing cells were incubated with various concentrations of GGPP for 1 h, followed by flow cytometry of the cultures or immunoblotting of lysates prepared as described under “Experimental Procedures.” In both assays, the approximate EC50 was 2 μM, and the lowest maximally effective concentration was ~11 μM.

**GGPP Enhances Hmg2p Ubiquitination**—Regulated degradation of Hmg2p proceeds by the HRD ER-associated degradation pathway, which employs the Hrd1p ubiquitin ligase to ubiquitinate Hmg2p (4). The effect of GGPP on Hmg2p-GFP similarly required an intact HRD pathway and was absent in a hrd1Δ mutant.

Increasing the signal for Hmg2p degradation with agents such as ZA causes increased ubiquitination. The effect of GGPP on Hmg2p-GFP ubiquitination was evaluated by immunoprecipitation followed by immunoblotting for Hmg2p-GFP or ubiquitin. Identical cultures of cells were treated with GGPP, ZA, or their respective vehicles for just 10 min, followed by lysis and immunoprecipitation/immunoblotting analysis for ubiquitination. Like ZA, GGPP added directly to the yeast cells causes sudden and profound ubiquitination of Hmg2p-GFP (Fig. 3B).

**Effect of GGPP Meets Hallmarks of Hmg2p-regulated Degradation**—An extensive mutagenic analysis showed that lysines 6 and 357 of Hmg2p are each required for Hmg2p regulated degradation (21). Loss of either lysine rendered GGPP ineffective in stimulating Hmg2p degradation (Fig. 4A). Similarly, the highly stable Hmg1p was not affected by GGPP (Fig. 4B).

A number of distinct Hmg2p mutants undergo HRD-dependent degradation but are unregulated by the FPP-derived signal. That is, their HRD-dependent degradation is the same at all levels of the FPP-derived signal. We examined two of these mutants, NR1 and 6myc-Hmg2p, to further test the specificity of the GGPP effect. NR1-Hmg2p-GFP contains a five-amino acid change in the sterol sensing domain within the transmembrane domain of Hmg2p, and 6myc-Hmg2p-GFP has a segment of the lumenal transmembrane domain replaced with six Myc epitopes (7). Each of these proteins undergoes HRD-dependent degradation but are unregulated by the FPP-derived signal. GGPP incubation did not influence NR1-Hmg2p-GFP or 6myc-Hmg2p-GFP levels in conditions where the wild type Hmg2p-GFP underwent the expected degradation (Fig. 5). Taken together, these in cis studies indicated that the effect of

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GGPP shows the same high specificity as the physiological signal derived from FPP.

GGPP Action Is Not through Squalene Synthase Inhibition—The squalene synthase inhibitor ZA induces Hmg2p degradation by causing FPP levels to build-up and acts very rapidly (5). GGPP addition had a similar rapid effect on stability (Fig. 2E) and ubiquitination (Fig. 3B). Thus, it was important to test whether GGPP was also a potent inhibitor of squalene synthase or, alternatively, operated in a manner distinct from inhibition of this enzyme. Because ZA has a structure that is reminiscent of isoprenoids, this is a formal possibility. We tested this idea by several criteria convincingly demonstrating that GGPP acts directly on Hmg2p regulation rather than through squalene synthase inhibition.

The effect of ZA is dependent on its inhibition of squalene synthase, encoded by the yeast 

\[ \text{ERG9} \]

 gene (Ref. 22 and Fig. 1). Not surprisingly, overexpression of squalene synthase shifts the dose-response curve for ZA-induced stimulation of Hmg2p degradation strongly to the right. Specifically, we have shown that stimulation of Hmg2p degradation requires significantly higher concentrations of ZA when the squalene synthase gene is overexpressed by using the strong 

\[ \text{TDH3} \]

 promoter to drive the 

\[ \text{ERG9} \]

 gene (5). This provided a useful test for assessing whether GGPP was also an inhibitor of squalene synthase.

We directly compared the effects of squalene synthase overexpression on ZA or GGPP stimulation of Hmg2p-GFP degradation. The indicated concentrations of GGPP or ZA were incubated with otherwise identical strains expressing normal genomic 

\[ \text{ERG9} \]

 or 

\[ \text{ERG9} \]

 driven by the strong 

\[ \text{TDH3} \]

 promoter. Although the ZA showed the expected decrease in potency as a stimulant of Hmg2p-GFP degradation in the strains with its overexpressed target enzyme, GGPP had identical potency in each strain, indicating that its effects were not mediated by inhibition of squalene synthase (Fig. 6A).

Mevalonate Pathway Inhibition Does Not Block Effect of GGPP—The action of ZA depends on flux through the mevalonate pathway, because stimulation of Hmg2p degradation by ZA is caused by a build-up of the pathway intermediate FPP (Fig. 1). Thus, the action of ZA is inhibited by the upstream inhibitor lovastatin, which at sufficient doses blocks the production of FPP needed for stimulation of Hmg2p degradation (5, 23). We wondered whether the action of GGPP was independent of sterol pathway flux; this would be further evidence that GGPP does not operate by inhibition of squalene synthase or any other downstream enzyme. We tested the effect of lovastatin on GGPP-mediated lowering of Hmg2p-GFP. The cells were pretreated with lovastatin for 1 h and then incubated with either GGPP or ZA for an additional hour. As expected, ZA did not stimulate Hmg2p degradation in these conditions (Fig. 6B) or even when added at the same time as lovastatin (data not shown). In contrast, the GGPP effect was identical in the pres-
ence or absence ofLovastatin in these conditions. Taken together, these results indicate that GGPP acts directly on the degradation process of Hmg2p-GFP rather than by altering the production or levels of a sterol pathway-derived signal.

Intracellular Levels ofGGPP Influence Hmg2p Stability—We turned our attention to the possibility that endogenously made GGPP was involved in regulated degradation of Hmg2p. GGPP is synthesized from FPP by the geranylgeranyl pyrophosphate synthase encoded by BTS1 (Ref. 9 and Fig. 1A). We first tested whether overexpression of BTS1 would increase the degradation of Hmg2p, using a plasmid expressing BTS1 under the control of the strong galactose promoter. In galactose, Hmg2p-GFP levels were significantly lower in strains carrying the BTS1 plasmid. When these BTS1-expressing cells were treated with Lovastatin, Hmg2p-GFP levels rose (Fig. 7). As expected, overexpression of BTS1 had no effect on the unregulated NR1-Hmg2p-GFP and 6myc-Hmg2p-GFP versions of Hmg2p. The BTS1 overexpression test indicated that endogenously produced GGPP can promote Hmg2p degradation. We next addressed whether naturally made GGPP participated in Hmg2p-GFP levels by the addition of cycloheximide followed by flow cytometry at the indicated times. An untreated culture aliquot was also included in the run to indicate Hmg2p-GFP starting levels. Hmg2p-GFP degradation was reproducibly slower in the bts1Δ, showing a 1.6-fold slowing of degradation (Fig. 8, A and B). This altered rate of degradation was not observed when the identical experiment was done with unreg-
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FIGURE 5. Unregulated mutants of Hmg2p-GFP were unresponsive to GGPP. Hmg2p-GFP mutants NR1-Hmg2p-GFP (A) and 6myc-Hmg2p-GFP (B) were tested for an effect by GGPP. Strains expressing each mutant or WT Hmg2p-GFP were compared for effects of 1 h of incubation with 11 μM GGPP or vehicle.

FIGURE 6. GGPP action was distinct from zaragozic acid. A, overexpression of squalene synthase (ERG9) did not alter Hmg2p responsiveness to GGPP. Strains expressing WT or high levels of squalene synthase (from TDH3 promoter) were incubated with indicated concentrations of ZA or GGPP. Following 1 h of incubation, Hmg2p-GFP fluorescence was measured by flow cytometry. B, lovastatin preincubation did not block the GGPP effect. The cells were either untreated or preincubated with lovastatin (25 μg/ml) for 1 h before the addition of either ZA (13 μM) or GGPP (11 μM) for another additional hour of incubation, followed by GFP flow cytometry.

Prenylation Was Not Implicated in Hmg2p Degradation—We confirmed the independence of the
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**FIGURE 7.** Increasing endogenous production of GGPP synthase lowers Hmg2p levels. A, WT Hmg2p-GFP steady state levels are lower in cells expressing galactose-driven BTS1 compared with control, whereas lysine mutant K6R-Hmg2p-GFP remain unchanged. Lovastatin treatment for 4 h reversed BTS1 overexpression on Hmg2p-GFP levels. B, like A, unregulated NR1-Hmg2p-GFP levels were not influenced by high expression of Bts1p or inhibiting the pathway with lovastatin like its corresponding WT expressing strain.

effects of GGPP from protein synthesis. Cells expressing Hmg2p-GFP were treated with cycloheximide for 1 h, after which GGPP, ZA, or vehicle was added, followed by continued incubation and flow cytometry at various times. Despite this long period of protein synthesis inhibition, both GGPP and ZA caused an increased rate of Hmg2p degradation, showing that their regulatory effects work long after protein synthesis has ceased (Fig. 9A). Although this strongly implies that GGPP operates independently of prenylation, we also directly tested the effect of mutations in the geranylgeranyl transferase (GGTase) enzymes that transfer the isoprene group from GGPP to target proteins.

Yeast express two GGTases: Type I and Type II. Type I GGTase consists of two subunits, Cdc43p and Ram2p, and Type II consists of Bet2p, Bet4p, and Mrs6p (reviewed in Ref. 26). We employed temperature-sensitive alleles of the Type II GGTase subunit bet2-1 or the Type I GGTase subunit cdc43-2 to examine whether Hmg2p degradation was affected by deficiencies in either type of geranylgeranylation. We assayed Hmg2p-GFP degradation after stopping protein synthesis with cycloheximide at both permissive and restrictive temperatures, using both normally regulated Hmg2p-GFP and unregulated NR1-Hmg2p-GFP. Neither mutation, even after prolonged incubation at the nonpermissive temperatures, showed any defect in degradation specific for the regulated Hmg2p-GFP protein (Fig. 9, B and C). Furthermore, all of the prenylation mutants remained responsive to the addition of GGPP (data not shown).

**GGPP Affected Hmg2p in Vitro Proteolysis—**These studies indicate that the regulation of Hmg2p may involve a direct interaction of a sterol pathway-derived molecule with Hmg2p. Consistent with this idea, we have shown that a number of isoprene-containing molecules, principally farnesol, will reversibly alter the structure of Hmg2p to a more unfolded state that may be better recognized by the HRD quality control pathway (7). These studies were done using Myc$_1$-Hmg2p-GFP, a normally regulated version of Hmg2p-GFP with an added luminal Myc tag, to allow limited proteolysis of the microsome-bound protein to assess the changes in structure caused by the added isoprene. We confirmed that, like farnesol, GGPP directly altered the structure of Myc$_1$-Hmg2p-GFP to a similar extent when added directly to the microsomes (Fig. 10A) and that it did not have these effects on the NR1 variant of Myc$_1$-Hmg2p-GFP protein (data not shown), showing that by this structural transition assay that meets a number of criteria appropriate for relevance in

duced the mutant cdc48-3 allele into our strain background and confirmed that wild type Hmg2p-GFP and mutant NR1-Hmg2p-GFP were stable in this background by flow cytometry (supplemental Fig. S2). We also confirmed that this mutant strongly inhibits in vitro retrotranslocation of Hmg2p-GFP (17). The presence of the cdc48-3 allele had no effect on GGPP-stimulated ubiquitination of Hmg2p (Fig. 10C), despite it having a strong effect on Hmg2p-GFP stability in vivo (supplemental Fig. S2) and Hmg2p-GFP retrotranslocation in vitro. The NR1-Hmg2p-GFP was unaffected by GGPP in this pair of strains, confirming the maintained specificity of GGPP action in the presence of the Cdc48p deficiency. Consistent with this, the addition of GGPP directly to an in vitro retrotranslocation assay for Hmg2p had no effect on the process observed in this manner either (data not shown).

GGPP Enhanced Degradation of Full-length Hmg2p—The Hmg2p-GFP reporter protein is regulated identically to catalytically active Hmg2p in all of the ways tested. Therefore, we expected and confirmed that the full-length, catalytically active Hmg2p was similarly responsive to GGPP. Strains expressing full-length Hmg2p tagged with a single Myc epitope or its lysine 6 mutant K6R counterpart were exposed to GGPP for 0, 1, or 3 h. Wild type Hmg2p degradation was accelerated, and the stable lysine mutant was resistant, precisely as determined with alterations of cellular GGPP (21) (Fig. 10D).

DISCUSSION

In these studies we demonstrate that the mevalonate-derived molecule GGPP can function as a positive regulator of Hmg2p stability and is, in all likelihood, the FPP-derived molecule that controls Hmg2p stability in response to changes in the mevalonate pathway. This work was initiated by the still surprising observation that GGPP added directly to living yeast cells caused a high potency (low micromolar EC50), immediate change in Hmg2p stability, resulting in enhanced ubiquitination and rapid degradation similar to the response to the squalene synthase inhibitor zaragozic acid. The effect of GGPP was highly specific for regulated Hmg2p. Nonregulated and stable variants of Hmg2p or normally stable Hmg1p were all entirely unaffected by GGPP. Intracellular elevation of GGPP by overexpression of the BTS1 gene, encoding GGPP synthase, similarly caused a drastic and specific hastening of Hmg2p degradation, again consistent with its role as an intracellular regulator of Hmg2p.
The striking similarities between the addition of GGPP and the addition of SS inhibitor ZA led us to examine the idea that GGPP was in fact a potent inhibitor of this key sterol pathway enzyme. However, the potency of GGPP was unaffected by elevation of SS to levels that drastically affected that of ZA, indicating that it does not work through inhibition of this enzyme. Furthermore, exogenous GGPP still stimulated Hmg2p degradation in the presence of lovastatin concentrations that fully blocked the effects of ZA, again consistent with GGPP operating directly as opposed to acting through inhibition of squalene synthase or any sterol pathway enzyme downstream of HMGR for that matter.

Because GGPP is naturally derived from FPP, it seems reasonable that this molecule is generated from FPP to control Hmg2p stability. In fact, naturally generated GGPP similarly altered stability of Hmg2p, because the $bts1\Delta$ null allele showed reproducible increased stability of Hmg2p specific for the normally regulated version of the protein. Furthermore, alteration of GGPP pools by overexpression of wild type or mutant versions of the prenylation cofactor Mrs6p also showed the expected changes in Hmg2p stability consistent with endogenous GGPP playing a role in Hmg2p regulation. We were initially surprised that the effects of the $bts1\Delta$ null were not stronger. If this were the only source of GGPP, and if GGPP were the key regulator, then we would have expected the $bts1\Delta$ null to cause complete stabilization of Hmg2p, similar to the effects of lovastatin treatment or mutations in Hmg2p that render it refractory to regulatory signals (21). A recent study of prenyl synthetases in both Schizosaccharomyces pombe and S. cerevisiae offers a possible explanation. It has recently been reported that sufficient expression of yeast FPP synthase (Erg20p), which is along the mevalonate pathway, can suppress specific phenotypes of the $bts1\Delta$, indicating that this enzyme can function as a GGPP synthase as well (25). Considering the great similarity in the two reactions, this is perhaps not terribly surprising. Thus, it is likely that the GGPP generated in the cell is a product of the actions of both the dedicated Bts1p enzyme and the bifunctional Erg20p. That redundancy may also explain why the viable $bts1\Delta$ null mutant has never been isolated in the several screens performed in our laboratory for mutants that stabilized normally regulated Hmg2p. Although these observations fit a model that normally made GGPP is the key FPP-derived regulator of Hmg2p, they will require future direct analysis to confirm this important idea.

Perhaps the most natural idea for the action of a regulatory isoprenoid like GGPP would be as part of a prenylation reaction that renders a regulatory protein functional. However, it seems unlikely that GGPP acts in this capacity in control of Hmg2p stability. First, even when protein synthesis is blocked for an hour, the response to added GGPP is still intact and observable (Fig. 9A), even though it is thought that prenylation occurs soon after translation in most situations. Furthermore, temperature-sensitive mutants of either

![FIGURE 9. Protein synthesis was not required for GGPP-mediated Hmg2p degradation. A, GGPP-stimulated Hmg2p-GFP degradation occurred independently of protein synthesis. Cells expressing Hmg2p-GFP were untreated (no CX) or preincubated with protein synthesis inhibitor cycloheximide (CX, 50 μg/ml) for 1 h before the addition of vehicle, 11 μM GGPP, or 13 μM ZA for incubation of an additional hour. B, normal geranylgeranylation was not required for GGPP-mediated Hmg2p-GFP degradation. Graphic representation of Hmg2p degradation evaluated in WT and GGTPase mutants bet2-1 (top panel) and cdc43-2 (bottom panel) at permissive (25 or 30 °C) and restrictive temperatures (37 °C) by cycloheximide chase. The cells were shifted to restrictive temperature 1 h before 2 h of cycloheximide exposure. The graphs display percentages of Hmg2p-GFP remaining in cells treated with cycloheximide with respect to cells treated in the same exact manner but without cycloheximide.](http://www.jbc.org/content/284/51/35377')
GGPP, a Potent Regulator of HMG-CoA Reductase Degradation

GGPP employing prenylation mutant had no effect on the stability of regulated Hmg2p. Both of these observations are consistent with prenylation not playing a role in Hmg2p regulation by GGPP.

The alternative model would be that GGPP or something derived from it, directly affects Hmg2p entry into the HRD pathway. In biochemical assays of Hmg2p structure, we showed that some isoprenoids could cause reversible misfolding of the Hmg2p transmembrane domain, which could render it more susceptible to detection by the HRD quality control pathway. The effect is highly specific for normally regulated Hmg2p, and the same structural transition can be observed when the in vivo signal is elevated prior to isolating the assay microsomes (7). The most effective molecule in this in vitro assay was farnesol, although direct addition of farnesol (or FPP) to cells has no effect on Hmg2p stability or regulation. In vitro results that indicate ubiquitination is rapidly and primarily elevated, and our in vitro results that indicate ubiquitination is not affected. There is an interesting question to address in the future.

There are several surprising features to our current work that warrant further study. The ease and potency with which direct addition of GGPP to living yeast cultures affects Hmg2p degradation was quite unexpected. Our normal experience has been that the addition of anionic organic molecules, such as mevalonate, is at best a poor route for delivery to the cytoplasm (3). In fact, GGPP was used as a negative control in our first experiments, because of our presumption that it could not get into the cells. Thus, either there is a high affinity transport system for GGPP or the intact yeast has a surprising hole in its permeability barrier for this molecule. Although we have not addressed this issue yet, we did attempt to test whether there was a transporter with an inhibition experiment. Because FPP added directly to cells had no effect, we tested whether very high concentrations of FPP would antagonize the effects of added GGPP. However, no concentration of FPP had any effect on added GGPP, even when in great excess to the active compound. Because FPP has no effect on its own, this observation does not inform us about the nature of GGPP influx, which, despite its inherent interest, remains a mystery demanding future study.

Our earlier work has strongly implicated endogenous FPP as a source for the principle regulator Hmg2p in vivo. Elevation of FPP by a number of means increases Hmg2p ubiquitination, and lowering FPP decreases ubiquitination (5). Those studies include both genetic and pharmacological means and form a highly consistent picture of a positive
regulator derived from FPP. Thus, we were surprised that the addition of FPP to intact cells has no effect on Hmg2p. It could be that the transport of 15-carbon FPP does not occur, whereas the very similar 20-carbon GGPP structure is transported. Although this seems unlikely, the question requires further investigation. If the FPP is indeed transported, then why is it ineffective at stimulating Hmg2p degradation, whereas endogenously produced FPP appears so central? We wondered whether added FPP could not be converted into GGPP because of a lack of the other precursor molecule isopentenyl pyrophosphate, but simultaneous addition had no effect either. One explanation may be that the synthesis of GGPP is tightly coupled to FPP production, perhaps by channeling between the enzymes that produce FPP and those that use it for GGPP synthesis, including both Bts1p and Erg20p (FPP synthase) itself. This would explain the striking difference between adding the product and the substrate of the GGPP reaction and warrants further study.

Tight coupling of FPP to GGPP production would mean that significant levels of GGPP should be expected during the normal operation of the sterol pathway and especially when FPP utilization is halted with ZA or by genetic down-modulation of squalene synthase. There are two lines of evidence that indicate that this is the case. In our own earlier work (20), we tested the effects of various manipulations of the mevalonate pathway on the labeling pattern of carbon 14 acetate as measured by thin layer chromatography. Although our primary interest in those studies was the detection of oxysterols produced by the “alternate pathway” that arises from a build-up of dioxidosqualene, the entire pattern of acetate products was delivered by this approach. Upon revisiting these TLC autoradiograms, we discovered that the most prominent sterol pathway product produced by inhibition of SS with ZA or by promoter shut-off had an identical \( R_t \) to GGGOH, to the complete exclusion of FOH, for which standards were run in those assays. We confirmed this by directly comparing standards of GOH, FOH, and GGGOH in identical TLC systems, which showed that the prominent band had the expected mobility of GGGOH. In supplemental Fig. S3, for the convenience of the reader, we have reproduced the picture of the TLC of one of these experiments from (20), with an arrow indicating the prominent band with the mobility of GGGOH. Clearly, this 20-carbon skeleton is abundantly produced in conditions where FPP utilization by SS is lessened. Similarly, in bulk lipid studies recently performed to evaluate the isoprenes produced when HMGR activity is elevated in yeast (29), the proportion of geranylgeranyl isoprenes produced is approximately the same as that of FPP, again indicating efficient conversion of FPP into GGPP when flux, and thus the degradation signal, is high.

Taken together, these results present a strong case for the FPP-derived signal that controls Hmg2p stability either being, or having as a direct precursor, GGPP. Because we are fairly confident that the GGPP is not employed in a prenylation reaction to make its regulatory hand felt, a product derived from GGPP would most likely be a more rare isoprenoid. It will be interesting to directly test some of the structural analogues of GGPP that cannot be hydrolyzed to the free alcohol to evaluate the effectiveness of the GGPP structure itself in this assay (30). Unraveling the nature of this striking effect of GGPP on Hmg2p regulation will allow us to understand the details of regulated HRD pathway degradation of Hmg2p, and perhaps the common facets of mechanism that connect the regulation of HMGR and Hmg2p, separated by over a billion years of evolution but joined by a common selective force to regulate the sterol pathway in highly divergent walks of eukaryotic life.

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REFERENCES

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